Charles University in Prague

Faculty of Science

Department of Genetics and Microbiology

Microbiology

Mgr. Michaela Urbanová

Vegetation of post-mining sites determines soil microbial community structure and soil processes

Vegetace na těžebních lokalitách určuje strukturu půdního mikrobiálního společenstva a průběh půdních procesů

Ph.D. Thesis

Supervisor: RNDr. Petr Baldrian, Ph.D.
Institute of Microbiology
Academy of Sciences of the Czech Republic

Prague, 2014

Declaration:

Here, I declare that I wrote this work on my own and all used sources and literature are properly citated. I also declare, that this work has not been used for obtaining any other academic degree.

Prague, 30. 09. 2014 Michaela Urbanová

Acknowledgements

First of all I would like to thank my supervisor RNDr. Petr Baldrian, PhD., the head of the Laboratory of Environmental Microbiology at the Institute of Microbiology ASCR, for his support, patience and advice.

I would also like to thank all other members of our lab for their advice, assistance, collaboration and fun; notably those who greatly contributed to soil sample collection in the hostile terrain of spoil heaps at the Sokolov mining district.

Further, I would like to thank Prof. Ing. Mgr. Jan Frouz, CSc., who is founder and supervisor of the whole project at Velká Podkrušnohorská spoil heap which I participate in, for introducing me into the spontaneous revegetation-vs.-reclamation issues.

This work was financially supported by the Czech Science Foundation (P504/12/1288 and 13-06763S) and by the Ministry of Education, Youth and Sports of the Czech Republic (LC06066 and LD12050).

Abstract

Post-mining sites are disrupted, man-made environments which are being created in the course of mining activity by heaping of the spoil material on deposits, resulting in the burial of the indigenous ecosystem under thick layers of the infertile mining waste material. The waste material is typically characterized by unfavourable biological, physical and chemical properties. In effort to rehabilitate the soil properties and ecosystem structure and functions, the vegetation must be re-introduced into the bare soil substrate. The restoration of the post-mining sites is achieved either using assisted afforestation which is more usual or using spontaneous revegetation which is often considered as insufficient for the full recovery of soil properties.

In this work, a variety of methodological approaches were employed in order to testify the effect of the vegetation on soil microbial processes, structure of soil microbial communities and to follow the chemical changes in the litter composition during its decomposition. Litter chemistry, enzymatic activities and microbial biomass were followed by standard biochemical procedures including spectrometry and HPLC. Bacterial microarray has been used for assessing the composition of bacterial communities in the soil of successional plots. 454-pyrosequencing has been employed for even deeper and more detailed identification of fungal and bacterial community composition in the litter layer and the bulk soil under different tree species.

It was shown, that the vegetation substantially affects the ecosystem development, soil biotic and abiotic properties. The importance of bacteria in the process of spontaneous succession has been demonstrated. While bacteria dominated the initial stage of succession and the late stage, fungi were virtually absent in the soil before the establishment of

vegetation and the vegetation was determined to be main driver of the changes in microbial community. Further it was revealed, that litter decomposition along successive series of post-mining sites was largely dependent on the initial composition of litters, while changes in fungal and bacterial biomass did not reflect the changes in litter chemistry, but responded to nutrient availability as well as the rate of decomposition did.

The tree effect, per se, examined at afforested sites was found to be the best predictor of fungal and bacterial communities and extracellular enzyme activities in litter and soil. In fact, fungi and activities of most extracellular enzymes were more affected by dominant vegetation type both in litter and the bulk soil than bacterial communities. Bacteria were influenced by pH and nutrient content and the tree effect on bacterial communities was mainly observable in the litter. Many fungal taxa were also strongly tree species-specific.

Comparison of soil processes and microbial communities developed at spontaneously revegetated sites and technically afforested sites did not reveal any substantial differences in examined aspects except for the sites revegetated with alder, because alder accelerated soil development due to the production of nitrogen-rich litter.

Abstrakt

Hnědouhelné výsypky jsou člověkem vytvořené lokality, na které bývá během těžby vyvážen a ukládán odpadní těžební materiál. Tím je pohřbena původní vegetace a existující ekosystém pod vrstvy neúrodné hlušiny s typicky nepříznivými biologickými, chemickými a fyzikálními vlastnosti. Pro obnovu vlastností půdy, a struktury a funkce ekosystému je nutné zajistit zdroj organického materiálu vstupujícího do půdy, nejčastěji rostlinného původu. Obnovy výsypek bývá dosaženo buď jejich rekultivací vybranými typy dřevin, což je způsob používanější nebo přirozenou revegetací, která ale bývá považována za nedostatečný způsob pro úplné obnovení vlastností půdy.

V této práci byly použity biochemické a molekulárně-biologické metody pro testování vlivu vegetace na mikrobiální procesy, strukturu mikrobiálních společenstev a pro sledování změn v chemickém složení opadu během jeho rozkladu. Chemické složení opadu, enzymové aktivity a mikrobiální biomasa byly stanovovány pomocí obvyklých biochemických metod zahrnujících spektrometrii a HPLC. Bakteriální microarray byl použit pro určení složení bakteriálního společenstva na sukcesních plochách. 454-pyrosekvenování bylo použito pro podrobnější identifikaci houbových a bakteriálních společenstev v opadu a půdě pod různými druhy stromů.

Bylo prokázáno, že vegetace podstatně ovlivňuje vývoj ekosystému, a půdní biotické a abiotické vlastnosti. Byla potvrzena důležitost bakterií v procesu spontánní sukcese. Zatímco bakterie dominovaly během počáteční a pozdní fáze sukcese, houby v počáteční fázi sukcese, díky absenci vegetace, úplně chyběly. Změny v mikrobiálním společenstvu během sukcese souvisely se změnou vegetačního pokryvu. Dále bylo zjištěno, že rozklad opadu v průběhu sukcese byl závislý na svém počátečním chemickém složení, zatímco změny

v houbové a bakteriální biomase neodrážely změny v chemickém složení opadu, ale odpovídaly dostupnosti živin stejně jako rychlost dekompozice.

Efekt stromů, studovaný na rekultivovaných plochách, byl hlavním faktorem určujícím složení houbových a bakteriálních společenstev a enzymových aktivit. Ve skutečnosti, houby a aktivity většiny extracelulárních enzymů byly více ovlivněny dominantní typem vegetace, jak v opadu, tak v půdě než bakteriální společenstva. Bakterie byly ovlivněny pH a obsahem živin a vliv vegetace byl pozorovatelný hlavně v opadu. Mnoho houbových taxonů se navíc specificky vyskytovaly jen pod určitým druhem stromu.

Porovnáním půdních procesů a mikrobiálních společenstev vyvinutých na plochách procházejících sukcesí s plochami rekultivovanými nebyly odhaleny žádné výrazné rozdíly ve studovaných aspektech kromě míst rekultivovaných olší, neboť olše urychluje vývoj půdy produkcí opadu s vyšším obsahem dusíku.

List of Abbreviations

A	*Alnus*
C	carbon
DNA	deoxyribonucleic acid
HPLC	high-performance liquid chromatography
L	*Larix*
N	nitrogen
Pc	*Picea*
PLFA	phospholipid fatty acid
Pn	*Pinus*
rDNA	ribosomal deoxyribonucleic acid
Q	*Quercus*
T	*Tilia*

List of Publications

This thesis is composed of these publications:

Paper I

Urbanová, M., Šnajdr, J., Brabcová, V., Merhautová, V., Dobiášová, P., Cajthaml, T., Vaněk, D., Frouz, J., Šantrůčková, H., Baldrian, P. (2014) Litter decomposition along a primary post-mining chronosequence. Biology and Fertility of Soils 50: 827-837. IF_{2013} = 3.396

Paper II

Urbanová, M., Kopecký, J., Valášková, V., Ságová-Marečková, M., Elhottová, D., Kyselková, M., Moënne-Loccoz, Y., Baldrian, P. (2011) Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. FEMS Microbiology Ecology 78: 59-69. IF_{2013} = 3.875

Paper III

Šnajdr, J., Dobiášová, P., Urbanová, M., Petránková, M., Cajthaml, T., Frouz, J., Baldrian, P. (2013) Dominant trees affect microbial community composition and activity in post-mining afforested soils. Soil Biology and Biochemistry 56: 105-115. IF_{2013} = 4.410

Paper IV

Urbanová, M., Šnajdr, J., Baldrian, P. (2014) Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. submitted for publication

Introduction

Mining areas arise as a result of human activities which usually cause a complete destruction of the affected ecosystems. During brown coal mining the spoil material, which represents a mixture of fertile topsoil, mineral soils and unfertile (and sometimes toxic) waste material, that may cover the desired deposits up to depth of several hundred meters, is usually removed and disposed in spoil heaps and thus the indigenous vegetation at these sites is buried. The existing ecosystem structures of mined areas as well as sites where spoil deposits are located are irreversibly affected.

In the region of the Central Europe there large areas were subject to mining, and even though the damage of the affected ecosystems may be seen as enormous, they nowadays offer a great opportunity for studying and evaluation of the natural or assisted successional development on the soil physical, chemical and biological properties.

The processes of the ecosystem restoration of post-mining sites have been characterized by the soil horizon development, accumulation of organic matter and nutrients, changes in soil chemistry and increase in soil biota biomass (Bardgett et al., 2005; Baldrian et al., 2008; Frouz et al., 2013); all changes are a result of the vegetation development (Bardgett and Walker, 2004), but apart from that they are also subject to the presence and activity of soil biota (Frouz and Nováková, 2005; Šourková et al., 2005a; Baldrian et al., 2008).

The manners of restoration of the post-mining sites

The restoration of the damaged structures of terrestrial soil ecosystems and the ecosystem functions is required to prevent their further devaluation, which lies namely in the continuing degradation of soil substrate by erosion, loss of soil productivity and deterioration

of the microclimatic conditions which represent the consequences of the exposition of sites to negative climatic impacts (Singh et al., 2002). The measures based on the re-introduction of plants into the barren soils enable to achieve the required ecosystem rehabilitation. The assisted afforestation and spontaneous revegetation belong to the most commonly selected restoration approaches.

The severe abiotic soil conditions, which could prevent the successful initial establishment of vegetation, have to be adjusted before the restoration processes are launched. The type of adjustment is selected according to the site-specific constrains and needs, and encompasses typically mechanical destabilization of the structure of surface soil by ripping in the case of the unfit and too tough structure of the exploit material or the application of organic residues such as mulch or chemical fertilizers in order to increase the soil moisture and the soil nutrient content and to protect the soil surface from the erosion, excessive solar radiation and water evaporation. In the case of very wet sites, it is advised to apply mechanical drainage to divert water and improve soil aeration. If the spoil substrate contains seriously toxic levels of metals or substrate pH is too low, the use of organic materials and the planting of tolerant cultivars or even chemical treatment are required to overcome these difficulties (Bradshaw, 1997).

The assisted afforestation is a usual restoration measure of the post-mining sites in the Central Europe (Chodak and Niklinska, 2010a; Insam and Domsh, 1988; Mudrák et al., 2010; Štýs, 1981; Šourková et al., 2005a). Different tree species can be used for this purpose. The other way, which is rather rare in this region, is leaving the vegetation to develop spontaneously (Prach and Pyšek, 2001; Prach and Lencova, 2013).

The assisted afforestation is typically performed by planting tree seedlings directly into the rough substrate and later results in the establishment of the forest patches which

resemble forest monocultures. In this approach, the preference is put on the selection of such plant species, which rapidly enhance biological functions of nutrient-poor or toxic soils. For instance, plants such as *Alnus spp*. which can symbiotically promote nitrogen fixation and produce nitrogen-rich organic materials, which consequently increase the nitrogen (N) content in the soil, are often preferred, since supply soils with limiting nutrients (Ekblad and HussDanell, 1995) and thus accelerate the development of the soil. Indeed, nitrogen deficiency of barren soils is a serious problem which prevents the vegetation establishment and can be solved by planting of such tree species (Bradshaw, 1997). It often happens during the afforestation processes that non-native plant species with the above properties are introduced into the environment and thereafter spread beyond the intended area of the species occurrence which may imply the threat of the native vegetation (Kowarik 1995; Pyšek et al., 2012) and changes of ecosystem functioning (van der Putten et al., 2007). This fact was confirmed by the work of Hodačová and Prach (2003), who have also declared, that the use of the allochthonous species has become a common praxis in reclamation processes of the post-mining sites in the Central Europe.

In contrast to such threats, spontaneous revegetation can be an alternative treatment which represents a low-cost approach resulting in the formation of complex, possibly highly valuable ecosystems which may even contribute to the conservation of biodiversity, in some cases. The vegetation development on excavated soil substrates of the post-mining sites typically starts with the initial colonisation by individual plants, followed by the development of herbal/grass vegetation cover and the establishment of woody species (Prach and Pyšek 2001) with variable composition of herbal understorey (Mudrák et al., 2010). Woodlands and forests represent the final climax stage in the Central Europe area. In this region, the typical pioneer colonizers are herbaceous plants and grasses, among them *Calamagrostis epigeios* often dominates, followed successively with woody species such as *Salix caprea*, *Betula*

pendula and *Populus tremuloides* (Frouz et al., 2008; Wiegleb and Felinks, 2001; Rebele and Lehman, 2002, Prach and Pyšek, 2001). Even if this way of the rehabilitation of the post-mining sites was not largely used in ecological engineering in the past, nowadays it is perceived as reasonable alternative to the assisted afforestation (Hodačová and Prach, 2003; Hendrychová et. al., 2012; Prach et al., 2013 ; Šálek, 2012; Tropek et al., 2012).

While the research on the soil development under the effect of the individual dominant plant species may be carried out at commercial forest plantations, the places which would provide the opportunity to study the effect of natural, successively developed vegetation are rare.

Phenomenon of spontaneous succession is widely known from polar and alpine regions, where it was also studied in detail (Duc et al., 2009; Noll and Wellinger, 2008; Schmidt et al., 2008; Yergeau et al., 2009). Despite the similar character of the ecosystem development processes taking part in the alpine/polar and temperate zones, such as the decrease of pH in the course of the succession as a result of the accumulation of organic matter and decomposition reactions or the increase of C:N ratio (De Kovel et al., 2000; Frouz et al., 2009; Kirmer and Mahn, 2001; Noll and Wellinger, 2008; Pennanen et al., 2001; Tscherko et al., 2004;), the alpine/polar regions may hardly serve as a model ecosystem for the temperate zones due to their extreme character and the large impact of the climate and the complicated initial establishment of the vegetation on the exposed barren substrates at the glacier forefields caused by strong nutrient limitations (Fierer et al., 2010). On the other hand, the post-mining sites can be considered as appropriate model sites because of their vastness and since the nutrient limitation of the plant colonization are not so strongly overwhelmed by nutrient deficiency (Baldrian et al., 2008; Frouz et al., 2009; Frouz and Nováková, 2005).

The effect of vegetation on soil environment

Plants greatly impact soil environment in several ways which are interconnected and thus influence soil structure as well as soil chemistry and soil processes. They affect soil mechanically via roots penetration, chemically via production of organic matter as well as biologically via multiple associations with soil biota. The plants impact is generally more pronounced in upper topsoil (0 – 10 cm) than in deeper mineral soil layers (20 – 30 cm) and is also partly dependent on other external site-specific factors such as soil mineralogy, clay and sand content, climatic factors, topography of the site or former and present land-use and management (Augusto et al., 2002; Bissett et al., 2011; Vesterdal et al., 2013).

Root system anchors the plants in the soil and enables the uptake of nutrients and water from the soil, but it also represents an ecologically interesting niche where the species-rich and plant species-specific microbial populations live (Buee et al., 2009a).

As it was indicated above, roots impact soil mechanically by root penetration to affect soil structure, soil aggregates formation, weathering of rocks and nutrient leaching. Roots expand through the pores changing soil porosity and increase bulk soil density, facilitate aeration and improve the soil water regime (Angers and Caron, 1998; Six et al., 2004). The resulting increase in air and water availability positively influences soil biological and biochemical processes, as many of them are aerobic and thus the nutrient availability is positively affected. Nevertheless, permanently higher water content may lead to the increase of microaerobic or anaerobic microbial processes and result in soil acidification and the release of nitrogen from soil as a consequence of the changes in microbial activities (Davidson and Janssens, 2006).

Apart from the mechanistic impact, plant roots affect soil environment also chemically by rhizodeposition, which encompasses a variety of plant-specific organic compounds, e.g.

carbohydrates, carboxylic acids, phenolics, amino acids or inorganic ions, that are released into the close surrounding of root-soil interface termed rhizosphere (Jones et al., 2004; Hartmann et al., 2009; Berg and Smalla, 2009). Rhizosphere, the compartment of the soil where the microbial populations and processes are under the strong influence of the plants, is the place of higher microbial biomass and activity compared to the bulk soil (Berg and Smalla, 2009; Raajimakers et al., 2009). Root-derived compounds (exudates) are the driving force in the selection of microbes from indigenous microbial populations inhabiting bulk soil (Bais et al., 2006, Haichar et al., 2008). The amounts and chemical composition of exudates vary considerably among plants, plant growth cycle stages and root segments (Jones et al., 2004). Because of their low rate of chemical diffusion, the root exudates do not affect the chemistry of the bulk soil directly, even if as the nutrient source for some groups of soil biota they may be transformed and translocated into the soil organic matter and humic compounds (Balasooriya et al., 2014; Hartmann et al., 2009) and thus affect the soil organic matter content, chemistry and soil structure (Brady and Weil, 2008).

Plant litter is a major source of soil nutrients and soil organic matter. The amount and quality of litter differs among plant species and affects the rate of decomposition (Aubert et al., 2010; Hättenschwiller and Jorgensen 2010; Talbot and Treseder, 2012). Chemical characteristics of soil, such as pH or nutrient content, change substantially (Augusto et al., 2002; Binkley and Valentine, 1991; Hagen-Thorn et al., 2004; Menyailo et al., 2002) when litter is processed and incorporated into the soil by soil biota (Frouz et al., 2007, 2009; Quideau et al., 2001; Rubino et al., 2010) whose composition is also influence by litter properties (Hendrychová et al., 2012; Chodak and Niklinska, 2010a; Iovieno et al., 2010; Lamb et al., 2011; Merilä et al., 2010). Aboveground part of litter is typically composed of dead plant residues such as leaves and twigs. The aboveground litter production is generally higher in forest ecosystems, however without any significant differences in the magnitude

between deciduous and coniferous tree species (Augusto et al., 2014), while the belowground part of litter encompasses dead roots and is relatively more important in agricultural ecosystems and grasslands.

Other factors which impact the soil environment

Although there is no doubt that plant species influence the structure and function of soil, there are also other factors, which contribute to that. However, the extent of such contribution is difficult to assess since they broadly vary across the ecosystems in their contribution (Berg and McClaugherty, 2003; Six et al., 2004).

Soil texture is very important factor since the content of the organic matter, nutrient availability and decomposition processes depend substantially on the presence of clay and sandy materials in soil substrate (Franzluebber et al, 1996; Müller and Höpper, 2004; Six et al., 2004). Both types of soil materials belong to nutrient poor materials, but with an opposite nutrient binding capacity. Clayey substrates bind the organic polymers, organic matter and ammonium to form colloids, therefore the decomposition of organic materials is often strongly retarded in such soils. Moreover, clay particles pronounce soil aggregate formation, which may also protect organic matter from decomposition and mineralization (Rice, 2002). On the other hand, sandy soils, which are well aerated, enhance nutrient decomposition and leaching.

External climatic factors such as temperature and the amount of precipitation also impact significantly soil microclimate and thus the microbial processes (Brockett et al., 2012; Franzluebbers et al., 2001; Thomsen et al., 2003). In common, higher soil temperature promotes organic matter decomposition, while precipitation affects it negatively (Post et al.,

1982). For example, the soils in the tropical climates have typically lower contents of organic matter and fast decomposition, while for the cooler climatic areas the pattern is opposite (Corteaux et al., 1995; Post et al., 1982). Lack of oxygen and available water can negatively modify the efficiency of soil biological processes (Thomsen et al., 2003; Davidson and Janssens, 2006). Linn and Doran (1984) reported about 60% of water filled pore spaces as about the optimal conditions for microbial activity. Too high water saturation leads to a poor aeration and increases anaerobic biological processes in soil, which results in the production of acidic waste products and the increase of soil acidity that inhibits organic matter decomposition and leads to the storage of organic compounds in the form of humic substances (Guggenberger, 1994).

Topography of the site, notably the slope and the aspect, affects the water dynamics and the organic matter accumulation. The organic matter accumulation is enhanced at the bottom of the slopes due to the organic matter runoff to the lower slope position and on the north faces of hills in the Northern Hemisphere and opposite in Southern Hemisphere because of the wetter conditions (Sariyildiz et al., 2005).

Also management practices, depending on their nature and extent, may have positive or negative effect on the soil properties, its fitness, nutrient availability and soil biota composition (Griven et al, 2003; Reiners et al., 1994).

Soil biota

Soil organisms represent an irreplaceable functional link between the aboveground and belowground environments since many of them play key roles in the biogeochemical cycles and processes (Prescott and Grayston, 2013; Wardle et al., 2003). Although the soil is inhabited by an enormous amount of microbiota such as bacteria, viruses, fungi, protozoa and

algae, as well as larger biota such as invertebrates and vertebrates, this work emphasizes bacteria and fungi which are able to degrade plant biopolymers and thus initiate and promote nutrient cycling and moreover these groups of organisms form ecologically important associations with plant roots (Aubert et al., 2010; Berg and McClaugherty, 2003; Hartmann et al., 2009) and are thus under the direct influence of the vegetation.

Several studies have already reported about the effect of plants on soil chemistry (Hagen-Thorn et al., 2004; Menyailo et al., 2002) and about the effect of soil chemistry on the microbial populations (Hackl et al., 2004; Högberg et al, 2007; Prescott and Grayston, 2013). The effect of the vegetation on microorganisms seems often to be rather mediated by soil chemical characteristic, although the litter properties itself are sometimes considered as the most important factor for the composition of litter-associated fungi and bacteria (Aneja et al., 2006; Bray et al., 2012), despite the effect of the underlaying forest floor, which was also identified as appreciable (Wallenstein et al., 2010). In the bulk soil, pH of the soil substrate seems to be highly important for the composition of the bacterial community exhibiting the highest diversity in circum neutral pH and the lowest in the acidic soils (Fierer and Jacksson, 2006; Lauber et al., 2009), while it seems to be of lower importance for fungi (Rousk et al., 2010). Moreover, both bacteria and fungi respond to soil nutrient content such as this of carbon, phosphorus or nitrogen (Cleveland and Liptzin, 2007; Fierer et al., 2009). Also the effects of land use and dominant vegetation seem to be important, although they probably more affect fungi than bacteria, being more significant for root-symbiotic fungal taxa than for saprotrophic ones (Martiny et al., 2006; Buee et al., 2009b; Zinger et al., 2011), because of their documented or expected plant-species specificity (Peay et al., 2008; Tedersoo et al., 2008).

Soil microbial populations encompasses autotrophs, which are important in the non-vegetated soils, where they support nutrient cycling and accumulation in the soil and help the

establishment of the vegetation; further saprotrophs, which are important for the decomposition of organic matter and nutrient cycling and the microbial plant-root symbionts living in the rhizosphere but spreading in some cases into the bulk soil (microorganisms with filamentous body structure).

Saprotrophic bacteria and fungi differ from root-symbiotic species by capability to produce a broad set of enzymes which are involved in the processes of decomposition of organic materials. Bacteria decompose organic substrates under both aerobic and anaerobic conditions, whereas fungi are exclusively aerobic or microaerobic degraders. Both groups are able to decompose all components of the plant cell walls, i.e. cellulose (10-50% of organic material), hemicellulose (30-40% of organic material) and lignin (15-40% of organic material) (Berg and McClaugherty, 2003), but with varying efficiency. Due to the filamentous life form of most taxa, fungi are considered to be more suitable for the penetration into the plant tissue structures and consequently more involved in the decomposition of polymeric, often recalcitrant compounds (de Boer et al., 2005) with high C:N ratio, while many bacterial taxa, preferentially utilizing low-molecular-mass organic compounds rather nutritionally rely on the products of fungal biopolymer decomposition (de Boer et al., 2005; Štursová et al., 2012). The coexistence of bacteria and fungi may result in the increase of the rate of decay of some organic substrates (Blanchette et al., 1990) and the bacterial decomposition may be of high importance in environments where fungi exist under stress (Berg and McClaugherty, 2003).

It was described in the previous section, that some bacteria and fungi inhabit the close vicinity of roots called rhizosphere (Hartmann et al., 2009); the soil compartment which is under major influence of plants (Buee et al., 2009a; Churchland and Grayston, 2014). These microorganisms originate from bulk soil pool, whence they are attracted by root exudates (Hartmann et al., 2009). Due to the selectivity of such attraction and establishment in or on

plant roots, rhizosphere communities are greatly distinct from bulk soil microbial communities (Koide et al., 2005; Buee et al., 2009a; Corneo et al., 2013). The fact that the properties of the plant rhizospheres, such as the specific composition of exudates, differ among plant species (Prescott and Grayston, 2013; Churchland and Grayston, 2014) should be reflected by the plant-specific differences among rhizosphere microbial communities as it was recently reported for bacteria associated with agricultural crops and grasses (Turner et al., 2013).

The microbe-plants interactions are based on nutrient and water exchange and are not exclusively mutualistic, some of the interactions are also neutral or pathogenic (Raaijmakers et al., 2008; Whipps, 2001). The decomposition capabilities of the microbes associated with roots are rather limited (Baldrian, 2009), contrarily they exert the influence on the plant fitness and physiology, as well as soil structure and the composition of other soil biota (Hartmann et al., 2009; Frey-Klett et al., 2007; Rillig and Mummey, 2006).

Different body structure of soil bacteria and fungi is a feature which has an important ecological aspect (de Boer et al., 2005). While the unicellular bacteria in the rhizosphere and the bulk soil inhabit one of these separate niches, the mycelia of root-associated fungi or saprotrophic fungi extend the area of their impact to facilitate the searching for the new host or nutrients. As a consequence, the effect of the host plant may be extended by their specific fungal symbionts into the bulk soil where the plant-symbiotic fungi were demonstrated to influence the local microbial community (Koide et al., 2005; Kluber et al., 2011) in this way extending the effect of their plant host into the bulk soil.

Study area

The study area was located at Velká Podkrušnohorská spoil heap in the Sokolov brown-coal mining district. The research focused on the various aspects of the ecosystem development and the changes in the ecosystem structure has been conducted here for already more than two decades. The simple fact of the substrate and climatic homogeneity of the whole area allows us to dissociate the impact of the external factors on the intended ecosystem characteristics and to follow the consequences of the plant introduction into the barren substrates. That makes this area unique and greatly valuable for examination of effect of vegetation on soil.

The area stretches over more than 9000 ha, at the altitude of 450 – 520 m a.s.l., with mean annual precipitation 650 mm and mean annual temperature 6.8°C. The substrate deposited is tertiary clay that consists of caolinite, montmorilonite and illite, with initially alkaline pH 8-9; spoil materials were further impregnated by calcite, siderite and by fossil organic matter, mainly type II kerogen (Rojík, 2004; Mudrák et al., 2010). After heaping, the soil horizon has not been developed neither on afforested nor spontaneously revegetated plots since the soil conditions (such as the low nutrient content) were highly unfavourable.

None of previously described adjustment of the excavated substrate had been used in the case of our experimental area except that part of spoil heap intended to the assisted afforestation. Here, the surface soils were levelled by earthmoving machinery before trees were planted unlike to the surface soil of plots left for spontaneous succession, where this treatment was not applied and were characterized by longitudinal depressions and elevations. Previous studies demonstrated that the depressions were hotspots of soil microbial activity since their microenvironment exhibited smaller humidity variations (Frouz and Nováková, 2005).

The afforested area covers the most of the spoil heap and the study area thus developed into a mosaic of forest patches, planted as a reclamation measure in a way that individual tree patches are randomly spread over the heap. This gives a "common garden" experiment of a landscape dimension. The reclaimed plantations, each dominated by one or two tree species of one genus: Alnus (A), *Alnus glutinosa* and *Alnus incana*; Larix (L), *Larix decidua*; Picea (Pc), *Picea omorica* and *Picea pungens*; Pinus (Pn), *Pinus contorta* and Pinus nigra; Quercus (Q), *Quercus robur*; and Tilia (T), *Tilia cordata*. Small parts of the heap had not been reclaimed and at various times it was left to spontaneous development. At the sites left to spontaneous development, vegetation was absent during the initial stages of soil development. Vegetation cover developed gradually after 5 years of the ecosystem development. Herbs and grasses (mainly *Calamagrostis epigeios*) were present on the plot at 12 years, shrubs (*Salix caprea*) at 21 years and tree cover (*Betula spp*. and *Populus tremuloides*) at 45 years. Shrubs shaded nearly the entire soil surface, resulting in a weak herb and grass cover. When the shrubs developed into a forest, the herb and grass cover reappeared.

Substrate conditions were homogeneous across most of the area in all measured aspects into the depth of 0.75 m (Šourková et al., 2005), with negligible initial microbial activity and very low content of organic carbon of plant origin (Elhottová et al, 2006; Frouz and Nováková, 2005; Helingerová et al., 2010). However, the rate of accumulation of nutrients increased with time as vegetation developed (Šourková et al., 2005b). For instance, the organic C content in the topsoil layer at the successional sites increased form 2.9% (early) to more than 5.7% (middle) and then to 7.7% (late) (Helingerová et al., 2010). Along the increasing nutrients contents and nitrogen availability, the activity of decomposition-related extracellular enzymes positively correlated (Baldrian et al., 2008).

At the sites of study area, the amount of carbon in soil depends on the aboveground plant biomass than on the quantity of litter because there are huge differences in the fate of the senescent plant material. In fact, the litter with higher N content and soils with low C:N exhibited higher activities of extracellular enzymes, higher activities of soil fauna and the undeveloped organic horizon layer, but developed organo-mineral soil horizon (Baldrian et al., 2008; Frouz et al., 2009).

All surveyed processes of soil formation and ecosystem development were greatly subject to the prevailing vegetation type or dominant vegetation tree species even if to some extent may be affected by the understorey vegetation, whose cover ranges from 16% at the *Tillia* plots to 51% in *Pinus* and *Quercus* plots; and under *Alnus*, the understorey vegetation covers 100% of the soil surface (Mudrák et al., 2010).

Aims

Despite the wide acceptance of the existence of the interactions among plant traits and microbial community structure (Aponte et al., 2013; Grayston et al., 1998; Hackl et al., 2004; Hobbie et al., 2006; Ushio et al., 2008), there is currently a little knowledge about the extent to which tree species affect the diversity and structure of bacterial and fungal communities in the soil horizon. Previously published studies are often limited by used methods with the low phylogenetic resolution, such as the comparison of PLFA profiles, or they consider only a part of the microbial community (typically only bacteria or only fungi) or their interpretation is limited by the experimental design, i.e. low number of replications or the inability to exclude tree-independent external factors (Prescott and Grayston, 2013).

Also the processes characterizing the spontaneous revegetation and natural succession are more frequently studied in the alpine and polar regions, but not at the temperate zones due to the limited occurrence of suitable experimental plots. There is thus a lack of data about the effect of changes in vegetation cover on the soil ecosystem.

This work was carried out to broaden the existing knowledge about the effect of different types of the vegetation on soil properties, microbial processes and microbial communities using bacterial microarray, high-throughput sequencing methods, phospholipid fatty acid (PLFA) analysis, enzyme assays and gas chromatography to explore the possible ecological role of microbes in the studied ecosystems.

The aim of the first study was to describe the development of the bacterial communities in the soil along the spontaneous revegetation series and to compare them with the bacterial communities from the plots planted with *Alnus* in order to evaluate the success of the natural revegetation. Due to the fact that the plants can shape the composition of microbial communities in soil, it was expected, that the structure of bacterial community from the initial

pre-plant stages of the succession will be most distinct from the rest of the sites which were either colonized by pioneer plants or the shrubs and dominant trees were developed. We hypothesized that the development of bacterial community along the primary succession chronosequence will be influenced by vegetation when considering the importance of plant litter chemistry and rhizosphere effects (Hartmann et al., 2009). The transitions of key plant species will result in the discontinuous development of bacterial community, which will reflect vegetation traits of the site specific age. The relative importance of the gradual accumulation of organic matter and changes in soil chemistry will be likely less important.

The aim of the second study was to track the changes in the chemical composition of different litter types corresponding with the early, middle and late stage of succession and the experiment had been conducted at the same successional series as the previous work. In the two years experiment, it was identified which properties of the litter most probably affected its decomposability and transformation and how these changes influence microbial abundance, their composition and activity. Here, we hypothesized that plant litter with the higher nutrient content and lower content of the recalcitrant biopolymers such as lignin will support larger biomass of soil microorganisms and exhibit higher activity of decomposition-related enzymes resulting ultimately in faster rates of decomposition.

The aim of the third and fourth study was to evaluate the effect of the dominant tree species on the microbial activities and the diversity and structure of the bacterial and fungal communities both in the litter layer and the soil and also to examine seasonal dynamics of their abundance and the activities of extracellular enzymes. The sites, where the studies were conducted, were 22-33 years old and had been established on the same initial substrate by planting with six tree genera or leaving for spontaneous revegetation. It was hypothesized that the effects of vegetation on extracellular enzyme activity will be more pronounced in soil where the accumulation of nutrients and plant-specific products leads to the development of

plant-specific organic matter and where the microbial community contains a large proportion of specific, root-associated microbiota (Hartmann et al., 2009). In addition, we expected that the effect of vegetation will more affect fungal community composition than bacterial community composition both in the litter and in the soil. Since fungi as primary decomposers of litter biopolymers should more reflect the properties of litter and also mycelia of root-symbiotic fungi are common that extend from plant roots. Due to the complex nature of plant-symbiotic plant-fungal symbiosis, which bacteria miss, symbiotic fungi are expected to be more plant species-specific and this higher level of specificity should be still detectable in the bulk soil. Bacteria are, on other hand due to the prevailing unicellular bacteria, doom to inhabit single soil niches of a very small scale (Vos et al., 2013) that often have no direct connection to the plant root or the rhizosphere.

List of methods

Soil sample collection

Enzyme assays

Quantification of microbial biomass

DNA extraction

Polymerase Chain Reaction

Library preparation for tag-encoded amplicon pyrosequencing

Bioinformatic analysis of pyrosequencing data

Statistical analysis

Discussion

This work summarizes the observations about the effect of the dominant vegetation types on the composition of soil microbial communities, soil microbial processes and soil characteristics at the post-mining sites in the Sokolov brown coal mining district. In this area, the different tree species as well as spontaneously developed vegetation were employed as a measure of rehabilitation of the excavated spoil material. As the research has been conducted in this area for the several decades with the effort to monitor the processes of *de novo* developing ecosystems, two types of the research plots were created as results of different restoration approaches. The experimental plots at the forest patches which are similar to the commercial forest monocultures allow us to study the effect of individual tree species on soil biotic and abiotic properties, while the plots at the spoil heaps left for spontaneous development allow us to monitor the processes of primary ecosystem succession and the effects of a more complex vegetation cover.

In the recent past due to the increasing pace of the global climate changes, the succession processes began to be massively studied in the alpine and polar regions at the forefields of the receding glaciers (Bardgett and Walker, 2004; Kastovska et al., 2005; Noll and Wellinger, 2008). The succession in these ecosystems is substantially governed by the harsh climate and physical constrains of the exposed substrate (Schmidt et al., 2008) as well as by strong nutrient limitation which all together interfere with successful vegetation establishment and slow down the potential ecosystem development (Fierer et al., 2010). However, once the plant colonization at these sites starts, vegetation becomes an important factor determining the ecosystem development processes. Apart from that, the post-mining sites as well as coastal up-lifts or sites affected by volcanic eruptions represent ecosystems where the impact of climate and physical properties of the barren substrates on the trajectory

of succession is rather minor or reduced by relatively fast vegetation establishment. The subsequent changes in vegetation cover and increasing nutrient availability belong to the drivers of the successional processes at these sites (De Kovel et al., 2000; Merilä et al., 2010; Pennanen et al., 2001). Specifically, the post-mining sites due to their vast distribution in the region of the Central Europe and temperate zone represent the convenient model for the tracking of the impact of the gradual development of the natural vegetation on the ecosystem succession. Unlike the forest monocultures, where the individual tree species affect the soil environment from the initial point, successional ecosystem development is impacted by changing, but complex vegetation.

The soil structure and the nutrient deficiency, notably nitrogen, belong to the main constrains of the immediate plant colonization. It was shown that soils with low C:N are typically readily colonized (Chodak et al., 2009) in comparison to the clayey substrates with the initially high C:N ratio (Knelman et al., 2012; Kastovska et al., 2005; Knelman et al., 2014). Similarly unfavourable properties characterize also the barren substrates of our experimental area. Here, the first herbaceous plants showed up after 6 years since the heaps formation and low nitrogen content, high pH and compact structure of deposits consisting of clay were considered to be the main reason of the late arrival of plants (Paper I).

Independently on climatic zones, the exposed substrates undergo predictable successional changes with similar patterns. These changes are mostly caused by the gradual accumulation of soil organic matter of plant origin (Frouz et al., 2009). With the age of the sites, substrate pH typically decreases, while the C:N ratio increases together with the nutrient content and their availability (Baldrian et al., 2008; Frouz and Novaková, 2005; Chodak et al., 2009; Kastovska et al., 2005; Noll and Wellinger, 2008; Pennanen et al., 2001). The composition of the soil biota changes as well. Microbial biomass and microbial activity increase (Baldrian et al., 2008; Kastovska et al., 2005; Ohtonen et al., 1999; Tscherko et al.,

2003; Pennanen et al., 2001; Merilä et al., 2010), which seems to be apparently promoted not only by the vegetation development (Bardgett and Walker, 2004; Tscherko et al., 2005) but as it was recently suggested also by the amount of the available nutrients alone. The independence of bacterial community development on the occurrence of vegetation cover and other factors was recently reported by Knelman et al. (2014). The nitrogen and phosphorus fertilization of recently deglaciated plots led to the massive acceleration of the bacterial community development. However in nature, the nutrient delivery into the soil in sufficient amount is almost exclusively connected to plant litter input, less to air deposition.

Results from the area of our study showed that the changes in the soil development greatly depend on the occurrence and abundance of the plants, are species-specific to the certain extent and impact the soil microbial community composition and their activities (Baldrian et al., 2008) (Paper I, II, III, IV).

The importance of the vegetation for the microbial community development in the barren soils had been proven by the analysis of the microbial community structure along the successional chronosequence established at the post-mining sites. The non-vegetated plots representing the initial stage of succession had been compared with plots representing the early, middle and late stage of succession. The different vegetation types characterized the successional stages. Particularly *C. epigeios* occurred at the early successional sites, at the middle successional sites dominated shrubs composed of *S. caprea*, and *B. pendula* with *P. tremuloides* grew at the late successional sites. The amount of microbial biomass was strongly affected by the vegetation. Fungi absented at the initial sites without vegetation cover. At the early and middle stages of succession, the fungal biomass increased and dropped substantially at the late successional stage. Bacterial biomass was higher than fungal along the whole successional series, but notably dominated at the initial and the late stages of succession. The detailed analysis of the bacterial community succession demonstrated distinct composition on

the sites without vegetation cover from the sites where the plants occurred (Paper I). This is consistent with the theoretical predictions as well as results from other ecosystems (Fierer et al., 2010). The substantial part of the bacterial community at the initial, pre-plant stage of the succession in the study area was composed of the N_2-fixers. The importance of the autotrophic microbial fixation of nitrogen had been documented for the glacier forefields with long-term absence of the vegetation cover (Fierer et al., 2010; Kastovska et al., 2005; Nemergut et al., 2007; Knelmann et al., 2012). The potential appearance of N_2 -fixers was indicated by high signals of probes for *Cyanobacteria* and *Rhizobiaceae* and *Bradyrhizobiaceae*, but also for the genera *Thiobacillus* and *Acidithiobacillus,* which were typical for this site. Both genera are autotrophs fixing CO_2 using reduced sulphur compounds as electron donors and oxygen or nitrogen oxides as electron acceptors (Robertson and Kuene, 2006). Indeed, the clayey material of the Sokolov mine deposits exhibits suitable living conditions. The higher water retention capacity of the clay (Šourková et al., 2005b) with the content of sulphur 0.31-0.33% may potentially form anaerobic conditions necessary for sulphur oxidations with the sufficient source of sulphur. In addition, the fact that both genera differ in pH optima being between 6 – 8 pH for thiobacilli and around 4 and less for acidithiobacilli (Johnson et al., 2001; Johnson and Hallberg, 2003) suggests the existence and the importance of environmental gradients at the microstructure level of the soil supporting the microbial diversity (Vos et al., 2013). The presence of cyanobacteria, acidithiobacilli and thiobacilli as N_2-fixing autotrophs in the non-vegetated soil contributes to the accumulation of both organic C and N and reduction of nitrogen deficiency for plants (Paper I). Bacterial communities developed at the older successional sites under different vegetation types did not exhibit such profound differences (Paper I). Their composition was affected by soil chemical properties such as pH or nutrient content, typical environmental factors affecting bacterial community structure (Lauber et al., 2009; Rousk et al., 2010).

In the two-year litterbag experiment conducted at the successional plots, it was observed, that litter chemical characteristics are highly dependent on the initial chemical composition of the litter. The decomposition of grass litter with prevailing *C. epigeios* senescent leaves collected at the early successional sites, *S. caprea*-dominated leaf litter collected at the middle successional sites and litter with prevailing leaves of *B. pendula* and *P. tremuloides* from the late succession site was followed to track the changes in litter chemistry along their decomposition. The initial chemical composition of litter differed notably in pH, nutrient content and their availability expressed as the cold- and hot-water-extractable nutrients (Table 1, Paper II). The highest amount of both hot-water-extractable and cold-water-extractable available nutrients was found in the litter of the middle successional sites which lost the most of its dry mass during the two year. The initial composition of the litters differed also with respect to the composition of the plant biopolymers as well as the monosaccharide composition of plant polysaccharides. The distinct composition of the individual litters remained preserved and kept its specificity in the course of decomposition. Thus, soil organic matter which is substantially formed by decomposed and transformed litter residues still carries the chemical legacy of the litter and it is thus likely that the also the composition of soils reflects largely the composition of vegetation (Quideau et al., 2001; Thevenot et al., 2013) (Paper II). Interestingly, microbial communities were less responsive to the litter composition specificity than it was expected. The decreasing fungal abundance in all litters through the decomposition process suggested that the decomposers rather reflected the nutrient availability, i.e. the stage of decay, than the specific changes in litter chemistry (Paper II). Glucose contained in cellulose disappeared faster than the monosaccharides within hemicellulose which was in agreement with previous observations by Šnajdr et al. (2011a) on *Quercus petrea* litter. The results suggest that the initial composition of the litter as well as the plant biopolymers determine the trajectory of the decomposition (Wickings et al., 2012).

The soil properties, which are known to be under the influence of the dominant vegetation (Menyailo et al., 2002; Augusto et al., 2014) impact the composition of the microbial communities inhabiting litter and soil (Brockett et al., 2012; Fierer and Jackson, 2006; Fierer et al., 2009; Grayston and Prescott, 2005; Rousk et al., 2010;). However, several reports referred also about the vegetation effect alone as about the important factor explaining the variability in the soil environment (Mitchell et al., 2012; Prescott and Grayston, 2013; Ushio et al., 2010). Also, the existing reports focused on the enzyme activities is soil were often based on data from non-replicated stands (Baum and Hrynkiewicz, 2006; Chodak and Niklinska, 2010a; Niemi et al., 2007) and the effect of vegetation on microbial activities was thus difficult to distinguish from other potential factors. The plots technically revegetated with six different tree species (alder, larch, lime, oak, pine, spruce) in the combination with the middle stage site of the spontaneous succession chronosequence dominated with willow were choose for the evaluation of the tree species effect on the structure of the bacterial and fungal communities and the activities of extracellular enzymes both in the litter and the soil (Paper III). Significant differences both in the enzymatic activities and the microbial community composition as well as in the soil characteristics in the litter and the soils under different tree species were discovered. The observed differences in activities of extracellular enzymes were affected by the nutrient content of the various litters. The fresh litter of the trees differed substantially in N content (4-29 mg/g) as well as the content of P and lignin (Voříšková et al., 2011). The litters of broadleaved tree species with the low N content exhibited the lowest enzyme activities. The enzymatic activities were generally higher in the litter layer due to the higher nutrient content and availability, where the activities were more affected by the tree effect, than in the soil where the effect of soil chemistry was the most significant determinant (Paper III). Among the soil properties, pH and moisture were previously identified as important factors affecting enzyme activity (Baldrian et al., 2008; Štursová and Baldrian,

2011). Here, the effect of moisture was negligible probably due to the homogeneity of the soil substrate and low variation in the water content. The spatial stratification of the enzymatic activities and microbial biomass (microbial biomass was higher in the litter layer then in the soil) was in the agreement with previous studies, which revealed the clear stratification patterns in the soil biota composition and activities due to the decreasing availability of the nutrients with the soil depth. Specific changes have been observed among fungi. While the abundance of mycorrhizal taxa increases with the depth, saprotrophic fungi disappear (Baldrian et al., 2012; Lindahl et al., 2007; Šnajdr et al., 2008). The presence of the mycorrhizal taxa in the deeper layer of the soil horizon is bound to the symbiosis with the plant roots. Due to this association with roots, fungal communities in soils should be more affected by dominant vegetation than bacterial communities which rather respond to chemical properties of the soil.

Indeed, the detail identification of the bacterial and fungal communities within the soil profile under seven different tree species confirmed the effect of trees as the most important determinant of the fungal community composition in the whole soil horizon, while bacteria were affected by the trees mainly in the litter and to a lesser extent than fungi. In soil, the tree effect on bacteria was mediated by soil chemical properties, notably with pH and nutrient content (Paper IV). The effect of soil pH is besides other factors the most important determinant of the bacterial community composition worldwide (Fierer and Jackson, 2006; Lauber et al., 2009; Rousk et al., 2010) and was identified as one of the probable factors shaping the development of the communities of bacteria at the successional plots at the post-mining sites as well (Paper I).

Remarkable feature of the bacterial and fungal communities which turned out in this study is their distinct specificity to the dominant vegetation type. While most bacteria did not show specificity to one tree species, almost one third of soil fungi were exclusively associated

with one or two tree species. This specificity involved both root-symbiotic fungal taxa (approximately 15% of the fungal sequences) as well as saprotrophs. Because of the sampling of the bulk soil, the effect of the rhizosphere can not be considered as significant for the composition of the bacterial and fungal communities in our study. However, the nonspecific bacterial communities and partly specific fungal communities suggest the possible contribution of rhizosphere effect. Due to the filamentous form of mycorrhizal fungi, the rhizosphere specificity is likely extended from the rhizosphere to the bulk soil as proposed previously (Buee et al., 2009a; Lundberg et al., 2012; Churchland and Grayston, 2014).

Conclusions

Vegetation substantially affects the ecosystem development, soil biotic and abiotic properties. The importance of bacteria in the process of spontaneous succession has been demonstrated. Bacteria dominated the initial, pre-plant stage of succession and also the late stage. Fungi were virtually absent in the ecosystem before the establishment of vegetation. At this stage, bacterial autotrophic fixation is crucial for initial nutrient deficiency of exposed soil substrates and subsequent vegetation development.

Litter decomposition along successive series of post-mining sites is largely dependent on the initial composition of litters. Interestingly, initial chemical differences among litters were still apparent during decay. The soil organic matter thus probably keeps the legacy of the aboveground vegetation. Contrarily, changes in fungal and bacterial biomass did not reflect the changes in litter chemistry, but responded to nutrient availability, therefore microbial communities seem to be largely decay-stage-specific. The rate of decomposition of various litter types correlated positively with the contents of cold/hot-water-soluble C and N, not with the activities of extracellular enzymes.

In a detailed study of the factors influencing the fungal and bacterial communities and extracellular enzyme activities in litter and soil, the tree effect has been revealed as their best predictor. In fact, fungi and activities of most extracellular enzymes were more affected by dominant vegetation type both in litter and the bulk soil than bacterial communities. Bacteria were influenced by pH and nutrient content and the tree effect on bacterial communities was mainly observable in the litter. Fungi were also strongly tree species-specific and the observed specificity was not limited only to root-symbiotic fungal taxa, but included also saprotrophs.

Comparison of soil processes and microbial communities developed at spontaneously revegetated sites and technically afforested sites did not reveal any substantial differences in examined aspects except the sites revegetated with alder, because alder accelerates soil development due to the production of nitrogen-rich litter.

Although the results of this thesis have deepened the understanding of the plant influence on soil environment, the proposed mechanisms by which plants affect the soil microbiota composition and their activities have to be experimentally confirmed. Future research should be focused on the detailed recognition of the relationships between plants and microbes and also the identification of the individual players of these interactions to answer the question about who are the active and not active community members and what are their ecological requirements.

References

Aneja, M.K., Sharma, S., Fleischmann, F., Stich, S., Heller, W., Bahnweg, G., Munch, J.C., Schloter, M., 2006. Microbial colonization of beech and spruce litter - Influence of decomposition site and plant litter species on the diversity of microbial community. Microbial Ecology 52, 127-135.

Angers, D.A., Caron, J., 1998. Plant-induced changes in soil structure: Processes and feedbacks. Biogeochemistry 42, 55-72.

Aponte, C., García, L.V., Maranon, T., 2003. Tree species effects on nutrient cycling and soil biota: A feedback mechanism favouring species coexistence. Forest Ecology and Management 309, 36-46.

Aubert, M., Margerie, P., Trap, J., Bureau, F., 2010. Aboveground-belowground relationships in temperate forests: Plant litter composes and microbiota orchestrates. Forest Ecology and Management 259, 563-572.

Augusto, L., De Schrijver, A., Vesterdal, L., Smolander, A., Prescott, C., Ranger, J., 2014. Influences of evergreen gymnosperm and deciduous angiosperm tree species on the functioning of temperate and boreal forests. Biological Reviews, n/a-n/a.

Augusto, L., Ranger, J., Binkley, D., Rothe, A., 2002. Impact of several common tree species of European temperate forests on soil fertility. Annals of Forest Science 59, 233-253.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology 57, 233-266.

Balasooriya, W., Denef, K., Huygens, D., Boeckx, P., 2014. Translocation and turnover of rhizodeposit carbon within soil microbial communities of an extensive grassland ecosystem. Plant and Soil 376, 61-73.

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Ridl, J., Vlček, C., Voříšková, J., 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. Isme Journal 6, 248-258.

Baldrian, P., 2009. Microbial enzyme-catalysed processes in soils and their analysis. Plant Soil and Environment 55, 370-378.

Baldrian, P., Trögl, J., Frouz, J., Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., Herinková, J., 2008. Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. Soil Biology & Biochemistry 40, 2107-2115.

Bardgett, R.D., Bowman, W.D., Kaufmann, R., Schmidt, S.K., 2005. A temporal approach to linking aboveground and belowground ecology. Trends in Ecology and Evolution 11, 634-641.

Bardgett, R.D., Walker, L.R., 2004. Impact of coloniser plant species on the development of decomposer microbial communities following deglaciation. Soil Biology & Biochemistry 36, 555-559.

Baum, C., Hrynkiewicz, K., 2006. Clonal and seasonal shifts in communities of saprotrophic microfungi and soil enzyme activities in the mycorrhizosphere of *Salix* spp.. Journal of Plant Nutrition and Soil Science-Zeitschrift für Pflanzenernahrung and Bodenkunde 169, 481-487.

Berg, B., McClaugherty, C., 2003. Plant litter: Decomposition, Humus Formation, Carbon Sequestration. Berlin: Springer-Verlag.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. Fems Microbiology Ecology 68, 1-13.

Binkley, D., Valentine, D., 1991. Fifty-year biogeochemical effects of green ash, white pine, and Norway spruce in a replicated experiment. Forest Ecology and Management 40, 13-25.

Bissett, A., Richardson, A.E., Baker, G., Thrall, P.H., 2011. Long-term land use effects on soil microbial community structure and function. Applied Soil Ecology 51, 66-78.

Blanchette, R.A., Nilsson, T., Daniel, G., Abad, A., 1990. Biological degradtion of wood. Advances in Chemistry Series, 141-174.

Bradshaw, A., 1997. Restoration of mined lands - using natural processes. Ecological Engineering 8, 255-269.

Brady, N.C., Weil, R.C., 2008. The nature and properties of soils. Prentice Hall, Upper Saddle River, 992pp.

Bray, S.R., Kitajima, K., Mack, M.C., 2012. Temporal dynamics of microbial communities on decomposing leaf litter of 10 plant species in relation to decomposition rate. Soil Biology and Biochemistry 49, 30-37.

Brockett, B.F.T., Prescott, C.E., Grayston, S.J., 2012. Soil moisture is the major factor influencing microbial community structure and enzyme activities across seven biogeoclimatic zones in western Canada. Soil Biology & Biochemistry 44, 9-20.

Buee, M., De Boer, W., Martin, F., van Overbeek, L., Jurkevitch, E., 2009a. The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant and Soil 321, 189-212.

Buee, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., Martin, F., 2009b. 454-Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 184, 449-456.

Cleveland, C.C., Liptzin, D., 2007. C : N : P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? Biogeochemistry 85, 235-252.

Corneo, P.E., Pellegrini, A., Cappellin, L., Gessler, C., Pertot, I., 2013. Weeds influence soil bacterial and fungal communities. Plant and Soil 373, 107-123.

Couteaux, M.M., Bottner, P., Berg, B., 1995. Litter decomposition, climate and litter quality. Trends in Ecology & Evolution 10, 63-66.

Davidson, E.A., Janssens, I.A., 2006. Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. Nature 440, 165-173.

De Boer W., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. Fems Microbiology Reviews 29, 795-811.

De Kovel, C.G.F., Van Mierlo, A., Wilms, Y.J.O., Berendse, F., 2000. Carbon and nitrogen in soil and vegetation at sites differing in successional age. Plant Ecology 149, 43-50.

Duc, L., Noll, M., Meier, B.E., Burgmann, H., Zeyer, J., 2009. High Diversity of Diazotrophs in the Forefield of a Receding Alpine Glacier. Microbial Ecology 57, 179-190.

Elhottová, D., Krištůfek, V., Frouz, J., Nováková, A., Chroňáková, A., 2006. Screening for microbial markers in Miocene sediment exposed during open-cast brown coal mining. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 89, 459-463.

Ekblad, A. and HussDanell, K., 1995. Nitrogen fixation by *Alnus incana* and nitrogen transfer from *Alnus incana* to *Pinus sylvestris* influenced by macronutrients and ectomycorrhiza. New Phytologist 131, 453-459.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103, 626-631.

Fierer, N., Nemergut, D., Knight, R., Craine, J.M., 2010. Changes through time: integrating microorganisms into the study of succession. Research in Microbiology 161, 635-642.

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecology Letters 12, 1238-1249.

Franzluebbers, A.J., Haney, R.L., Honeycutt, C.W., Arshad, M.A., Schomberg, H.H., Hons, F.M., 2001. Climatic influences on active fractions of soil organic matter. Soil Biology and Biochemistry 33, 1103-1111.

Franzluebbers, A.J., Haney, R.L., Hons, F.M., Zuberer, D.A., 1996. Active fractions of organic matter in soils with different texture. Soil Biology & Biochemistry 28, 1367-1372.

Frey-Klett, P., Garbaye, J., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. New Phytologist 176, 22-36.

Frouz, J., Elhottová, D., Pižl, V., Tajovský, K., Šourková, M., Picek, T., Malý, S., 2007. The effect of litter quality and soil faunal composition on organic matter dynamics in post-mining soil: A laboratory study. Applied Soil Ecology 37, 72-80.

Frouz, J., Jílková, V., Cajthaml, T., Pižl, V., Tajovský, K., Háněl, L., Burešová, A., Šimáčková, H., Kolaříková, K., Franklin, J., Nawrot, J., Groninger, J.W., Stahl, P.D., 2013. Soil biota in post-mining sites along a climatic gradient in the USA: Simple communities in shortgrass prairie recover faster than complex communities in tallgrass prairie and forest. Soil Biology and Biochemistry 67, 212-225.

Frouz, J., Nováková, A., 2005. Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. Geoderma 129, 54-64.

Frouz, J., Pižl, V., Cienciala, E., Kalčík, J., 2009. Carbon storage in post-mining forest soil, the role of tree biomass and soil bioturbation. Biogeochemistry 94, 111-121.

Frouz, J., Prach, K., Pižl, V., Háněl, L., Starý, J., Tajovský, K., Materna, J., Balík, V., Kalčík, J., Řehounková, K., 2008. Interactions between soil development, vegetation and soil fauna during spontaneous succession in post mining sites. European Journal of Soil Biology 44, 109-121.

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M., Ball, A.S., 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Applied and Environmental Microbiology 69, 1800-1809.

Grayston, S.J., Prescott, C.E., 2005. Microbial communities in forest floors under four tree species in coastal British Columbia. Soil Biology & Biochemistry 37, 1157-1167.

Grayston, S.J., Wang, S.Q., Campbell, C.D., Edwards, A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biology and Biochemistry 30, 369-378.

Guggenberger, G., 1994. Acidification effects on dissolved organic-matter mobility in spruce forest ecosystems. Environment International 20, 31-41.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., Sessitsch, A., 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology 70, 5057-5065.

Haichar, F.E., Marol, C., Berge, O., Rangel-Castro, J.I., Prosser, J.I., Balesdent, J., Heulin, T., Achouak, W., 2008. Plant host habitat and root exudates shapes soil bacterial community structure. Isme Journal 2, 1221-1230.

Hagen-Thorn, A., Callesen, I., Armolaitis, K., Nihlgard, B., 2004. The impact of six European tree species on the chemistry of mineral topsoil in forest plantations on former agricultural land. Forest Ecology and Management 195, 373-384.

Hartmann, A., Schmid, M., van Tuinen, D., Berg, G., 2009. Plant-driven selection of microbes. Plant and Soil 321, 235-257.

Hattenschwiler, S., Jorgensen, H.B., 2010. Carbon quality rather than stoichiometry controls litter decomposition in a tropical rain forest. Journal of Ecology 98, 754-763.

Helingerová, M., Frouz, J., Šantrůčková, H., 2010. Microbial activity in reclaimed and unreclaimed post-mining sites near Sokolov (Czech Republic). Ecological Engineering 36, 768-776.

Hendrychová, M., Šálek, M., Tajovský, K., Řehoř, M., 2012. Soil Properties and Species Richness of Invertebrates on Afforested Sites after Brown Coal Mining. Restoration Ecology 20, 561-567.

Hobbie, S.E., Reich, P.B., Oleksyn, J., Ogdahl, M., Zytkowiak, R., Hale, C., Karolewski, P., 2006. Tree species effects on decomposition and forest floor dynamics in a common garden. Ecology 87, 2288-2297.

Hodačová, D., Prach, K., 2003. Spoil heaps from brown coal mining: Technical reclamation versus spontaneous revegetation. Restoration Ecology 11, 385-391.

Högberg, M.N., Högberg, P., Myrold, D.D., 2007. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia 150, 590-601.

Chodak, M., Niklinska, M., 2010. The effect of different tree species on the chemical and microbial properties of reclaimed mine soils. Biology and Fertility of Soils 46, 555-566.

Chodak, M., Pietrzykowski, M., Niklinska, M., 2009. Development of microbial properties in a chronosequence of sandy mine soils. Applied Soil Ecology 41, 259-268.

Churchland, C., Grayston, S.J., 2014. Specificity of plant-microbe interactions in the tree mycorrhizosphere biome and consequences for soil C cycling. Frontiers in Microbiology 5.

Insam, H., Domsch, K.H., 1988. Relationship between soil organic-carbon and microbial biomass on chronosequences of reclamation of sites. Microbial Ecology 15, 177-188.

Johnson, D.B., Hallberg, K.B., 2003. The microbiology of acidic mine waters. Research in Microbiology 154, 466-473.

Iovieno, P., Alfani, A., Baath, E., 2010. Soil microbial community sturcture and biomass as affected by *Pinus pinea* plantation in two Mediterranean areas. Applied Soil Ecology 45, 56-63.

Johnson, D.B., Rolfe, S., Hallberg, K.B., Iversen, E., 2001. Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. Environmental Microbiology 3, 630-637.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. Oikos 69, 373-386.

Jones, D.L., Hodge, A., Kuzyakov, Y., 2004. Plant and mycorrhizal regulation of rhizodeposition. New Phytologist 163, 459-480.

Kastovská, K., Elster, J., Štibal, M., Šantrůčková, H., 2005. Microbial assemblages in soil microbial succession after glacial retreat in Svalbard (high Arctic). Microbial Ecology 50, 396-407.

Kimer, A. and Mahn, E.G., 2001. Spontaneous and initiated succession on unvegetated slopes in the abandoned lignite-mining area of Goitsche, Germany. Applied Vegetation Science 4, 19-27.

Kluber, L.A., Smith, J.E., Myrold, D.D., 2011. Distinctive fungal and bacterial communities are associated with mats formed by ectomycorrhizal fungi. Soil Biology & Biochemistry 43, 1042-1050.

Knelman, J.E., Schmidt, S.K., Lynch, R.C., Darcy, J.L., Castle, S.C., Cleverland, C.C., Nemergut, D.R., 2014. Nutrient addition dramatically accelerates microbial community succession. Plos One 9, e102609.

Knelman, J.E., Legg, T.M., O'Neill, S.P., Washenberger, C.L., Gonzalez, A., Cleveland, C.C., Nemergut, D.R., 2012. Bacterial community structure and function change in association with colonizer plants during early primary succession in a glacier forefield. Soil Biology & Biochemistry 46, 172-180.

Koide, R.T., Xu, B., Sharda, J., 2005. Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytologist 166, 251-262.

Kowarik, I., 1995. Time lags in biological invasions with regard to the success and failure of alien species. S P B Academic Publ Bv, Amsterdam, pp. 15-38.

Lamb, E.G., Kennedy, N., Siciliano, S.D., 2011. Effects of plant species richness and evenness on soil microbial community diversity and function. Plant and Soil 338, 483-495.

Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. Applied and Environmental Microbiology 75, 5111-5120.

Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biology & Biochemistry 40, 2407-2415.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Hogberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611-620.

Linn, D.M. & Doran, J.W. 1984. Effect of water-filled pore space carbon dioxide and nitrous oxide production in tilled and nontilled soils. Soil Sci. Soc. Am. J., 48: 1267–1272.

Lugtenberg, B., Kamilova, F., 2009. Plant-Growth-Promoting Rhizobacteria, Annual Review of Microbiology. Annual Reviews, Palo Alto, pp. 541-556.

Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Furhman, J.A., Green, J.L., et al., 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology 4, 102-112.

Menyailo, O.V., Hungate, B.A., Zech, W., 2002. The effect of single tree species on soil microbial activities related to C and N cycling in the Siberian artificial afforestation experiment - Tree species and soil microbial activities. Plant and Soil 242, 183-196.

Merilä, P., Malmivaara-Lamsa, M., Spetz, P., Stark, S., Vierikko, K., Derome, J., Fritze, H., 2010. Soil organic matter quality as a link between microbial community structure and vegetation composition along a successional gradient in a boreal forest. Applied Soil Ecology 46, 259-267.

Mitchell, R.J., Hester, A.J., Campbell, C.D., Chapman, S.J., Cameron, C.M., Hewison, R.L., Potts, J.M., 2010. Is vegetation composition or soil chemistry the best predictor of the soil microbial community? Plant and Soil 333, 417-430.

Mudrák, O., Frouz, J., Velichová, V., 2010. Understory vegetation in reclaimed and unreclaimed post-mining forest stands. Ecological Engineering 36, 783-790.

Muller, T., Hoper, H., 2004. Soil organic matter turnover as a function of the soil clay content: consequences for model applications. Soil Biology & Biochemistry 36, 877-888.

Nemergut, D.R., Anderson, S.P., Cleveland, C.C., Martin, A.P., Miller, A.E., Seimon, A., Schmidt, S.K., 2007. Microbial community succession in an unvegetated, recently deglaciated soil. Microbial Ecology 53, 110-122.

Niemi, R.M., Vepsäläinen, M., Erkomaa, K., Ilvesniemi, H., 2007. Microbial activity during summer in humus layers under *Pinus silvestris* and *Alnus incana*. Forest Ecology and Management 242, 314-323.

Noll, M., Wellinger, M., 2008. Changes of the soil ecosystem along a receding glacier: Testing the correlation between environmental factors and bacterial community structure. Soil Biology & Biochemistry 40, 2611-2619.

Ohtonen, R., Fritze, H., Pennanen, T., Jumpponen, A., Trappe, J., 1999. Ecosystem properties and microbial community changes in primary succession on a glacier forefront. Oecologia 119, 239-246.

Peay, K.G., Kennedy, P.G., Bruns, T.D., 2008. Fungal community ecology: A hybrid beast with a molecular master. Bioscience 58, 799-810.

Pennanen, T., Strommer, R., Markkola, A., Fritze, H., 2001. Microbial and plant community structure across a primary succession gradient. Scandinavian Journal of Forest Research 16, 37-43.

Post, W.M., Emanuel, W.R., Zinke, P.J., Stangenberger, A.G., 1982. Soil carbon pools and world life zones. Nature 298, 156-159.

Prach, K., Lencova, K., Řehounková, K., Dvořáková, H., Jírová, A., Konvalinková, P., Mudrák, O., Novák, J., Trnková, R., 2013. Spontaneous vegetation succession at different central European mining sites: a comparison across seres. Environmental Science and Pollution Research 20, 7680-7685.

Prach, K., Pyšek, P., 2001. Using spontaneous succession for restoration of human-disturbed habitats: Experience from Central Europe. Ecological Engineering 17, 55-62.

Prescott, C.E., Grayston, S.J., 2013. Tree species influence on microbial communities in litter and soil: Current knowledge and research needs. Forest Ecology and Management 309, 19-27.

Pyšek, P., Danihelka, J., Sádlo, J., Chrtek, J., Chytrý, M., Jarošík, V., Kaplan, Z., Krahulec, F., Moravcová, L., Pergl, J., Stajerová, K., Tichý, L., 2012. Catalogue of alien plants of the Czech Republic (2nd edition): checklist update, taxonomic diversity and invasion patterns. Preslia 84, 155-255.

Quideau, S.A., Chadwick, O.A., Benesi, A., Graham, R.C., Anderson, M.A., 2001. A direct link between forest vegetation type and soil organic matter composition. Geoderma 104, 41-60.

Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C., Moenne-Loccoz, Y., 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321, 341-361.

Rebele, F., Lehmann, C., 2002. Restoration of a landfill site in Berlin, Germany by spontaneous and directed succession. Restoration Ecology 10, 340-347.

Reiners, W.A., Bouwman, A.F., Parsons, W.F.J., Keller, M., 1994. Tropical rain-forest conversion to pasture – changes in vegetation and soil properties. Ecological Applications 4, 363-377.

Rice, C.W. 2002. Organic matter and nutrient dynamics. In: Encyclopedia of soil science, pp. 925–928. New York, USA, Marcel Dekker Inc.

Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytologist 171, 41-53.

Robertson, L.A., Kuenen, J.G., 2006. The Genus Thiobacillus. Springer, New York, 812-827 pp.

Rojík, P., 2004. New stratigraphic subdivision of the Tertiary in the Sokolov Basin in Northwestern Bohemia. Journal of the Czech Geological Society 49, 173-185.

Rousk, J., Baath, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. Isme Journal 4, 1340-1351.

Rubino, M., Dungait, J.A.J., Evershed, R.P., Bertolini, T., De Angelis, P., D'Onofrio, A., Lagomarsino, A., Lubritto, C., Merola, A., Terrasi, F., Cotrufo, M.F., 2010. Carbon input belowground is the major C flux contributing to leaf litter mass loss: Evidences from a C-13 labelled-leaf litter experiment. Soil Biology & Biochemistry 42, 1009-1016.

Šálek, M., 2012. Spontaneous succession on opencast mining sites: implications for bird biodiversity. Journal of Applied Ecology 49, 1417-1425.

Sariyildiz, T., Anderson, J.M., Kucuk, M., 2005. Effects of tree species and topography on soil chemistry, litter quality, and decomposition in Northeast Turkey. Soil Biology & Biochemistry 37, 1695-1706.

Schmidt, S.K., Nemergut, D.R., Darcy, J.L., Lynch, R., 2014. Do bacterial and fungal communities assemble differently during primary succession? Molecular Ecology 23, 254-258.

Schmidt, S.K., Reed, S.C., Nemergut, D.R., Grandy, A.S., Cleveland, C.C., Weintraub, M.N., Hill, A.W., Costello, E.K., Meyer, A.F., Neff, J.C., Martin, A.M., 2008. The earliest stages of

ecosystem succession in high-elevation (5000 metres above sea level), recently deglaciated soils. Proceedings of the Royal Society B-Biological Sciences 275, 2793-2802.

Singh, A.N., Raghubanshi, A.S., Singh, J.S., 2002. Plantations as a tool for mine spoil restoration. Current Science 82, 1436-1441.

Six, J., Bossuyt, H., Degryze, S., Denef, K., 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil & Tillage Research 79, 7-31.

Šnajdr, J., Valášková, V., Merhautová, V., Herinková, J., Cajthaml, T., Baldrian, P., 2008. Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biology and Biochemistry 40, 2068-2075.

Šnajdr, J., Cajthaml, T., Valášková, V., Merhautová, V., Petránková, M., Špetz, P., Leppanen, K., Baldrian, P., 2011. Transformation of *Quercus petraea* litter: successive changes in litter chemistry are reflected in differential enzyme activity and changes in the microbial community composition. Fems Microbiology Ecology 75, 291-303.

Štursová, M., Žifčáková, L., Leigh, M.B., Burgess, R., and Baldrian, P. (2012) Cellulose utilization in forest litter and soil: identification of bacterial and fungal decomposers. *FEMS Microbiology Ecology* 80: 735-746.

Štursová, M., Baldrian, P., 2011. Effects of soil properties and management on the activity of soil organic matter transformint enzymes and the quantification of soil-bound and free activity. Plant and Soil 338, 99-110.

Štýs S., 1981. Rekultivace území postižných těžbou nerostných surovin. SNTL, Praha (1981).

Šourková, M., Frouz, J., Fettweis, U., Bens, O., Huttl, R.F., Šantrůčková, H., 2005a. Soil development and properties of microbial biomass succession in reclaimed post mining sites near Sokolov (Czech Republic) and near Cottbus (Germany). Geoderma 129, 73-80.

Šourková, M., Frouz, J., Šantrůčková, H., 2005b. Accumulation of carbon, nitrogen and phosphorus during soil formation on alder spoil heaps after brown-coal mining, near Sokolov (Czech Republic). Geoderma 124, 203-214.

Talbot, J.M., Treseder, K.K., 2012. Interactions among lignin, cellulose, and nitrogen drive litter chemistry-decay relationships. Ecology 93, 345-354.

Tedersoo, L., Jairus, T., Horton, B.M., Abarenkov, K., Suvi, T., Saar, I., Koljalg, U., 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barecoding and taxon-specific primers. New Phytologist 180, 479-490.

Thevenot, M., Dignac, M.F., Mendez-Millan, M., Bahri, H., Hatte, C., Bardoux, G., Rumpel, C., 2013. Ligno-aliphatic complexes in soils revealed by an isolation procedure: implication for lignin fate. Biology and Fertility of Soils 49, 517-526.

Thomsen, I.K., Schjonning, P., Olesen, J.E., Christensen, B.T., 2003. C and N turover in structurally intact soils of different texture. Soil Biology and Biochemistry 35, 765-774.

Tropek, R., Kadlec, T., Hejda, M., Kočárek, P., Skuhrovec, J., Malenovský, I., Vodka, S., Spitzer, L., Banar, P., Konvička, M., 2012. Technical reclamations are wasting the conservation potential of post-mining sites. A case study of black coal spoil dumps. Ecological Engineering 43, 13-18.

Tscherko, D., Hammesfahr, U., Marx, M.C., Kandeler, E., 2004. Shifts in rhizosphere microbial communities and enzyme activity of Poa alpina across an alpine chronosequence. Soil Biology & Biochemistry 36, 1685-1698.

Tscherko, D., Hammesfahr, U., Zeltner, G., Kandeler, E., Bocker, R., 2005. Plant succession and rhizosphere microbial communities in a recently deglaciated alpine terrain. Basic and Applied Ecology 6, 367-383.

Tscherko, D., Rustemeier, J., Richter, A., Wanek, W., Kandeler, E., 2003. Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. European Journal of Soil Science 54, 685-696.

Turner, T.R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., Osbourn, A., Grant, A., Poole, P.S., 2013. Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. Isme Journal 7, 2248-2258.

Ushio, M., Wagai, R., Balser, T.C., Kitayama, K., 2008. Variations in the soil microbial community composition of a tropical montane forest ecosystem: Does tree species matter? Soil Biology and Biochemistry 40, 2699-2702.

van der Putten, W.H., Klironomos, J.N., Wardle, D.A., 2007. Microbial ecology of biological invasions. Isme Journal 1, 28-37.

Vesterdal, L., Clarke, N., Sigurdsson, B.D., Gundersen, P., 2013. Do tree species influence soil carbon stocks in temperate and boreal forests? Forest Ecology and Management 309, 4-18.

Voříšková, J., Dobiášová, P., Šnajdr, J., Vaněk, D., Cajthaml, T., Šantrůčková, H., Baldrian, P., 2011. Chemical composition of litter affects the growth and enzyme production by the saprotrophic basidiomycete *Hypholoma fasciculare*. Fungal Ecology 4, 417-426.

Vos, M., Wolf, A.B., Jennings, S.J., Kowalchuk, G.A., 2013. Micro-scale determinants of bacterial diversity in soil. Fems Microbiology Reviews 37, 936-954.

Wallenstein, M.D., Hess, A.M., Lewis, M.R., Steltzerae, H., and Ayres, E. (2010). Decomposition of aspen leaf litter results in unique metabolomes when decomposed under different tree species. Soil Biology and Biochemistry 42, 484-490.

Wardle, D.A., Yeates, G.W., Williamson, W., Bonner, K.I., 2003. The response of a three trophic level soil food web to the identity and diversity of plant species and functional groups. Oikos 102, 45-56.

Whipps, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52, 487-511.

Wickings, K., Grandy, A.S., Reed, S.C., Cleveland, C.C., 2012. The origin of litter chemical complexity during decomposition. Ecology Letters 15, 1180-1188.

Wiegleb, G., Felinks, B., 2001. Primary succession in post-mining landscapes of Lower Lusatia - chance or necessity. Ecological Engineering 17, 199-217.

Yergeau, E., Schoondermark-Stolk, S.A., Brodie, E.L., Dejean, S., DeSantis, T.Z., Goncalves, O., Piceno, Y.M., Andersen, G.L., Kowalchuk, G.A., 2009. Environmental microarray analyses of Antarctic soil microbial communities. Isme Journal 3, 340-351.

Zinger, L., Lejon, D.P.H., Baptist, F., Bouasria, A., Aubert, S., Geremia, R.A., Choler, P., 2011. Constrasting diversity patterns of crenarchaeal, bacterial and fungal soil communities in an alpine landscape. Plos One 6, e19950.

I

RESEARCH ARTICLE

Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining

Michaela Urbanová[1], Jan Kopecký[2], Vendula Valášková[1], Markéta Ságová-Marečková[2], Dana Elhottová[3], Martina Kyselková[3,4,5,6], Yvan Moënne-Loccoz[4,5,6] & Petr Baldrian[1]

[1]Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, Praha, Czech Republic; [2]Crop Research Institute, Praha, Czech Republic; [3]Institute of Soil Biology, Biology Centre of the ASCR, České Budějovice, Czech Republic; [4]Université de Lyon, Lyon, France; [5]Université Lyon 1, Villeurbanne, France; and [6]CNRS, UMR5557, Ecologie Microbienne, Villeurbanne, France

Correspondence: Petr Baldrian, Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, Vídeňská 1083, 14220 Praha 4, Czech Republic.
Tel.: +420 723 770 570;
fax: +420 241 062 384;
e-mail: baldrian@biomed.cas.cz

Received 31 January 2011; revised 30 May 2011; accepted 20 June 2011.
Final version published online 21 July 2011.

DOI:10.1111/j.1574-6941.2011.01164.x

Editor: Michael Schloter

Keywords
Acidithiobacillus; bacteria; community composition; microarray; primary succession; soil development.

Abstract

Changes in the abundance of bacteria and fungi and in the composition of bacterial communities during primary succession were investigated in a brown coal mine deposit area near Sokolov, the Czech Republic, using phospholipid fatty acids analysis, microarray and 16S rRNA gene sequencing. The study considered a chronosequence of sites undergoing spontaneous succession: 6-, 12-, 21- and 45-year-old and a 21-year-old site revegetated with *Alnus glutinosa*. During succession, organic carbon and the total nitrogen content increased while the pH and the C/N ratio decreased. Microbial biomass and bacterial diversity increased until 21 years and decreased later; bacteria dominated over fungi in the initial and late phases of succession. Bacterial community composition of the 6-year-old site with no vegetation cover largely differed from the older sites, especially by a higher content of *Gammaproteobacteria*, *Cyanobacteria* and some *Alphaproteobacteria*. Bacteria belonging to the genera *Acidithiobacillus*, *Thiobacillus* and related taxa, the CO_2 and N_2 fixers, dominated the community at this site. In the later phases, bacterial community development seemed to reflect more the changes in soil nutrient content and pH than vegetation with a decrease of *Actinobacteria* and an increase of *Acidobacteria*. The site revegetated with *A. glutinosa* resembled the 45-year-old primary succession site and exhibited an even lower pH and C/N ratio, indicating that recultivation is able to accelerate soil development.

Introduction

During open-cast coal mining, large amounts of spoil material overlying coal layers are excavated and deposited aboveground. Although this process often adversely affects existing ecosystems that are either destroyed or buried under the deposited spoil material, it also offers the possibility to study primary succession processes at a substrate with very low initial biological activity (Frouz *et al.*, 2001; Frouz & Novakova, 2005; Elhottova *et al.*, 2006). For example, the spoil material excavated from mines in the Sokolov area mining district, the Czech Republic, comes from depths of up to 200 m and contains only trace amounts of microbial biomarkers or living microorganisms (Elhottova *et al.*, 2006; Chronakova *et al.*, 2010). While the processes of soil formation in reclaimed postmining sites are known to some detail (Frouz *et al.*, 2001; Frouz & Novakova, 2005; Šourková *et al.*, 2005) and recent studies also focused on soil formation and microbial activity (Baldrian *et al.*, 2008), detailed studies of microbial succession on postmining sites are scarce and often limited to markers with low taxonomic resolution such as the phospholipid fatty acids (PLFAs) (Baldrian *et al.*, 2008; Chodak *et al.*, 2009; Elhottova *et al.*, 2009). This is in contrast to the primary succession of deglaciated areas in the polar and alpine regions, where detailed studies exist (Noll & Wellinger, 2008; Schmidt *et al.*, 2008; Duc *et al.*, 2009; Yergeau *et al.*, 2009).

Microbial succession on newly available substrates (e.g. deposited sediments, lava flows or soils formed during glacier retreat) is often subject to nutrient limitation (Fierer *et al.*, 2010) and that defines the probable directions of microorganisms-catalysed processes. For example, N

deficiency limitation of plant growth was demonstrated from deglaciated soils and the vegetation establishment in the later phases of succession was linked to the preceding activity of N_2-fixing microorganisms inhabiting the initial ecosystems (Nemergut *et al.*, 2007; Duc *et al.*, 2009). Further development of the ecosystems where organic compounds become available during succession showed the important role of extracellular enzymes in soil formation (Schipper *et al.*, 2001; Tscherko *et al.*, 2004, 2005). Measurements of microorganisms-catalysed processes using enzymes, however, offer only summary estimates on selected aspects of the successional development of microbial communities and do not identify the microorganisms involved, their diversity and factors shaping their communities with time.

In our previous paper, which also considered some of the study sites, we described the development of soil structure of postmining deposits in the Sokolov mining area and characterized the changes in the rates of enzyme-catalysed reactions during spontaneous succession (Baldrian *et al.*, 2008). We showed that despite seasonal changes, affecting mainly the fungal biomass in the ecosystem, enzyme activities in both the litter and the topsoil generally increase with primary succession, but may decline at sites $>$ 40 years of age. The aim of this study was to assess the development of bacterial communities along the succession with a specific focus on the initial stage where the soil did not support plant growth. We hypothesized that given the importance of plant litter chemistry and rhizosphere effects (Hartmann *et al.*, 2009), the development of bacterial community during primary succession will be mainly shaped by vegetation, which, together with transitions of key plant species, will result in discontinuous development of the bacterial community that will reflect vegetation traits of the site of specific age. The relative importance of the gradual accumulation of organic matter and changes in soil chemistry will be likely less important. In addition to the primary succession chronosequence, the analysis of a site reclaimed by revegetation with *Alnus glutinosa* has been studied to consider the relative importance of succession age and vegetation cover on soil development and bacterial community composition.

Materials and methods

Study site and soil sampling

The study was carried out at a large postmining area in the Sokolov brown-coal mining district, the Czech Republic (50°14′21″N, 12°39′24″E). The average altitude of the spoil heaps is about 500–600 m a.s.l. The mean annual precipitation is 650 mm and the mean annual temperature is 6.8 °C (Baldrian *et al.*, 2008; Helingerová *et al.*, 2010). This study was performed on four postmining sites 6, 12, 21 and 45 years old, covered by spontaneously developed vegetation, and a 21-year-old site revegetated with *A. glutinosa*. The spoil dumps were formed by tertiary clay material with pH about 8; the prevailing minerals were kaolinite, illite, calcium carbonate and quartz. The surface was characterized by longitudinal depressions and elevations formed during the spoil deposition. Consistent with previous studies, the depressions were studied because these represent hotspots of soil microbial activity and their microenvironment exhibits smaller humidity variations (Frouz & Novakova, 2005).

The 6-year-old site had not developed vegetation cover. Herbs and grasses (mainly *Calamagrostis epigeios*) were present on the 12-year-old site, shrubs with prevailing *Salix caprea* occurred on the 21-year-old site and tree cover (*Betula* spp. and *Populus tremuloides*) had developed on the 45-year-old site. Shrubs shaded nearly the entire soil surface at the 21-year-old site, resulting in the virtual absence of the understorey. When the shrubs developed into forest on the 45-year-old site, dense grass and herb understorey appeared again. The site with *A. glutinosa* had a sparse undergrowth of perennial herbs.

Soils were sampled in October in the period of greatest microbial activity (Frouz & Novakova, 2005; Baldrian *et al.*, 2008). Six subsamples (soil cores of 45 mm diameter taken at 2-m intervals) were collected from a single depression and combined into one sample; three depressions were sampled at each site. Soil was transported to the laboratory, and the soil core material comprising the upper 5 cm of soil after litter removal was sieved through a 2-mm mesh, homogenized and frozen immediately. Samples for DNA extraction were stored at -80 °C until extraction. Dry mass was estimated after drying at 85 °C to a constant mass. The elemental composition of soils was determined in three independent samples per site in an external laboratory using the NC 2100 Soil Analyser (Thermo-Quest, Italia); the total S content in the soil of the 6-year-old site was measured using TOX100 Trace Sulfur Analyzer (Mitsubishi, Japan). Soil pH was determined using an electronic pH-meter in 1 g soil to 10 mL slurries in 1 M KCl.

Quantification of microbial biomass

The biomass of the total microbial community and its bacterial and fungal parts were assessed by total PLFAs and specific bacterial and fungal PLFA biomarkers, respectively. The procedure of the PLFA analysis has been described previously (Elhottova *et al.*, 2009). The bacterial (a15:0, 15:0, i16:0, 16:1ω7, i17:0, 18:1ω7 and cy19:0) and fungal (18:2ω6,9) PLFA bioindicators were selected according to Frostegard & Bååth (1996). The ratio of fungi-specific to bacteria-specific PLFA (F/B) was also calculated.

DNA extraction and analysis

Total DNA was isolated from soil samples (0.3 g, fresh weight) using the modified Miller method (Sagova-Mareckova

et al., 2008), in which a phenol–chloroform approach is combined with the addition of $CaCl_2$, followed by purification using the Geneclean Turbo Kit (Biogenic). DNA was stored at $-20\,^{\circ}C$ before further analysis.

Bacterial diversity was assessed using a 16S rRNA gene taxonomic microarray. The microarray contained 1033 probes and targeted 19 bacterial phyla in a hierarchical structure (Kyselková *et al.*, 2009). The microarray has been validated previously (Kyselková *et al.*, 2009). Probes were custom synthesized (Eurogentec, Seraing, Belgium) with a 5′-C6-NH_2-group, allowing a covalent attachment onto aldehyde slides AL (Schott Nexterion AG, Mainz, Germany). Spotting of slides was performed by the DTAMB/Génopôle Rhône-Alpes gene array platform at IFR41, Université Lyon 1, as described previously (Sanguin *et al.*, 2006). Each probe was replicated four times per slide. In this study, one microarray slide was hybridized per sample. Briefly, the universal bacterial primers T7-pA (forward: *TAATACGACTCACTATAG*AGA GTTTGATCCTGGCTCAG) and pH (reverse: AAGGAGGTG ATCCAGCCGCA) (Bruce *et al.*, 1992) were used to amplify 16S rRNA genes from soil DNA extracts. Primer T7-pA includes at its 5′-end the sequence of T7 promoter (in italics above), which enabled a subsequent T7 RNA polymerase-mediated *in vitro* transcription. Fluorescence labelling by *in vitro* transcription was performed as in Stralis-Pavese *et al.* (2004), RNA purification and fragmentation was carried out as described by Sanguin *et al.* (2008). Overnight hybridization at $57\,^{\circ}C$ was carried out in a custom-tailored aluminium block used as an insert for a temperature-controlled Belly Dancer (Stovall Life Sciences, Greensboro, NC) set at maximum bending. Slide washing and handling were carried out as described previously (Sanguin *et al.*, 2008). The slides were scanned at 532 nm with 10 μm resolution, using a GeneTac LS IV scanner (Genomic Solutions, Huntingdon, UK). Images were analysed using the GENEPIX 4.01 software (Axon, Union City, CA). Data filtering was conducted using the R 2.2.0 statistical computing environment (http://www.r-project.org). A given spot was considered hybridized when 80% of the spot pixels had intensity higher than the median local background pixel intensity plus twice the SD of the local background. The intensity signals (median of signal minus background) were replaced by their square root value and the intensity of each spot was then expressed as a fraction of the total intensity signal of the basic pattern it belonged to (Sanguin *et al.*, 2006). Finally, a given feature probe was considered positive when (1) hybridization signals were superior to the mean signal of the negative controls and (2) at least three of four replicate spots were hybridized. The list of probes is available in a previous publication (Kyselková *et al.*, 2009) and at the ProbeBase site (http://www.microbial-ecology.net/probebase/).

Isolated DNA from the 6-year-old site was used for assessment via clone library analysis. PCR reactions targeting the 16S rRNA gene with the primers pA (forward: AGAGTTTGATCCTGGCTCAG) and pH (reverse: AAGG AGGTGATCCAGCCGCA) (Bruce *et al.*, 1992) were used. PCR reactions (25 μL) consisted of $1\times$ PCR buffer with $MgCl_2$ (Roche), 200 μM dNTPs (Amersham Biosciences), 0.6 μM of each primer, 1.4 U of Fast Start High Fidelity DNA Polymerase (Roche) and 1.2 μL DNA. Cycling conditions were: $94\,^{\circ}C$ for 2 min, 35 cycles ($94\,^{\circ}C$ for 30 s, $55\,^{\circ}C$ for 1 min, $72\,^{\circ}C$ for 90 s + 1 s per cycle) and $72\,^{\circ}C$ for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned using a CloneJET PCR cloning kit (Fermentas) following the manufacturer's instructions. Ligated plasmids were transformed into *Escherichia coli* XL1-Blue cells by electroporation. Transformants were screened for insertion by colony PCR using vector primers pJET1.2 F/pJET1.2 R. PCR products from 20 colonies per sample were used for single extension sequencing from the primer pJET1.2 F by Macrogen Inc. (Korea) using an ABI 3730 XL DNA Analyzer (Applied Biosystems, Carlsbad).

Sequences were checked and edited using STADEN PACKAGE version 1.7.0 (Bonfield *et al.*, 1995). Chimeric 16S rRNA gene sequences were identified using the chimera detection programs BELLEROPHON version 3 (http://greengenes.lbl.gov/; Huber *et al.*, 2004) and PINTAIL version 1.0 from Bioinformatics Toolkit (University of Cardiff, Cardiff, UK; http://www.bioinformatics-toolkit.org/; Ashelford *et al.*, 2002), and putative chimeric clones were discarded. Sequence affiliation of the 53 nonchimeric sequences was performed using the Ribosomal database project classifier tool (http://rdp.cme.msu.edu/classifier/; Wang *et al.*, 2007) and algorithm BLASTN with default parameters at NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences are available at GenBank (accession nos JF267661–JF267713).

To quantify the abundance of *Acidithiobacillus ferrooxidans* at all sites, qPCR was performed with species-specific primers F1_Thio (ATGCGTAGGAATCTGTCTTT) and R1_Thio (GGACTTAACCCAACATCTCA) and universal bacterial primers 1108F (ATGGYTGTCGTCAGCTCGTG) and 1132R (GGGTTGCGCTCGTTGC) as described previously (Escobar *et al.*, 2008; Větrovský *et al.*, 2010). Amplifications were performed on a StepOne Plus cycler (Applied Biosystems) using optical grade 96-well plates. Each 20-μL reaction mixture contained 10 μL SYBR Green Master Mix (Applied Biosystems), 0.9 μL bovine serum albumin ($10\,mg\,mL^{-1}$), 1.35 μL of each primer, 1.5 μL of template and 6.1 μL of water. The PCR cycling protocol for bacterial DNA quantification was $56\,^{\circ}C$ for 2 min; $95\,^{\circ}C$ for 10 min; and $95\,^{\circ}C$ for 15 s/$60\,^{\circ}C$ for 1 min (40 cycles), and for *A. ferrooxidans*, it was $56\,^{\circ}C$ for 2 min; $95\,^{\circ}C$ for 10 min; and $95\,^{\circ}C$ for 1 min/$58.5\,^{\circ}C$ for 1 min (40 cycles). Genomic DNA from *Streptomyces lincolnensis* DNS 40335 and *A. ferrooxidans* CCM3973 were used as standards.

Statistics

Statistical tests were conducted using the software package STATISTICA 7 (StatSoft). Differences between groups were tested by a one-way ANOVA, followed by Tukey's *post hoc* test. Principal component (PC) analysis was used to analyse the differences in the response of microarray probes and cluster analysis with 1-Pearson r as a distance measure was used to evaluate the similarity of microarray data among samples. PC analysis of hybridization data was performed with ADE-4 (Thioulouse *et al.*, 1997) in the R environment (http://www.r-project.org), based on the correlation matrix. This was followed by a comparison of the treatments by one-way ANOVA along each of the first two coordination axes. The correlations between microarray data and soil pH, contents of organic C, total N and K were tested using Spearman's rank correlation coefficient of the distance matrices. Mantel test (or null correlation of the Spearman's correlation coefficient) was used to conduct a formal test of the null correlation against a positive correlation. The correlations between soil characteristics were assessed using Pearson's correlation coefficient. In all cases, differences at $P < 0.05$ were regarded as statistically significant.

Results

Soil properties and microbial biomass at the studied sites

Soil properties changed substantially along the chronosequence of the studied sites, ranging from the nonvegetated 6-year-old site over the 12-year-old site covered by grasses to the shrub and tree-covered sites developing for 21 and 45 years since their establishment. Most notably, the organic carbon and total nitrogen content increased by factors of two and three, respectively (Table 1). The soil C/N ratio decreased from >100 in the 6-year-old site to 60 in the oldest site and pH of soil decreased with site age. The site covered with *A. glutinosa* after 21 years from revegetation exhibited the highest N content (approximately twice as much as the 21-year-old site undergoing primary succession). The *Alnus* site also showed the lowest C/N ratio and more acidic pH than the sites of the primary succession series (Table 1). Soil pH was negatively correlated with organic C and N contents and a positive correlation between N and C contents was also observed.

Table 1. Properties of postmining sites undergoing primary succession and of the site reclaimed with *Alnus glutinosa*

Site	pH (KCl)	C_{org} (%)	N_{tot} (%)	C/N
6 years	7.4 ± 0.1 a	15.2 ± 0.3 d	0.15 ± 0.01 d	101.3
12 years	6.9 ± 0.1 b	16.9 ± 0.0 c	0.17 ± 0.01 d	99.0
21 years	6.3 ± 0.1 c	23.2 ± 0.1 b	0.33 ± 0.03 c	70.3
45 years	6.2 ± 0.0 c	29.6 ± 0.6 a	0.52 ± 0.05 b	59.9
Alnus glutinosa	5.7 ± 0.3 d	24.6 ± 2.3 b	0.63 ± 0.04 a	39.1

The data represent the averages and SDs of three replicate measurements. Different letters denote statistically significant differences ($P < 0.05$).

Microbial biomass increased from 12 nmol PLFA g^{-1} soil dry mass at the 6-year-old site to 187 nmol g^{-1} at the 21-year-old site and declined later to 116 nmol g^{-1} at the 45-year-old site. The same development was also observed for the bacteria-specific PLFA with the initial concentration of 5.1 nmol g^{-1} and a peak concentration of 75 nmol g^{-1}. The development of fungal biomass was slower in the beginning of succession, where only 0.6 nmol g^{-1} of fungi-specific PLFA and a low F/B ratio were recorded at the 6-year-old site. The soil at the youngest site was thus highly dominated by bacteria. Fungal biomass sharply peaked after 21 years, with 22 nmol g^{-1}, as well as the F/B ratio with 0.29. The *Alnus* site had 2.5 × less total microbial biomass than the 21-year-old primary succession site and showed the highest relative amount of bacterial biomass (Fig. 1).

Bacterial community development during primary succession

The microarray analysis of bacterial communities showed clear distinctions among the sites of different succession ages. In the PC analysis of microarray data, the 6-year-old site was most dissimilar from the other sites, significantly separated along the first axis that explained 47% of the total variability. Older sites were significantly separated only along the second PC axis, explaining 15% of the total variability of hybridization signals among biological replicates (Fig. 2). The three plots of the *A. glutinosa* site differed in its community composition, but were close to the plots of the 45-year-old and 21-year-old sites of the primary

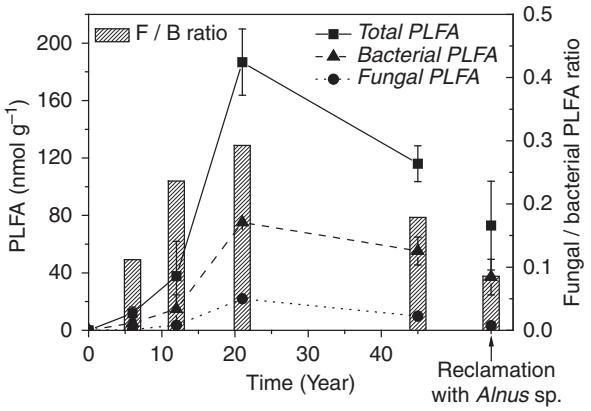


Fig. 1. Microbial biomass content on postmining sites undergoing primary succession and on the site reclaimed with *Alnus glutinosa*. The data represent the averages and SDs of three replicate measurements.

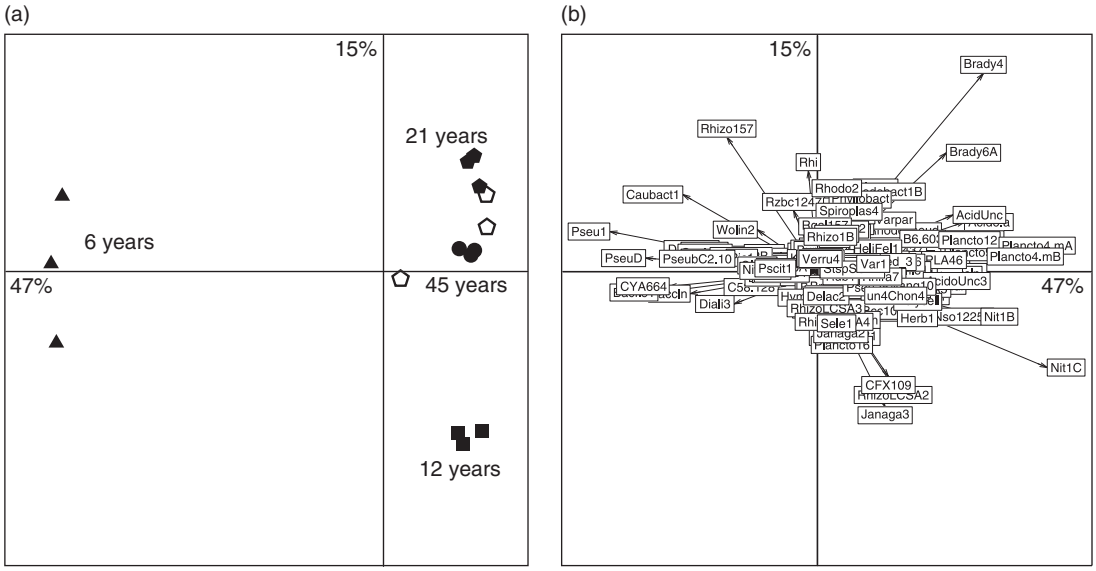


Fig. 2. PC analysis of hybridization data obtained from postmining sites undergoing primary succession and on the site reclaimed with *Alnus glutinosa*. (a) PC coordinates of individual sites: 6 years (triangles), 12 years (squares), 21 (pentagons) and 45 years (circles) and the site reclaimed with *A. glutinosa* (open pentagons). Numbers indicate individual plots within the sites. (b) The corresponding results for individual probes.

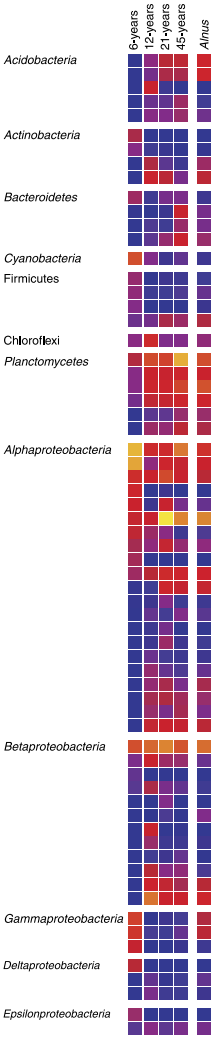
succession chronosequence. The relationships between the soil characteristics and microarray community profiles revealed a strong effect of soil pH ($P < 0.001$) and organic C content ($P = 0.003$); yet the effect of N content on the community composition was not significant ($P = 0.069$) in spite of its correlation with the organic C content.

In a total, 246 probes yielded a significant signal in at least one of the studied sites. In the primary succession series, 228 probes were positive. The diversity of bacterial community estimated indirectly as the number of positive probes increased during succession from 70 at the 6-year-old site and 134 in the 12-year-old site to 179 in the 21-year-old site; 155 positive probes were recorded at the 45-year-old site. Out of the probes occurring in the succession series, 31% were positive at the youngest site, while 79% were positive at the most diverse 21-year-old site. The site reclaimed with *A. glutinosa* showed 185 positive probes, 18 of which were not found in the primary succession series, but all of these were probes with low response. According to the results of cluster analysis, the composition of bacterial community at this site was most similar to the oldest site of the primary succession because the samples from these two sites clustered together. This was also supported by the fact that the PC analysis positions of samples from the *Alnus* site were significantly different from the samples of the 21-year-old site, but not from these of the 45-year-old site, along the second PC axis.

A total of 129 probes in the primary succession series showed statistically significant differences in the relative intensity with time (Table 1; Supporting Information, Table S1). Sixteen probe signals out of the total of 70 (i.e. 23%) were only recorded at the nonvegetated, 6-year-old site. Out of these, probes Propb3 specific for *Propionibacterium* and Caulo1A (*Caulobacteraceae*) showed strong signals. The 12-year-old site had only 10 site-specific probes, none of which exhibited high intensity. At this site, the probes for *Chloroflexi* and several *Betaproteobacteria* (*Janthinobacterium*, *Herbaspirillum* and *Nitrospira*) were more intensive than at the other sites. Thirty-five probe signals (19%) were only recorded at the richest 21-year-old site, among which the probes Alca1b and Alca2 for *Alcaligenaceae* showed the strongest signal. The probes for *Rhizobiaceae*, *Bradyrhizobiaceae* and the genus *Agrobacterium* were highly intensive at this site. At the oldest site, 18 probes were site specific, but all of them showed a low signal intensity and while the signal intensities of most dominant probes were similar to the 21-year-old site, *Bacteroidetes* probes were increased here (Table S1). The site reclaimed with *A. glutinosa* exhibited a similar hybridization pattern for most probes with the oldest site of the spontaneous succession series. The major differences were found in probes for *Pseudomonas* spp. that were more intensive here than at the late succession stage sites and the high intensity of probes targeting cluster 6 *Acidobacteria* at this site with the most acidic soil.

Bacterial community in the initial succession stage

As mentioned above, the bacterial community at the youngest site of the primary succession chronosequence with high pH and undeveloped vegetation cover was largely distinct



	Probe	6-years	12-years	21-years	45-years	*Alnus*	Target
Acidobacteria	AcidUnc	5.6 c	18.2 b	24.4 b	25.0 b	36.8 a	Uncultured *Acidobacteria (Acidobacteria*_6 cluster)
	Acido-a	0.0 d	16.0 c	22.1 b	21.7 b	37.0 a	Uncultured *Acidobacteria (Acidobacteria*_6 cluster)
	RhizoLCSA2	0.0 c	29.0 a	10.7 b	3.9 bc	5.4 bc	Maize rhizosphere clones affiliated to *Acidobacteria (Acidobacteria_7* cluster)
	AcidoUnc3	0.0 b	17.6 a	17.2 a	19.5 a	15.6 a	Uncultured *Acidobacteria (Acidobacteria*_4 cluster)
	Acido-c	0.0 c	15.3 ab	14.6 ab	20.1 a	12.6 b	Uncultured *Acidobacteria (Acidobacteria*_4 cluster)
Actinobacteria	Propb3	21.9 a	0.0 b	0.0 b	0.0 b	0.0 b	Propionibacterium
	Kisp9	17.5 a	11.8 ab	8.6 ab	7.7 b	8.5 ab	*Kitasatospora griseola*
	HGC236-m	0.0 c	22.6 a	14.2 b	10.9 b	20.1 ab	Most Actinobacteria
	ActORD1	0.0 d	31.6 a	24.5 b	16.3 c	24.7 b	Actinomycetales
Bacteroidetes	CFB562B	20.2 a	11.4 b	16.3 ab	16.7 ab	10.6 b	Most *Bacteroidales* (excepted Porphyromonas et rel.)
	Blatta1	8.0 ab	3.4 b	9.1 ab	28.9 a	16.3 ab	*Blattabacterium*
	Flavo2	0.0 b	13.4 ab	12.4 ab	19.7 a	16.3 a	*Flavobacterium* columnare
	CFB562C	0.0 b	14.2 ab	19.2 a	29.0 a	19.5 a	Few *Bacteroidetes*
Cyanobacteria	CYA664	46.5 a	17.2 b	9.7 b	14.6 b	10.5 b	Most Cyanobacteria (excepted some subsections) and some Chloroplasts
Firmicutes	LGC354B	19.2 a	7.3 b	10.1 ab	10.8 ab	12.5 ab	Firmicutes (excepted *Clostridia*, and some Lactobacillales)
	LGC354A	18.7	12.1	12.1	13.3	15.0	Firmicutes (excepted *Clostridia*, and some Lactobacillales)
	Bacfle1	17.3	0.0	0.0	0.0	9.2	*Bacillus flexus*
	Bacpsf1	15.5 b	15.8 b	21.9 a	20.0 ab	22.7 a	*Bacillus pseudofirmus*
Chloroflexi	CFX109	18.0 b	39.8 a	16.9 b	17.1 b	18.7 b	Most Chloroflexi
Planctomycetes	Plancto4-mB	23.1 b	45.5 ab	44.8 ab	58.3 a	46.0 ab	Most Planctomycetes
	Plancto9	17.7 b	29.5 a	28.1 a	36.3 a	32.6 a	Gemmata cluster
	Plancto4-mA	17.5 b	38.1 a	37.5 a	45.1 a	46.7 a	Most Planctomycetes
	Plancto12	16.0 b	26.0 a	27.2 a	34.7 a	34.0 a	Gemmata cluster
	PLA46	0.0 b	14.2 a	14.5 a	19.3 a	19.5 a	Planctomycetes, *Lentipshaerae* and OP3 candidate phylum
	EUB338II	0.0 c	19.9 ab	18.9 b	24.7 a	22.2 ab	Most Planctomycetes
Alphaproteobacteria	Alpha685	59.6 a	39.4 b	51.3 ab	42.1 b	48.9 ab	Most *Alphaproteobacteria*, most *Deltaproteobacteria*, and *Fusobacteria*
	Rhizo157	57.3 a	18.3 c	37.8 b	27.0 bc	31.1 c	*Rhizobiaceae* (excepted *Agrobacterium*), *Bradyrhizobiaceae*, *Brucellaceae* and *Brevundimonas*
	Rzbc1247	41.8 a	29.5 b	45.8 a	28.8 b	24.4 b	*Rhizobiaceae*, *Brucellaceae*, *Bartonella*, *Phyllobacteriaceae*, *Blastochloris*, *Azospirillum irakense* and A. *amazonense*
	Caubact1	38.3 a	0.0 c	10.4 b	0.0 c	2.3 c	Caulobacter
	Rhi	29.3 a	9.8 b	28.1 a	16.3 b	15.0 b	*Rhizobiaceae* (excepted *Agrobacterium)*
	Brady4	27.4 b	28.2 b	67.6 a	52.8 a	53.2 a	*Bradyrhizobiaceae*
	Methylo1	25.5 a	20.0 b	17.9 b	10.8 c	13.3 bc	*Methylobacterium*
	Rgal157	21.3 ab	18.3 b	26.9 a	19.1 b	18.4 b	Some *Rhizobium, Mesorhizobium, Phyllobacterium, Methylobacterium, Acetobacter, Gluconacetobacter, Rhodospirillacae*
	Caulo1A	20.9 a	0.0 b	0.0 b	0.0 b	0.0 b	Some *Caulobacteraceae*
	Rhodobact1B	20.2 c	24.0 b	40.7 a	34.5 ab	33.4 ab	*Rhodobacteraceae* (excepted *Paracoccus, Amaricoccus, Rhodobacter* and *Rhodovulum)*
	Brady6A	8.1 c	12.5 bc	34.1 a	27.5 ab	27.4 ab	Most *Bradyrhizobium* and *Afipia*
	Rhodopseud	7.8	10.1	17.4	12.4	11.1	Rhodopseudomonas
	Aceto2	5.7 b	14.9 ab	12.8 ab	17.2 a	14.1 ab	*Acetobacteraceae* (excepted *Acidiphilium, Acidocella, Acidisphaera, Paracraurococcus, Craurococcus,* Roseococcus)
	Ochro3	0.0 c	10.7 ab	16.1 a	3.0 bc	7.5 b	Ochrobactrum
	B6-603	0.0 c	10.5 b	19.8 a	10.0 b	12.0 b	*Agrobacterium* (G1, G3, G4, G7), A. rubi, A. larrymoorei, some Rhizobium and some Brevundimonas
	Azo5	0.0 c	15.3 a	12.8 ab	11.3 ab	10.6 b	Some *Azospirillum*, some *Roseomonas* spp., some *Rhodospirillum* spp., some *Rhodocista* spp., some *Skermanella* spp.
	Azo1	0.0 c	19.2 a	14.9 ab	13.3 b	15.0 ab	Some *Azospirillum*, some *Roseomonas* spp., some *Rhodospirillum* spp., some *Rhodocista* spp., some *Skermanella* spp.
	Xan	0.0 b	18.9 a	21.5 a	16.5 a	21.4 a	Xanthobacter
	Cow1	0.0 b	21.3 a	22.6 a	20.7 a	19.3 a	Cowdria
	Glud1	0.0 b	19.3 a	16.4 a	21.1 a	16.8 a	*Gluconacetobacter diazotrophicus*
	Acidocella1	0.0 b	26.4 a	32.3 a	27.5 a	24.5 a	*Acidocella/Acidiphilium*
Betaproteobacteria	BET940	46.2	49.1	52.9	47.1	49.8	Betaproteobacteria (excepted *Comamonadaceae, Nitrosomonadaceae and Methylophilaceae)*
	Beta2	16.6 ab	29.2 a	20.1 ab	19.3 ab	15.1 b	Betaproteobacteria
	Pic1	14.7 a	0.0 b	0.0 b	0.0 b	0.0 b	*Ralstonia pickettii*
	Beta3	13.9 b	22.1 a	16.4 ab	14.8 ab	11.5 b	Betaproteobacteria
	Alca1b	0.0 b	0.0 b	17.1 a	0.0 b	0.0 b	*Alcaligenaceae*
	Nit1A	0.0 b	6.5 ab	13.2 ab	6.0 ab	17.0 a	Most Nitrosospira
	Janaga3	0.0 b	28.5 a	3.9 b	8.2 b	2.2 b	*Janthinobacterium agaricidamnosum*
	Herb1	0.0 c	19.4 a	10.7 b	11.1 b	9.7 b	Herbaspirillum
	Burcep	0.0	12.6	9.9	14.9	7.4	Some Burkholderia and some Herbaspirillum
	Nso1225b	0.0 c	24.1 a	17.7 b	18.6 ab	13.2 b	Betaproteobacterial ammonia-oxidizing bacteria
	Nit1B	0.0 c	33.5 a	26.3 b	21.1 b	23.9 b	Most *Nitrosospira*
	Nit1C	0.0 c	50.7 a	32.3 b	30.0 b	34.8 b	Some *Nitrosospira*
Gammaproteobacteria	Pseu1	44.4 a	6.1 c	10.4 c	12.8 c	22.9 b	*Pseudomonas*
	PseuD	42.9 a	13.1 c	12.2 c	14.9 c	24.6 b	*Pseudomonas*
	PseubC4-6	27.5 a	6.8 b	2.5 b	9.1 b	10.8 b	*Pseudomonas* corrugata
Deltaproteobacteria	DSC193	24.1 a	0.0 b	0.0 b	0.0 b	0.0 b	Desulfosarcina
	Nancs8	4.6 b	14.7 a	8.4 ab	12.2 a	14.8 a	Nannocystis
	Polycell	0.0 c	16.5 a	11.5 b	11.8 b	9.7 b	*Polyangium cellulosum*
Epsilonproteobacteria	Wolin2	19.4 a	0.0 b	4.3 b	2.3 b	4.1 b	*Wolinella succinogenes*
	Campy	13.9	17.7	14.3	16.4	16.1	*Campylobacter*

Fig. 3. Microarray analysis of bacterial communities in postmining sites for probes showing the highest response. Probe response intensity increases from dark blue over red to yellow. Only probes with significant site age effect with abundance above 1.5% of the plate total at one of the sites. The values for individual sites represent means of values from three replicate plots. Different letters denote statistically significant differences among sites (ANOVA, followed by the Tukey *post hoc* test, $P < 0.05$).

from the older sites. Microbial biomass was generally low, but bacteria highly dominated over fungi, which demonstrated their important role in the soil processes of the young substrate. In addition to the low proportion of positive probes, this site also exhibited a high percentage of probes positive exclusively at this site (23% of the total). The microarray analysis showed the presence of all major bacterial phyla at the youngest site (Fig. 3). *Acidobacteria* were only represented by one probe with a low relative signal intensity (5.6 compared with 109.9/seven probes 6 years later) and *Actinobacteria* by two probes. Probes targeting generally all or most *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Alphaproteobacteria* and *Betaproteobacteria* were positive at the initial site. The probes for general *Cyanobacteria*, the alphaproteobacterial genus *Caulobacter* and several probes for *Gammaproteobacteria*, especially those designed to target *Pseudomonas* spp., were significantly more intensive here than at older sites and contributed to the distinct localization of the 6-year-old site plots in the PC analysis (Fig. 2).

Sequencing of cloned PCR products was used to further characterize the bacterial community at the initial succession site. *Actinobacteria* were represented by five clones out of the total of 53, of which three closely related ones belonged to *Acidimicrobiaceae*, two sequences belonged to *Firmicutes* and one to *Gemmatimonadetes* (Table S2). The vast majority of clones – more than 80% of the total, however, belonged to *Gammaproteobacteria* (28), *Betaproteobacteria* (14) and *Alphaproteobacteria* (3). Among *Betaproteobacteria*, four sequences were identified as members of the genus *Thiobacillus*. The *Gammaproteobacteria*

were dominated by a large cluster with sequences identified as *Acidithiobacillus* (22), showing that the bacterial community at the 6-year-old site – despite the presence of members of different microbial taxa – was highly uneven and dominated by members of this single genus comprising 40% of all clones. Within the genus, clone sequences formed separate subclusters that clustered closely with sequences identified previously as either the *A. ferrooxidans*, the *Acidithiobacillus ferrivorans* or with sequences of uncultured acidithiobacilli, while sequences related to *Acidithiobacillus thiooxidans* were not recovered (Fig. S1). Another cluster within the *Gammaproteobacteria* comprised five clones with closely related sequences showing similarity values of only $< 95\%$ for the closest GenBank entries that likely represent a so far unknown taxon. This group was probably responsible for the positive signals of the probes PseuD and Pseu1 designed to target *Pseudomonas* spp. (weighted mismatch 2.2, $\Delta G^0 = -34.0\,\text{kcal mol}^{-1}$ for Pseu1, and weighted mismatch 2.5, $\Delta G^0 = -42.4\,\text{kcal mol}^{-1}$ for PseuD). Quantitative PCR showed that the *A. ferrooxidans* was virtually absent at other sites than the youngest one. While the 6-year-old site contained 0.052–0.143 ng *A. ferrooxidans* DNA ng^{-1} of total bacterial DNA, at all other sites, *A. ferrooxidans* DNA was never recorded in all samples and its content was always $< 0.0004\,\text{ng ng}^{-1}$ bacterial DNA.

Discussion

In the last decades, the study of succession processes in soils became highly important in ecosystems that are vulnerable to an increase of global temperature. These ecosystems include the forefields of receding glaciers in different parts of the world in both the mountainous and the polar regions (Kastovska *et al.*, 2005; Bardgett *et al.*, 2007; Noll & Wellinger, 2008; Duc *et al.*, 2009; Fierer *et al.*, 2010). Succession in these ecosystems is severely shaped by the climatic factors and often also by the specific physical quality of the substrate (Kastovska *et al.*, 2005; Schmidt *et al.*, 2008). These extra factors that, for example select the microorganisms able to colonize drying surfaces or are prone to long-term freezing and low temperatures, make it difficult to understand the successional limitations imposed by the nutrient resources of the ecosystem. In this comparison, the postmining sites represent areas where the impact of climate and physical properties does not impose major limitations and where, thus, the nutritional factors are the main drivers of successional processes. Moreover, these ecosystems are quantitatively important in the Central Europe, where postmining deposits are still being created.

While the succession on sandy postmining deposits with a relatively high C/N ratio proved to be relatively fast and the soil was readily colonized by tree species typical for the climax ecosystem (Chodak *et al.*, 2009), the fate of deep subsurface sediments deposited in brown coal mines of the Sokolov mining area represents the situation where initial plant colonization is very slow. The reason is that the deposited clays exhibit a very high C/N ratio and are almost devoid of microbial life, containing only around $0.54\,\text{ng g}^{-1}$ PLFA, i.e. approximately $22\times$ less than the nonvegetated 6-year-old site of this study. The fungal PLFA biomarker 18:2ω6,9 was not detected in the initial material (Elhottova *et al.*, 2006). After the establishment of vegetation after > 8 years, the spontaneous succession proceeds through a series of distinct vegetation communities. These changes are accompanied by the accumulation and transformation of organic materials in the topsoil, whose quality is vegetation dependent (Frouz & Novakova, 2005).

Our results show that the initial changes in the soil nutrient contents were relatively slow during the initial phases of plant cover development between years 6 and 12 and accelerated later, which corresponded with microbial biomass development. Also, the previous analysis of enzyme-catalysed processes along the same primary succession series of sites showed that the activity of decomposition-related extracellular enzymes increased with site age to peak after 21 years of succession as did the microbial biomass in our study (in the case of β-glucosidase, cellobiohydrolase, β-xylosidase and arylsulphatase) or continued to increase until 45 years (*N*-acetylglucosaminidase and phosphatase). The earliest succession stage exhibited very low enzyme activities compared with the 12-year-old site. Consistent with the absence of plant material left for decomposition, the activities of polysaccharide hydrolases were 14–35-fold lower at the 4-year-old site than at the 12-year-old site; activities of phosphatase and *N*-acetylglucosaminidase were lower by factors of 4 and 8 (Baldrian *et al.*, 2008). In most other succession studies on poor substrates, microbial biomass accumulated gradually over the entire period (Tscherko *et al.*, 2004; Fierer *et al.*, 2010). Although the initial appearance of vegetation seemed to promote microbial biomass development in several succession series (Tscherko *et al.*, 2004; Kastovska *et al.*, 2005), it was either negligible or plant species dependent in others (Bardgett & Walker, 2004; Tscherko *et al.*, 2005). Here, the fastest increase of both total microbial and bacterial biomass appeared during early vegetation development from grassland to shrubland. The probable cause is the rapid accumulation of soil organic matter and N during this period. In the later phases of succession, bacterial biomass slightly decreased, but fungal biomass decreased rapidly, possibly due to the fact that the decrease of the C/N ratio favoured bacterial taxa (de Boer *et al.*, 2005).

The number of positive microarray probes increased rapidly until 21 years of succession and slightly decreased later following the pattern of microbial biomass development. The community inhabiting the 6-year-old site with

barren soil showed a low amount of positive probes, with many of them specific for this stage. Missing vegetation cover pointed to nutrient limitation resulting in autotrophic microbial succession as defined by Fierer *et al.* (2010). This development is typically imposed by N availability limitations to plant growth. The autotrophic succession is typically characterized by slow N accumulation by N_2-fixing microorganisms (Nemergut *et al.*, 2007; Schmidt *et al.*, 2008; Duc *et al.*, 2009).

At the 6-year-old site of our study, the potential appearance of N_2 fixers was indicated by high signals of probes for *Cyanobacteria* and selected *Alphaproteobacteria* including the families *Rhizobiaceae* and *Bradyrhizobiaceae*. However, the most striking feature of the site was the dominance of bacteria formerly belonging to the genus *Thiobacillus* (now *Thiobacillus* and *Acidithiobacillus*). Both genera are colourless sulphur bacteria using reduced sulphur compounds as electron donors with oxygen or nitrogen oxides as electron acceptors. While thiobacilli are obligate or facultative autotrophs, the more frequently found acidithiobacilli are obligate CO_2 fixers (Robertson & Kuenen, 2006). Sulphur oxidation occurs at the oxidation front where sulphide and oxygen coexist, for example in the anaerobic pockets of sediments or wet soils. The clay material of the Sokolov mine deposits exhibits a high water retention capacity with a potential to form such anaerobic surfaces (Šourková *et al.*, 2005) and contains considerable amount of sulphur; the total S content in the soil of the 6-year-old site ranged 0.31–0.33%. While the neutral pH optimum for thiobacilli between 6 and 8 is consistent with the soil pH of the 6-year-old site, acidithiobacilli are obligate acidiphiles reported from acidic mine drainage waters, biofilms or mine sediments (Johnson *et al.*, 2001; Johnson & Hallberg, 2003; Macalady *et al.*, 2007) with pH between 0 and 4. In such highly acidic environments, *A. ferrooxidans* was reported to dominate bacterial communities, with abundances ranging up to 70% (Gonzalez-Toril *et al.*, 2003; Mendez *et al.*, 2008; Yang *et al.*, 2008). This study is the first report on *A. ferrooxidans* being highly abundant in an apparently neutral pH and indicates the probable existence of pH gradients associated with acidic microniches (Robertson & Kuenen, 2006). The presence of the neutrophilic *Thiobacillus* seems to prove this concept of pH microvariation at the 6-year-old site. It should be noted that the microarray included three probes targeting the genus *Acidithiobacillus*, Thio2, ACT465a and TF539 (Demanéche *et al.*, 2008), but the signal was not detected. While the first one targets a specific *Acidithiobacillus* not present among cloned sequences, the other two probes showed high ΔG^0 values and might thus have been insufficient for signal detection in environmental samples (Kyselková *et al.*, 2008).

The clone sequences obtained from the early succession site showed several clusters with close relation to *A. ferrooxidans*, but also *A. ferrivorans*, a species with lower tolerance for extremely acidic pH (Hallberg *et al.*, 2010). The presence of *Acidithiobacillus* as an N_2-fixing autotroph in the nonvegetated spoil deposit soil should contribute to the gradual accumulation of both organic C and N in the ecosystem, alleviating its limitations for plant establishment.

During the primary succession in vegetated soils represented here by the 12-year-old, the 21-year-old and the 45-year-old site, we presumed that bacterial community will exhibit both gradual changes as a consequence of changing soil pH and the increase of N and the C/N ratio as well as more abrupt changes caused by the changes of plant community composition and the effect of rhizosphere and chemical traits of litter. Significant effects of plant species on the amount and composition of microbial communities were reported previously from glacier forefields (Bardgett & Walker, 2004; Tscherko *et al.*, 2005) as well as coastal uplift sites (Pennanen *et al.*, 2001; Merilä *et al.*, 2002, 2010) representing chronosequences where pH decreases while the C/N ratio changes in opposite directions. In our study, the rhizosphere development and litter production changed considerably. The root systems at the 12-year-old site were exclusively formed by grasses and scarce herbs, at the 21-year-old site, the understorey was missing and the roots of *Salix* and *Populus* were relatively scarce in the topsoil horizon and the 45-year-old site comprised trees as well as a rich understorey of grasses and herbs. The soil was also subjected to different amounts of nutrients allocated via roots and litter, respectively. The aboveground litter production increased from 190 g m^{-2} at the 12-year-old site over 770 g m^{-2} at the 21-year-old site to 510 g m^{-2} at the oldest site (Chronakova *et al.*, 2010) and it was 470 g m^{-2} at the *Alnus* site (Helingerová *et al.*, 2010). Despite the above differences, the changes of bacterial community composition revealed by the microarray analysis were rather gradual and rarely concerned the probes with high intensity (Fig. 3, Table S1). Statistical analyses showed that those changes follow pH and C_{org} content during soil development.

Soil pH tends to decrease during plant succession on alkaline or neutral substrates (Pennanen *et al.*, 2001; Tscherko *et al.*, 2004; Noll & Wellinger, 2008; Chodak *et al.*, 2009) due to organic acid production, the consequence of litter transformation and production of these compounds by saprotrophic litter-decomposing and mycorrhizal fungi (Baldrian, 2008). pH was previously reported to be a major factor influencing the composition and diversity of soil bacteria (Lauber *et al.*, 2009; Rousk *et al.*, 2010). The shift of pH in the succession series from 7.4 to 5.7–6.3 as seen in our study may lead to significant changes in bacterial diversity. Some important phyla, namely the *Acidobacteria* and *Actinobacteria*, were reported to change their relative abundances dramatically between these pH values (Lauber *et al.*, 2009).This is consistent with the increase of the signal

intensity of probes targeting *Acidobacteria* and the decrease of those targeting *Actinobacteria* along the decreasing pH of the primary succession. The changes in soil pH and the C/N ratio may also affect the composition of bacterial communities indirectly through their effect on fungi because pH decrease tends to decrease the bacterial to fungal DNA ratio while N enrichment acts in the opposite direction (de Boer *et al.*, 2005).

As an alternative to primary succession, reclamation by planting the trees of *A. glutinosa* has been applied in the Sokolov area. The idea behind the treatment is to fasten soil development by the support of soil microbial community. The reason for reclamation is the limitation of N in the initial substrate, which can be potentially decreased due to the activity of symbiotic N_2 fixers occurring on the *Alnus* roots (Šourková *et al.*, 2005). This is supported by the positive signal of the microarray probe Frank16, targeting *Frankia* spp., in two of three samples from the *Alnus* site. Although the input of litter in the unreclaimed sites is not lower than in the reclaimed sites, the microbial C content development during the initial soil development until 15 years was found to be faster in the sites reclaimed by *Alnus* than in the unreclaimed sites in a previous study of Helingerová *et al.* (2010). Here, we show that after 21 years, the C content is not substantially different among the sites, but the N content is approximately twofold under *Alnus*. This would be in agreement with a closer similarity of the bacterial community composition at this and the 45-year-old site likely due to the soil pH that was identified as the most important determinant of bacterial community composition and is in line with the observation that the total microbial biomass and the relative amount of fungal PLFA were lower at the *Alnus* site than at the 45-year-old site. This observation supports the results of statistical analyses showing that soil pH and C_{org} content significantly affect bacterial community development and in this way the soil nutrient content and pH, which are independent on the actual composition of vegetation at the studied sites, are likely of higher importance for bacterial community development than the changes in vegetation cover.

In conclusion, our results show that bacterial communities are especially important during primary succession in its initial and late phases, when they dominate over soil fungi. The initial soil development is hindered by the long absence of vegetation, the phase of autotrophic succession when bacterial N_2 fixers increase the soil N content. Part of this process might be performed by acidophilic *Proteobacteria* belonging or related to *Acidithiobacillus* spp., because they dominate the early succession stage. After N limitation is subdued, vegetation cover develops, which brings about rapid changes in bacterial diversity and community composition. Further changes of bacterial communities seem to be mainly affected by soil pH and C_{org} content. *Alnus glutinosa* planting speeded up the pH decrease and N accumulation in the soil. Thus, planting of this tree species proved successful in terms of postmining substrate recultivation. In the future, however, more research would be still needed to assess the relative roles of vegetation composition and soil development in the structure of bacterial communities and microbial processes in soils.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (LC06066, OC10064, LA10001), by the Grant Agency of the ASCR (IAA603020901) and by the Institutional Research Concepts of the Institute of Microbiology of the ASCR, v.v.i. and the Crop Research Institute (AV0Z50200510, MZE 0002700604). Marek Omelka (Charles University, Prague) is acknowledged for his help with statistical analyses of the data.

References

Ashelford KE, Weightman AJ & Fry JC (2002) PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res* **30**: 3481–3489.

Baldrian P (2008) Enzymes of saprotrophic basidiomycetes. *Ecology of Saprotrophic Basidiomycetes* (Boddy L, Frankland JC & van West P, eds), pp. 19–41. Academic Press, Amsterdam.

Baldrian P, Trogl J, Frouz J, Snajdr J, Valaskova V, Merhautova V, Cajthaml T & Herinkova J (2008) Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. *Soil Biol Biochem* **40**: 2107–2115.

Bardgett RD & Walker LR (2004) Impact of coloniser plant species on the development of decomposer microbial communities following deglaciation. *Soil Biol Biochem* **36**: 555–559.

Bardgett RD, Richter A, Bol R *et al.* (2007) Heterotrophic microbial communities use ancient carbon following glacial retreat. *Biol Lett* **3**: 487–490.

Bonfield JK & Staden R (1995) The application of numerical estimates of base calling accuracy to DNA sequencing projects. *Nucleic Acids Res* **23**: 1406–1410.

Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P & Ritchie DA (1992) Amplification of DNA from native populations of soil bacteria by using the polymerase chain-reaction. *Appl Environ Microb* **58**: 3413–3416.

Chodak M, Pietrzykowski M & Niklinska M (2009) Development of microbial properties in a chronosequence of sandy mine soils. *Appl Soil Ecol* **41**: 259–268.

Chronakova A, Kristufek V, Tichy M & Elhottova D (2010) Biodiversity of streptomycetes isolated from a succession

sequence at a post-mining site and their evidence in Miocene lacustrine sediment. *Microbiol Res* **165**: 594–608.

de Boer W, Folman LB, Summerbell RC & Boddy L (2005) Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev* **29**: 795–811.

Demanéche S, Sanguin H, Pote J, Navarro E, Bernillon D, Mavingui P, Wildi W, Vogel TM & Simonet P (2008) Antibiotic-resistant soil bacteria in transgenic plant fields. *P Natl Acad Sci USA* **105**: 3957–3962.

Duc L, Noll M, Meier BE, Burgmann H & Zeyer J (2009) High diversity of diazotrophs in the forefield of a receding alpine glacier. *Microb Ecol* **57**: 179–190.

Elhottova D, Kristufek V, Frouz J, Novakova A & Chronakova A (2006) Screening for microbial markers in Miocene sediment exposed during open-cast brown coal mining. *Anton Leeuw Int J G* **89**: 459–463.

Elhottova D, Kristufek V, Maly S & Frouz J (2009) Rhizosphere effect of colonizer plant species on the development of soil microbial community during primary succession on postmining sites. *Comm Soil Sci Plant Anal* **40**: 758–770.

Escobar B, Bustos K, Morales G & Salazar O (2008) Rapid and specific detection of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* by PCR. *Hydrometallurgy* **92**: 102–106.

Fierer N, Nemergut D, Knight R & Craine JM (2010) Changes through time: integrating microorganisms into the study of succession. *Res Microbiol* **161**: 635–642.

Frostegard A & Bååth E (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fert Soils* **22**: 59–65.

Frouz J & Novakova A (2005) Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. *Geoderma* **129**: 54–64.

Frouz J, Keplin B, Pizl V *et al.* (2001) Soil biota and upper soil layer development in two contrasting post-mining chronosequences. *Ecol Eng* **17**: 275–284.

Gonzalez-Toril E, Llobet-Brossa E, Casamayor EO, Amann R & Amils R (2003) Microbial ecology of an extreme acidic environment, the Tinto River. *Appl Environ Microb* **69**: 4853–4865.

Hallberg KB, Gonzalez-Toril E & Johnson DB (2010) *Acidithiobacillus ferrivorans*, sp. nov.; facultatively anaerobic, psychrotolerant iron-, and sulfur-oxidizing acidophiles isolated from metal mine-impacted environments. *Extremophiles* **14**: 9–19.

Hartmann A, Schmid M, Tuinen D & Berg G (2009) Plant-driven selection of microbes. *Plant Soil* **321**: 235–257.

Helingerová M, Frouz J & Santruckova H (2010) Microbial activity in reclaimed and unreclaimed post-mining sites near Sokolov (Czech Republic). *Ecol Eng* **36**: 768–776.

Huber T, Faulkner G & Hugenholtz P (2004) Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.

Johnson DB & Hallberg KB (2003) The microbiology of acidic mine waters. *Res Microbiol* **154**: 466–473.

Johnson DB, Rolfe S, Hallberg KB & Iversen E (2001) Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. *Environ Microbiol* **3**: 630–637.

Kastovska K, Elster J, Stibal M & Santruckova H (2005) Microbial assemblages in soil microbial succession after glacial retreat in Svalbard (high Arctic). *Microb Ecol* **50**: 396–407.

Kyselková M, Kopecký J, Felfoldi T, Čermák L, Omelka M, Grundmann GL, Moenne-Loccoz Y & Ságová-Marečková M (2008) Development of a 16S rRNA gene-based prototype microarray for the detection of selected actinomycetes genera. *Anton Leeuw Int J G* **94**: 439–453.

Kyselková M, Kopecký J, Frapolli M, Defago G, Ságová-Marečková M, Grundmann GL & Moënne-Loccoz Y (2009) Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J* **3**: 1127–1138.

Lauber CL, Hamady M, Knight R & Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microb* **75**: 5111–5120.

Macalady JL, Jones DS & Lyon EH (2007) Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. *Environ Microbiol* **9**: 1402–1414.

Mendez MO, Neilson JW & Maier RM (2008) Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. *Appl Environ Microb* **74**: 3899–3907.

Merilä P, Strömmer R & Fritze H (2002) Soil microbial activity and community structure along a primary succession transect on the land-uplift coast in western Finland. *Soil Biol Biochem* **34**: 1647–1654.

Merilä P, Malmivaara-Lämsä M, Spetz P, Stark S, Vierikko K, Derome J & Fritze H (2010) Soil organic matter quality as a link between microbial community structure and vegetation composition along a successional gradient in a boreal forest. *Appl Soil Ecol* **46**: 259–267.

Nemergut DR, Anderson SP, Cleveland CC, Martin AP, Miller AE, Seimon A & Schmidt SK (2007) Microbial community succession in an unvegetated, recently deglaciated soil. *Microb Ecol* **53**: 110–122.

Noll M & Wellinger M (2008) Changes of the soil ecosystem along a receding glacier: testing the correlation between environmental factors and bacterial community structure. *Soil Biol Biochem* **40**: 2611–2619.

Pennanen T, Strommer R, Markkola A & Fritze H (2001) Microbial and plant community structure across a primary succession gradient. *Scand J Forest Res* **16**: 37–43.

Robertson LA & Kuenen JG (2006) The genus *Thiobacillus*. *The Prokaryotes*, *Vol. 5* (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), pp. 812–827. Springer, New York.

Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R & Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J* **4**: 1340–1351.

Sagova-Mareckova M, Cermak L, Novotna J, Plhackova K, Forstova J & Kopecky J (2008) Innovative methods for soil DNA purification tested in soils with widely differing characteristics. *Appl Environ Microb* **74**: 2902–2907.

Sanguin H, Remenant B, Dechesne A, Thioulouse J, Vogel TM, Nesme X, Moenne-Loccoz Y & Grundmann GL (2006) Potential of a 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on *Agrobacterium* spp. and bacterial communities. *Appl Environ Microb* **72**: 4302–4312.

Sanguin H, Kroneisen L, Gazengel K, Kyselková M, Remenant B, Prigent-Combaret C, Grundmann GL, Sarniguet A & Moënne-Loccoz Y (2008) Development of a 16S rRNA microarray approach for the monitoring of rhizosphere *Pseudomonas* populations associated with the decline of take-all disease of wheat. *Soil Biol Biochem* **40**: 1028–1039.

Schipper LA, Degens BP, Sparling GP & Duncan LC (2001) Changes in microbial heterotrophic diversity along five plant successional sequences. *Soil Biol Biochem* **33**: 2093–2103.

Schmidt SK, Reed SC, Nemergut DR *et al.* (2008) The earliest stages of ecosystem succession in high-elevation (5000 metres above sea level), recently deglaciated soils. *P Roy Soc B-Biol Sci* **275**: 2793–2802.

Šourková M, Frouz J & Šantrůčková H (2005) Accumulation of carbon, nitrogen and phosphorus during soil formation on alder spoil heaps after brown-coal mining, near Sokolov (Czech Republic). *Geoderma* **124**: 203–214.

Stralis-Pavese N, Sessitsch A, Weilharter A, Reichenauer T, Riesing J, Csontos J, Murrell JC & Bodrossy L (2004) Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. *Environ Microbiol* **6**: 347–363.

Thioulouse J, Chessel D, Doledec S & Olivier JM (1997) ADE-4: a multivariate analysis and graphical display software. *Stat Comput* **7**: 75–83.

Tscherko D, Hammesfahr U, Marx MC & Kandeler E (2004) Shifts in rhizosphere microbial communities and enzyme activity of *Poa alpina* across an alpine chronosequence. *Soil Biol Biochem* **36**: 1685–1698.

Tscherko D, Hammesfahr U, Zeltner G, Kandeler E & Bocker R (2005) Plant succession and rhizosphere microbial communities in a recently deglaciated alpine terrain. *Basic Appl Ecol* **6**: 367–383.

Větrovský T, Voříšková J, Šnajdr J, Gabriel J & Baldrian P (2010) Ecology of coarse wood decomposition by the saprotrophic fungus: *Fomes fomentarius*. *Biodegradation* **22**: 709–718.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb* **73**: 5261–5267.

Yang Y, Shi WY, Wan MX, Zhang YF, Zou LH, Huang JF, Qiu GZ & Liu XD (2008) Diversity of bacterial communities in acid mine drainage from the Shen-bu copper mine, Gansu province, China. *Electronic J Biotechnol* **11**: 6.

Yergeau E, Schoondermark-Stolk SA, Brodie EL, Dejean S, DeSantis TZ, Goncalves O, Piceno YM, Andersen GL & Kowalchuk GA (2009) Environmental microarray analyses of Antarctic soil microbial communities. *ISME J* **3**: 340–351.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogeny tree of DNA sequences closely related to *Acidithiobacillus* spp. (*Gammaproteobacteria*) obtained from the 6-year-old postmining site.

Table S1. Microarray analysis of bacterial communities in postmining sites – data for all probes.

Table S2. Identification of DNA sequences obtained from the 6-year-old postmining site and best hits obtained by the BLASTN search.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

II

Biol Fertil Soils
DOI 10.1007/s00374-014-0905-z

ORIGINAL PAPER

Litter decomposition along a primary post-mining chronosequence

Michaela Urbanová · Jaroslav Šnajdr · Vendula Brabcová · Věra Merhautová · Petra Dobiášová · Tomáš Cajthaml · Daniel Vaněk · Jan Frouz · Hana Šantrůčková · Petr Baldrian

Received: 28 August 2013 / Revised: 15 January 2014 / Accepted: 17 January 2014

Abstract The aim of this study was to describe the decomposition of litter along a successive series of sites developed at a post-mining overburden deposit over 12, 21 and 45 years, representing the early, mid and late stages of succession. Litter decomposition was largely dependent on the initial composition of the litters. The tree litter of the mid and late stages decomposed faster than the grass litter of the early stage, with 64, 60 and 35 % of mass lost over 2 years, respectively. The contents of hot-water-soluble C and N, which were the best predictors of litter decay rates, were relatively stable over time in all litters. Neither the nutrient content nor the plant biopolymer composition exhibited convergence during decay, indicating that the litter-derived soil organic matter most likely carries a legacy of the original vegetation. In contrast to the litter chemistry, the development of the microbial community was largely specific to the decay stage and consistent among the litters, showing decreasing fungal dominance in time. Extracellular enzymes were found to be of limited value in the prediction of litter decay rates, chemical transformation or microbial community composition.

Keywords Litter decomposition · Primary succession · Microbial community · Extracellular enzymes · Vegetation development

Introduction

During open-cast coal mining, large amounts of soil material above the coal layer are often removed and deposited on spoil heaps. The soil material excavated from the deep subsurface typically does not contain organic carbon derived from recent plant material decomposition (Baldrian et al. 2008; Šourková et al. 2005a; Urbanová et al. 2011). If left to develop on their own, post-mining sites represent excellent models of de novo soil formation not constrained by extreme climatic conditions as are, for example, glacial retreat sites.

Soil formation typically depends on a complex interplay of vegetation and soil biota. Vegetation affects soil development both directly and indirectly. The direct effects result from root growth, which supports the formation of soil pores and soil aggregates (Angers and Caron 1998), and from the production of aboveground and belowground litter and root exudates, which greatly affect the soil organic matter content, chemistry and soil structure (Brady and Weil 2008). The indirect effects of vegetation on soils are mediated by the associated soil microorganisms and soil fauna. While the microfauna is a key player in decomposition, the soil fauna substantially affects the soil structure by bioturbation (Frouz et al. 2007; Lavelle et al. 1997).

It is well established that vegetation affects the composition of microbial communities in the litter and in the soil (Grayston et al. 1998), as well as their functioning (Šnajdr et al. 2013). The vegetation effects might be mediated by pH, soil organic matter and nutrient stoichiometry (Kiikkila et al. 2006; Quideau et al. 2001; Štursová and Baldrian 2011; Urbanová et al. 2011), which are largely mediated by C allocation, either

Electronic supplementary material The online version of this article (doi:10.1007/s00374-014-0905-z) contains supplementary material, which is available to authorized users.

M. Urbanová · J. Šnajdr · V. Brabcová · V. Merhautová · P. Dobiášová · T. Cajthaml · P. Baldrian (✉)
Institute of Microbiology of the ASCR, v.v.i., Vídeňská 1083, 14220 Praha 4, Czech Republic
e-mail: baldrian@biomed.cas.cz

D. Vaněk · H. Šantrůčková
Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic

J. Frouz
Institute of Environmental Studies, Charles University in Prague, Benátská 2, 12800 Praha 2, Czech Republic

Published online: 02 February 2014

in the form of simple compounds delivered by plant roots or in the forms of above- and belowground litter input. It is the aboveground litter input in particular that affects soil formation as a source of the C accumulation in the upper organic layers of soil.

Spontaneous development of ecosystems on barren soils includes organic matter accumulation accompanied by successive changes in vegetation, as well as changes in the associated microbial community (Baldrian et al. 2008; Frouz et al. 2008; Urbanová et al. 2011). Vegetation development at post-mining sites starts with initial colonisation by individual plants, followed by the development of herbal/grass vegetation and the establishment of woody species (Prach and Pyšek 2001). These general trends are similar to those that occur at other types of succession sites (De Kovel et al. 2000; Kirmer and Mahn 2001; Pennanen et al. 2001) and can thus serve as a model of ecosystem development. Vegetation development also increases the microbial biomass in soils as demonstrated by comparisons of barren versus vegetated soils (Bardgett and Walker 2004; Knelman et al. 2012) and tends to increase the fungal to bacterial biomass ratio (Bardgett et al. 2005).

The amount and quality of litter differs among plants (Quideau et al. 2001; Shi et al. 2013). Part of the litter C is lost as CO_2 from the ecosystem; considerable amounts of C are incorporated into soil organic matter. In a recent study with ^{13}C-poplar litter, over two thirds of C lost from the litter was incorporated into soil organic matter (SOM) in a process mediated by soil microorganisms (Rubino et al. 2010). Not surprisingly, the SOM carries the legacy of the composition of the litter, and the composition of the soils thus reflects the composition of the vegetation (Quideau et al. 2001; Thevenot et al. 2013).

The development of SOM from litter is mainly mediated by soil microorganisms, which have been found to be essential in leaf litter decomposition (Aubert et al. 2010; Hättenschwiler and Jørgensen 2010). Reports from post-mining sites show that the dominant vegetation affects soil microbial community composition and function (Chodak and Niklinska 2010; Šnajdr et al. 2013) and consequently the decomposition of its own litter. However, previous studies also showed that these effects are not mediated by the bulk chemistry of litter (Šnajdr et al. 2013) and that the minute vegetation-specific chemical differences, including, theoretically, the composition of biopolymers (e.g. polysaccharides, pectins or lignin) in the litter (Talbot and Treseder 2012), may be important for the litter decomposition process.

The goal of this study was to describe the transformation of litter along a successive series of sites developed at a post-mining overburden deposit site and to identify which properties of litter most affect its transformation during decomposition and the associated microbial community and how this is reflected in soil development described in previous studies (Baldrian et al. 2008; Urbanová et al. 2011). The present study was unique in that it combined the analysis of nutrient content and availability with a detailed analysis of the composition of plant biopolymers contained in the litter. We hypothesised that plant litter with higher nutrient content and lower content of the recalcitrant biopolymers such as lignin will support larger biomass of soil microorganisms and exhibit higher activity of decomposition-related enzymes resulting ultimately in faster rates of decomposition. Due to the limited amount of nutrients in soils of the early successional stages, N or P content can be limiting for the decomposition of the early stage litter while the effects of the successional stage on the lignin content are difficult to predict. Early, middle and late succession stages developed over 12, 21 and 45 years were considered. These stages represented the transformation from grass and herbal vegetation to shrubs and then to a young forest. The study sites were extensively studied in the past, so the development of the vegetation and soil is well known (Baldrian et al. 2008; Helingerová et al. 2010; Kuráž et al. 2012; Mudrák et al. 2010).

Materials and methods

Study site and sampling

This study considered the chronosequence of three post-mining plots 12, 21 and 45 years old, developed on the same initial material (Frouz and Nováková 2005) and covered by spontaneously developed vegetation, located in the Sokolov coal mining district (Czech Republic). The same sites were used in a previous study of soil development (Baldrian et al. 2008). The altitude of the surveyed sites is 500–600 m a.s.l. The mean annual precipitation is 650 mm, and the mean annual temperature is 6.8 °C (Helingerová et al. 2010). Soil dumps were formed by the same initial material of tertiary clay with a pH of approximately 8. The prevailing minerals were kaolinite, illite, calcium carbonate and quartz. The surface, which was not levelled after the heaping process, is characterised by longitudinal depressions and elevations. Consistent with previous studies, the depressions were studied because these represent hotspots of soil microbial activity, and their microenvironments exhibit smaller humidity variations than elevations (Frouz and Nováková 2005).

Vegetation cover developed gradually after 5 years of ecosystem development. Herbs and grasses (mainly *Calamagrostis epigeios*) were present on the plot at 12 years (early), shrubs (*Salix caprea*) at 21 years (mid) and tree cover (*Betula* spp. and *Populus tremuloides*) at 45 years (late). Shrubs shaded nearly the entire soil surface, resulting in a weak herb and grass cover. When the shrubs developed into a forest, the herb and grass cover reappeared (Fig. 1) (Frouz et al. 2008). The studied sites also differed in the amount of aboveground litter input (early, 128 g m^{-2} $year^{-1}$; mid,

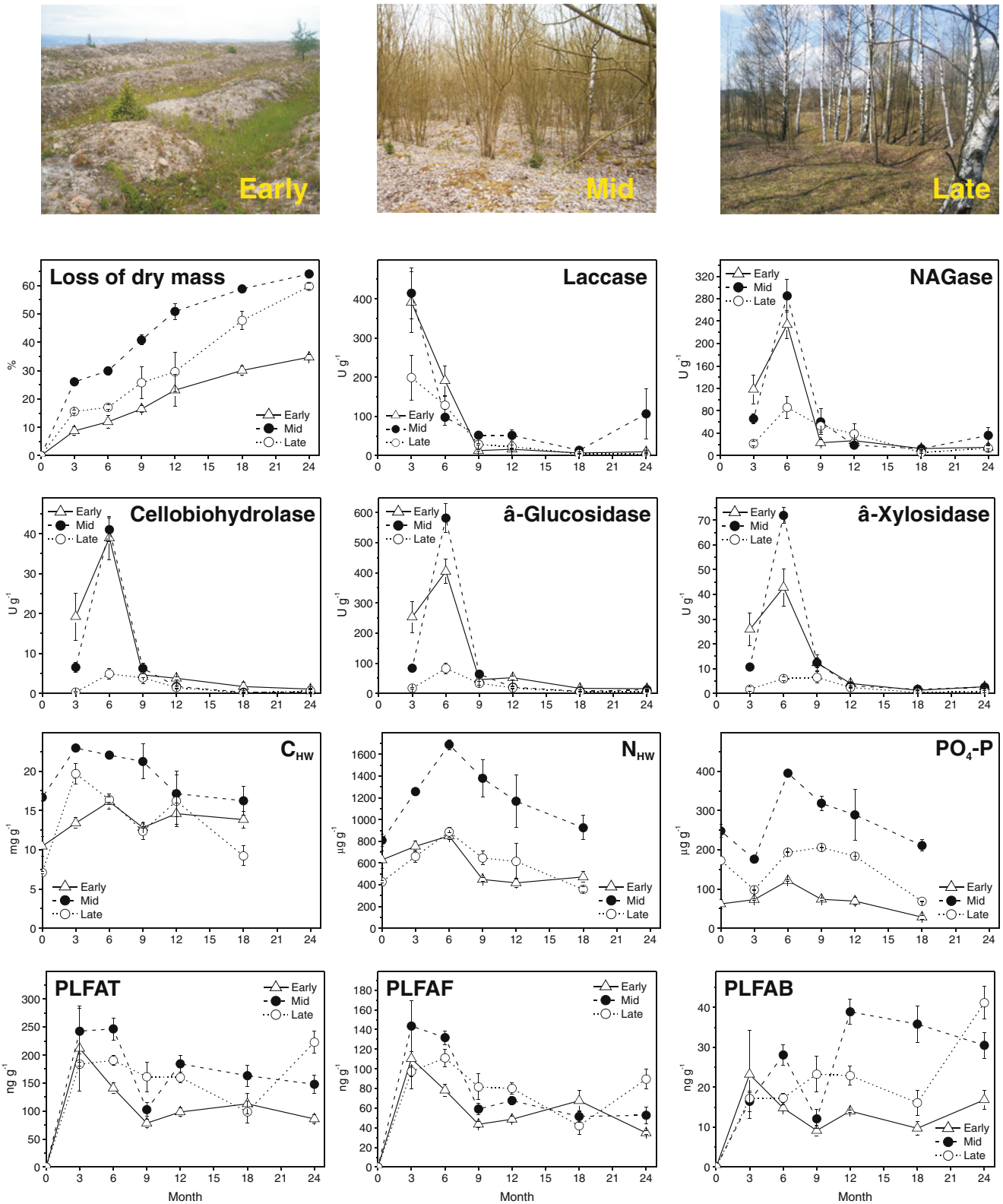


Fig. 1 Loss of dry mass, enzyme activity, litter chemistry and microbial biomass content during decomposition of aboveground plant litter at various stages of primary succession at post-mining sites. The data represent means and standard errors. Abbreviations: C_{HW} hot-water-extractable C, N_{HW} hot-water-extractable N, PO_4-P phosphorus in phosphate, *PLFAT* total PLFA, *PLFAF* fungal PLFA and *PLFAB* bacterial PLFA

184 g m^{-2} $year^{-1}$ and late, 314 g m^{-2} $year^{-1}$) and plant root density (28.5, 1,050 and 520 g m^{-2} of the whole soil profile, respectively) (Helingerová et al. 2010; Kuráž et al. 2012).

The previous year's litter was collected at each site immediately after litterfall (or senescence in the case of *C. epigeios*) and allowed to dry in air at 20 °C. Litterbags containing 6 g of air-dried autochthonous litter (10×20 cm, 1-mm nylon mesh size) were placed on the top of the litter horizon at each study site in November 2006. The mesh size of the litterbags was chosen to ensure a low level of potential mixing with allochtonous material and to allow the activity of microbiota and microfauna, the groups responsible for most of the litter mass loss among the soil fauna (Aubert et al. 2010). At each site, litterbags were placed within a layer of recent litter. Litterbags were removed after 3, 6, 9, 12, 18 and 24 months (seven litterbags per site at each sampling time) and used for the analyses. The initial material was analysed in triplicate.

The collected litterbags were transferred to the laboratory and processed immediately. The litterbags were cleaned and opened, and the material in four of the litterbags was cut into approximately 0.25-cm^2 pieces and used immediately for enzyme activity assays and phospholipid fatty acid (PLFA) analyses. The remaining litterbags were used to measure the loss of dry mass and the lignin content and to analyse the polysaccharide composition. The dry mass content in all litterbags was measured.

Litter chemistry

The dry mass of the litter was determined after drying it at 85 °C to a constant mass, and the pH was measured in distilled water (1:10). The litter material was finely milled before the subsequent analyses. The content of acid-insoluble residues (the equivalent of "Klason lignin") was measured as the dry mass of solids after hydrolysis with 72 % (*w*/*w*) H_2SO_4 (Kirk and Obst 1988).

The carbohydrate composition of hemicelluloses (including pectin) and the concentration of cellulose were determined from dry samples (10–20 mg in weight) collected after months 0, 6, 12 and 24. The concentration of neutral and acidic sugar units in non-cellulosic polysaccharides was determined by acid methanolysis, followed by gas chromatography (GC), as described previously (Šnajdr et al. 2011). The content of hemicellulose was calculated by adding the contents of individual sugars—arabinose, rhamnose, xylose, mannose, galactose and glucose (except glucose contained within cellulose)—and that of pectin, by summing the contents of glucuronic acid, galacturonic acid and 4-*O*-Me-glucuronic acid. The content of cellulose was determined from the concentration of glucose measured by GC after acid hydrolysis and silylation (Sundberg et al. 2003). The amount of non-cellulosic glucose determined by acid methanolysis was subtracted from the concentration determined by acid hydrolysis to obtain the concentration of glucose units derived from cellulose.

The analysis of extractable compounds was conducted using samples reflecting 0–18 months of decomposition, as described previously (Šantrůčková et al. 2006; Voříšková et al. 2011). The available P (Pox) was determined by extraction of 0.5 g of the litter by 50 mL of acid ammonium oxalate solution (0.2 M $H_2C_2O_4$+0.2 M $(NH_4)_2$ C_2O_4 at pH 3). Water-extractable compounds were extracted from the litter step by step in cold water (CW; water/litter, 10:1, *v*/*w*, 30 min at 20 °C) and then by hot water (HW; water/litter, 10:1, *v*/*w*, 16 h at 80 °C). The contents of extractable C and N were then determined using a FormacsHT TOC analyser (Skalar, the Netherlands), and the NH_4^+ and NO_3^-contents were determined using a Tecator 5042 flow injection analyser (Foss, Denmark). All results were expressed on a dry mass basis.

Enzyme assays

Homogenised samples of litter were extracted at 4 °C for 2 h on an orbital shaker (100 rpm) with 100 mM phosphate buffer at a pH of 7 (16:1 *w*/*v*), filtered through Whatman # 5 filter paper and desalted using PD-10 desalting columns (Pharmacia, Sweden), according to the supplier's protocol, to remove inhibitory small-molecular-mass compounds (Šnajdr et al. 2008).

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid in a citrate–phosphate (100 mM citrate, 200 mM phosphate) buffer (pH 5.0) at 420 nm (Bourbonnais and Paice 1990). Manganese peroxidase (MnP, EC 1.11.1.13) was assayed using a succinate–lactate buffer (100 mM, pH 4.5). 3-Methyl-2-benzothiazolinone hydrazone and 3,3-dimethylaminobenzoic acid were oxidatively coupled by enzymes, and the resulting purple indamine dye was detected at 595 nm (Ngo and Lenhoff 1980). The results were corrected by the activities of the samples without manganese (for MnP): the addition of manganese sulphate was substituted by an equimolar amount of ethylenediaminetetraacetate.

Endocellulase (EC 3.2.1.4) and endoxylanase (EC 3.2.1.8) activities were routinely measured with azo-dyed carbohydrate substrates (carboxymethyl cellulose and birchwood xylan, respectively) using the protocol of the supplier (Megazyme, Ireland). The reaction mixture contained 0.2 mL 2 % dyed substrate in a 200-mM sodium acetate buffer (pH 5.0) and 0.2 mL of sample. The reaction mixture was incubated at 40 °C for 60 min; then, the reaction was stopped by adding 1 mL of ethanol, followed by 10 s of vortexing and 10 min of centrifugation (10,000×*g*) (Valášková et al. 2007). The amount of released dye was 595 nm, and the enzyme activity was calculated according to standard curves correlating the dye release with the release of reducing sugars.

Cellobiohydrolase (EC 3.2.1.91) activity was assayed in microplates using *p*-nitrophenyl-β-D-cellobioside (PNPC). The reaction mixture consisted of 0.16 mL of 1.2 mM PNPC in a 50-mM sodium acetate buffer (pH 5.0) and a 0.04-mL sample. The reaction mixture was incubated at 40 °C for 90–120 min. The reaction was stopped by adding 0.1 mL of 0.5 M sodium carbonate, and the absorbance was read at 400 nm. 1,4-β-Glucosidase (EC 3.2.1.21), 1,4-β-xylosidase (EC 3.2.1.37) and 1,4-β-*N*-acetylglucosaminidase (EC 3.2.1.52) activities were assayed using *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, respectively (Valášková et al. 2007). Phosphomonoesterase (EC 3.1.3.1) was assayed using 2 g L^{-1} *p*-nitrophenylphosphate in a 50-mM sodium acetate buffer (pH 5.0), as described previously (Šnajdr et al. 2008).

All spectrophotometric measurements were performed using a microplate reader (Infinite, Tecan) or a UV–VIS spectrophotometer (Lambda 11, PerkinElmer) and expressed per gramme of dry mass of the litter sample. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute.

Quantification of microbial biomass

The samples used for PLFA analysis were extracted by a mixture of chloroform-methanol-phosphate buffer (1:2:0.8), according to Bligh and Dyer (1959). Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck), and the samples were subjected to mild alkaline methanolysis, as described previously (Šnajdr et al. 2008). The free methyl esters of PLFAs were analysed by gas chromatography–mass spectrometry (Varian 3400; ITS-40, Finnigan). The total content of all PLFA molecules (PLFAT) was used as a measure of the total microbial biomass. The fungal biomass was quantified as the 18:2ω6,9 content (PLFAF), and the bacterial biomass was quantified as the sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10 Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10 Me-17:0, 10 Me-18:0 and cy19:0 (PLFAB). The fatty acids found in both bacteria and fungi, 15:0, 16:0 and 18:1ω7, were excluded from the analysis. The fungal/bacterial biomass (F/B) ratio was calculated as PLFAF/PLFAB.

Statistical analysis

Statistical tests were conducted using the software package Statistica 7 (StatSoft, USA). A one-way analysis of variance with Fisher's least significant difference *post hoc* test was used to analyse the statistical significance of differences among groups of samples, and Pearson's correlation coefficients and *t* values were calculated for linear regressions and general linear models applied to assess the effects of multiple factors. Differences and correlations at $P<0.05$ were regarded as statistically significant. Principal component analysis was used to analyse the variability in the relative contents of individual PLFA molecules and in the chemical composition of the litter during decomposition.

Results

The aboveground plant litter composition differed among the sites. At the early site, the bulk of the litter (>90 %) was senescent *C. epigeios* and at the mid site, it was almost exclusively *S. caprea* leaves. At the late site, the litter composition was more diverse, with the majority consisting of tree leaves of *Betula* and *Populus* and a small amount of senescent herbs and grasses.

The differences in litter origin were also reflected in substantial differences in the initial litter composition (Table 1). The litter from the mid site contained the highest amount of both hot-water-extractable and cold-water-extractable available nutrients. Litter from the early site was especially low in available Pox—three to four times less than the other two litters. The litters also differed significantly in pH (Table 1, supplementary Table S1). With respect to the content of plant biopolymers, the grass litter of the early site was considerably different from the other two litter types, with a high content of cellulose and hemicelluloses and a low amount of pectin (Table 1). In addition, the monosaccharide composition of the hemicellulose was similar in the mid and late site litters and differed from the early site litter, in which xylose was the dominant monosaccharide (Fig. 2).

The highest loss of dry mass was observed at the mid site (64±1 % after 2 years)—slightly but significantly higher than at the late site (60±1 %) and substantially higher than at the early site (35±1 %; Fig. 1). The mean turnover rates in the first year were 0.25, 0.60 and 0.32 $year^{-1}$ at the early, mid and late sites, respectively. In the second year, the rates decreased at the early and mid sites to 0.16 and 0.29 $years^{-1}$, while the decomposition rate increased to 0.49 $years^{-1}$ at the late site.

During decomposition, the quantity of hot-water extractable compounds peaked in months 3 through 6 and decreased significantly afterward. In all phases of decomposition, the mid site exhibited the highest N_{HW} and Pox contents (Fig. 1). The decrease in N_{HW} was due primarily to the decrease in the dominant $N_{org\text{-}HW}$; the mineral forms $NH_{4\pm HW}$ and NO_3^--HW were not affected. The C/N ratio did not change significantly, except at the early site, where it increased over time (Fig. 1, supplementary Table S1). The relative contents of plant biopolymers in the litter also changed over time. The cellulose content decreased substantially in all litters, with 13–36 % of its initial mass remaining after 2 years. Hemicellulose degraded more slowly, with 27–46 % remaining after 2 years. Notably, the composition of hemicellulose also changed over time: while most of the xylose was removed, galactose,

Table 1 Initial composition of plant litter from various stages of primary succession at post-mining sites

	Early		Mid		Late	
pH	5.4±0.0	c	6.7±0.0	a	6.5±0.0	b
PO_4^--P (μg g^{-1})	62±11	c	248±16	a	173±3	b
C_{HW} (μg g^{-1})	10,400±1,300	b	16,700±400	a	7,100±100	c
N_{HW} (μg g^{-1})	628±80	b	808±57	a	427±11	c
NH_4^+-HW (μg g^{-1})	52±6	b	126±13	a	53±3	b
NO_3^--HW (μg g^{-1})	4.4±0.4	b	8.0±0.5	a	1.9±0.1	c
C/N	16.6±0.4	b	20.6±1.6	a	16.6±0.4	b
C/P	168±12	a	67±2	b	41±1	c
Lignin (mg g^{-1})	496±17	a	431±5	b	523±39	a
Cellulose (mg g^{-1})	204±10	a	59±8	b	40±6	c
Hemicellulose (mg g^{-1})	329±22	a	154±6	b	116±3	c
Pectin (mg g^{-1})	14±1	c	54±2	a	28±1	b

The data represent means and standard errors. Letters indicate significant differences among sites at $P<0.05$

mannose and glucose decreased substantially less (Fig. 2). The rate of lignin decomposition differed among sites: while its decomposition was as fast as that of the hemicellulose at the late site, it decomposed slowly at the early and mid sites, with 64 and 58 % of the initial content still left after 2 years (Fig. 2).

Principal component analysis of the chemical properties of the litters during decomposition clearly showed that the nutrient contents separated the samples along the first canonical axis, explaining as much as 66 % of the total variability (Fig. 3). The samples were clearly grouped by litter type: the mid site litter had the highest nutrient contents and negative PC1 loads and the early site had the lowest nutrient contents and positive PC1 loads. The second axis, which explained another 15 % of the variability, separated the early samples (months 3–6) characteristic with higher $N_{org\text{-}HW}$ and C_{HW}, from the late ones (months 12–18) with a higher pH and more $N_{min\text{-}HN}$. High enzyme activity and fungal biomass coincided with the early stages of decomposition, while high microbial biomass was associated with samples with high nutrient contents (Fig. 3).

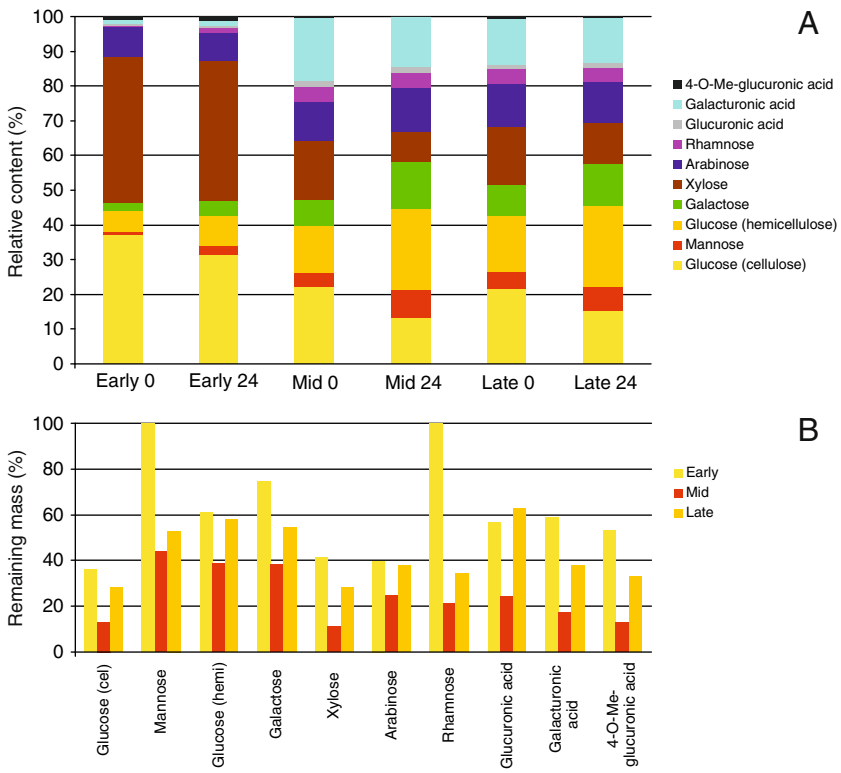


Fig. 2 Initial composition of polysaccharides in aboveground plant litter from various stages of primary succession at post-mining sites (**a**) and changes in monosaccharide composition during 2 years of decomposition in situ (**b**). The data represent mean values

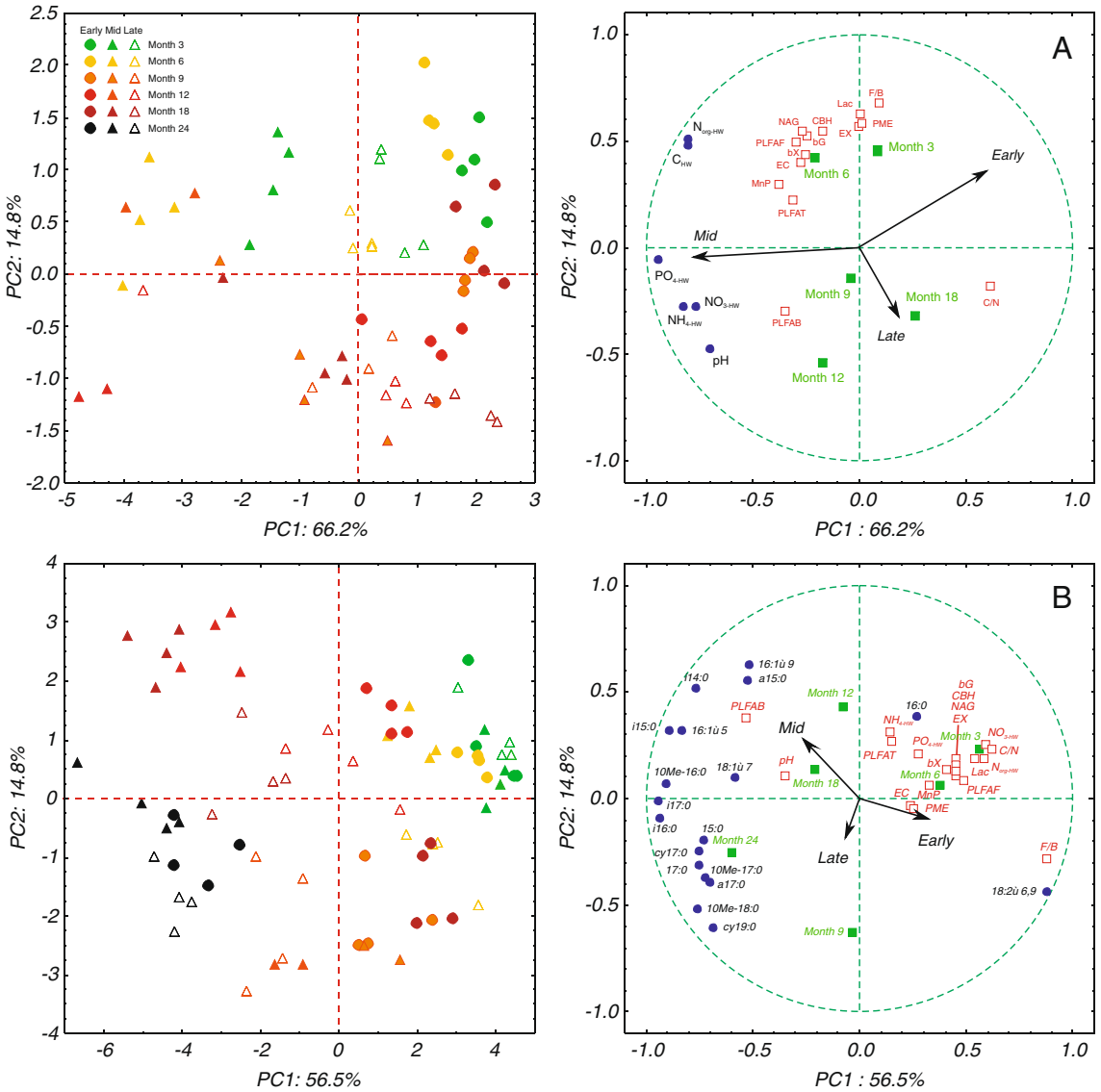


Fig. 3 Principal component analysis of litter chemistry (**a**) and microbial community composition (relative contents of individual PLFA molecules) (**b**) during decomposition of aboveground plant litter at various stages of primary succession at post-mining sites. PCA loads of analysed variables are in *blue*, and those of covariables are in *red*. Abbreviations: *bG* beta-glucosidase, *bX* beta-xylosidase, *CBH* cellobiohydrolase, *EC* endocellulase, *EX* endoxylanase, *Lac* laccase, *MnP* Mn peroxidase, *NAG* N-acetylglucosaminidase and *PME* phosphomonoesterase

The activity of all enzymes in all litters was high during a short initial period (3–6 months) generally peaking at 6 months. The activity of most enzymes was higher in the litters of the early and mid sites litters than in the litter of the late site. Later, the activity of all enzymes dropped to much lower levels. The initial period of high enzyme activity coincided with an elevated content of fungal biomass. The litter of the early site supported lower total microbial biomass than the litters of the mid and late sites, particularly for bacterial biomass in the second year of decomposition; on average, the late site contained twice as much PLFAB as the early site, and the mid site contained 2.6 times more than the early site (Fig. 1). Neither the total biomass nor the bacterial biomass exhibited significant trends over time, but fungal PLFA decreased over time at all sites. While the F/B ratio was high (5.8–8.7) after 3 months, it was only 1.7–2.2 after 24 months (supplementary Table S1).

Cold-water-extractable C and N did not significantly affect microbial biomass in the litter, in a contrast to the positive effects of hot-water-extractable C, N and available Pox. N_{HW} and Pox, which most affected PLFA content, are not independent. Therefore, general linear models were used to assess the effect of these two variables on microbial biomass. In these models, PLFAB was only significantly affected by Pox (R^2= 0.140, P=0.003), while PLFAT and PLFAF were only significantly affected by N_{HW} (R^2=0.147, P=0.002 and R^2=0.155, P=0.002). Subsequent analyses of the effects of Pox, $N_{org\text{-}HW}$ and $N_{min\text{-}HW}$ indicated that PLFAF responds only significantly

to N_{org} (R^2=0.285, P=0.0008) and not to N_{min}. All these trends were significant for the whole dataset as well as for the individual litters.

Principal component analysis demonstrated that the major source of variability in the microbial community composition among the litters was the time of decomposition. The first canonical axis explained 57 % of the total variability, with samples in the initial stages of decomposition having positive loads and samples in the late stages having negative loads. Interestingly, the composition of microbial community during initial decomposition was similar among the litters and the same was also true for microbial community after 2 years of decomposition. The major trend in the data was due to the gradual decrease in fungal biomass and the increase in bacterial biomass. The secondary axis explained 15 % of the total variability, distinguishing litter samples in later stages of decomposition based on the composition of the bacterial community represented by various PLFA (Fig. 3).

Discussion

Soils developing on initial substrates tend to exhibit the same trends in development, the most obvious of them being the accumulation of C and N within the soil profile. This process was also observed in the soil of the study area: the rates of nutrient accumulation were initially high and decreased over time (Šourková et al. 2005b). At the sites considered in this study, the organic C content in the top 5 cm increased from 2.9 % (early) to more than 5.7 % (mid) and then to 7.7 % (late) (Helingerová et al. 2010). This accumulation is largely due to the input of litter, which is one of the major sources of C and other nutrients. The quantity and composition of litter substantially affects soil formation. Litter input varies with time, depending on the vegetation: in the studied sites, litter input increased substantially with site age. This is consistent with the observed effects of the establishment of trees on sand dunes, which increased C and N accumulation compared to the grassland stage, due to higher litter production (De Kovel et al. 2000).

Litter chemistry has been shown to affect both litter-associated microbiota (Šnajdr et al. 2013) and faunal assemblages (Dunger et al. 2001; Frouz et al. 2001). Faunal assemblages affect litter mixing with soil and thus affect the incorporation of organic matter into deeper soil (Frouz et al. 2007). Earthworms have been found to be particularly important in this process (Frouz et al. 2013). The litters from various development stages of this study differed substantially in their composition: those from the tree-covered mid and late stages exhibited higher pH and P contents than the litter from the early stage, and the polysaccharide compositions of the mid and late states were much more similar to each other than they were to that of the early stage (Table 1, Fig. 2). On the same substrate, afforested with different types of trees, sites with litters with low C/N ratios tend to lack an organic horizon, while deep organomineral horizon develops (Frouz et al. 2007). This is consistent with the findings of this study: the mid site had a high C/N ratio in litter and a deep organic horizon (Baldrian et al. 2008), while the late site with a low C/N ratio lacked an organic horizon and instead developed an organomineral horizon, due to the abundance of earthworms that incorporated litter material into the soil (Frouz et al. 2001).

Litter decomposition rates are largely determined by the traits of the living plants, according to a gradient of slow to fast return from invested C (Cornwell et al. 2008; Tilston et al. 2013). The decomposition rate of litter also depends on the properties of soil in which the decomposition occurs, as demonstrated by a litter decomposition test on volcanic soils from various succession stages (Hopkins et al. 2007). The data from ecosystems undergoing succession are, however, not consistent. For example, in one study, the litters of herbaceous plants of the early and intermediate stages of succession of a Mediterranean grassland series exhibited higher initial decomposition rates than plants from the advanced succession stage (Kazakou et al. 2006). In another study, the older sites of a glacier retreat chronosequence exhibited higher initial litter decomposition rates than earlier sites (Esperschütz et al. 2011). Our results, showing the fastest litter decomposition in the mid stage ecosystem, support the idea that the ecosystem development stage is not per se an important determinant of litter turnover. Rather, decomposition is more related to characteristics of the litter and hence the vegetation than the stage.

Litter chemistry seems to be the most important factor affecting litter decomposition rates. Its effect is probably more significant than the site of the decomposition, which was demonstrated in a recent metaanalysis of litter decay constants from a wide set of litters that showed the same ranking across several biomes (Makkonen et al. 2012); a litter transplant experiment would be, however, necessary to provide a final proof. The effects of litter chemistry can be due to nutrient limitations and chemical recalcitrance, both of which decrease decomposition rates. Berg (2000) suggested that macronutrient limitations (e.g. the lack of N) have a considerable effect on the initial decomposition rate of plant litter. The correction of limited N and P by adding these nutrients has been shown to increase the rate of cellulose decomposition (Güsewell and Gessner 2009). Our study indicates that it is the availability of nutrients rather than the total content of nutrients that is important. The mid site litter had higher C/N and C/P ratios than the late site, but it still exhibited the fastest decomposition rate. The high C_{HW} and N_{HW} contents in this litter throughout the experiment and the low values in the early litter are likely to have mostly affected the rate of decomposition. The recalcitrance of litter was proposed to depend on the lignin/N ratio

which has repeatedly been found to be a good predictor of litter decomposition rates (Melillo et al. 1982; Trofymow et al. 2002). In this study, the lignin/N ratio was found to be particularly high in the litter from the late site, which exhibited an intermediate decomposition rate. All of the above observations tend to indicate that the availability of C and N is more important than the total C and N contents. This is in line with the findings of a previous study comparing the litter mass loss rate across 14 tree leaf litters, in which decomposition increased with the available content of water-extractable polysaccharides (Osono and Takeda 2005), which parallels the effects of C_{HW} observed here. Because our previous study showed different rates of disappearance of various polysaccharides in decomposing litter (Šnajdr et al. 2011), the effects of monosaccharidic composition on litter transformation were also examined. There was a consistent trends in the rate of disappearance of individual monosaccharides, since arabinose, xylose and glucose contained within cellulose disappeared rapidly, while mannose and glucose contained within hemicelluloses were largely retained in all three litters (Fig. 2). This confirms the trend observed in *Quercus petraea* litter (Šnajdr et al. 2011) and indicates that the initial polysaccharide composition of litter may also affect its decomposition.

The chemical composition of litter during and after decomposition was found to be mainly dependent on the initial chemistry. Neither nutrient content nor plant biopolymer composition showed convergent development during decomposition (Figs. 2 and 3). This is consistent with the results of a recent analysis performed after prolonged decomposition in multiple litters (Wickings et al. 2012). If this trend is consistent during decomposition, it would indicate that the properties of SOM likely reflect the original litter composition and thus plant leaf traits.

Weak relationships between litter chemistry, litter decay rates and the activities of β-glucosidase, *N*-acetylglucosaminidase and phosphomonoesterase have been detected during incubation of various plant litters (Güsewell and Freeman 2005). In this study, enzyme activities also turned out to be poor predictors of either the litter fate (decomposition rate) or litter chemistry changes, in contrast to the successive changes in enzyme activity reported for other litters (Fioretto et al. 2005; Šnajdr et al. 2011). The high degree of activity at the beginning of decomposition is most likely due to the activity of an initial decomposer community with a distinct composition, dominated by fungi (Fig. 3). The lack of correlation between enzyme activity and mass loss indicates that the enzyme efficiency varies substantially during decomposition. This observation indicates that estimates of in situ turnover rates based on enzyme activity measurements should be made with great caution.

Microbial succession on litter during decomposition is a well-known phenomenon that has been studied repeatedly (Frankland 1998; Voříšková and Baldrian 2013), but the question of whether common series of decomposer organisms may exist in different litters has not been adequately answered. In this study, the microbial community composition depended more on the litter decomposition stage than on the litter source and was strikingly similar among litters at both the beginning and the end of decomposition (Fig. 3). These differences in microbial community composition were largely due to the decrease in the ratio of fungal to bacterial biomass, a trend that has also been observed in oak litter (Šnajdr et al. 2011), but the pattern of gradual development of litter decomposition over time was also observable for bacteria-specific PLFA alone (data not shown). These results seem to indicate that despite profound differences in substrate chemistry, successional development of microbial communities in various litters follow similar patterns at least with respect to the resolution provided by the PLFA analysis.

In this study, litter chemistry affected microbial abundance more than community composition: bacterial PLFA were positively (P=0.006) correlated to the Pox content, while fungal PLFA content was positively (P=0.002) correlated to the N content. This is consistent with the findings of experiments in which cellulose incubated at high N/P ratios exhibited high fungal biomass, while those incubated at low N/P ratios exhibited high bacterial biomass (Güsewell and Gessner 2009). The C/N/P ratio was also found to affect the structure of the microbial decomposer community in beech litter (Schneider et al. 2012).

In the sites considered in this study, the microbial biomass, expressed as PLFAT, was 38, 187 and 116 nmol g^{-1} in the top 5 cm of the soil of the early, mid and late sites, respectively (Baldrian et al. 2008). Tree species effects were also identified as important for the abundance, community composition and activity of soil microorganisms in the same study area planted with various trees (Frouz et al. 2013; Šnajdr et al. 2013). From the present study it seems possible that the litter traits of the individual trees mediate this plant effect— at least partly. Its relative importance compared to other tree-dependent factors (e.g. faunal activity and root effects) is, however, difficult to assess. The composition of the soil bacterial community at the studied sites developed gradually over time, the differences being minor compared to the distinct community of barren soil before the arrival of vegetation (Urbanová et al. 2011). The effect of litter-mediated soil traits on the bacterial community composition thus seems to be rather limited.

The results of this study show that litter decomposition is largely dependent on the initial composition of the litter and that chemical differences among litter types persist during decay. On the other hand, the development of the microbial community follows a largely similar pattern of decreasing fungal dominance and is more decay-stage specific than litter specific. The activities of extracellular enzymes were of limited value in predicting litter decay rates or chemical transformation which confirmed the reservations upon such predictions that were recently published (Nannipieri et al. 2012).

Future research should be focused on the identification of the microorganisms mediating litter decomposition of various litters and on the identification of their ecological needs in order to better understand the mechanisms underlying the plant-soil feedback in developing soils. Furthermore, comparison of data from other succession series would be required before generalisations of the observed trends can be made.

Acknowledgement This work was supported by the Czech Science Foundation (P504/12/1288), the Ministry of Education, Youth and Sports of the Czech Republic (LC06066) and the Research Concept of the Institute of Microbiology ASCR (RVO61388971). We also thank the Sokolovská Uhelná a.s. coal mining company for the research permit.

References

Angers DA, Caron J (1998) Plant-induced changes in soil structure: processes and feedbacks. Biogeochemistry 42:55–72

Aubert M, Margerie P, Trap J, Bureau F (2010) Aboveground-belowground relationships in temperate forests: plant litter composes and microbiota orchestrates. Forest Ecol Manag 259:563–572

Baldrian P, Trögl J, Frouz J, Šnajdr J, Valášková V, Merhautová V, Cajthaml T, Herinková J (2008) Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. Soil Biol Biochem 40:2107–2115

Bardgett RD, Walker LR (2004) Impact of coloniser plant species on the development of decomposer microbial communities following deglaciation. Soil Biol Biochem 36:555–559

Bardgett RD, Bowman WD, Kaufmann R, Schmidt SK (2005) A temporal approach to linking aboveground and belowground ecology. Trends Ecol Evol 20:634–641

Berg B (2000) Litter decomposition and organic matter turnover in northern forest soils. Forest Ecol Manag 133:13–22

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102

Brady NC, Weil R (2008) The nature and properties of soils. Prentice Hall, Upper Saddle River, 992 pp

Chodak M, Niklinska M (2010) The effect of different tree species on the chemical and microbial properties of reclaimed mine soils. Biol Fertil Soils 46:555–566

Cornwell WK, Cornelissen JHC, Amatangelo K, Dorrepaal E, Eviner VT, Godoy O, Hobbie SE, Hoorens B, Kurokawa H, Perez-Harguindeguy N, Quested HM, Santiago LS, Wardle DA, Wright IJ, Aerts R, Allison SD, van Bodegom P, Brovkin V, Chatain A, Callaghan TV, Diaz S, Garnier E, Gurvich DE, Kazakou E, Klein JA, Read J, Reich PB, Soudzilovskaia NA, Vaieretti MV, Westoby M (2008) Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. Ecol Lett 11:1065–1071

De Kovel CGF, Van Mierlo A, Wilms YJO, Berendse F (2000) Carbon and nitrogen in soil and vegetation at sites differing in successional age. Plant Ecol 149:43–50

Dunger W, Wanner M, Hauser H, Hohberg K, Schulz HJ, Schwalbe T, Seifert B, Vogel J, Voigtlander K, Zimdars B, Zulka KP (2001) Development of soil fauna at mine sites during 46 years after afforestation. Pedobiologia 45:243–271

Esperschütz J, Pérez-De-Mora A, Schreiner K, Welzl G, Buegger F, Zeyer J, Hagedorn F, Munch JC, Schloter M (2011) Microbial food web dynamics along a soil chronosequence of a glacier forefield. Biogeosciences 8:3283–3294

Fioretto A, Di Nardo C, Papa S, Fuggi A (2005) Lignin and cellulose degradation and nitrogen dynamics during decomposition of three leaf litter species in a Mediterranean ecosystem. Soil Biol Biochem 37:1083–1091

Frankland JC (1998) Fungal succession—unravelling the unpredictable. Mycol Res 102:1–15

Frouz J, Nováková A (2005) Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. Geoderma 129:54–64

Frouz J, Keplin B, Pižl V, Tajovský K, Starý J, Lukešová A, Nováková A, Balík V, Hánel L, Materna J, Duker C, Chalupský J, Rusek J, Heinkele T (2001) Soil biota and upper soil layer development in two contrasting post-mining chronosequences. Ecol Eng 17:275–284

Frouz J, Elhottová D, Pižl V, Tajovský K, Šourková M, Picek T, Malý S (2007) The effect of litter quality and soil faunal composition on organic matter dynamics in post-mining soil: a laboratory study. Appl Soil Ecol 37:72–80

Frouz J, Prach K, Pižl V, Háněl L, Starý J, Tajovský K, Materna J, Balík V, Kalčík J, Řehounková K (2008) Interactions between soil development, vegetation and soil fauna during spontaneous succession in post mining sites. Eur J Soil Biol 44:109–121

Frouz J, Livečková M, Albrechtová J, Chroňáková A, Cajthaml T, Pižl V, Háněl L, Starý J, Baldrian P, Lhotáková Z, Šimáčková H, Cepáková Š (2013) Is the effect of trees on soil properties mediated by soil fauna? A case study from post-mining sites. Forest Ecol Manag 309:87–95

Grayston SJ, Wang SQ, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378

Güsewell S, Freeman C (2005) Nutrient limitation and enzyme activities during litter decomposition of nine wetland species in relation to litter N: P ratios. Funct Ecol 19:582–593

Güsewell S, Gessner MO (2009) N:P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. Funct Ecol 23:211–219

Hättenschwiler S, Jørgensen HB (2010) Carbon quality rather than stoichiometry controls litter decomposition in a tropical rain forest. J Ecol 98:754–763

Helingerová M, Frouz J, Šantrůčková H (2010) Microbial activity in reclaimed and unreclaimed post-mining sites near Sokolov (Czech Republic). Ecol Eng 36:768–776

Hopkins DW, Badalucco L, English LC, Meli SM, Chudek JA, Ioppolo A (2007) Plant litter decomposition and microbial characteristics in volcanic soils (Mt Etna, Sicily) at different stages of development. Biol Fertil Soils 43:461–469

Kazakou E, Vile D, Shipley B, Gallet C, Garnier E (2006) Co-variations in litter decomposition, leaf traits and plant growth in species from a Mediterranean old-field succession. Funct Ecol 20:21–30

Kiikkila O, Kitunen V, Smolander A (2006) Dissolved soil organic matter from surface organic horizons under birch and conifers: degradation in relation to chemical characteristics. Soil Biol Biochem 38:737–746

Kirk TK, Obst JR (1988) Lignin determination. Meth Enzymol 161:87–101

Kirmer A, Mahn EG (2001) Spontaneous and initiated succession on unvegetated slopes in the abandoned lignite-mining area of Goitsche, Germany. Appl Veget Sci 4:19–27

Knelman JE, Legg TM, O'Neill SP, Washenberger CL, González A, Cleveland CC, Nemergut DR (2012) Bacterial community structure and function change in association with colonizer plants during early primary succession in a glacier forefield. Soil Biol Biochem 46:172–180

Kuráž V, Frouz J, Kuráž M, Mako A, Shustr V, Cejpek J, Romanov OV, Abakumov EV (2012) Changes in some physical properties of soils in the chronosequence of self-overgrown dumps of the Sokolov quarry-dump complex, Czechia. Eurasian Soil Sci 45:266–272

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson P, Heal OW, Dhillion S (1997) Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur J Soil Biol 33:159–193

Makkonen M, Berg MP, Handa IT, Hättenschwiler S, van Ruijven J, van Bodegom PM, Aerts R (2012) Highly consistent effects of plant litter identity and functional traits on decomposition across a latitudinal gradient. Ecol Lett 15:1033–1041

Melillo JM, Aber JD, Muratore JF (1982) Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63:621–626

Mudrák O, Frouz J, Velichová V (2010) Understory vegetation in reclaimed and unreclaimed post-mining forest stands. Ecol Eng 36:783–790

Nannipieri P, Giagnoni L, Renella G, Puglisi E, Ceccanti B, Masciandaro G, Fornasier F, Moscatelli MC, Marinari S (2012) Soil enzymology: classical and molecular approaches. Biol Fertil Soils 48:743–762

Ngo TT, Lenhoff HM (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal Biochem 105:389–397

Osono T, Takeda H (2005) Decomposition of organic chemical components in relation to nitrogen dynamics in leaf litter of 14 tree species in a cool temperate forest. Ecol Res 20:41–49

Pennanen T, Strömmer R, Markkola A, Fritze H (2001) Microbial and plant community structure across a primary succession gradient. Scand J Forest Res 16:37–43

Prach K, Pyšek P (2001) Using spontaneous succession for restoration of human-disturbed habitats: experience from central Europe. Ecol Eng 17:55–62

Quideau SA, Chadwick OA, Benesi A, Graham RC, Anderson MA (2001) A direct link between forest vegetation type and soil organic matter composition. Geoderma 104:41–60

Rubino M, Dungait JAJ, Evershed RP, Bertolini T, De Angelis P, D'Onofrio A, Lagomarsino A, Lubritto C, Merola A, Terrasi F, Cotrufo MF (2010) Carbon input belowground is the major C flux contributing to leaf litter mass loss: evidences from a ^{13}C labelled-leaf litter experiment. Soil Biol Biochem 42:1009–1016

Šantrůčková H, Krištůfková M, Vaněk D (2006) Decomposition rate and nutrient release from plant litter of Norway spruce forest in the Bohemian Forest. Biologia 61:S499–S508

Schneider T, Keiblinger KM, Schmid E, Sterflinger-Gleixner K, Ellersdorfer G, Roschitzki B, Richter A, Eberl L, Zechmeister-Boltenstern S, Riedel K (2012) Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME J 6:1749–1762

Shi AD, Penfold C, Marschner P (2013) Decomposition of roots and shoots of perennial grasses and annual barley-separately or in two residue mixes. Biol Fertil Soils 49:673–680

Šnajdr J, Valášková V, Merhautová V, Herinková J, Cajthaml T, Baldrian P (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biol Biochem 40:2068–2075

Šnajdr J, Cajthaml T, Valášková V, Merhautová V, Petránková M, Spetz P, Leppanen K, Baldrian P (2011) Transformation of *Quercus petraea* litter: successive changes in litter chemistry are reflected in differential enzyme activity and changes in the microbial community composition. FEMS Microbiol Ecol 75:291–303

Šnajdr J, Dobiášová P, Urbanová M, Petránková M, Cajthaml T, Frouz J, Baldrian P (2013) Dominant trees affect microbial community composition and activity in post-mining afforested soils. Soil Biol Biochem 56:105–115

Šourková M, Frouz J, Fettweis U, Bens O, Hüttl RF, Šantrůčková H (2005a) Soil development and properties of microbial biomass succession in reclaimed post mining sites near Sokolov (Czech Republic) and near Cottbus (Germany). Geoderma 129:73–80

Šourková M, Frouz J, Šantrůčková H (2005b) Accumulation of carbon, nitrogen and phosphorus during soil formation on alder spoil heaps after brown-coal mining, near Sokolov (Czech Republic). Geoderma 124:203–214

Štursová M, Baldrian P (2011) Effects of soil properties and management on the activity of soil organic matter transforming enzymes and the quantification of soil-bound and free activity. Plant Soil 338:99–110

Sundberg A, Pranovich AV, Holmbom B (2003) Chemical characterization of various types of mechanical pulp fines. J Pulp Paper Sci 29: 173–178

Talbot JM, Treseder KK (2012) Interactions among lignin, cellulose, and nitrogen drive litter chemistry-decay relationships. Ecology 93:345–354

Thevenot M, Dignac MF, Mendez-Millan M, Bahri H, Hatte C, Bardoux G, Rumpel C (2013) Ligno-aliphatic complexes in soils revealed by an isolation procedure: implication for lignin fate. Biol Fertil Soils 49:517–526

Tilston EL, Halpin C, Hopkins DW (2013) Decomposition of tobacco roots with modified phenylpropanoid content by fungi with contrasting lignocellulose degradation strategies. Biol Fertil Soils 49: 305–311

Trofymow JA, Moore TR, Titus B, Prescott C, Morrison I, Siltanen M, Smith S, Fyles J, Wein R, Camir TC, Duschene L, Kozak L, Kranabetter M, Visser S (2002) Rates of litter decomposition over 6 years in Canadian forests: influence of litter quality and climate. Can J Forest Res 32:789–804

Urbanová M, Kopecký J, Valášková V, Ságová-Marečková M, Elhottová D, Kyselková M, Moënne-Loccoz Y, Baldrian P (2011) Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. FEMS Microbiol Ecol 78:59–69

Valášková V, Šnajdr J, Bittner B, Cajthaml T, Merhautová V, Hoffichter M, Baldrian P (2007) Production of lignocellulose-degrading enzymes and degradation of leaf litter by saprotrophic basidiomycetes isolated from a *Quercus petraea* forest. Soil Biol Biochem 39:2651–2660

Voříšková J, Baldrian P (2013) Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME J 7:477–486

Voříšková J, Dobiášová P, Šnajdr J, Vaněk D, Cajthaml T, Šantrůčková H, Baldrian P (2011) Chemical composition of litter affects the growth and enzyme production by the saprotrophic basidiomycete *Hypholoma fasciculare*. Fungal Ecol 4:417–426

Wickings K, Grandy AS, Reed SC, Cleveland CC (2012) The origin of litter chemical complexity during decomposition. Ecol Lett 15: 1180–1188

III

Soil Biology & Biochemistry 56 (2013) 105–115

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Dominant trees affect microbial community composition and activity in post-mining afforested soils

Jaroslav Šnajdr [a], Petra Dobiášová [a], Michaela Urbanová [a], Mirka Petránková [a], Tomáš Cajthaml [a], Jan Frouz [b], Petr Baldrian [a,*]

[a] Institute of Microbiology of the ASCR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Institute of Soil Biology, Biology Centre of the ASCR, v.v.i., Na Sádkách 7, 370 05 České Budějovice, Czech Republic

ARTICLE INFO

Article history:
Received 26 February 2012
Received in revised form
3 May 2012
Accepted 8 May 2012
Available online 23 May 2012

Keywords:
Enzyme activity
Litter
Microbial biomass
Spontaneous succession
Vegetation
Seasonality

ABSTRACT

The aim of this work was to quantify the effects of vegetation on the activity of extracellular enzymes in the litter and soil. To achieve this, we investigated a set of post-mining sites in a brown-coal mine deposit area near Sokolov, Czech Republic. The sites were 22–33 years old and had been established on the same initial substrate by planting with six tree genera or leaving for spontaneous revegetation, with four replicate sites per vegetation type. The activity of extracellular hydrolytic and oxidative enzymes and the microbial community composition of the litter and topsoil were compared in the spring, summer and autumn using the dominant tree, pH, soil nutrient content and soil moisture as the explanatory variables. Sites under individual trees exhibited significant differences in the chemical properties of both the litter and soil, and the tree effect was identified as the most important factor affecting the activity of extracellular enzymes either directly or in the interaction with seasonal effects, although not all pairs of tree species were significantly different from each other. Seasonal effects on enzyme activity were only important in the litter. The effects of dominant trees and of seasons contributed equally to the variation in the microbial community composition at individual sites. Only a minor part of the tree effect could be explained by differences in the litter or soil chemistry. Among the chemical variables, the N content most affected the microbial biomass content, increasing fungal (but not bacterial) biomass in the litter and bacterial (but not fungal) biomass in the soil. The results indicate that other factors, such as nutrient quality or the specific association of microorganisms with rhizospheres of different trees or the understory, are likely important mediators of the vegetation effects. When comparing the revegetated sites with sites under spontaneous succession, the enzyme activities and microbial biomass were similar except for the sites revegetated with *Alnus* which may indicate similar rates of soil development at revegetated and succession sites. Spontaneous succession in temperate Europe may thus be a suitable option for land restoration.

1. Introduction

Open-cast coal mining has a large impact on the ecosystem. During mining, large amounts of soil material above the coal layer are removed and deposited on spoil heaps. The spoil material is excavated from deep subsurface (e.g., in the Sokolov area from as deep as 200 m), and it typically does not contain organic carbon derived from recent plant material. The amounts of other macronutrients, such as nitrogen and phosphorus, can be limiting factors for biological development of this environment (Šourková et al., 2005). Spoil material with such properties has very low biological activity (Baldrian et al., 2008; Frouz and Nováková, 2005; Urbanová et al., 2011; Frouz et al., 2011) and thus represents an excellent model of *de novo* soil development, including the role of the microbiota in the soil processes.

The newly created ecosystems may be left to develop spontaneously, but the restoration of functional ecosystems on post-mining sites is often manipulated by revegetation intended to accelerate the stabilization and development of the soil surface. Soil formation and reestablishment of soil biological functions are among the basic preconditions for the recovery of ecologically sound ecosystems on spoil heaps (Bradshaw, 1997). Organic matter

* Corresponding author. Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, v.v.i., Vídeňská 1083, 142 20 Praha 4, Czech Republic. Tel.: +420 723770570; fax: +420 241062384.
E-mail address: baldrian@biomed.cas.cz (P. Baldrian).

0038-0717/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2012.05.004

can enter the soil via the leaf litter, which mainly accumulates on the surface, or by the root exudate flux through plant roots to root-associated microbes and the turnover of fine roots in the upper layers of the soil. Therefore, afforestation is a common way of reclaiming spoil areas. The growing trees enhance soil-forming processes, contributing to the buildup of organic matter and development of microbial and microfaunal communities (Frouz et al., 2009; Chodak and Niklinska, 2010a; Lamb et al., 2011; Merilä et al., 2010). The ratio of carbon sources of litter or root origin is species specific, and different plant species differ with respect to the amount of organic matter they add to the soil (Finér et al., 2007; Hooper et al., 2000). In addition, tree species may differently influence soil chemical properties, such as the pH or the relative content and chemical form of macronutrients (Augusto et al., 2000; Binkley and Valentine, 1991; Frouz et al., 2009; Hagen-Thorn et al., 2004; Iovieno et al., 2010; Menyailo et al., 2002). For example, the tree species like *Alnus* spp. can promote nitrogen fixation and produce nitrogen-rich litter, which consequently increases the N content in the topsoil (Ekblad and HussDanell, 1995). Trees also influence the extent and composition of the understory vegetation largely through their effects on soil formation and through other soil biota, including invertebrates (Frouz et al., 2008; Mudrák et al., 2010).

The effects of vegetation on ecosystem properties change the environment of the microbiota inhabiting litter and soil. This group of organisms is substantially involved in the decomposition of organic matter and contributes to the biogeochemical cycles of all important nutrients. As a consequence, soil development is a process modulated by both the vegetation and the microbial inhabitants of the soil and litter. Previous studies from forested ecosystems indicate that vegetation can affect the composition of microbial communities in the litter and in the soil (Grayston et al., 1998; Osono and Takeda, 2007; Priha et al., 2001). These changes are affected by pH, soil organic matter, N content or the association of specific microbes, including symbiotic fungi, with certain plants (Kiikkila et al., 2006; Quideau et al., 2001; Šťursová and Baldrian, 2011; Urbanová et al., 2011). Less is currently known on the effects of dominant vegetation on the biogeochemical processes in soils, including the rates of enzyme-catalyzed processes in soils. Although such studies have been published (Hättenschwiler and Jørgensen, 2010; Chodak and Niklinska, 2010a; Niemi et al., 2007; Weand et al., 2010), they have usually been limited to comparisons of a limited set of vegetation types, limited replication of study sites or had sampling limited to a certain season of the year.

The aim of this work was to quantify the effects of vegetation on the activity of extracellular enzymes in the litter and soil. We investigated the activity of hydrolytic enzymes as well as the amount and composition of soil microbial biomass at a set of post-mining sites under six different trees introduced by recultivation and at sites left to spontaneous succession for the same time. Although comparison studies between soils under different tree species have been made previously, here we used the unique opportunity that the set of tree plantations was established in field size trials yet on a very similar substrate which was not previously affected by another tree species. The materials deposited in the area are tertiary clays of Miocene age. Similar minerals belong to the most common overburden materials in the Czech Republic, Poland, and Germany and are thus good representatives of common overburden materials worldwide. The set of studied sites covered seven types of dominant vegetation, six of which were represented by tree genera used for recultivation and the last being subject to spontaneous revegetation. For each of the seven vegetation types, four independent sites were analyzed, each of them developing on the same initial substrate for approximately the same time (22–33 years). Previous studies concerning this study area have shown vegetation-dependent differences in the C accumulation both aboveground and belowground (Frouz et al., 2009), the extent and type of understory vegetation (Mudrák et al., 2010), the differences in litter composition of individual dominant trees (Voříšková et al., 2011) and, among some of the sites, tree-specific differences in soil chemistry (Helingerová et al., 2010). The sites were analyzed during three seasons: in October, when fresh litter was available; in April, when most of the available carbon in litter had been decomposed and the abundance of root-associated symbiotic fungi was low; and in August, a period of temperature optimum with the lowest quality of litter and a high abundance of mycorrhizal fungi (Baldrian et al., 2008; Kaiser et al., 2010). Spontaneously revegetated sites in the study area have previously been found to differ during these seasons in soil microbial biomass content and extracellular enzyme activity (Baldrian et al., 2008). We hypothesized that the effects of vegetation on extracellular enzyme activity, if any, would be more pronounced in soil where the accumulation of nutrients and plant-specific products leads to the development of plant-specific organic matter and where the microbial community contains a large proportion of specific, root-associated microbiota (Hartmann et al., 2009). Despite the differences in litter quality among trees, we expect that enzyme activity in litter is more affected by seasonal changes reflecting the changes in litter chemistry during decomposition (Šnajdr et al., 2011). Furthermore, we propose that enzyme in individual treatments reflects the extent of the site development with high enzyme activity indicating faster soil development. This is anticipated due to the fact that enzyme activity was found to increase with time during the development of soils under spontaneous succession in the area of study (Baldrian et al., 2008) and the values of enzyme activity after 25 years of spontaneous succession do not yet reach the values typically observed in undisturbed forest soils (Šťursová and Baldrian, 2011).

2. Materials and methods

2.1. Study site

The study was carried out at the Velká Podkrušnohorská spoil heap in the Sokolov brown-coal mining district (in the western part of the Czech Republic). The study area of 1900 ha was located at an altitude of 450–520 m a.s.l. with a mean annual precipitation of 650 mm and a mean annual temperature of 6.8 °C. The spoil heaps were formed by Tertiary clays of the so-called cypris formation. When dumped on the heap, this material had an alkaline pH of 8–9, and its prevailing minerals were kaolinite, montmorilonite and illite. These minerals may be impregnated by calcite, siderite and fossil organic matter, namely type II kerogen (Mudrák et al., 2010).

The area is mostly covered by a mosaic of forest patches, planted as a reclamation measure in a way that individual tree patches are randomly spread over the heap. This gives a “common garden” experiment of a landscape dimension. Some of the heap had not been reclaimed and was dedicated to spontaneous revegetation. Seven types of forest stands were chosen for the study. One type was unreclaimed stands undergoing spontaneous succession (S) dominated by the birch *Betula pendula* and the willow *Salix caprea*. Six types were reclaimed plantations, each dominated by one or two tree species of one genus: *Alnus* (A), *Alnus glutinosa* and *Alnus incana*; *Larix* (L), *Larix decidua*; *Picea* (Pc), *Picea omorica* and *Picea pungens*; *Pinus* (Pn), *Pinus contorta* and *Pinus nigra*; *Quercus* (Q), *Quercus robur*; and *Tilia* (T), *Tilia cordata*. Four replicated patches per tree plus four replicated patches left to spontaneous succession were selected for this study. Each patch had at least 1 ha (typically 2–5 ha). The study sites were randomly spread on the heap and in all cases; patches of other trees are closer to each patch than a patch

of the same tree. Typical distance between replication of the same species is 1–4 km while other species are usually located less that 1 km apart. In each patch, a study area was chosen to be at least 25 m from a patch edge.

The surface of the unreclaimed sites consisted of parallel ridges (1 m high) and depressions formed by the heaping machinery. Reclaimed sites had been leveled by earthmoving machinery before the tree seedlings were planted at 10 000 seedlings per ha. No additional reclamation measures were performed. The age of each site was considered to be the time since the major disturbance (i.e., heaping in the case of the unreclaimed sites and leveling in the case of the reclaimed sites) and was determined based on historical data supplied by the coal mining company. All sites ranged from 22 to 33 years in age (Frouz et al., 2009).

2.2. Sample collection

The samples were collected in late October 2007 (immediately after litter fall), in April 2008 (shortly after snowmelt) and in August 2008. Six spatially independent subsamples (soil cores of 45-mm diameter) were collected from each study site at the patches of litter without understory vegetation except for the *Alnus* sites. The samples were transported to the laboratory, the soil cores were separated into the litter including the fermentation layer and the remaining soil to a depth of 5 cm, and these subsamples were homogenized by cutting the litter into 0.25 cm^2 pieces or sieving the soil through a 2-mm mesh. Samples for the enzyme activity assays were stored at 4 °C until analyzed (within one week); samples for PLFA analysis were processed immediately. The elemental composition of the soil was determined in three independent samples per site using an elemental analyzer (NC 2100 Soil Analyzer, Thermo-Quest Italia). Two types of litter were quantified: woody litter and herbaceous litter. To measure the litter input from woody vegetation, the litter fall was collected using nylon sacks of 0.5 mm mesh size, fixed on an iron frame of 0.5 × 0.5 m erected above the soil surface. The collectors were exposed for a whole year, and the litter was collected at the end of December. To estimate the herbaceous litter input, six quadrates (0.25 × 0.25 m) were harvested in each plot to estimate the aboveground herbaceous biomass in August, which is the period of maximal development of the herbaceous vegetation. The total annual litter input was estimated as the sum of the woody litter input and the herbaceous layer biomass during the period of maximum vegetation development.

Sample pH was measured in soil water extract (1 g soil and 10 mL deionized water were mixed and left to stand overnight at room temperature), and the soil moisture content was assessed by drying the soil at 85 °C until a constant mass was reached. Soil moisture content was 0.38 ± 0.09 in April, 0.30 ± 0.10 in August and 0.33 ± 0.05 in October; litter moisture content was 0.54 ± 0.08 in April, 0.48 ± 0.14 in August and 0.45 ± 0.09 in October.

2.3. Enzyme assays

For the exo-cleaving hydrolytic enzyme assays, 0.5 g of fresh soil or litter was homogenized in 50 mL of 50 mM sodium acetate buffer (pH 5.0) using an UltraTurrax (IKA Labortechnik, Germany) for 3 min at 8000 rev min^{-1} in an ice bath. The homogenate was used as a sample (Šturšová and Baldrian, 2011). The activities of arylsulfatase (EC 3.1.6.1), 1,4-α-glucosidase (EC 3.2.1.20), cellobiohydrolase (CBH; EC 3.2.1.91), 1,4-β-glucosidase (EC 3.2.1.21), 1,4-β-xylosidase (EC 3.2.1.37), N-acetylglucosaminidase (NAGase, EC 3.2.1.30), phosphomonoesterase (PME, EC 3.1.3.2), alanine aminopeptidase (EC 3.4.11.12) and leucine aminopeptidase (EC 3.4.11.1) were then measured using 4-methylumbelliferol- (MUF) or 7-amido-4-methylcoumarin- (AMC) based substrates as described previously (Baldrian, 2009). The substrates (40 μL in DMSO) at a final concentration of 500 μM were combined with three technical replicates of 200 μL of extracts in a 96-well plate. For the background fluorescence measurement, 200 μL of 50 mM sodium acetate buffer (pH 5.0) was combined with 40 μL of MUF standards to correct the results for fluorescence quenching. The multiwell plates were incubated at 40 °C, and the fluorescence was recorded from 5 min to 125 min using a microplate reader (Infinite, TECAN, Austria) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The quantitative enzymatic activities after blank subtraction were calculated based on a standard curve of MUF or AMC. One unit of enzyme activity was defined as the amount of enzyme releasing 1 nmol of MUF or AMC per min and expressed per g soil dry mass.

To analyze the activity of endo-cleaving polysaccharide hydrolases and ligninolytic enzymes, homogenized samples were extracted at 4 °C for 2 h on an orbital shaker (100 rpm) with 100 mM phosphate buffer, pH 7 (16:1 w/v), filtered through Whatman #5 filter paper and desalted using PD-10 desalting columns (Pharmacia, Sweden) according to the supplier's protocol to remove inhibitory small-molecular-mass compounds (Baldrian, 2009). Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid in citrate–phosphate (100 mM citrate, 200 mM phosphate) buffer, pH 5.0, at 420 nm (Bourbonnais and Paice, 1990). Manganese peroxidase (MnP, EC 1.11.1.13) was assayed in succinate–lactate buffer (100 mM, pH 4.5). 3-Methyl-2-benzothiazolinone hydrazone and 3,3-dimethylaminobenzoic acid were oxidatively coupled by the enzymes, and the resulting purple indamine dye was detected spectrophotometrically at 595 nm (Ngo and Lenhoff, 1980). The results were corrected for using the activities of the samples without manganese (for MnP); the addition of manganese sulfate was substituted by an equimolar amount of ethylenediaminetetraacetic acid (EDTA). Endo-1,4-β-glucanase (endocellulase; EC 3.2.1.4) and endo-1,4-β-xylanase (EC 3.2.1.8) activities were measured with azo-dyed carbohydrate substrates (carboxymethyl cellulose and birchwood xylan, respectively) using the protocol of the supplier (Megazyme, Ireland). The reaction mixture contained 0.2 mL of 2% dyed substrate in 200 mM sodium acetate buffer (pH 5.0) and 0.2 mL of the sample. The reaction mixture was incubated at 40 °C for 60 min, and the reaction was stopped by adding 1 mL of ethanol followed by vortexing for 10 s and centrifuging for 10 min at 10 000 × g (Baldrian, 2009). The amount of released dye was measured at 595 nm, and the enzyme activity was calculated according to standard curves correlating the dye release with the release of reducing sugars. One unit of enzyme activity was defined as the amount of enzyme releasing 1 mmol reducing sugars per minute. Enzyme activity was expressed per g soil dry mass.

2.4. Quantification of microbial biomass

The samples for phospholipid fatty acid (PLFA) analysis were extracted by a mixture of chloroform-methanol-phosphate buffer (1:2:0.8) according to Bligh and Dyer (1959). Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck), and the samples were subjected to mild alkaline methanolysis as described previously (Šnajdr et al., 2008). The free methyl esters of phospholipid fatty acids were analyzed using gas chromatography–mass spectrometry (Varian 3400; ITS-40, Finnigan). Fungal biomass was quantified based on the 18:2ω6,9 content (PLFAF), and bacterial biomass was quantified as a sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 (PLFAB). The fatty

acids found in both bacteria and fungi, 15:0, 16:0 and 18:1ω7, were excluded from the analysis. The relative content of individual PLFA molecules was also calculated. The total content of all PLFA molecules (PLFAT) was used as a measure of total microbial biomass. The fungal/bacterial biomass (F/B) ratio was calculated as PLFAF/PLFAB.

2.5. Statistics

Statistical analyses were performed using the software package Statistica 7 (StatSoft, USA). Differences among soil horizons were tested by the Wilcoxon paired test. Differences among treatments were tested using one-way analysis of variance (ANOVA) followed by the Fisher LSD *post-hoc* test. To study the effects of trees, seasons and their interactions on enzyme activities and microbial community composition, two-way ANOVA was used; general linear regression models (GLM, Statistica 7, StatSoft USA) were used when soil moisture content was used as an additional explanatory variable. Dunnett test was used to evaluate the significance of differences in enzyme activities and microbial biomass between the spontaneous succession sites (S) and revegetated sites. In all cases, differences of $P < 0.05$ were regarded as statistically significant. Principle component analyses (PCAs) were performed on the dataset of enzyme activities from all sampling times to visualize the complex differences in enzyme activities among tree species and seasons; the relative content of PLFA was also analyzed using PCA with litter and soil chemistry data as additional explanatory variables.

3. Results

3.1. Effects of woody vegetation on soil properties and enzyme activity in litter and soil

Litter and soil under different trees exhibited variable physicochemical properties. This variation was most apparent for the pH, which ranged between 4.6 and 6.2 in the litter. The litter was the most acidic under *Larix*, and spontaneous succession sites had the highest litter pH. The litter horizons also differed widely in N content, which ranged from 0.8% under *Pinus* to 2.1% under *Alnus* (Table 1). The pH in soils was more homogeneous and higher than in the litter, typically ranging between 6.6 and 7.2 except at *Larix* sites, where it was only 5.5. The differences in the N content were less pronounced than in the litter. The soil properties at all sites showed significant differences from the initial values of deposited material as reported by Krištůfek et al. (2005). During site development, the pH decreased from a high initial value of 7.6, carbon accumulated to a 4–7 times higher content and soil N content increased by 30–140% (Table 1). Interestingly, both litter and soil exhibited only very low variation in P content. The P content did not change substantially from the original value in the deposited material, and the C/P ratio in the soil was substantially lower than in the litter.

The microbial biomass varied substantially among trees and seasons but was always higher in the litter horizon than in the soil. Between 100 and 400 ng g^{-1} of PLFAT with maxima of up to 1000 ng ng^{-1} were typical for the litter horizon, while 15–50 ng g^{-1} PLFAT were recorded in the soil. The highest microbial biomass content was recorded in October in the litter horizon and in August in the soil (Fig. 1). The differences in PLFAT between the litter and the soil were mainly due to fungi. The content of PLFAF was 50–100 ng g^{-1} in the litter but only 2–10 ng g^{-1} in the soil. The differences in PLFAB content were much less pronounced; the PLFAB content in the litter was only 2–3 fold higher than that in the soil (Fig. 1). In terms of the tree species, the highest microbial biomass was found in the litter under *Alnus* and *Tilia* and in the soil under succession and *Pinus* (in October). *Picea* showed the lowest PLFAT content of all trees. The F/B ratio was quite consistent among different trees with the exception of soil under *Alnus* and *Larix* trees, in which bacteria were much more abundant than fungi. The relative content of fungi always decreased from the litter to the soil.

Higher activity of hydrolytic enzymes was always recorded in the litter than in the soil. The ratios of activity in the litter and soil ranged from <5 in the case of arylsulfatase and <10 for alanine aminopeptidase and phosphomonoesterase to >20 for cellobiohydrolase and α-glucosidase. The differences among horizons were typically highest in April and lowest in August.

PCA showed that enzyme activity in both horizons varies considerably among study sites and is shaped by the combined effects of tree species and season. The effects of trees, tested as the differences of PC1 and PC2 loads by ANOVA are statistically significant in both the litter and soil (Fig. 2). The differences among trees and seasons were more pronounced in the litter where the first canonical axis separated the samples based on the activity of most enzymes that correlated strongly negatively with the PC1. Only the alanine and leucine aminopeptidases showed stronger association with the PC2. The first axis also weakly correlated with the PLFAB and PLFAF concentrations, while the samples with different F/B ratios were separated along the second axis. The litter samples from *Alnus* sites showed high activity for most enzymes except the aminopeptidases, while these from *Picea* and *Pinus* sites

Table 1
Properties of litter and soil input at post-mining sites revegetated by different trees (October). A – *Alnus*, L – *Larix*, Pc – *Picea*, Pn – *Pinus*, Q – *Quercus*, S – succession, T – *Tilia*. The means and standard errors of four replicate sites per tree are shown. Statistically homogeneous values are marked by the same letter ($P < 0.05$).

		pH	C_{ox} (%)	N (%)	P (ppm)	C:N	Litter input (g $m^{-2}y^{-1}$)
Litter	A	5.44 ± 0.16bc	82.4 ± 2.3a	2.05 ± 0.13a	130 ± 24a	40.5 ± 1.9c	480 ± 120a
	L	4.63 ± 0.31d	59.2 ± 3.5b	1.21 ± 0.13b	127 ± 7a	49.5 ± 2.3bc	216 ± 14b
	Pc	5.65 ± 0.20ab	66.2 ± 2.6b	0.95 ± 0.03bc	134 ± 10a	70.0 ± 4.4ab	206 ± 71b
	Pn	4.84 ± 0.41cd	65.5 ± 5.4b	0.79 ± 0.06c	123 ± 4a	83.6 ± 6.5a	354 ± 64ab
	Q	5.42 ± 0.05bc	79.7 ± 4.4a	1.05 ± 0.16bc	125 ± 7a	80.2 ± 11.0a	246 ± 58b
	S	6.23 ± 0.27a	82.5 ± 4.5a	1.22 ± 0.20bc	134 ± 11a	73.7 ± 13.2a	238 ± 55b
	T	6.06 ± 0.17ab	84.2 ± 2.3a	1.07 ± 0.08bc	130 ± 17a	79.6 ± 4.7a	188 ± 19b
Soil	A	6.62 ± 0.16a	16.2 ± 1.9c	0.54 ± 0.10ab	14 ± 2a	34.1 ± 7.4c	
	L	5.49 ± 0.51b	25.6 ± 1.7a	0.49 ± 0.08ab	11 ± 2a	54.9 ± 6.5ab	
	Pc	7.22 ± 0.24a	22.7 ± 2.2ab	0.39 ± 0.05b	14 ± 1a	59.7 ± 4.0ab	
	Pn	6.59 ± 0.34a	20.8 ± 2.0abc	0.35 ± 0.07b	11 ± 1a	62.6 ± 6.7a	
	Q	6.84 ± 0.20a	18.7 ± 3.5bc	0.44 ± 0.01ab	11 ± 0a	42.3 ± 7.9bc	
	S	6.93 ± 0.22a	22.3 ± 1.7abc	0.44 ± 0.08ab	11 ± 2a	54.2 ± 7.4ab	
	T	6.75 ± 0.47a	25.6 ± 0.9a	0.62 ± 0.06a	14 ± 2a	41.9 ± 2.7bc	
	I[a]	7.57 ± 0.04	3.7 ± 1.6	0.26 ± 0.03	12 ± 1	13.9 ± 3.9	

[a] I – initial substrate deposited on the heaps; data from Krištůfek et al. (2005).

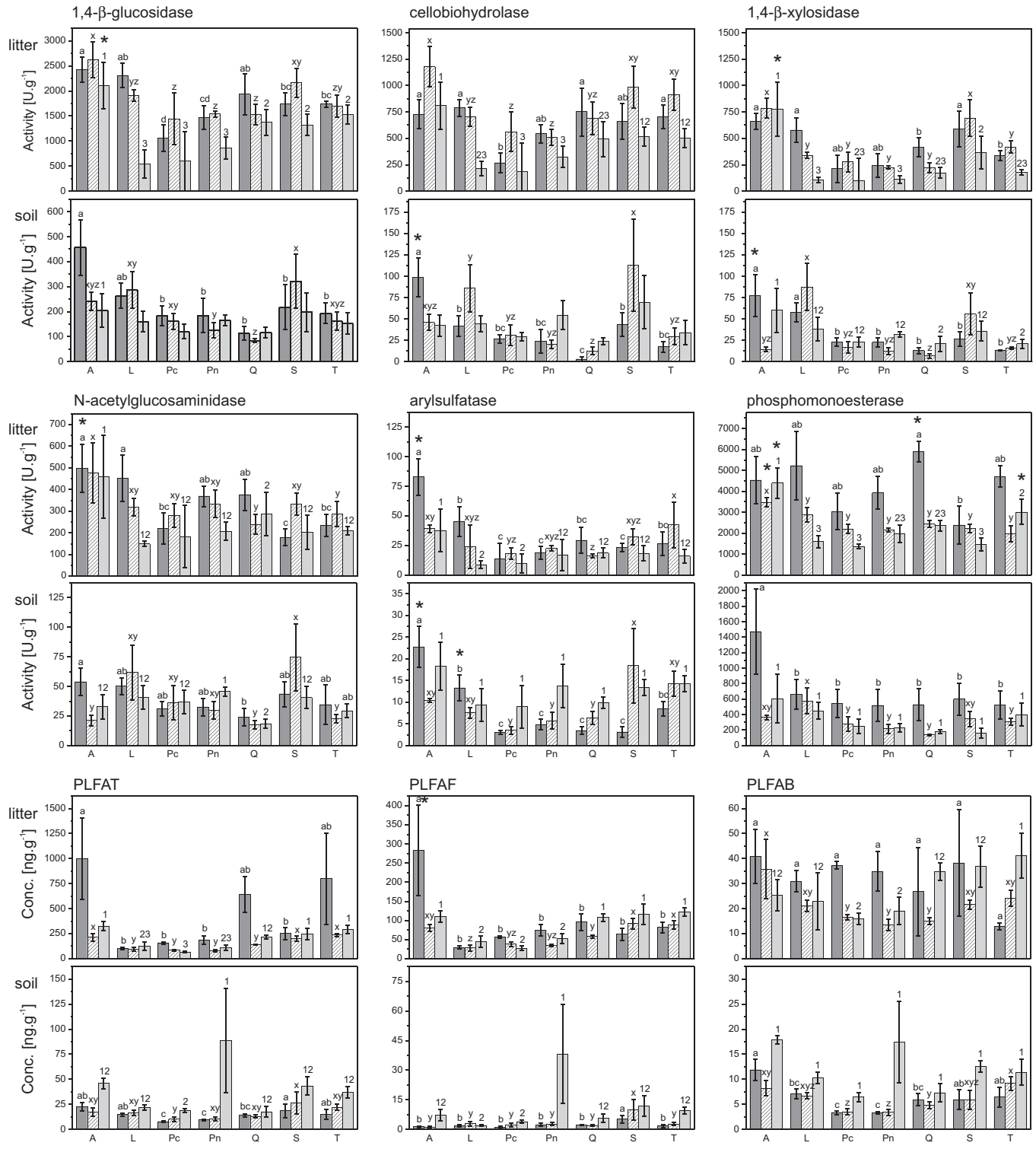


Fig. 1. Enzyme activities and microbial biomass content in the litter and soil from post-mining sites revegetated by different trees. Sampling season: October – dark gray, April – hatched, August – light gray. A – *Alnus*, L – *Larix*, Pc – *Picea*, Pn – *Pinus*, Q – *Quercus*, S – succession, T – *Tilia*. The means and standard errors of four replicate sites per tree are shown. Statistically homogeneous values of individual properties within a season are marked by the same letter (Fisher LSD, $P < 0.05$); values in revegetated sites significantly higher than in the spontaneous succession sites (S) are indicated with an asterisk (Dunnett test, $P < 0.05$). PLFAT – Total PLFA, PLFAF – fungi-specific PLFA, PLFAB – bacteria-specific PLFA.

typically showed lower activity of hydrolytic enzymes (Fig. 1, Table 2). Correlation analysis of data from the October sampling showed that in the litter, enzyme activity under individual trees is mediated by the amount of N in the litter and also by the C/N ratio, while the effect of pH is of minor importance. β-glucosidase, NAGase, arylsulfatase, β-xylosidase and leucine aminopeptidase activity all significantly increased with increasing N content and decreased with the C/N ratio, while pH only affected NAGase

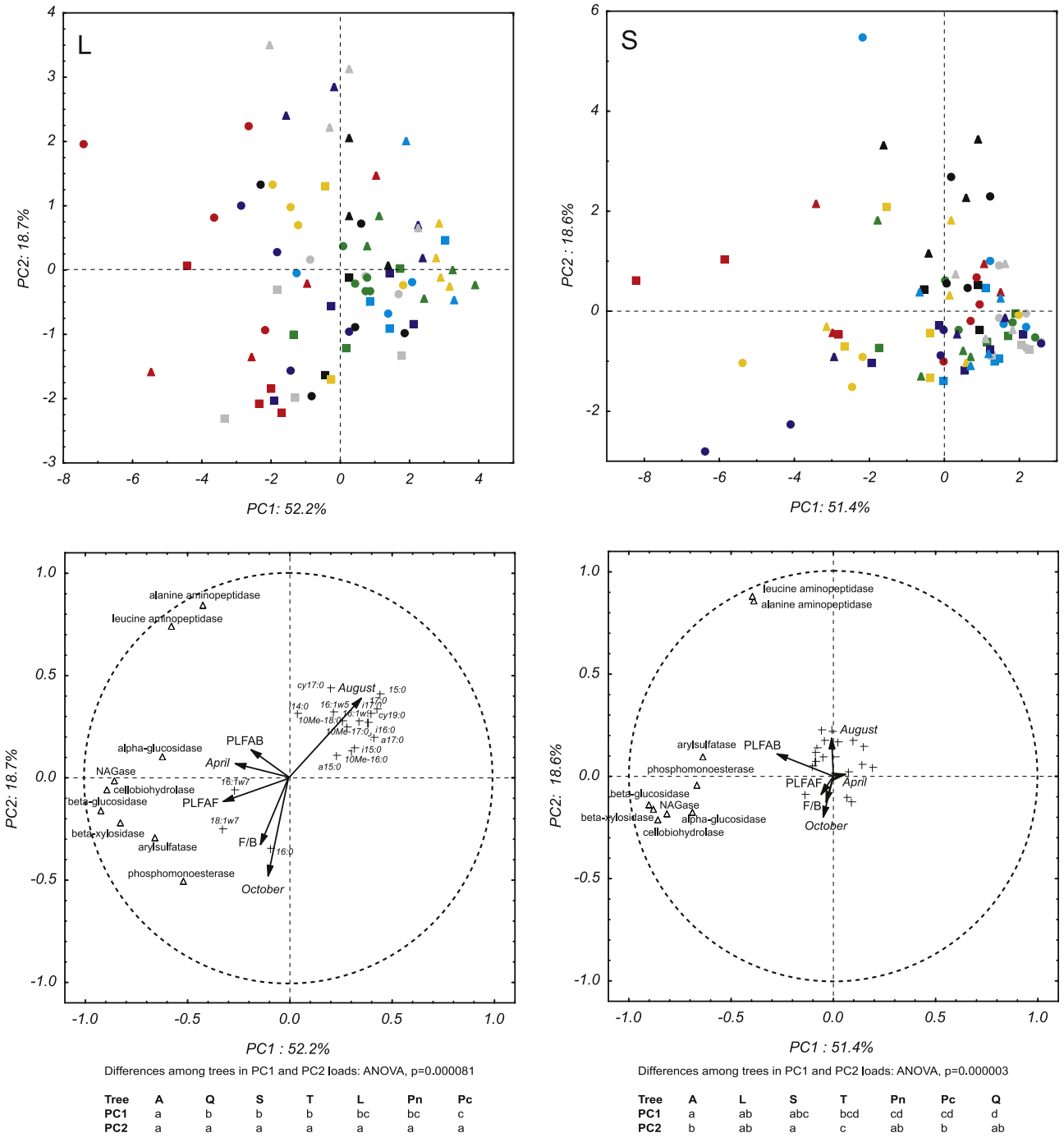


Fig. 2. Principal component analysis of enzyme activities in the litter and soil from post-mining sites revegetated by different trees. Sampling season: October – squares, April – circles, August – triangles. *Alnus* – red, *Larix* – yellow, *Picea* – light blue, *Pinus* – green, *Quercus* – gray, succession – dark blue, *Tilia* – black. The effects of individual enzymes, seasons, microbial biomass and relative contents of individual PLFA molecules are shown. Differences of PC1 and PC2 loads among trees were tested by one-way ANOVA and a Fisher LSD post-hoc test. Significant differences in PC loads among trees are marked with different letters ($P < 0.05$). L – litter, S – soil.

activity, which increased with increasing pH. The sites undergoing spontaneous succession generally showed comparable levels of enzyme activity as revegetated sites. Activity of certain enzymes was significantly higher at sites revegetated with *Alnus*, but none of the increased activities was consistently present in all seasons (Fig. 1).

The soil samples showed less distinct patterns of enzyme activity associated with trees or seasons than the litter samples in the PCA (Fig. 2). Again, the activity of most hydrolases and the bacterial PLFA content were associated with the first axis, while the activity of the aminopeptidases was associated with the second axis. The effects of fungal biomass content and seasons were much less pronounced then in the litter. Among individual trees, soils under *Quercus* typically showed low enzyme activity (Fig. 1). Only arylsulfatase responded significantly to the soil N content and increased with increasing N; β-xylosidase activity decreased with pH. Compared to the litter, the effects of seasons were less important.

Table 2
Activities of oxidative enzymes and endo-cleaving hydrolases in the litter from post-mining sites revegetated by different trees. A – *Alnus*, L – *Larix*, Pc – *Picea*, Pn – *Pinus*, Q – *Quercus*, S – succession, T – *Tilia*. The means and standard errors of four replicate sites per tree are shown. Statistically homogeneous values of individual properties within a season are marked by the same letter ($P < 0.05$).

		October	April	August
Laccase	A	7.1 ± 0.9a	23.4 ± 6.2a	4.9 ± 1.8bc
	L	18.7 ± 6.1a	22.3 ± 4.8a	15.7 ± 4.3c
	Pc	11.7 ± 7.4a	25.1 ± 5.5a	9.2 ± 2.2c
	Pn	18.3 ± 7.4a	47.4 ± 13.4a	39.3 ± 7.5ab
	Q	8.2 ± 2.3a	148.3 ± 101.9a	64.2 ± 18.2a
	S	16.1 ± 2.4a	26.1 ± 4.0a	12.6 ± 4.2b
	T	19.6 ± 6.9a	23.5 ± 4.9a	52.1 ± 13.6ab
Mn-peroxidase	A	10.0 ± 2.0ab	18.0 ± 13.8a	5.3 ± 0.5a
	L	5.4 ± 2.8ab	11.8 ± 4.8a	2.8 ± 0.1ab
	Pc	4.0 ± 2.0ab	4.8 ± 4.3a	1.7 ± 1.4ab
	Pn	14.9 ± 10.1a	14.2 ± 4.6a	5.1 ± 1.1a
	Q	8.8 ± 2.8ab	5.4 ± 2.7a	4.4 ± 1.3ab
	S	0.3 ± 0.2b	2.0 ± 1.4a	2.0 ± 0.4b
	T	2.6 ± 1.6ab	2.5 ± 0.9a	3.8 ± 1.0ab
Endocellulase	A	23.3 ± 3.0a	58.9 ± 16.7a	24.0 ± 2.1b
	L	28.5 ± 13.7a	32.6 ± 4.4ab	22.2 ± 3.4b
	Pc	29.2 ± 7.4a	40.1 ± 5.4ab	18.7 ± 4.6b
	Pn	34.0 ± 12.5a	49.4 ± 7.2ab	47.5 ± 10.0a
	Q	26.1 ± 7.7a	37.9 ± 5.4ab	26.5 ± 8.0b
	S	8.0 ± 1.6a	22.4 ± 5.0b	10.3 ± 2.5b
	T	9.8 ± 2.0a	21.9 ± 4.2b	6.9 ± 3.6b
Endoxylanase	A	73.8 ± 6.3a	42.4 ± 5.7bc	43.2 ± 8.9bc
	L	130.8 ± 91.6a	48.6 ± 9.8bc	62.6 ± 7.2bc
	Pc	91.3 ± 39.4a	54.8 ± 6.8ab	89.4 ± 51.2bc
	Pn	112.8 ± 48.8a	91.4 ± 5.2a	199.8 ± 71.3ab
	Q	70.1 ± 6.1a	55.2 ± 20.8b	265.9 ± 87.8a
	S	13.8 ± 5.6a	31.4 ± 1.4bc	36.7 ± 14.1c
	T	18.5 ± 9.4a	16.8 ± 4.5c	32.0 ± 6.0c

Because the chemical composition of the litter and soil are largely mediated by the tree species used for recultivation (Table 1), only the effects of tree, season and moisture content were used as independent variables to explain the observed differences in enzyme activities among sites. Of these variables, moisture content showed little variation among sites and its effect was only mediated as a part of the seasonal effect. Only the effects of tree, season and their interaction were thus quantified (Table 3). In the litter, effects of tree were significant for all enzymes except α-glucosidase and alanine aminopeptidase and all enzymes were significantly affected by tree in at least one season. The season effects were important for all enzymes. In the soil, effects of tree were significant for all enzymes except N-acetylglucosaminidase, but the tree effect on enzyme activities varied seasonally. Seasonal effects were only significant in the case of phosphomonoesterase (Table 3).

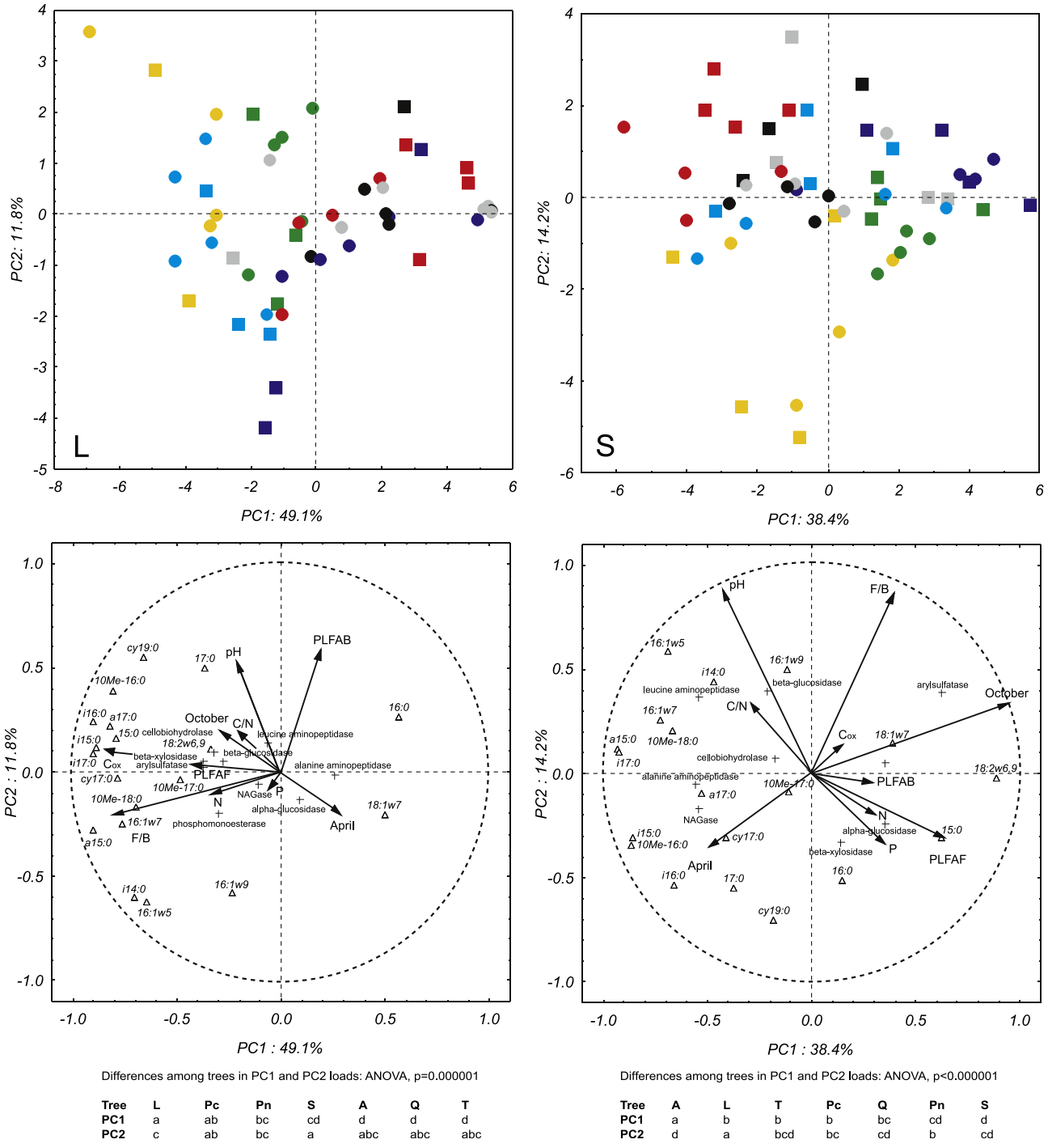
3.2. Microbial community composition at revegetated sites

The PC analysis of microbial biomass showed that the composition of microbial communities differed among trees and also exhibited seasonal differences (Fig. 3). The latter were more important in the soil than in the litter. The effects of trees, tested as the differences of PC1 and PC2 loads by ANOVA were statistically significant in both the litter and soil. Litter samples from coniferous tree sites were separated from those of most hardwood trees along the first canonical axis, and these differences were mostly due to the C content and fungal biomass. The PLFAB and pH correlated with the second axis. In the soil, the first axis separated samples with a high relative content of fungal PLFA from those that were more rich in bacterial PLFA. The pH correlated most strongly with the second canonical axis. Among the chemical parameters contributing to the composition of soil microbial communities, the N content was equally important in the litter and soil, while the pH and P content were more important in the soil.

In the litter, both the effects of tree and season significantly affected the relative content of the fungal PLFA as well as bacteria-specific PLFA and all PLFA molecules. Tree effect was also significant in each individual sampling season except fungal PLFA in October. In the soil, tree effect was significant for PLFAF, PLFAB and PLFAT

Table 3
Probabilities of significance of the effects of tree and season on enzyme activity and relative abundance of PLFA molecules in the litter and soil at post-mining sites revegetated by different trees (two-way ANOVA) and significance of tree effect in all seasons. Values with $P > 0.05$ were regarded as statistically insignificant and are indicated as "ns".

	Enzyme/PLFA	Effects of tree and season			Tree effect in seasons		
		Tree	Season	Tree × season	October	April	August
Litter	β-glucosidase	0.0000	0.0000	ns	0.0062	0.0063	0.0001
	α-glucosidase	ns	0.0001	0.0200	ns	ns	0.0203
	cellobiohydrolase	0.0000	0.0000	ns	ns	0.0067	0.0061
	β-xylosidase	0.0000	0.0004	ns	0.0124	0.0001	0.0002
	N-acetylglucosaminidase	0.0004	0.0285	ns	0.0049	ns	ns
	Arylsulfatase	0.0000	0.0007	0.0096	0.0000	ns	ns
	Phosphomonoesterase	0.0020	0.0000	ns	ns	0.0050	0.0012
	Alanine aminopeptidase	ns	0.0011	0.0060	ns	ns	0.0427
	Leucine aminopeptidase	0.0007	0.0146	0.0148	ns	0.0097	0.0258
	All enzymes	0.0000	0.0000	0.0001	0.0011	0.0001	0.0001
	Fungi-specific PLFA	0.0010	0.0000	0.0209	ns	0.0011	0.0006
	Bacteria-specific PLFA	0.0000	0.0000	0.0000	0.0003	0.0000	0.0070
	All PLFA	0.0000	0.0000	0.0000	0.0016	0.0001	0.0489
Soil	β-glucosidase	0.0073	ns	ns	0.0454	ns	ns
	α-glucosidase	0.0041	ns	ns	0.0024	ns	ns
	Cellobiohydrolase	0.0019	ns	ns	0.0010	ns	ns
	β-xylosidase	0.0009	ns	0.0235	0.0027	0.0051	ns
	N-acetylglucosaminidase	ns	ns	ns	ns	ns	ns
	Arylsulfatase	0.0181	ns	0.0231	0.0000	ns	ns
	Phosphomonoesterase	0.0321	0.0016	ns	ns	0.0491	ns
	Alanine aminopeptidase	0.0443	ns	ns	ns	ns	0.0109
	Leucine aminopeptidase	0.0051	ns	ns	ns	ns	ns
	All enzymes	0.0000	0.0034	ns	0.0006	0.0009	ns
	Fungi-specific PLFA	0.0000	ns	ns	0.0248	0.0079	0.0072
	Bacteria-specific PLFA	0.0000	0.0000	0.0191	0.0258	0.0004	0.0213
	All PLFA	0.0000	0.0000	0.0148	ns	0.0032	0.0150



Differences among trees in PC1 and PC2 loads: ANOVA, p=0.000001

Tree	L	Pc	Pn	S	A	Q	T
PC1	a	ab	bc	cd	d	d	d
PC2	c	ab	bc	a	abc	abc	abc

Differences among trees in PC1 and PC2 loads: ANOVA, p<0.000001

Tree	A	L	T	Pc	Q	Pn	S
PC1	a	b	b	b	bc	cd	d
PC2	d	a	bcd	bc	cd	b	cd

Fig. 3. Principal component analysis of microbial biomass composition (relative content of individual PLFA molecules) in the litter and soil from post-mining sites revegetated by different trees. Sampling season: October – squares, April – circles. *Alnus* – red, *Larix* – yellow, *Picea* – light blue, *Pinus* – green, *Quercus* – gray, succession – dark blue, *Tilia* – black. The effects of individual enzymes, seasons, microbial biomass and chemical properties are shown. Differences of PC1 and PC2 loads among trees were tested by one-way ANOVA and a Fisher LSD post-hoc test. Significant differences in PC loads among trees are marked with different letters ($P < 0.05$). C_{ox} – oxidizable carbon.

while season only affected PLFAB and PLFAT. Significant effects of tree on the relative content of fungal and bacterial PLFA was also observed in each season separately (Table 3).

4. Discussion

During soil development on barren soils, microbial biomass increases as a consequence of organic matter accumulation. Along with this increase, the microbial community composition has also been demonstrated to change during spontaneous succession at post-mining sites in the area of this study, and enzyme activities have been shown to increase during early succession (Baldrian et al., 2008). When the soils are subject to revegetation, their development is largely influenced by the dominant tree species, including the effects on the quality and quantity of litter and soil organic matter.

At our study sites, carbon storage in the top 20 cm of soil organic matter varied among trees from 4.5 to 38.0 t ha^{-1}, and the rate of C accumulation in the soil organic matter varied between 0.15 and 1.28 t ha^{-1} $year^{-1}$, increasing in the order succession < *Picea* < *Pinus*, *Quercus* < *Larix* < *Alnus* < *Tilia*. Carbon storage in the soil was positively correlated with aboveground tree biomass, but no significant correlation was found between C storage in the soil and aboveground litter input (Frouz et al., 2009) due to different rates of litter mineralization and differences in C allocation belowground via photosynthate flow and bioturbation. The dominant tree also influences the soil via its effects on the understory vegetation, with understory cover ranging from 16% in *Tilia* to 51% in *Pinus* and *Quercus*; under *Alnus*, vegetation covers 100% of the soil surface (Mudrák et al., 2010).

For this study, samples were taken under patches of litter without understory vegetation except for the *Alnus* site. The litter horizon was thus mostly affected by the chemical composition of tree litter representing the dominant source of nutrients. The fresh litter of the trees from this study differed substantially in N content (4–29 mg g^{-1}) as well as the content of P or lignin (Voříšková et al., 2011).

Although the litter horizons comprise materials deposited over long periods of time, substantial variation in litter horizon chemistry among trees was recorded in this study, especially in the pH and N content. The carbon quality and litter N and P contents have been experimentally demonstrated to control the quality of dissolved organic matter and consequently litter mass loss under environmental conditions (Hättenschwiler and Jørgensen, 2010; Kiikkila et al., 2006; Sariyildiz et al., 2005) as well as during decomposition by an individual fungus (Voříšková et al., 2011). The lower activity of enzymes in the litter of evergreen trees with low N content observed in this study was the most probable reason for the high accumulation of organic matter under these trees. Indeed, the mass of the litter and the fermentation layer under *Picea* and *Pinus* ranged from 1.0 to 1.4 kg m^{-2} and was higher than that under deciduous trees (0.03–0.34 kg m^{-2}) (Frouz et al., 2009), indicating accumulation of C from non-decomposed litter.

The litter and upper soil differ substantially in microbial biomass content due to the higher content and quality of organic matter in the litter than in the soil. The differences in the nutrient availability are demonstrated by the decrease of extracellular enzyme activity with soil depth as well as the relative decrease of saprotrophic fungi and increase in symbiotic mycorrhizal fungi with soil depth (Baldrian et al., 2012; Lindahl et al., 2007; Šnajdr et al., 2008). Thus, microbial community composition should be theoretically a better predictor of enzyme activity in litter than in soil, as was observed in this study; while the effects of tree and season on microbial community composition were comparable in both horizons (Fig. 3), enzyme activities were more strongly affected by the vegetation in the litter than in the soil (Fig. 2).

Despite the longer residence time, the nutrient content and pH in soils under different trees still varied to a similar extent as in the litter horizon (Table 1). This finding is in agreement with earlier observations that the tree effect on soil OM quality is superior to other effects, including that of the climate (Quideau et al., 2001), although the differences in soil pH and N content observed in other studies were lower, likely due to the longer site development time (Binkley and Valentine, 1991; Niemi et al., 2007). Among forest soil properties, pH was previously identified as the most important factor affecting enzyme activity (Štursová and Baldrian, 2011), but the effect of tree species on soil pH is often low (Chodak and Niklinska, 2010a, b; Niemi et al., 2007). Here, we observed particularly low pH in soils under *Larix*, but the PCA did not show any particular effect of this tree on enzyme activity (Fig. 2). Previous papers reporting on the effects of trees on enzyme activity in soils are unfortunately based on data from non-replicated stands and do not sufficiently address seasonal differences (Baum and Hrynkiewicz, 2006; Chodak and Niklinska, 2010a, b; Niemi et al., 2007; Selmants et al., 2005). Generally, only the activity of certain enzymes was found to be affected by trees depending on the study setup and the ecosystem explored. Here, we show that tree species is the most important explanatory variable for the activity of all tested hydrolytic enzymes both in the litter and the soil, explaining as much as 25–35% of the total variation, although not all pairs of tree species are significantly different. Most of this effect is independent of the season, but the interaction of tree and season is also significant for certain enzymes (Table 3). Interestingly, soil moisture content, which was previously demonstrated to be an important factor affecting enzyme activity and litter decomposition rate (Baldrian et al., 2010; Dilly and Munch, 1996), did not significantly affect enzyme activity or microbial community composition in this study, likely due to the relatively low seasonal and spatial variation in moisture content.

The compositions of microbial communities associated with litters and soils under different trees have been previously reported to differ (Grayston and Prescott, 2005; Osono and Takeda, 2007; Priha et al., 2001; Söderberg et al., 2002), and these structural differences were more important than differences in function estimated by community-level physiological profiling (Priha et al., 2001; Söderberg et al., 2002). Previous studies have also indicated that the tree effect on soil microbes was often mediated by soil chemistry. Fungal biomass typically increased with the C/N content, while the relative abundance of bacteria increased with pH (Grayston and Prescott, 2005; Högberg et al., 2007). During spontaneous succession, also considering the sites of this study, bacterial community composition developed gradually with no dramatic transitions caused by the change of dominant vegetation. The most important variables correlating with bacterial community composition were pH and C content (Urbanová et al., 2011). In this study, however, the response of soil microbial community composition to the environmental variables was relatively low. In the litter, only the contents of three PLFA molecules were affected by pH; one was affected by the N content and one by the C/N ratio, and the amount of P was not significant. Neither of the variables affected the ratio of bacteria to fungi. The total fungal biomass (but not bacterial biomass) increased with the litter N content. This observation extends the earlier finding of the positive effect of litter N on fungal biomass production from a single-species level (Voříšková et al., 2011) to the level of the whole community. The effects of chemistry on microbial communities in the soil were stronger than in the litter (Fig. 3). The N content and C/N ratio, which varied substantially among trees, affected four and one PLFA molecules, respectively, and unlike in the litter, total bacterial biomass (but not fungal biomass) increased with increasing N. The pH and P content had significant effects on five and four PLFA molecules, respectively, but these effects are unlikely to explain the differences among trees because the variation in soil P content and pH was largely independent from trees.

Despite the limited effects of soil chemistry, the tree effect explained most of the variation in the microbial community composition, and the tree × season interaction was also important in the litter horizon (Table 3). It is likely that these vegetation-dependent effects are either due to differences in finer soil chemistry, e.g., the quality of C, or due to specific effects of tree roots on the rhizosphere microorganisms, factors not considered in this study. The PLFA molecules 16:1ω5 and 18:2ω6,9, previously reported as markers for arbuscular mycorrhizal fungi and total fungi, respectively, contributed substantially to the PCA separation of soil samples among individual trees (Fig. 3). While 16:1ω5 was highly

abundant under *Alnus* with a dense understory of herbs and grasses possessing AM fungi, 18:2ω6,9 was more abundant under *Pinus* and in the succession sites where an herbal layer and AM fungi were virtually missing.

Specific microbes are not only associated with individual trees but may also contribute to the formation of soil traits by supplying N and P to plants and thus affecting plant production and litter quality. In temperate forests, up to 75% of N and P is supplied to plants via N-fixing bacteria or symbiotic microbes efficient in N acquisition, e.g., the mycorrhizal fungi (van der Heijden et al., 2008). Among the trees considered in this study, the association of *Alnus* with symbiotic N_2-fixing bacteria is the ultimate cause of high N content in its litter (Ekblad and HussDanell, 1995; Selmants et al., 2005). The N-rich litter of *Alnus* is also likely the cause of the lowest C/N ratio in soils under *Alnus*. Relatively high N content under *Tilia* is likely due to the high abundance of earthworms burrying the litter thus increasing both the C and N allocation belowground (Frouz et al., 2009; Table 1).

Differences in enzyme production under individual trees show only limited effects of microbial community composition (Fig. 2) mainly in the litter, where cellobiohydrolase, β-glucosidase and β-xylosidase respond to the relative content of the fungal PLFA 18:2ω6,9. Differential expression due to the differences in substrate pH and C/N ratio is probably more important. The observed effects of the litter and soil C/N contents on several enzymes are consistent with previous results that showed that the N content in litter determines the production of the same enzymes by a single fungus, *Hypholoma fasciculare*, growing on different litter types (Voříšková et al., 2011).

Despite being subject to the dominant vegetation effects, seasonal variations in the enzyme activity and especially in the microbial community composition were also important in this study. This finding is not surprising considering that 40–90% of *A. glutinosa* litter and 50% of *Quercus petraea* litter can be decomposed within a single year (Dilly and Munch, 1996; Šnajdr et al., 2011) and that the properties of the litter horizon thus change substantially throughout the year. In the deeper soil, seasonal differences were found to mainly reflect the seasonal climatic changes and C allocation belowground, the latter factor mainly affecting the abundance of ectomycorrhizal fungi (Kaiser et al., 2010; Rasche et al., 2011). Here, the microbial community composition was demonstrated to substantially respond to seasonal effects in both horizons to an extent almost equally important to the effects of trees. The seasonal effects on enzyme activity were only important in the litter, where the quality of organic matter changes more substantially (Table 3).

In post-mining sites or other sites affected by human activity, revegetation is often considered to improve soil quality and function, but this effect may be only temporary. In soils revegetated with *Alnus* at sites also encompassing some of the sites of this study, microbial activity was higher than at the unreclaimed sites. This difference was, however, most evident at young sites (Helingerová et al., 2010). Here we show that after 22–33 years, the properties of soils undergoing spontaneous succession are comparable to revegetated sites in terms of soil nutrient content and microbial biomass as well as soil microbial activity (Table 1, Fig. 1). In all spontaneous succession series considered in the comparative study of Prach and Pyšek (2001), continuous vegetation was always formed before year 15. Additionally, the spontaneously developed sites show higher plant diversity (Hodačová and Prach, 2003; Mudrák et al., 2010), which might be desired in certain cases. The important advantage of revegetation would thus be mainly the accelerated development of vegetation in the early phases of development which may be susceptible to soil erosion (Mudrák et al., 2010).

This paper shows that dominant tree vegetation substantially affects the rates of microbial decomposition processes both in the litter and in soil. When comparing the revegetated sites with sites under spontaneous succession, the enzyme activities and microbial biomass were similar except for the elevated enzyme activity at sites revegetated with *Alnus* which may indicate similar rates of soil development at revegetated and succession sites. Spontaneous succession in temperate Europe may thus be a suitable option for land restoration.

Acknowledgments

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (OC10064, 2B8023) and by the Czech Science Foundation (P504/12/1288). We also thank the Sokolovská uhelná for the research permit.

References

Augusto, L., Ranger, J., Ponette, Q., Rapp, M., 2000. Relationships between forest tree species, stand production and stand nutrient amount. Annals of Forest Science 57, 313–324.

Baldrian, P., 2009. Microbial enzyme-catalyzed processes in soils and their analysis. Plant Soil and Environment 55, 370–378.

Baldrian, P., Trögl, J., Frouz, J., Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., Herinková, J., 2008. Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. Soil Biology & Biochemistry 40, 2107–2115.

Baldrian, P., Merhautová, V., Petránková, M., Cajthaml, T., Šnajdr, J., 2010. Distribution of microbial biomass and activity of extracellular enzymes in a hardwood forest soil reflect soil moisture content. Applied Soil Ecology 46, 177–182.

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Rídl, J., Vlček, Č., Voříšková, J., 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME Journal 6, 248–258.

Baum, C., Hrynkiewicz, K., 2006. Clonal and seasonal shifts in communities of saprotrophic microfungi and soil enzyme activities in the mycorrhizosphere of *Salix* spp. Journal of Plant Nutrition and Soil Science 169, 481–487.

Binkley, D., Valentine, D., 1991. Fifty-year biogeochemical effects of green ash, white pine, and Norway spruce in a replicated experiment. Forest Ecology and Management 40, 13–25.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911–917.

Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Letters 267, 99–102.

Bradshaw, A., 1997. Restoration of mined lands – using natural processes. Ecological Engineering 8, 255–269.

Chodak, M., Niklinska, M., 2010a. The effect of different tree species on the chemical and microbial properties of reclaimed mine soils. Biology and Fertility of Soils 46, 555–566.

Chodak, M., Niklinska, M., 2010b. Effect of texture and tree species on microbial properties of mine soils. Applied Soil Ecology 46, 268–275.

Dilly, O., Munch, J.C., 1996. Microbial biomass content, basal respiration and enzyme activities during the course of decomposition of leaf litter in a black alder (*Alnus glutinosa* (L.) Gaertn.) forest. Soil Biology & Biochemistry 28, 1073–1081.

Ekblad, A., HussDanell, K., 1995. Nitrogen fixation by *Alnus incana* and nitrogen transfer from *A. incana* to *Pinus sylvestris* influenced by macronutrients and ectomycorrhiza. New Phytologist 131, 453–459.

Finér, L., Helmisaari, H.S., Lohmus, K., Majdi, H., Brunner, I., Børja, I., Eldhuset, T., Godbold, D., Grebenc, T., Konopka, B., Kraigher, H., Möttönen, M.R., Ohashi, M., Oleksyn, J., Ostonen, I., Uri, V., Vanguelova, E., 2007. Variation in fine root biomass of three European tree species: beech (*Fagus sylvatica* L.), Norway spruce (*Picea abies* L. Karst.), and Scots pine (*Pinus sylvestris* L.). Plant Biosystems 141, 394–405.

Frouz, J., Nováková, A., 2005. Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. Geoderma 129, 54–64.

Frouz, J., Prach, K., Pižl, V., Háněl, L., Starý, J., Tajovský, K., Materna, J., Balík, V., Kalčík, J., Rehounkova, K., 2008. Interactions between soil development, vegetation and soil fauna during spontaneous succession in post mining sites. European Journal of Soil Biology 44, 109–121.

Frouz, J., Pižl, V., Cienciala, E., Kalčík, J., 2009. Carbon storage in post-mining forest soil, the role of tree biomass and soil bioturbation. Biogeochemistry 94, 111–121.

Frouz, J., Cajthaml, T., Kříbek, B., Schaeffer, P., Bartuška, M., Galertová, R., Rojík, P., Krištůfek, V., 2011. Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter. Folia Microbiologica 56, 389–396.

Grayston, S.J., Prescott, C.E., 2005. Microbial communities in forest floors under four tree species in coastal British Columbia. Soil Biology & Biochemistry 37, 1157–1167.
Grayston, S.J., Wang, S.Q., Campbell, C.D., Edwards, A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biology & Biochemistry 30, 369–378.
Hagen-Thorn, A., Armolaitis, S., Callesen, I., Stjernquist, I., 2004. Macronutrients in tree stems and foliage: a comparative study of six temperate forest species planted at the same sites. Annals of Forest Science 61, 489–498.
Hartmann, A., Schmid, M., Tuinen, D., Berg, G., 2009. Plant-driven selection of microbes. Plant and Soil 321, 235–257.
Hättenschwiler, S., Jørgensen, H.B., 2010. Carbon quality rather than stoichiometry controls litter decomposition in a tropical rain forest. Journal of Ecology 98, 754–763.
Helingerová, M., Frouz, J., Šantrůčková, H., 2010. Microbial activity in reclaimed and unreclaimed post-mining sites near Sokolov (Czech Republic). Ecological Engineering 36, 768–776.
Hodačová, D., Prach, K., 2003. Spoil heaps from brown coal mining: technical reclamation versus spontaneous revegetation. Restoration Ecology 11, 385–391.
Högberg, M.N., Högberg, P., Myrold, D.D., 2007. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia 150, 590–601.
Hooper, D.U., Bignell, D.E., Brown, V.K., Brussaard, L., Dangerfield, J.M., Wall, D.H., Wardle, D.A., Coleman, D.C., Giller, K.E., Lavelle, P., Van Der Putten, W.H., De Ruiter, P.C., Rusek, J., Silver, W.L., Tiedje, J.M., Wolters, V., 2000. Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: patterns, mechanisms, and feedbacks. BioScience 50, 1049–1061.
Iovieno, P., Alfani, A., Bååth, E., 2010. Soil microbial community structure and biomass as affected by *Pinus pinea* plantation in two Mediterranean areas. Applied Soil Ecology 45, 56–63.
Kaiser, C., Koranda, M., Kitzler, B., Fuchslueger, L., Schnecker, J., Schweiger, P., Rasche, F., Zechmeister-Boltenstern, S., Sessitsch, A., Richter, A., 2010. Belowground carbon allocation by trees drives seasonal patterns of extracellular enzyme activities by altering microbial community composition in a beech forest soil. New Phytologist 187, 843–858.
Kiikkila, O., Kitunen, V., Smolander, A., 2006. Dissolved soil organic matter from surface organic horizons under birch and conifers: degradation in relation to chemical characteristics. Soil Biology & Biochemistry 38, 737–746.
Krištůfek, V., Elhottová, D., Chroňáková, A., Dostálková, I., Picek, T., Kalčík, J., 2005. Growth strategy of heterotrophic bacterial population along successional sequence on spoil of brown coal colliery substrate. Folia Microbiologica 50, 427–435.
Lamb, E.G., Kennedy, N., Siciliano, S.D., 2011. Effects of plant species richness and evenness on soil microbial community diversity and function. Plant and Soil 338, 483–495.
Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611–620.
Menyailo, O.V., Hungate, B.A., Zech, W., 2002. Tree species mediated soil chemical changes in a Siberian artificial afforestation experiment: tree species and soil chemistry. Plant and Soil 242, 171–182.
Merilä, P., Malmivaara-Lämsä, M., Spetz, P., Stark, S., Vierikko, K., Derome, J., Fritze, H., 2010. Soil organic matter quality as a link between microbial community structure and vegetation composition along a successional gradient in a boreal forest. Applied Soil Ecology 46, 259–267.
Mudrák, O., Frouz, J., Velichová, V., 2010. Understory vegetation in reclaimed and unreclaimed post-mining forest stands. Ecological Engineering 36, 783–790.
Ngo, T.T., Lenhoff, H.M., 1980. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Analytical Biochemistry 105, 389–397.
Niemi, R.M., Vepsäläinen, M., Erkomaa, K., Ilvesniemi, H., 2007. Microbial activity during summer in humus layers under *Pinus silvestris* and *Alnus incana*. Forest Ecology and Management 242, 314–323.
Osono, T., Takeda, H., 2007. Microfungi associated with *Abies* needles and *Betula* leaf litter in a subalpine coniferous forest. Canadian Journal of Microbiology 53, 1–7.
Prach, K., Pyšek, P., 2001. Using spontaneous succession for restoration of human-disturbed habitats: experience from Central Europe. Ecological Engineering 17, 55–62.
Priha, O., Grayston, S.J., Hiukka, R., Pennanen, T., Smolander, A., 2001. Microbial community structure and characteristics of the organic matter in soils under *Pinus sylvestris, Picea abies* and *Betula pendula* at two forest sites. Biology and Fertility of Soils 33, 17–24.
Quideau, S.A., Chadwick, O.A., Benesi, A., Graham, R.C., Anderson, M.A., 2001. A direct link between forest vegetation type and soil organic matter composition. Geoderma 104, 41–60.
Rasche, F., Knapp, D., Kaiser, C., Koranda, M., Kitzler, B., Zechmeister-Boltenstern, S., Richter, A., Sessitsch, A., 2011. Seasonality and resource availability control bacterial and archaeal communities in soils of a temperate beech forest. ISME Journal 5, 389–402.
Sariyildiz, T., Anderson, J.M., Kucuk, M., 2005. Effects of tree species and topography on soil chemistry, litter quality, and decomposition in Northeast Turkey. Soil Biology & Biochemistry 37, 1695–1706.
Selmants, P.C., Hart, S.C., Boyle, S.I., Stark, J.M., 2005. Red alder (*Alnus rubra*) alters community-level soil microbial function in conifer forests of the Pacific Northwest, USA. Soil Biology & Biochemistry 37, 1860–1868.
Söderberg, K.H., Olsson, P.A., Bååth, E., 2002. Structure and activity of the bacterial community in the rhizosphere of different plant species and the effect of arbuscular mycorrhizal colonisation. FEMS Microbiology Ecology 40, 223–231.
Šnajdr, J., Valášková, V., Merhautová, V., Herinková, J., Cajthaml, T., Baldrian, P., 2008. Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biology & Biochemistry 40, 2068–2075.
Šnajdr, J., Cajthaml, T., Valášková, V., Merhautová, V., Petránková, M., Spetz, P., Leppanen, K., Baldrian, P., 2011. Transformation of *Quercus petraea* litter: successive changes in litter chemistry are reflected in differential enzyme activity and changes in the microbial community composition. FEMS Microbiology Ecology 75, 291–303.
Šourková, M., Frouz, J., Šantrůčková, H., 2005. Accumulation of carbon, nitrogen and phosphorus during soil formation on alder spoil heaps after brown-coal mining, near Sokolov (Czech Republic). Geoderma 124, 203–214.
Štursová, M., Baldrian, P., 2011. Effects of soil properties and management on the activity of soil organic matter transforming enzymes and the quantification of soil-bound and free activity. Plant and Soil 338, 99–110.
Urbanová, M., Kopecký, J., Valášková, V., Ságová-Marečková, M., Elhottová, D., Kyselková, M., Moënne-Loccoz, Y., Baldrian, P., 2011. Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. FEMS Microbiology Ecology 78, 59–69.
van der Heijden, M.G.A., Bardgett, R.D., van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296–310.
Voříšková, J., Dobiášová, P., Šnajdr, J., Vaněk, D., Cajthaml, T., Šantrůčková, H., Baldrian, P., 2011. Chemical composition of litter affects the growth and enzyme production by the saprotrophic basidiomycete *Hypholoma fasciculare*. Fungal Ecology 4, 417–426.
Weand, M.P., Arthur, M.A., Lovett, G.M., McCulley, R.L., Weathers, K.C., 2010. Effects of tree species and N additions on forest floor microbial communities and extracellular enzyme activities. Soil Biology & Biochemistry 42, 2161–2173.

IV

Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees

Michaela Urbanová, Jaroslav Šnajdr, Petr Baldrian*

Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, Videnska 1083, 14220 Praha 4, Czech Republic

Regular paper

Date of preparation: July 30, 2014.

Pages: 26; Figures: 6; Tables: 0

*Corresponding author: Petr Baldrian, Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, Videnska 1083, 14220 Praha 4, Czech Republic, e-mail: baldrian@biomed.cas.cz, phone: +420723770570, fax: +420241062384

Research highlights

- Forest trees affect community composition of bacteria and fungi in soil and litter.
- More fungi than bacteria are tree-specific, especially in the litter.
- Effects of trees on bacteria are likely mediated by litter or soil pH.
- Both root-symbiotic and saprotrophic fungi are tree-specific.
- Litter and soils under different trees are dominated by different fungi.

Abstract

In forest ecosystems, trees represent the major primary producers and affect the chemical composition and microbial processes in the ecosystem via specific litter chemistry and rhizodeposition. Effects of trees on the abundance of soil microorganisms were observed but the extent to which trees affect the composition of microbial communities remained unknown. Here we analyze the factors affecting the composition of bacterial and fungal communities in forest litter and soil under seven tree species studied at twenty-eight spatially independent sites of similar age developed on the same initial substrate. Microbial communities differed between litter and soil. Bacterial communities were more diverse than fungal communities, especially in litter, and exhibited higher evenness. Eighty percent of the bacterial sequences belonged to the 200-250 most dominant operational taxonomic units (OTUs), and 80% of the fungal sequences were composed of only 23-28 OTUs. The effect of tree species on the microbial-community composition was significant in both litter and soil for fungi as well as bacteria. In bacteria, the tree effect was likely partly mediated by litter and soil pH. Fungal taxa showed a greater tendency to be tree-specific: 35-37% of the dominant fungal OTUs but only 0-3% of the bacterial OTUs were restricted to 1 or 2 trees, and 15-45% of the fungi and 80% of the bacteria were common under 6 or 7 trees. As a consequence, the numbers of observed dominant fungal OTUs in the study area increased faster with an increasing numbers of trees, indicating high β-diversity. Although the proportion of the arbuscular mycorrhizal and ectomycorrhizal fungi differed among trees, the tree-specific fungal taxa were both root-symbiotic and saprotrophic.

Keywords: forest soil; litter; bacteria; fungi; plant-microbe interactions; diversity

1. Introduction

The interactions between soil and litter microbiota and plants receive considerable attention. Soil microbiota are responsible for a wide range of biogeochemical processes, including nutrient mobilisation, decomposition and gas fluxes and are connected to the aboveground part of the terrestrial ecosystems through plants. Plants, as primary producers, mediate the bulk of the organic-carbon input into the ecosystem (Wardle et al., 2004). In the forest ecosystems that cover a substantial part of the Earth's surface, trees play a prominent role in these aboveground/belowground interactions because they are responsible for the bulk of the total primary production and because, as dominant organisms, they often shape the character of the rest of the vegetation. The functional traits of

dominant trees (or vegetation as a whole) may affect microbial communities in several ways, including through the production of aboveground and belowground litter, rhizodeposition, direct interactions with root-symbiotic microorganisms, indirect biotic effects on soil microorganisms mediated by herbivores or soil fauna or through the alteration of the microclimate (shading, interception of precipitation, water uptake etc.) (Augusto et al., 2014; Prescott and Grayston, 2013).

In forest ecosystems, carbon fixed by trees enters the soil either via the accumulation of aboveground litter on the soil surface or through root litter and exudates. The differences in the chemistry of these carbon pools among trees affect the abundance and composition of soil fungal and bacterial communities to various extents (Prescott and Grayston, 2013). Plant litter is mostly composed of recalcitrant biopolymers, which are represented by polysaccharides and polyphenols that are utilised by decomposer microorganisms. Due to the filamentous form of most taxa, fungi are often considered better suited for and consequently more involved in the decomposition of polymeric compounds (de Boer et al., 2005), and many bacterial taxa that preferentially utilise low-molecular-mass organic compounds may rely on the products of fungal-biopolymer decomposition for nutrition (Štursová et al., 2012). As a consequence, the litter traits should have stronger effects on the community of the saprotrophic fungi than on bacteria. For example, in a comparative study using DNA-fingerprint profiles from *Fagus*, *Quercus*, *Pseudotsuga* and *Picea* litter, tree species explained almost 50% of the variability in the fungal community (Kubartova et al., 2009). Unfortunately, data on the effect of trees on litter bacteria is relatively scarce. The properties of the litter itself are more important than other environmental variables for the composition of litter-associated fungi and bacteria, as evidenced by the similarity of litter communities transplanted into other environmental contexts (Aneja et al., 2006; Bray et al., 2012), but an effect of the underlying forest floor has also been observed (Wallenstein et al., 2010).

In bulk soil, pH is highly important to the composition of the bacterial community, but it seems to be less important for fungi (Lauber et al., 2009; Rousk et al., 2010). Both bacteria and fungi may respond to soil nutrients such as phosphorus or nitrogen. The latter was reported to specifically affect the abundance of ectomycorrhizal (ECM) fungi (Lauber et al., 2008). On the other hand, the effects of land use and dominant vegetation affect fungi more strongly than bacteria. These factors affect both the saprotrophic and root-symbiotic taxa but seem to be much more important for the latter (Buéé et al., 2009b; Martiny et al., 2006; Zinger et al., 2011) because many root symbionts are tree-specific (Peay et al., 2008; Tedersoo et al., 2008).

Plant roots and the rhizosphere represent unique environments strongly influenced by plants whose existence contributes to the spatial heterogeneity of soils (Buéé et al., 2009a; Churchland and Grayston, 2014). Several factors, such as the quality and availability of C compounds of plant origin or the presence of sites for microbial attachment distinguish the rhizosphere from bulk soil. The rhizosphere is typically richer in plant-derived C but depleted of P and N and affected by root respiration (Hinsinger et al., 2005; Philippot et al., 2013). Bacterial and fungal communities in the

rhizosphere thus differ from those in the bulk soil, with the former containing more microbial biomass and a greater share of symbiotic microorganisms and r-strategists (Buéé et al., 2009a; Corneo et al., 2013; Koide et al., 2005). The differences in properties of plant rhizospheres, such as the specific composition of exudates, among plant species (Churchland and Grayston, 2014; Prescott and Grayston, 2013) should be reflected by the plant-specific differences among rhizosphere microbial communities like those recently reported for bacteria associated with agricultural crops and grasses (Turner et al., 2013).

Although the unicellular bacteria in the rhizosphere and bulk soil inhabit one of these separate niches, the mycelia of root-associated fungi may extend from roots and the rhizosphere into the bulk soil. Due to this connection and the physiological activity of the mycorrhizal symbiosis, the effect of plant roots may be extended by their specific fungal symbionts into the bulk soil where it affects the local microbial community (Kluber et al., 2011; Koide et al., 2005). Ectomycorrhizal mycelial mats, whose formation is dependent on tree roots in this way, create a specific niche that is often characterised by high oxalate content and low pH (Kluber et al., 2010), which supports different microbial communities than the soil outside these ectomycorrhizal mats (Poole et al., 2001; Timonen et al., 2004).

Despite the wide acceptance of the abovementioned general relationships among tree traits and microbial community structure and multiple reports of tree effects on microbial communities (Aponte et al., 2013; Grayston et al., 1998; Hackl et al., 2004; Hobbie et al., 2006; Ushio et al., 2008), there is currently little knowledge about the quantitative extent to which tree species affect bacterial and fungal diversity and community composition. This is mainly because most of the previous results were obtained using low resolution methods, such as the comparison of PLFA profiles, considered only a part of the microbial community (such as bacteria or ectomycorrhizal fungi) or because their interpretation was limited by their experimental design, such as, most typically, low replication or the inability to exclude tree-independent external factors (Prescott and Grayston, 2013).

The aim of this work was to analyse the effects of dominant tree species on the composition of bacterial and fungal communities in the litter and in the soil and to investigate the extent to which the dominant tree vegetation shapes microbial-community composition. The study was performed at a set of post-mining sites under six different tree species introduced by recultivation and at sites left to spontaneous development for the same length of time, which developed into *Salix*-dominated forests. We used the rare opportunity presented by a set of spatially independent tree plantations established as field-size plots on the same initial substrate, which had not been previously affected by other tree species. Moreover, the plots had developed for approximately the same length of time (22-33 years), and a wide variety of additional data are available. Previous studies on the same plots demonstrated vegetation-dependent differences in the C accumulation above- and belowground (Frouz et al., 2009), the extent and type of understory vegetation (Mudrák et al., 2010), the biomass and composition of soil fauna (Frouz et al., 2013) and the differences in litter and soil composition, microbial biomass and

the relative abundance of fungi and bacteria (Šnajdr et al., 2013). These previous studies performed at the same or similar plot ages gave us information about factors shaped by the effect of the dominant tree species that might have affected the microbial diversity and community composition.

We hypothesised that the effects of vegetation will affect fungal-community composition more strongly than bacterial-community composition in both the litter and the soil. Fungi, as primary decomposers of litter biopolymers, should reflect the properties of litter. Due to the complex nature of plant-fungal symbiosis, symbiotic fungi are expected to be more tree-species-specific, and this higher level of specificity should remain detectable in the bulk soil. In contrast, bacterial taxa, especially the prevailing unicellular bacteria, inhabit soil niches on a very small scale (Vos et al., 2013) that often have no direct connection to the plant root or the rhizosphere. The tree-species effects on the bulk-soil bacteria were expected to be indirect and thus less pronounced.

2. Materials and Methods

2.1. Study site

The study was carried out at the Velká Podkrušnohorská spoil heap in the Sokolov brown-coal mining district (in the western part of the Czech Republic). The study area of 1900 ha was located at an altitude of 450-520 m a.s.l., with a mean annual precipitation of 650 mm and a mean annual temperature of 6.8°C. The spoil heap was formed from Tertiary clays of the so-called cypris formation. When dumped on the heap, this material had an alkaline pH of 8-9, and the dominant minerals were kaolinite, montmorilonite and illite. These minerals were also accompanied by calcite, siderite and fossil organic matter, namely type-II kerogen (Mudrák et al., 2010).

The homogeneity of the initial composition of the deposited material and the climate across the study area ensured that the vegetation development was the major driving source in the development of soil. The area was mostly covered by a mosaic of forest patches, planted as a reclamation measure such that individual tree patches were randomly spread over the heap. This procedure resulted in a “common garden” experiment on the landscape scale. Some of the heap had not been reclaimed and was dedicated to spontaneous revegetation. Seven types of forest stands were chosen for the study. One type was unreclaimed stands undergoing spontaneous succession dominated by the willow *Salix caprea* (S). Six types were reclaimed plantations, each dominated by one or two tree species of one genus: *Alnus glutinosa* and *A. incana* (A); *Larix decidua* (L); *Picea omorica* and *Picea pungens* (P); *Pinus contorta* and *Pinus nigra* (N); *Quercus robur* (Q); and *Tilia cordata* (T). Four replicated patches per treatment were selected for this study. Each patch covered at least 1 ha (typically 2-5 ha). The study sites were randomly spread over the heap, and patches of other tree

species were closer to each patch than any patch of the same tree species. The typical distance between replication of the same species was 1-4 km, and other species were usually located less that 1 km apart. In each patch, a study area was chosen at least 25 m from a patch edge. The age of each site was considered to be the time since the last major disturbance (i.e., heaping in the case of S and planting of trees in the case of the reclaimed sites) and ranged from 22 to 33 years (Frouz et al., 2009).

2.2. Sample collection and characterisation

The samples were collected in late October 2007, immediately after litter fall and during the period of the highest microbial activity (Baldrian et al., 2008; Frouz and Nováková, 2005). Six spatially independent subsamples (soil cores of 45-mm diameter) were collected from each study site at the litter patches without understory vegetation, except for the *Alnus* sites where ground vegetation was omnipresent. The samples were transported to the laboratory and separated into (1) the litter, including the fermentation layer and (2) the upper 5 cm of soil. These composite samples from each site were homogenised by cutting the litter into 0.25-cm^2 pieces or passing the soil through a 2-mm sieve. Samples for DNA extraction were frozen and stored at -80°C before processing. The same samples were also analysed for litter and soil chemistry, microbial biomass and composition using PLFA analysis and the activity of extracellular enzymes. These data were published by (Šnajdr et al., 2013), and a summary of soil and litter characteristics is provided in Supplementary Table 1.

2.3. DNA extraction and amplicon pyrosequencing of fungal and bacterial communities

Total genomic DNA was extracted from 250 mg of fresh soil material or litter material using a modified Miller method (Sagova-Mareckova et al., 2008). The fungi-specific primers ITS1/ITS4 (White et al., 1990) were used to amplify the ITS region, the 5.8S ribosomal DNA and the ITS2 region of the fungal ribosomal DNA. The bacteria-specific primers eub530f/eub1100br (modified from (Dowd et al., 2008) were used to amplify the V4-V6 region of the bacterial 16S rRNA gene, as described by (Baldrian et al., 2012).

PCR amplifications were performed in two steps. In the first step, each of three independent 25-µl reactions per DNA sample contained 2.5 µl of 10 × polymerase buffer, 1.5 µl of 10 mg ml^{-1} bovine serum albumin, 1 µl of each primer (0.01 mM), 0.5 µl of PCR Nucleotide Mix (10 mM), 0.75 µl polymerase (2 U/ul; Pfu DNA polymerase:DyNAZyme II DNA polymerase, 1:24) and 1 µl of template DNA. Cycling conditions were 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 62 °C for 50 s, 72 °C for 30 s, followed by 72 °C for 10 min for primers eub530f/eub1100br; 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 10 min for primers ITS1/ITS4.

Pooled PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The product of the first PCR was used as a template for the second PCR. In the second step, each 50-µl reaction per DNA sample contained 5 µl of 10x polymerase buffer, 1.5 µl of DMSO for PCR, 0.4 µl of forward fusion primer (ITS1, tag sequence, 454-specific sequence; (Baldrian et al., 2012)), 0.4l µ of reverse fusion primer (ITS4, 454-specific sequence), 1 µl of PCR Nucleotide Mix, 1.5 µl of polymerase (2 U/ul Dynazyme DNA polymerase, 1:24) and 100 ng of template DNA. The cycling conditions were 94°C for 5 min; 10 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min; followed by a final extension at 70°C for 10 min.

PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Beverly, MA, USA). The concentration of PCR products was quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and an equimolar mix of PCR products from all samples was prepared. The mixture of PCR products was separated by electrophoresis and gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), followed by purification using AMPure XP and the MinElute PCR Purification Kit to remove primer-dimers. The amplicons were sequenced on a GS Junior 454-pyroseqeuncer (Roche, Basel, Switzerland).

2.4. Bioinformatic analysis and statistics

The pyrosequencing data were processed using the pipeline SEED with the proposed procedures of standardised data analysis (Nilsson et al., 2011; Větrovský and Baldrian, 2013). Pyrosequencing noise reduction was performed using the Denoiser 0.851 (Reeder and Knight, 2010), and chimeric sequences were detected using UCHIME (Edgar et al., 2011) and deleted. The sequences were shortened to 300 bases, and shorter sequences were removed. Bacterial and fungal sequences were independently clustered using Usearch (Edgar, 2010) at 98% similarity. Consensus sequences were constructed for each cluster, and Operational Taxonomic Units (OTUs) were constructed by clustering these consensus sequences at 97% identity (Lundberg et al., 2012). The abundance data reported in this paper are based on this dataset of sequence abundances and should be taken as proxies of taxon abundances only with caution (Lindahl et al., 2013). The closest hits to fungal consensus sequences were identified using the PlutoF pipeline (Tedersoo et al., 2010), and nonfungal sequences (< 1%) were disregarded. The Ribosomal Database Project (Cole et al., 2009) and BLASTn hits against GenBank were used to generate best hits (Altschul et al., 1997) for bacterial consensus sequences. The sequence data were deposited in the MG-RAST public database (http://metagenomics.anl.gov/, data set numbers XXXX.X and YYYY.Y).

2.5. Diversity and statistical analyses

The Shannon-Wiener index, evenness index, species richness index and the most abundant OTUs that represented 80% of all the sequences were used as diversity estimates. These estimates were calculated for a data set containing 700 randomly chosen sequences from each sample. Because the majority of taxa were represented by a very small number of reads and because such read counts were demonstrated not to be technically reproducible (Lundberg et al., 2012), only taxa with higher relative abundances (≥ 1% in ≥ 3 samples) were tested for variations in abundance. The relative abundances of these taxa were square-root-transformed to ensure normality of the dataset, tested for treatment effects and subjected to a Principal Component Analysis with tree type and environmental metadata as additional variables. The Jaccard Index, as calculated for all OTUs with relative abundances ≥ 0.5% in at least one sample, was used as a measure of community similarity (Koleff et al., 2003). The Jaccard Index is calculated as A/(A+B+C), where A is the number of species found in both of the samples, and B and C represent the number of species unique to either of the two samples analysed. The Jaccard Index ranges from 0 (no species shared) to 1 (all species shared). A one-way analysis of variance with Fisher's least-significant-difference *post hoc* test was used to analyse the statistical significance of the differences among groups of samples. Differences with a $P < 0.05$ were regarded to be statistically significant. To explore the relative effects of trees and other environmental variables, general linear models (GLM) were used. To explore the effects of tree diversity on the diversity of fungi and bacteria, species richness was calculated for all combinations of trees. For microbial OTUs with relative abundances ≥ 1% in ≥ 3 samples, the number of associated tree species was calculated as those with abundances > 0.1%. The pipeline SEED (Větrovský and Baldrian, 2013) was used for data pre-processing and diversity calculations, and Statistica 7 (Statsoft, Tulsa, USA) was used for statistical analyses.

3. Results

3.1. Bacterial communities in forest litter and soil

In total 72 996 bacterial sequences remained from the whole dataset after quality trimming and the removal of chimeras (in average, 1303 per sample) that were clustered into 12279 OTUs including 8255 singletons. The bacterial communities were clearly different between litter and soil: out of the

dominant OTUs with >1% abundance in three or more samples, only 8 were shared; 27 were dominant only in the litter, and 22 were dominant only in the soil (Supplementary Table 2).

Litter was dominated by Proteobacteria (58.9%), followed by Actinobacteria (13.8%), Bacteroidetes (13.8%) and Acidobacteria (6.0%); most of the Proteobacteria belonged to the Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria (Fig. 1). On the species level, *Sphingomonas*, *Erwinia*, *Mucilaginigacter*, *Burkholderia* and *Pedobacter* were the most common. Although *Sphingomonas*, *Mucilaginibacter* and *Burkholderia* were common in litter of all tree taxa, *Erwinia* was highly dominant in *Alnus* litter and common in *Quercus* and *Tilia* litter but virtually absent in other litters. *Pedobacter* was rare in the litter of *Larix* and *Pinus*. In general, the composition of dominant bacterial taxa was similar in the *Larix* and *Pinus* litter and in the *Quercus* and *Tilia* litter, and the *Alnus* litter differed the most from the litters of the other tree species (Fig. 1). The specificity of bacterial taxa to certain litter types was most apparent on the OTU level: out of the 35 dominant OTUs, only 2 (6%) did not show significant differences in abundance across litter types; however, litter type-specific taxa were typically abundant in litters of multiple tree species (Supplementary Table 2).

Principal component analysis of the abundances of bacterial genera showed that samples of the same litter type typically clustered together. Samples from the coniferous litter, especially *Larix* and *Pinus* were separated from the broadleaf-litter samples along the first canonical axis that explained 38% of the total variability. Samples from Alnus sites and Salix sites showed high loads along the second axis that explained an additional 19% of the total variance (Fig. 2). The first axis was associated with differences in the C content of the soil, total microbial biomass, the fungal/bacterial biomass ratio and pH. All of these values were lower in coniferous litter types than in the litter of broadleaved trees. The second axis tended to separate the samples based on the N content or the C/N ratio, resulting in the separation of the *Alnus* sites. The PCA also indicated that the Acidobacteria preferentially inhabited the litters with low pH and low C and that the Actinobacteria were better represented in litters with a high C/N content (Fig. 2).

In the soil, Proteobacteria was the major phylum (50.7%), followed by Actinobacteria (16.7%), Bacteroidetes (8.7%) and Acidobacteria (8.4%); most Proteobacteria belonged to the Alphaproteobacteria and Betaproteobacteria (Fig. 1). *Bradyrhizobium*, *Sphingomonas*, *Burkholderia*, *Rhodoplanes* and *Chthoniobacter* were the most common genera, and all were abundant in all soil types. Most of the next-most-abundant bacterial genera were present in the soils under all trees (Fig. 1). Differences in abundance among soils were significant for only 10 of the 30 dominant OTUs (33%), and none of these OTUs was particularly abundant in the soil under any single tree species (Supplementary Table 2).

Although the percentage of the variance explained by the first two PCA axes was relatively high (37.7%), soil samples from individual trees were only rarely separated; for example, the soils of *Alnus* were distinguishable from those of *Pinus*, and the soils under *Larix* or *Salix* were relatively

heterogeneous (Fig. 2). The first axis was associated with differences in the content of P and N in the soil, and pH was associated with both axes. The Acidobacteria were associated with acidic soils, and the Firmicutes showed higher loading along the first axis, indicating their association with soils with high N and P content (Fig. 2).

3.2. Fungal communities in forest litter and soil

In total, 85 394 fungal sequences that survived quality trimming and chimera removal (on average, 1525 per sample) were clustered into 4915 OTUs, including 2738 singletons. The fungal communities in litter and soil showed very low similarity: only two of the dominant OTUs with >1% abundance in three or more samples (*Cadophora* and *Thysanophora*) were shared, and 25 and 27 were dominant in only litter and soil, respectively (Supplementary Table 3).

The litter was dominated by Ascomycota (71.4%) and Basidiomycota (19.2%), but their abundances differed among trees: Ascomycota represented 96 and 94% of the sequences in *Alnus* and *Tilia* soils but only 38% in *Larix* soils. Coniferous trees and *Salix* showed higher abundances of Basidiomycota, and Mortierellomycotina were abundant in the *Larix* and *Picea* litters, representing >10% of the sequences. In addition, >10% of the sequences associated with the litters of *Larix* and *Pinus* were Chytridiomycota. On the level of fungal orders, the composition of litter communities was highly tree-species-specific, with Capnodiales, Agaricales, Saccharomycetales and Helotiales being the most abundant in different litter types (Fig. 3). On the species level, the relative abundances of major genera showed litter similarity, with *Candida*, *Mortierella*, *Mycosphaerella*, *Ramularia* and *Tetracladium* being the most abundant in specific litter types (Fig. 3). Fungal OTUs were also litter-specific; only three dominant OTUs out of 27 (11%) did not show significant differences in abundance across litter types. Moreover, 37% of the dominant OTUs showed significantly higher abundances on a specific litter type, evidencing a high level of tree-species specificity (Supplementary Table 3).

Principal component analysis of the abundances of fungal genera showed that samples from the same litter type clustered together in many cases, as for *Larix*, *Alnus* and *Tilia*. Samples from coniferous litter tended to be separated from the broadleaf litter, especially those of *Alnus* and *Tilia*, along the first canonical axis, which explained 24% of the total variability. The second axis explained an additional 11% of the total variance but did not separate litter types from each other (Fig. 4). The first axis was associated with differences in carbon and nitrogen content in the litter, total microbial biomass and the fungal/bacterial biomass ratio, reflecting differences in the results between the coniferous and broadleaf litters. pH showed high loads on both PC axes, separating the more acidic litters of *Larix* and *Pinus* (Fig. 4).

The sequences of the Ascomycota and Basidiomycota were also the most abundant in the soil, representing 70.2% and 17.8% of all sequences, respectively. The Glomeromycota were the third-

most-abundant, ranging from <2% abundance in the *Pinus* soil to more than 7% in the *Salix* and *Alnus* soils. Consistently across the soils, Capnodiales and Verrucariales were the major orders within the Ascomycota and the Agaricales, and Russulales were most abundant among the Basidiomycota. Among fungi, *Cryptococcus* and *Candida* were abundant in all soil types, but the rest of the communities were tree-species-specific (Fig. 3). Among fungal OTUs, 93% exhibited significant differences in abundance among the soils of different tree species; 28% of the fungal OTUs, both saprotrophic and ectomycorrhizal taxa, were highly abundant in the soil of one specific tree (Supplementary Table 3).

The first two PCA axes explained 32.5% of the variance in the abundance of fungal genera in the soil. The samples from soils of the same tree typically clustered close together. The first axis separated the soils under *Alnus* and *Tilia* with high N and low C/N content and high total and bacterial biomass from other soils. pH was most closely associated with the second axis, separating the acidic soils under *Larix* and *Pinus* from the higher-pH *Picea* soil (Fig. 4).

When the observed fungal taxa were divided into arbuscular mycorrhizal, ectomycorrhizal, nonmycorrhizal taxa or yeasts, soil samples exhibited significantly higher proportions of the arbuscular mycorrhizal and ectomycorrhizal taxa and yeasts. The relative proportion of mycorrhizal taxa in litter and soil also differed among trees. In both litter and soil, the highest abundances of ectomycorrhizal fungi were observed under coniferous trees, and arbuscular mycorrhizal fungi were most common in the *Alnus* and *Salix* soils. Relative abundances of yeasts were found to be highly variable across samples (Supplementary Table 4).

3.3. Effect of trees on litter and soil microorganisms

Fungal communities show significantly lower diversity than bacterial communities as measured by the Shannon index or the species richness index. The values of the latter were 117 ± 8 and 131 ± 9 for fungal communities in litter and soil but 331 ± 18 and 396 ± 8 for bacterial communities, respectively. Fungal communities also showed lower evenness, with 80% of all the sequences represented by 23 ± 4 of the most abundant OTUs in the litter and 28 ± 8 of the most abundant OTUs in the soil; these values were 194 ± 17 and 256 ± 8 for bacteria, respectively. Bacterial communities exhibited significantly higher diversity and evenness in the soil than in the litter (Fig. 5).

When the effect of tree species on the genus-level composition of microbial communities was tested, it was found to be significant in all cases, being stronger in fungi than in bacteria and stronger in the litter than in soil. The probability (P) that tree effect was absent was < 0.00001 for fungi in litter, 0.0003 for fungi in soil, < 0.0001 for bacteria in litter and 0.0393 for bacteria in soil. General linear models were applied that included both trees and soil-chemistry data (Cox, N, P, and pH) to explore whether this effect is mediated by the effects of trees on soil chemistry. These complete models

showed that in fungi, only the effects of trees were significant, with $P = 0.000148$ in the litter and $P = 0.031$ in the soil, and the effects of C, N, P, and pH were insignificant ($P > 0.13$ invariably). For bacteria, the complete models including all variables showed no significant effect of any variable alone. When the complete model was reduced on the combined effects of pH and tree, the two factors with the lowest P values in both litter and soil, the effect of trees was significant in litter ($P < 0.0001$) and had the lowest but still non-significant, p-value in soil ($P = 0.23$). These analyses show that, unlike in fungi, the effect of trees on bacteria is likely partly mediated by the tree effects on soil and litter chemistry, most apparently the pH.

The effect of trees on the composition of microbial communities was also demonstrated by the fact that fungal communities were more similar among sites with the same tree species then among sites with different tree species. Expressed as the Jaccard index, the OTUs similarity was 0.47 ± 0.20 and 0.26 ± 0.20 in the litter ($P < 0.00001$) and 0.55 ± 0.19 and 0.42 ± 0.14 in the soil ($P < 0.00001$) for the sites with the same and different tree species, respectively. This effect was observed for bacteria in the litter (0.71 ± 0.10 and 0.62 ± 0.15, $P < 0.00001$) but not in the soil, where the samples from under the same tree species were not significantly more similar (0.58 ± 0.19 and 0.61 ± 0.20, $P = 0.94$). The JI values also showed that the similarity of bacterial communities was in general higher than that of fungal communities because the dominant fungal OTUs were more tree-specific: 37% of fungal OTUs in the litter and 35% of those in the soil were found under only one or two tree species, but these "specialist" taxa in bacteria were absent from the soil and only represented in 3% of the litter samples. On the other hand, 77% of the dominant bacterial OTUs in litter and 83% of those in soil were found under six or seven trees, but there were no such "generalist" fungal OTUs in the litter and only 21% in the soil (Fig. 6). Another important consequence of this strong association of fungi with certain tree species was the observation that the counts of abundant OTUs, those that represent > 0.5% of the sequences in at least one sample, scaled almost linearly with the number of tree species analysed, but the increase in bacterial OTUs with increasing tree-species counts slowed down rapidly (Fig. 6).

4. Discussion

Trees may affect ecosystem properties through a multitude of processes, including alteration of the microclimate (temperature and moisture), production of litter, production of root exudates or direct interactions with root-symbiotic and root-associated microorganisms (Prescott and Grayston, 2013). The importance of individual mechanisms is not well known, but recent results show a strong relationships between fungal and bacterial community structures and soil factors such as pH, texture, organic matter and C:N ratio (Brockett et al., 2012; Fierer and Jackson, 2006; Fierer et al., 2009; Rousk et al., 2010), and thus, the effects of trees on soil chemistry are likely significant, especially in developing soils where the bulk of carbon is of plant origin (Baldrian et al., 2008). In our experimental

system, the dominant tree vegetation developed on barren soil with very low initial C content (3.8%), and each plot was thus under the almost exclusive influence of a single dominant tree. The quantitative extent of this influence is demonstrated by the fact that soil C increased to 15-25% before analysis (Šnajdr et al., 2013).

In a previous study conducted at the same sites, dominant trees significantly affected bacterial and fungal biomass and their ratio. Only a small proportion of these tree effects could be explained by differences in the litter or soil chemistry. Among the chemical variables, the N content had the strongest effect on the microbial biomass, increasing fungal (but not bacterial) biomass in the litter and bacterial (but not fungal) biomass in the soil. The results indicated that other factors, such as nutrient quality or the specific association of microorganisms with rhizospheres of different trees or the understory, are likely important mediators of vegetation effects (Šnajdr et al., 2013). This observation has now been extended to microbial-community composition.

Our results show that the responses of fungal and bacterial communities to the dominant tree species differ and that the extent of their influence differs between litter and soil. This result is not surprising, considering that litter and soil microbial communities have been demonstrated to differ (Baldrian et al., 2012; Lindahl et al., 2007) as in this study (Fig. 1, 3). In agreement with previous reports (e.g., (Baldrian et al., 2012; Rousk et al., 2010), plot-level bacterial diversity was higher than fungal diversity and bacterial communities were more even, with 80% of the sequences typically belonging to the 200-250 most dominant bacterial OTUs compared to only 23-28 fungal OTUs. Bacterial communities in litter showed higher diversity and evenness than those in soil.

In the litter, the similarity of communities was higher among plots associated with the same tree species than among those with different trees for both bacteria and fungi. Especially in fungi, a high level of litter-specificity was observed, which applied at the OTU level as well as the levels of the genus, order and class. Dominant bacterial OTUs were typically observed in multiple litters. In the dominant OTUs, 37% of the fungi and 28% of the bacteria showed a significant preference for one specific litter, with higher abundance in that litter than in all other litters (Supplementary Tables 2-3). Analysis using general linear models (GLM) that included tree type and litter chemistry indicated that tree identity is significant for the community composition of both bacteria and fungi. In bacteria, but likely not in fungi, this effect is partly due to litter chemistry, especially pH, which was also the dominant factor shaping community composition, as indicated by PCA (Fig. 2). The high abundance of Acidobacteria in acidic litters and of Actinobacteria in litters with high C/N ratios is consistent with the previously observed traits of these taxa (Lauber et al., 2009; Lauber et al., 2008; Rousk et al., 2010).

In the soil, 28% of fungal OTUs (both ectomycorrhizal root symbionts and saprotrophic taxa) preferred one specific tree species, but there were no such OTUs in bacteria (Supplementary Tables 2-3). In bacteria, the effect of trees on soil-community composition was less pronounced, and the communities under the same tree species were not more similar than communities under different

species. The bacterial community in the soil, according to PCA, seemed to mostly reflect soil chemistry, especially pH, P, and N content. Sequences of the Acidobacteria were more abundant in acidic soils, but the Firmicutes tended to prefer soils with high N, P and pH. This finding seems to confirm the importance of soil pH to bacterial-community composition (Lauber et al., 2009; Rousk et al., 2010). In fungi, the effect of trees was the only statistically significant factor in the general linear model. Soil-chemical factors were insignificant in the GLM, and their small PCA loads indicated their limited effects on fungal-community composition (Fig. 4).

It is well documented that rhizosphere microbial communities differ from bulk soil in many respects, most obviously the availability of root exudates comprised of carbohydrates, organic acids, amino acids and other compounds. This difference is reflected by the specific composition of fungal and bacterial communities in the rhizosphere soil (Buéé et al., 2009a; Churchland and Grayston, 2014; Lundberg et al., 2012). In recent papers, high-throughput sequencing was used to demonstrate that rhizospheres of agricultural crops harbour distinct bacterial and eukaryotic communities. Furthermore, the strength of the rhizosphere effect depends on the plant species and may range from communities that are highly specific to those composed by generalist taxa shared by multiple plants (Botnen et al., 2014; Turner et al., 2013).

The tree-specific communities of bulk-soil fungi and much less specific communities of bacteria suggest that the root-associated filamentous fungi, unlike bacteria, may extend from the rhizosphere and carry the legacy of the plant traits into the bulk soil. Filamentous microorganisms are stronger decomposers of bulky substrates in a spatially heterogeneous environment such as soil (de Boer et al., 2005) and can better cope with a dry environment with limited connectivity (Wolf et al., 2013). Their ability to extend plant influences into the bulk soil, if verified, would represent another important ecological feature of the filamentous lifestyle. In our system, sequences of mycorrhizal fungi represented 15% of the total fungal sequences and dominant ECM OTUs typically showed strong host preferences (Supplementary Table 3, 4). In boreal forests, ectomycorrhizal mycelia may be much more important and account for up to 80% of the fungal community and 30% of the total microbial biomass (Högberg and Högberg, 2002). In combination with a recent report that 34% of ECM-community variation across several biomes can be explained by the host-tree family (Tedersoo et al., 2012) and that specific microorganisms are associated with ECM mycelia (Kluber et al., 2011; Warmink and van Elsas, 2008), the potential strength of the linkage between plant roots and bulk soil seems obvious.

Due to the unicellularity and size of most bacteria, they are expected to be exposed to the conditions of their immediate surroundings, microniches often the size of a single soil pore (Vos et al., 2013), often with very specific conditions that differ from the average properties of their environmental matrix (Urbanová et al., 2011). Due to their size, most of these microniches are likely separated from the direct influence of plant roots and may be the reason why bacterial communities in soils under different trees are not significantly different. Although unicellular eukaryotes are larger,

our observation that the abundant unicellular fungi *Candida* and *Cryptococcus* were universally present in soils of all trees seems to support the importance of body shape for microbial distributions.

At least a part of the tree effect on soil microorganisms could be due to indirect effects mediated through specific understorey vegetation and soil fauna, both of which were documented in the studied system (Frouz et al., 2013; Mudrák et al., 2010). The fauna-mediated effect can be observed in the case of *Tilia,* where the abundant earthworms (Frouz et al., 2009) mix litter into topsoil, thus increasing both the C and N content of the soil (Supplementary Table 1).

In addition to the tree effect, specific microbes associated with certain trees may also contribute to the development of specific litter or soil traits. In temperate forests, up to 75% of N and P is supplied to plants via N-fixing bacteria or symbiotic mycorrhizal fungi (van der Heijden et al., 2008). The high N content and low C/N ratio in the *Alnus* litter and soil (Supplementary Table 1) is most likely the result of the activity of N_2-fixing bacteria associated with the *Alnus* (Ekblad and HussDanell, 1995; Selmants et al., 2005). The content of N and P can also be affected by the differences in the relative abundance of the ectomycorrhizal and arbuscular mycorrhizal fungi among trees with the former being most abundant under *Larix* and *Pinus* and the latter under *Alnus* and *Salix,* the only two tree species that may host AM fungi on their roots (Supplementary Table 4).

One important question concerning microbial communities under different trees is whether they are species-specific or shared by multiple trees with specific litter or soil traits. Evergreen gymnosperm trees and deciduous angiosperm trees were reported to represent distinct groups in many respects, including litter properties such as litter and soil acidification and lower N mobility due to accumulation in recalcitrant complexes under evergreen gymnosperms (Augusto et al., 2014), and these two groups may theoretically represent such groups with common traits. In our study of community composition, however, evergreen gymnosperms do not seem to represent a separate group because *Pinus* and *Picea*-associated communities seem to be less similar than those under *Pinus* and the deciduous conifer *Larix*. For example, the similarity of bacterial communities in the litters of *Larix* and *Pinus* may be due to their low pH (Supplementary Table 1). Although the extent of this study does not allow definite conclusions, comparisons of community composition among trees (e.g., using the Jaccard Index) tend to indicate that microbial communities are tree-species-specific rather than tree group-specific (data not shown).

Although specific plant-microbe associations were described, most dominant microbial taxa are shared among soils under multiple tree species. Here, we show that the proportion of this shared diversity is relatively higher in bacteria than in fungi: only 0-3% of dominant bacteria OTUs but 35-37% of fungal OTUs were tree-specific (1-2 trees), and 80% of bacterial OTUs were common to 6 or seven trees; only 15-45% of fungal OTUs behaved similarly (Figure 6). Moreover, in our study, fungal diversity (expressed as numbers of OTUs with high abundance in at least one sample) increased more rapidly with increasing tree diversity than did bacterial diversity. Furthermore, the trend of increasing fungal diversity with increasing diversity of trees was also demonstrated at mixed forest sites with

high tree species diversity in the Amazon (Peay et al., 2013). If this trend can be extrapolated even further, it may indicate why fungal diversity is expected to be as high as 1.5 mil. species (Hawksworth, 2001) and why the global diversity of fungi is higher than that of bacteria despite the lower alpha diversity at any single site.

The effect of tree species at the study sites was the most important factor affecting the activity of extracellular enzymes (Šnajdr et al., 2013). Because extracellular enzymes are produced by soil microorganisms, differences in enzyme activities may be partly due to differences in microbial-community composition that we show here to be tree-specific. The effect of dominant trees on microbial-community composition can thus mediate their effect on the enzyme-mediated ecosystem processes.

Our paper confirms the hypothesis that fungal communities are more affected by dominant vegetation than the communities of bacteria. Interestingly, the specificity of the fungal community in the soil is not limited to root-symbiotic taxa. Although differences in litter chemistry likely contribute to the tree effect in bacteria, no such observations were made in fungi. One explanation for this pattern might be the extension of the plant rhizosphere-specific fungal mycelia into the bulk soil. The comparisons of tree-root rhizospheres and bulk soils would also shed more light on the question of whether the tree effects are direct or indirect (e.g., through alteration of the ground vegetation). Despite lower C allocation, herbal layer plants were demonstrated to affect the fungal community in bulk soil in a microcosm study (Corneo et al., 2013), and so their effect should be considered. Future studies should examine the upscaling of fungal diversity with increasing plant diversity and should extend our studies to mixed-forest ecosystems, where much more complex relationships between vegetation and microbiota should to be expected.

Acknowledgments

This work was supported by the Czech Science Foundation (P504/12/1288, 13-06763S), the Ministry of Education, Youth and Sports of the Czech Republic (LD12050), and the Research Concept of the Institute of Microbiology ASCR (RVO61388971). We also thank the Sokolovská Uhelná a.s. coal-mining company for the research permit.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Aneja, M.K., Sharma, S., Fleischmann, F., Stich, S., Heller, W., Bahnweg, G., Munch, J.C., Schloter, M., 2006. Microbial colonization of beech and spruce litter - Influence of decomposition site and plant litter species on the diversity of microbial community. Microbial Ecology 52, 127-135.

Aponte, C., García, L.V., Marañón, T., 2013. Tree species effects on nutrient cycling and soil biota: A feedback mechanism favouring species coexistence. Forest Ecology and Management 309, 36-46.

Augusto, L., De Schrijver, A., Vesterdal, L., Smolander, A., Prescott, C., Ranger, J., 2014. Influences of evergreen gymnosperm and deciduous angiosperm tree species on the functioning of temperate and boreal forests. Biological Reviews in press, doi: 10.1111/brv.12119.

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Rídl, J., Vlček, C., Voříšková, J., 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME Journal 6, 248-258.

Baldrian, P., Trögl, J., Frouz, J., Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., Herinková, J., 2008. Enzyme activities and microbial biomass in topsoil layer during spontaneous succession in spoil heaps after brown coal mining. Soil Biology & Biochemistry 40, 2107-2115.

Botnen, S., Vik, U., Carlsen, T., Eidesen, P.B., Davey, M.L., Kåuserud, H., 2014. Low host specificity of root-associated fungi at an Arctic site. Molecular Ecology 23, 975-985.

Bray, S.R., Kitajima, K., Mack, M.C., 2012. Temporal dynamics of microbial communities on decomposing leaf litter of 10 plant species in relation to decomposition rate. Soil Biology & Biochemistry 49, 30-37.

Brockett, B.F.T., Prescott, C.E., Grayston, S.J., 2012. Soil moisture is the major factor influencing microbial community structure and enzyme activities across seven biogeoclimatic zones in western Canada. Soil Biology & Biochemistry 44, 9-20.

Buéé, M., De Boer, W., Martin, F., van Overbeek, L., Jurkevitch, E., 2009a. The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant and Soil 321, 189-212.

Buéé, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., Martin, F., 2009b. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 184, 449-456.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37, D141-D145.

Corneo, P.E., Pellegrini, A., Cappellin, L., Gessler, C., Pertot, I., 2013. Weeds influence soil bacterial and fungal communities. Plant and Soil 373, 107-123.

de Boer, W., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. Fems Microbiology Reviews 29, 795-811.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics, btq461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Ekblad, A., HussDanell, K., 1995. Nitrogen fixation by Alnus incana and nitrogen transfer from A-incana to Pinus sylvestris influenced by macronutrients and ectomycorrhiza. New Phytologist 131, 453-459.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103, 626-631.

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecology Letters 12, 1238-1249.

Frouz, J., Livečková, M., Albrechtová, J., Chroňáková, A., Cajthaml, T., Pižl, V., Háněl, L., Starý, J., Baldrian, P., Lhotáková, Z., Šimáčková, H., Cepáková, S., 2013. Is the effect of trees on soil properties mediated by soil fauna? A case study from post-mining sites. Forest Ecology and Management 309, 87-95.

Frouz, J., Nováková, A., 2005. Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. Geoderma 129, 54-64.

Frouz, J., Pižl, V., Cienciala, E., Kalčík, J., 2009. Carbon storage in post-mining forest soil, the role of tree biomass and soil bioturbation. Biogeochemistry 94, 111-121.

Grayston, S.J., Wang, S.Q., Campbell, C.D., Edwards, A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biology & Biochemistry 30, 369-378.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., Sessitsch, A., 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology 70, 5057-5065.

Hawksworth, D.L., 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycological Research 105, 1422-1432.

Hinsinger, P., Gobran, G.R., Gregory, P.J., Wenzel, W.W., 2005. Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. New Phytologist 168, 293-303.

Hobbie, S.E., Reich, P.B., Oleksyn, J., Ogdahl, M., Zytkowiak, R., Hale, C., Karolewski, P., 2006. Tree species effects on decomposition and forest floor dynamics in a common garden. Ecology 87, 2288-2297.

Högberg, M.N., Högberg, P., 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. New Phytologist 154, 791-795.

Churchland, C., Grayston, S.J., 2014. Specificity of plant-microbe interactions in the tree mycorrhizosphere biome and consequences for soil C cycling. Frontiers in Microbiology 5, 261.

Kluber, L.A., Smith, J.E., Myrold, D.D., 2011. Distinctive fungal and bacterial communities are associated with mats formed by ectomycorrhizal fungi. Soil Biology & Biochemistry 43, 1042-1050.

Kluber, L.A., Tinnesand, K.M., Caldwell, B.A., Dunham, S.M., Yarwood, R.R., Bottomley, P.J., Myrold, D.D., 2010. Ectomycorrhizal mats alter forest soil biogeochemistry. Soil Biology & Biochemistry 42, 1607-1613.

Koide, R.T., Xu, B., Sharda, J., 2005. Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytologist 166, 251-262.

Koleff, P., Gaston, K.J., Lennon, J.J., 2003. Measuring beta diversity for presence-absence data. Journal of Animal Ecology 72, 367-382.

Kubartova, A., Ranger, J., Berthelin, J., Beguiristain, T., 2009. Diversity and Decomposing Ability of Saprophytic Fungi from Temperate Forest Litter. Microbial Ecology 58, 98-107.

Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. Applied and Environmental Microbiology 75, 5111-5120.

Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biology & Biochemistry 40, 2407-2415.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Hogberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611-620.

Lindahl, B.D., Nilsson, R.H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J., Kåuserud, H., 2013. Fungal community analysis by high-throughput sequencing of amplified markers – a user's guide. New Phytologist 199, 288-299.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., del Rio, T.G., Edgar, R.C., Eickhorst, T., Ley, R.E., Hugenholtz, P., Tringe, S.G., Dangl, J.L., 2012. Defining the core Arabidopsis thaliana root microbiome. Nature 488, 86-90.

Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine, M.C., Kane, M., Krumins, J.A., Kuske, C.R., Morin, P.J., Naeem, S., Ovreas, L., Reysenbach,

A.L., Smith, V.H., Staley, J.T., 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology 4, 102-112.

Mudrák, O., Frouz, J., Velichová, V., 2010. Understory vegetation in reclaimed and unreclaimed post-mining forest stands. Ecological Engineering 36, 783-790.

Nilsson, R.H., Tedersoo, L., Lindahl, B.D., Kjoller, R., Carlsen, T., Quince, C., Abarenkov, K., Pennanen, T., Stenlid, J., Bruns, T., Larsson, K.H., Koljalg, U., Kåuserud, H., 2011. Towards standardization of the description and publication of next-generation sequencing datasets of fungal communities. New Phytologist 191, 314-318.

Peay, K.G., Baraloto, C., Fine, P.V.A., 2013. Strong coupling of plant and fungal community structure across western Amazonian rainforests. ISME Journal 7, 1852-1861.

Peay, K.G., Kennedy, P.G., Bruns, T.D., 2008. Fungal Community Ecology: A Hybrid Beast with a Molecular Master. Bioscience 58, 799-810.

Philippot, L., Raaijmakers, J.M., Lemanceau, P., van der Putten, W.H., 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology 11, 789-799.

Poole, E.J., Bending, G.D., Whipps, J.M., Read, D.J., 2001. Bacteria associated with Pinus sylvestris-Lactarius rufus ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytologist 151, 743-751.

Prescott, C.E., Grayston, S.J., 2013. Tree species influence on microbial communities in litter and soil: Current knowledge and research needs. Forest Ecology and Management 309, 19-27.

Reeder, J., Knight, R., 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nature Methods 7, 668-669.

Rousk, J., Baath, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME Journal 4, 1340-1351.

Sagova-Mareckova, M., Cermak, L., Novotna, J., Plhackova, K., Forstova, J., Kopecky, J., 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Applied and Environmental Microbiology 74, 2902-2907.

Selmants, P.C., Hart, S.C., Boyle, S.I., Stark, J.M., 2005. Red alder (Alnus rubra) alters community-level soil microbial function in conifer forests of the Pacific Northwest, USA. Soil Biology & Biochemistry 37, 1860-1868.

Šnajdr, J., Dobiášová, P., Urbanová, M., Petránková, M., Cajthaml, T., Frouz, J., Baldrian, P., 2013. Dominant trees affect microbial community composition and activity in post-mining afforested soils. Soil Biology & Biochemistry 56, 105-115.

Štursová, M., Žifčáková, L., Leigh, M.B., Burgess, R., Baldrian, P., 2012. Cellulose utilization in forest litter and soil: identification of bacterial and fungal decomposers. FEMS Microbiology Ecology 80, 735-746.

Tedersoo, L., Bahram, M., Toots, M., Diedhiou, A.G., Henkel, T.W., Kjoller, R., Morris, M.H., Nara, K., Nouhra, E., Peay, K.G., Polme, S., Ryberg, M., Smith, M.E., Koljalg, U., 2012. Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. Molecular Ecology 21, 4160-4170.

Tedersoo, L., Jairus, T., Horton, B.M., Abarenkov, K., Suvi, T., Saar, I., Koljalg, U., 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. New Phytologist 180, 479-490.

Tedersoo, L., Nilsson, R.H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., Bahram, M., Bechem, E., Chuyong, G., Koljalg, U., 2010. 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. New Phytologist 188, 291-301.

Timonen, S., Christensen, S., Ekelund, F., 2004. Distribution of protozoa in Scots pine mycorrhizospheres. Soil Biology & Biochemistry 36, 1087-1093.

Turner, T.R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., Osbourn, A., Grant, A., Poole, P.S., 2013. Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. Isme Journal 7, 2248-2258.

Urbanová, M., Kopecký, J., Valášková, V., Ságová-Marečková, M., Elhottová, D., Kyselková, M., Moenne-Loccoz, Y., Baldrian, P., 2011. Development of bacterial community during spontaneous succession on spoil heaps after brown coal mining. FEMS Microbiology Ecology 78, 59-69.

Ushio, M., Wagai, R., Balser, T.C., Kitayama, K., 2008. Variations in the soil microbial community composition of a tropical montane forest ecosystem: Does tree species matter? Soil Biology and Biochemistry 40, 2699-2702.

van der Heijden, M.G.A., Bardgett, R.D., van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296-310.

Větrovský, T., Baldrian, P., 2013. Analysis of soil fungal communities by amplicon pyrosequencing: current approaches to data analysis and the introduction of the pipeline SEED. Biology and Fertility of Soils 49, 1027-1037.

Vos, M., Wolf, A.B., Jennings, S.J., Kowalchuk, G.A., 2013. Micro-scale determinants of bacterial diversity in soil. Fems Microbiology Reviews 37, 936-954.

Wallenstein, M.D., Hess, A.M., Lewis, M.R., Steltzerae, H., Ayres, E., 2010. Decomposition of aspen leaf litter results in unique metabolomes when decomposed under different tree species. Soil Biology & Biochemistry 42, 484-490.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setala, H., van der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Warmink, J.A., van Elsas, J.D., 2008. Selection of bacterial populations in the mycosphere of Laccaria proxima: is type III secretion involved? ISME Journal 2, 887-900.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: Innins, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. Academic Press, San Diego, CA, pp. 315-322.

Wolf, A.B., Vos, M., de Boer, W., Kowalchuk, G.A., 2013. Impact of Matric Potential and Pore Size Distribution on Growth Dynamics of Filamentous and Non-Filamentous Soil Bacteria. PLoS One 8, e83661.

Zinger, L., Lejon, D.P.H., Baptist, F., Bouasria, A., Aubert, S., Geremia, R.A., Choler, P., 2011. Contrasting Diversity Patterns of Crenarchaeal, Bacterial and Fungal Soil Communities in an Alpine Landscape. PLoS One 6, e19950.

Fig. 1: Phylogenetic assignment of bacterial sequences from the litter and soil of forests with different dominant trees. The data represent mean values from four sites for higher bacterial taxa and for the twenty-four most-abundant genera.

Fig. 2: Principal component analysis of the composition of bacterial communities in the litter and soil of forests with different dominant trees. *Alnus* – blue, *Larix* – yellow, *Picea* – light green, *Pinus* – green, *Quercus* – brown, *Salix* – red, *Tilia* – black. The association of bacterial genera, microbial biomass and soil or litter chemistry are shown. Underlined genera show statistically significant differences in abundance among trees: yellow, Acidobacteria; green, Actinobacteria; red, Bacteroidetes; cyan, Firmicutes; blue, Proteobacteria; brown, Verrucomicrobia. Only genera with >1% abundance in >2 samples were considered.

Fig. 3: Phylogenetic assignment of fungal sequences from the litter and soil of forests with different dominant trees. The data represent the mean values from four sites for the twenty-four most-abundant genera and orders.

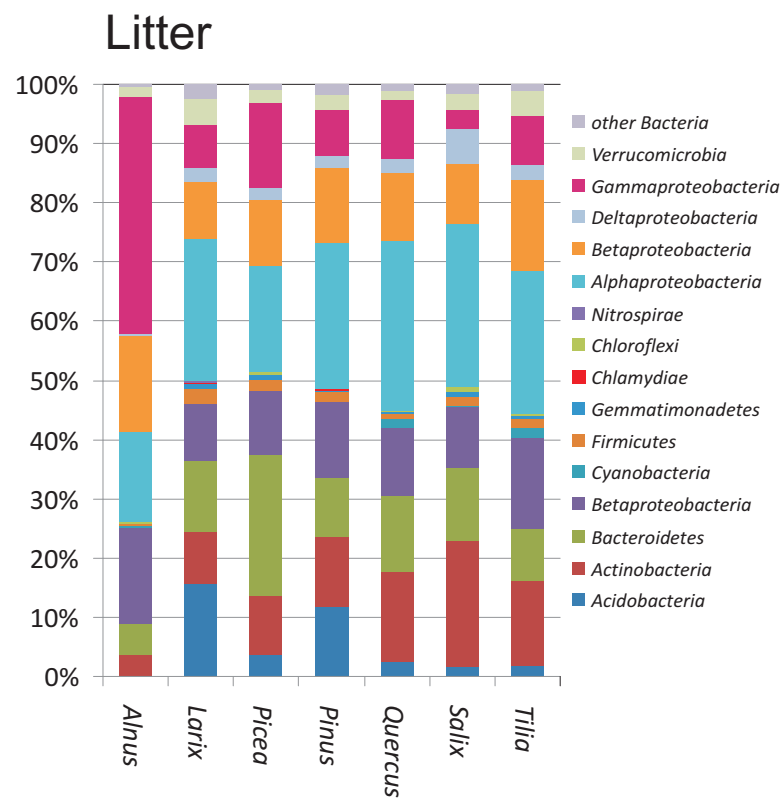
Fig. 4: Principal component analysis of the composition of fungal communities in the litter and soil of forests with different dominant trees. *Alnus* – blue, *Larix* – yellow, *Picea* – light green, *Pinus* – green, *Quercus* – brown, *Salix* – red, *Tilia* – black. The association of fungal genera, microbial biomass and soil or litter chemistry are shown. Underlined genera show statistically significant differences in abundance among trees: blue, Ascomycota; green, Basidiomycota; black, Chytridiomycota; yellow, Glomeromycota; magenta, Mortierellomycotina; cyan, Mucoromycotina. Only genera with >1% abundance in >2 samples were considered.

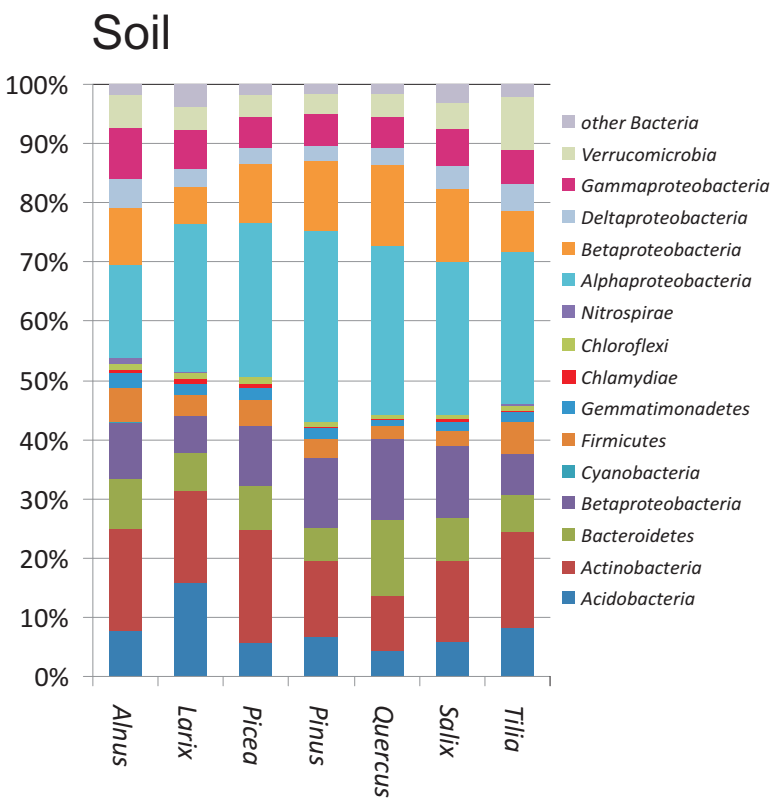
Fig. 5: Diversity of bacteria and fungi in the litter and soil of forests with different dominant trees. Data represent the means of all samples (seven trees x four replicates) with standard deviations. Statistically significant differences among groups are indicated with different letters.

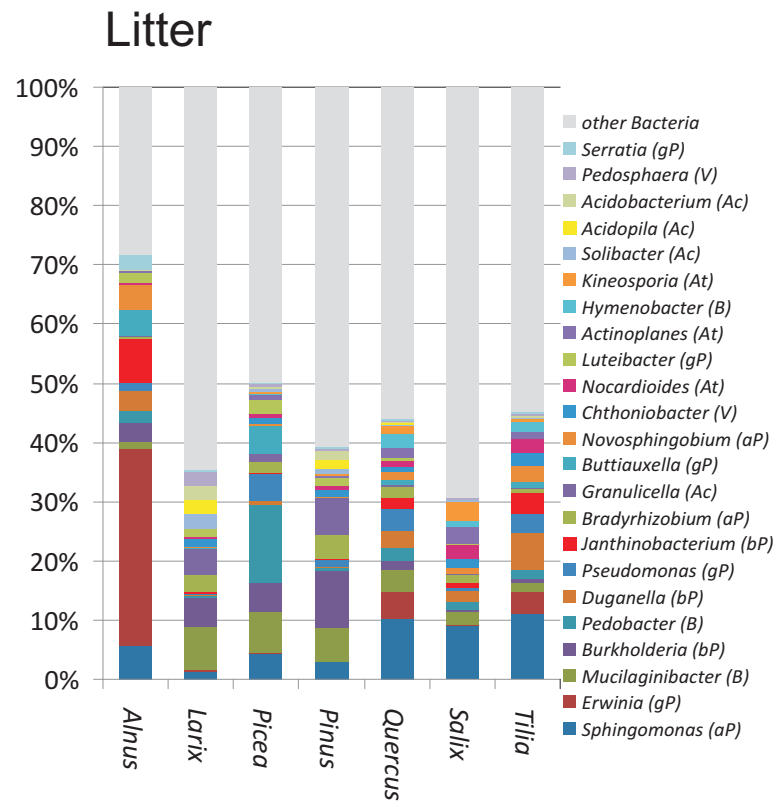
Fig. 6: Effect of dominant trees on the diversity of bacteria and fungi in the litter and soil. A: Increase of the numbers of dominant microbial taxa with increasing diversity of trees. Only microbial OTUs with >1% abundance in at least one sample were considered. The data represent averages, and standard deviations are omitted for clarity. B: The specificity of the association of microbial taxa with a single dominant tree or multiple tree taxa. Association was defined as the >0.1% abundance in the

litter or soil of a given tree. Only microbial OTUs with >1% abundance in >2 samples were considered.

Figure 1


Litter
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alnus
Larix
Picea
Pinus
Quercus
Salix
Tilia
other Bacteria
Verrucomicrobia
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Nitrospirae
Chloroflexi
Chlamydiae
Gemmatimonadetes
Firmicutes
Cyanobacteria
Betaproteobacteria
Bacteroidetes
Actinobacteria
Acidobacteria


Soil
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alnus
Larix
Picea
Pinus
Quercus
Salix
Tilia
other Bacteria
Verrucomicrobia
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Nitrospirae
Chloroflexi
Chlamydiae
Gemmatimonadetes
Firmicutes
Cyanobacteria
Betaproteobacteria
Bacteroidetes
Actinobacteria
Acidobacteria


Litter
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alnus
Larix
Picea
Pinus
Quercus
Salix
Tilia
other Bacteria
Serratia (gP)
Pedosphaera (V)
Acidobacterium (Ac)
Acidopila (Ac)
Solibacter (Ac)
Kineosporia (At)
Hymenobacter (B)
Actinoplanes (At)
Luteibacter (gP)
Nocardioides (At)
Chthoniobacter (V)
Novosphingobium (aP)
Buttiauxella (gP)
Granulicella (Ac)
Bradyrhizobium (aP)
Janthinobacterium (bP)
Pseudomonas (gP)
Duganella (bP)
Pedobacter (B)
Burkholderia (bP)
Mucilaginibacter (B)
Erwinia (gP)
Sphingomonas (aP)

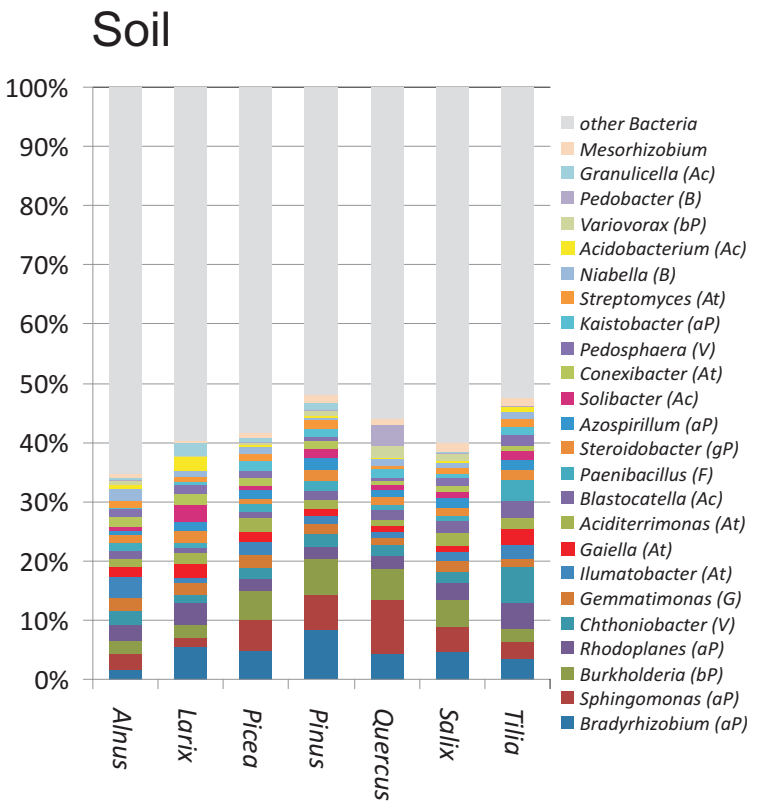
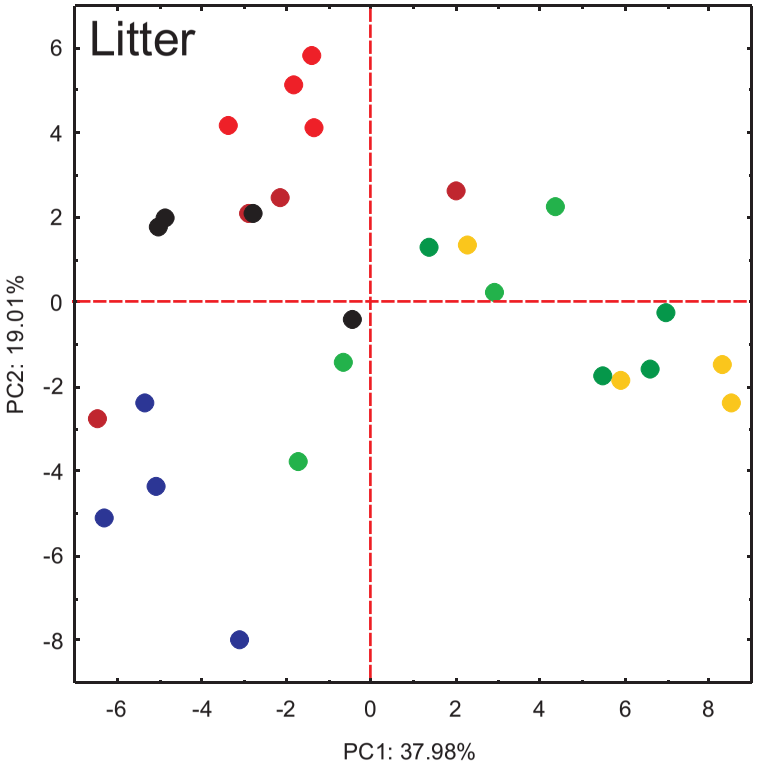
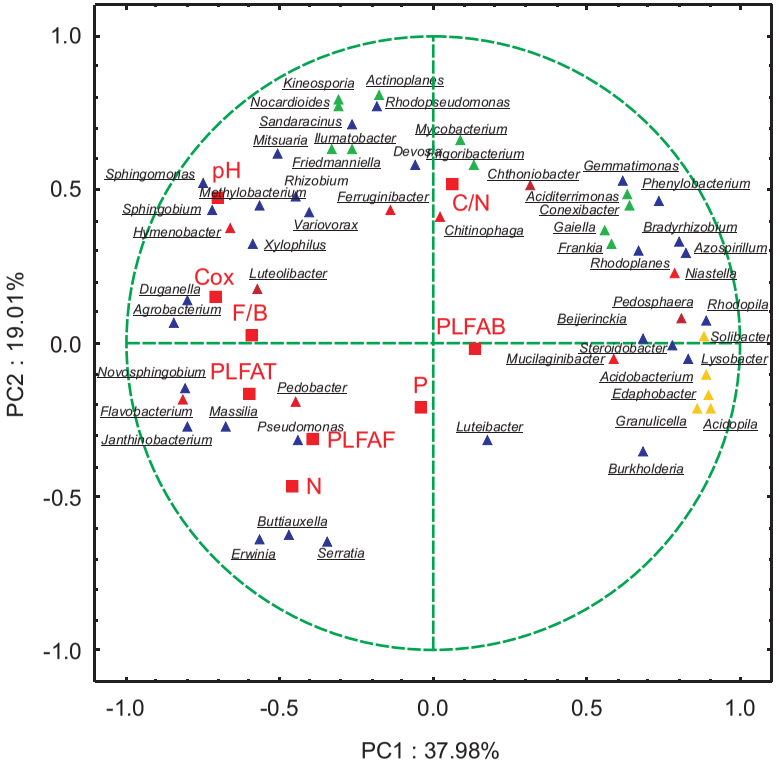
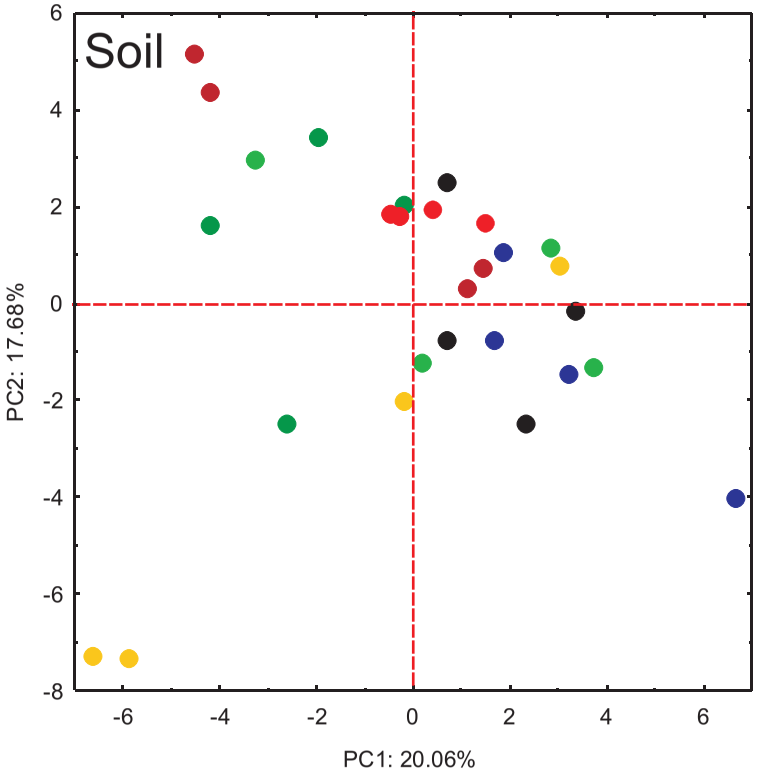

Soil
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alnus
Larix
Picea
Pinus
Quercus
Salix
Tilia
other Bacteria
Mesorhizobium
Granulicella (Ac)
Pedobacter (B)
Variovorax (bP)
Acidobacterium (Ac)
Niabella (B)
Streptomyces (At)
Kaistobacter (aP)
Pedosphaera (V)
Conexibacter (At)
Solibacter (Ac)
Azospirillum (aP)
Steroidobacter (gP)
Paenibacillus (F)
Blastocatella (Ac)
Aciditerrimonas (At)
Gaiella (At)
Ilumatobacter (At)
Gemmatimonas (G)
Chthoniobacter (V)
Rhodoplanes (aP)
Burkholderia (bP)
Sphingomonas (aP)
Bradyrhizobium (aP)

Figure 2


Litter
PC2: 19.01%
PC1: 37.98%


PC2 : 19.01%
PC1 : 37.98%
Kineosporia
Actinoplanes
Nocardioides
Rhodopseudomonas
Sandaracinus
Mycobacterium
Mitsuaria
Ilumatobacter
Friedmanniella
Devosia
Frigoribacterium
Sphingomonas
pH
Rhizobium
Chthoniobacter
Gemmatimonas
Methylobacterium
Ferruginibacter
C/N
Aciditerrimonas
Phenylobacterium
Sphingobium
Conexibacter
Variovorax
Chitinophaga
Gaiella
Bradyrhizobium
Hymenobacter
Frankia
Azospirillum
Xylophilus
Rhodoplanes
Niastella
Cox
Luteolibacter
Duganella
Pedosphaera
Rhodopila
Agrobacterium
F/B
Beijerinckia
PLFAB
Solibacter
Steroidobacter
Mucilaginibacter
Lysobacter
Novosphingobium
PLFAT
Acidobacterium
Pedobacter
P
Edaphobacter
Flavobacterium
Massilia
Granulicella
Acidopila
Pseudomonas
Luteibacter
Janthinobacterium
PLFAF
Burkholderia
N
Buttiauxella
Erwinia
Serratia


Soil
PC2: 17.68%
PC1: 20.06%

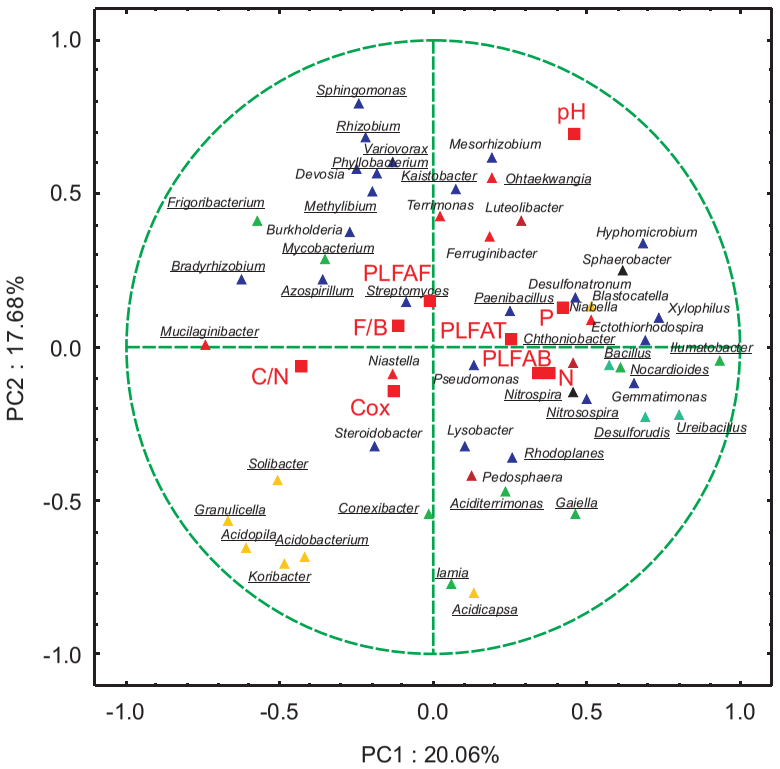

PC2 : 17.68%
PC1 : 20.06%
Sphingomonas
pH
Rhizobium
Mesorhizobium
Variovorax
Phyllobacterium
Devosia
Kaistobacter
Ohtaekwangia
Frigoribacterium
Methylibium
Terrimonas
Luteolibacter
Burkholderia
Hyphomicrobium
Mycobacterium
Ferruginibacter
Sphaerobacter
Bradyrhizobium
PLFAF
Desulfonatronum
Azospirillum
Streptomyces
Paenibacillus
Blastocatella
Niabella
Xylophilus
Mucilaginibacter
F/B
P
Ectothiorhodospira
PLFAT
Chthoniobacter
Ilumatobacter
Bacillus
Niastella
PLFAB
Nocardioides
C/N
Pseudomonas
N
Nitrospira
Gemmatimonas
Cox
Nitrosospira
Steroidobacter
Lysobacter
Desulforudis
Ureibacillus
Solibacter
Rhodoplanes
Pedosphaera
Granulicella
Conexibacter
Aciditerrimonas
Gaiella
Acidopila
Acidobacterium
Koribacter
Iamia
Acidicapsa

Figure 3

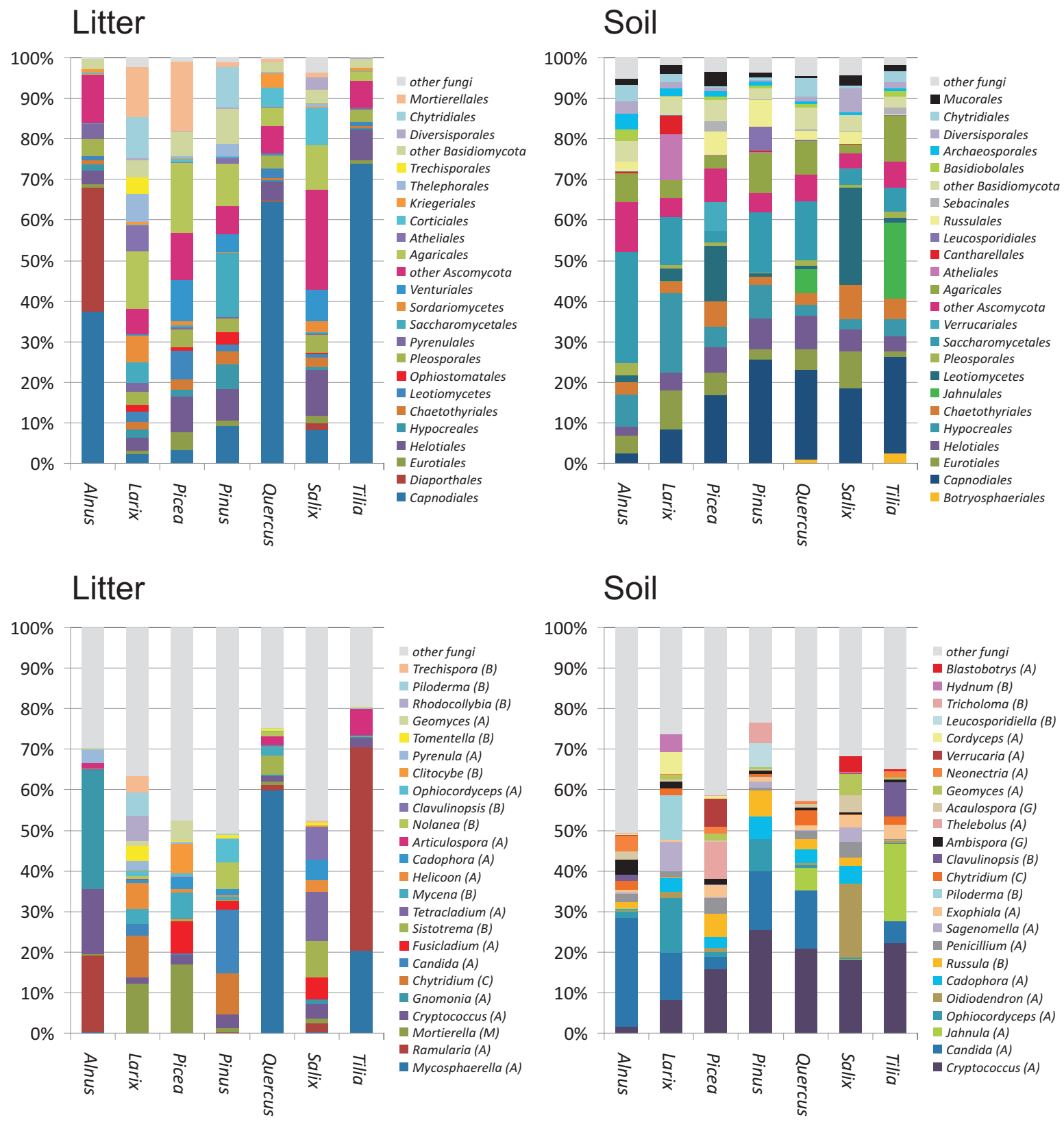
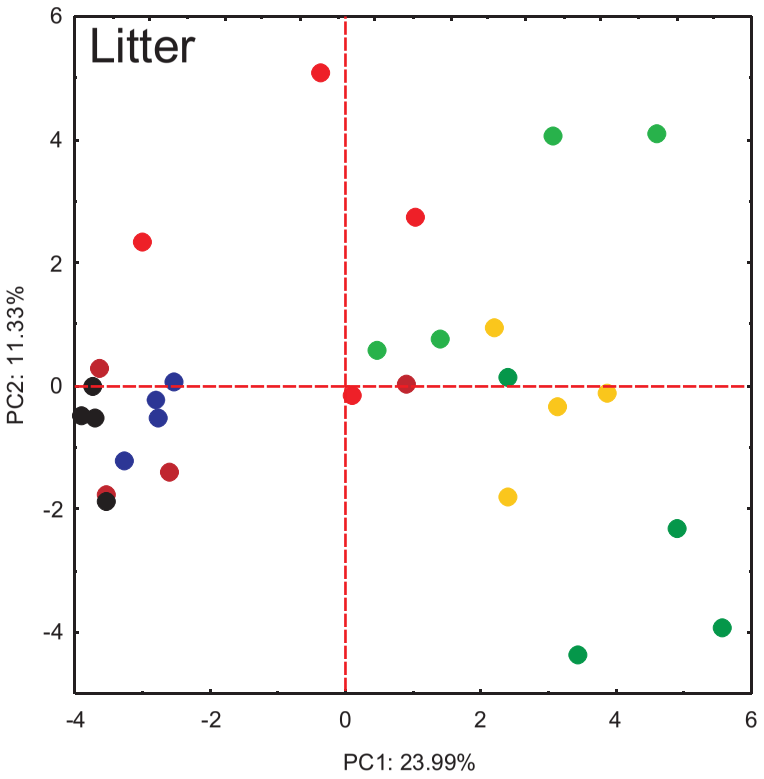
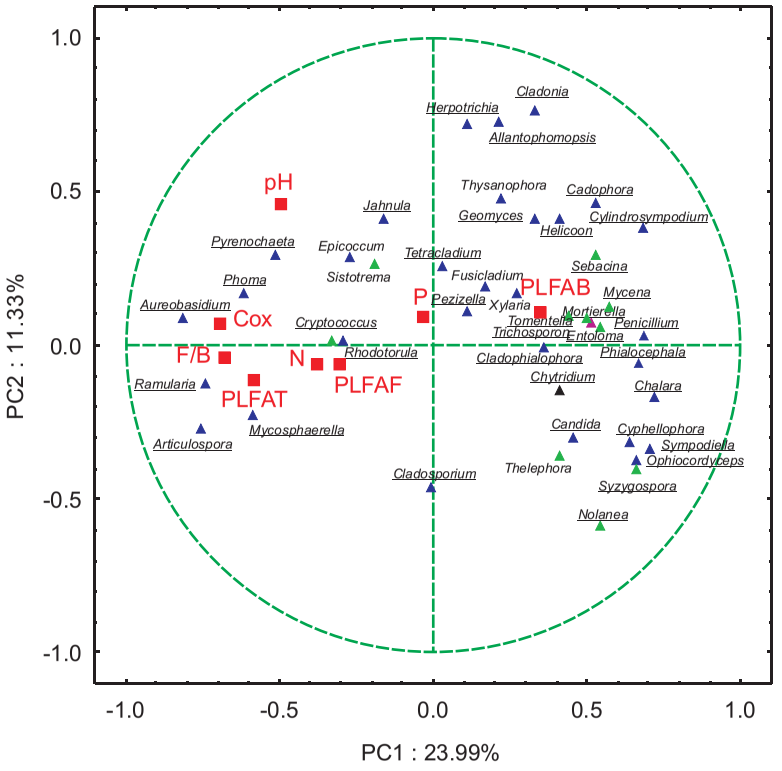
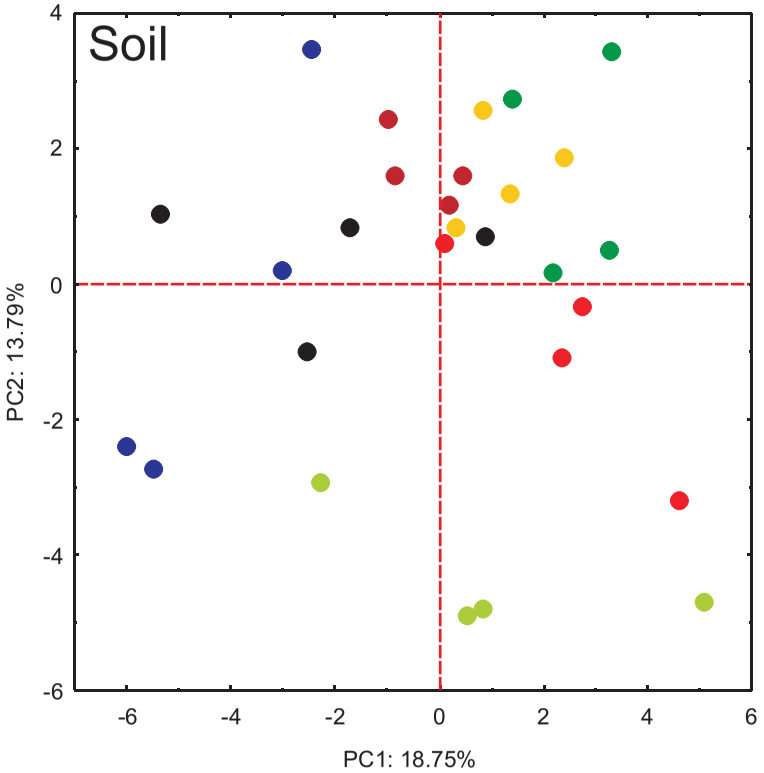

Litter
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alnus
Larix
Picea
Pinus
Quercus
Salix
Tilia
other fungi
Mortierellales
Chytridiales
Diversisporales
other Basidiomycota
Trechisporales
Thelephorales
Kriegeriales
Corticiales
Atheliales
Agaricales
other Ascomycota
Venturiales
Sordariomycetes
Saccharomycetales
Pyrenulales
Pleosporales
Ophiostomatales
Leotiomycetes
Chaetothyriales
Hypocreales
Helotiales
Eurotiales
Diaporthales
Capnodiales
Soil
other fungi
Mucorales
Chytridiales
Diversisporales
Archaeosporales
Basidiobolales
other Basidiomycota
Sebacinales
Russulales
Leucosporidiales
Cantharellales
Atheliales
Agaricales
other Ascomycota
Verrucariales
Saccharomycetales
Pleosporales
Leotiomycetes
Jahnulales
Chaetothyriales
Hypocreales
Helotiales
Eurotiales
Capnodiales
Botryosphaeriales
Litter
other fungi
Trechispora (B)
Piloderma (B)
Rhodocollybia (B)
Geomyces (A)
Tomentella (B)
Pyrenula (A)
Clitocybe (B)
Ophiocordyceps (A)
Clavulinopsis (B)
Nolanea (B)
Articulospora (A)
Cadophora (A)
Helicoon (A)
Mycena (B)
Tetracladium (A)
Sistotrema (B)
Fusicladium (A)
Candida (A)
Chytridium (C)
Gnomonia (A)
Cryptococcus (A)
Mortierella (M)
Ramularia (A)
Mycosphaerella (A)
Soil
other fungi
Blastobotrys (A)
Hydnum (B)
Tricholoma (B)
Leucosporidiella (B)
Cordyceps (A)
Verrucaria (A)
Neonectria (A)
Geomyces (A)
Acaulospora (G)
Thelebolus (A)
Ambispora (G)
Clavulinopsis (B)
Chytridium (C)
Piloderma (B)
Exophiala (A)
Sagenomella (A)
Penicillium (A)
Russula (B)
Cadophora (A)
Oidiodendron (A)
Ophiocordyceps (A)
Jahnula (A)
Candida (A)
Cryptococcus (A)

Figure 4


Litter
PC2: 11.33%
PC1: 23.99%


PC2 : 11.33%
PC1 : 23.99%
pH
Cox
F/B
N
PLFAT
PLFAF
P
PLFAB
Cladonia
Herpotrichia
Allantophomopsis
Thysanophora
Cadophora
Jahnula
Geomyces
Cylindrosympodium
Helicoon
Pyrenochaeta
Epicoccum
Tetracladium
Sebacina
Sistotrema
Fusicladium
Phoma
Pezizella
Xylaria
Mycena
Aureobasidium
Cryptococcus
Mortierella
Tomentella
Trichosporon
Penicillium
Entoloma
Cladophialophora
Phialocephala
Rhodotorula
Chytridium
Ramularia
Chalara
Mycosphaerella
Candida
Cyphellophora
Articulospora
Sympodiella
Ophiocordyceps
Cladosporium
Thelephora
Syzygospora
Nolanea


Soil
PC2: 13.79%
PC1: 18.75%

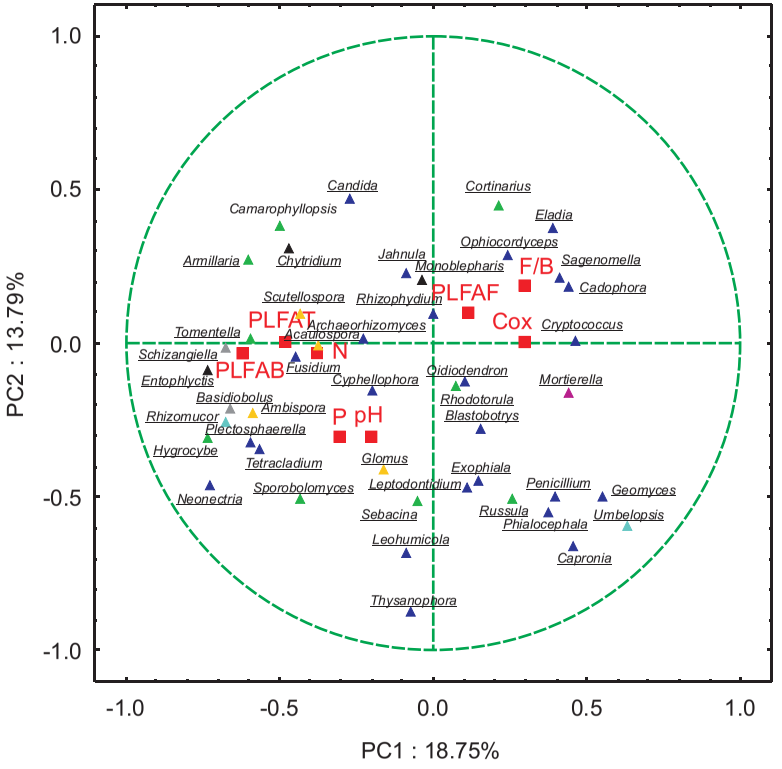
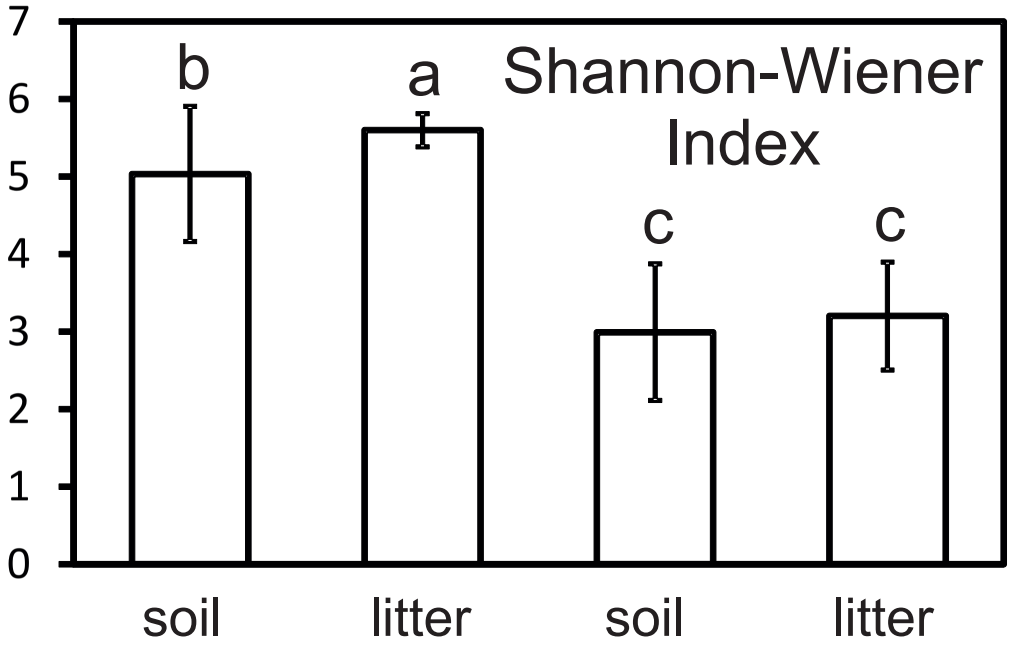
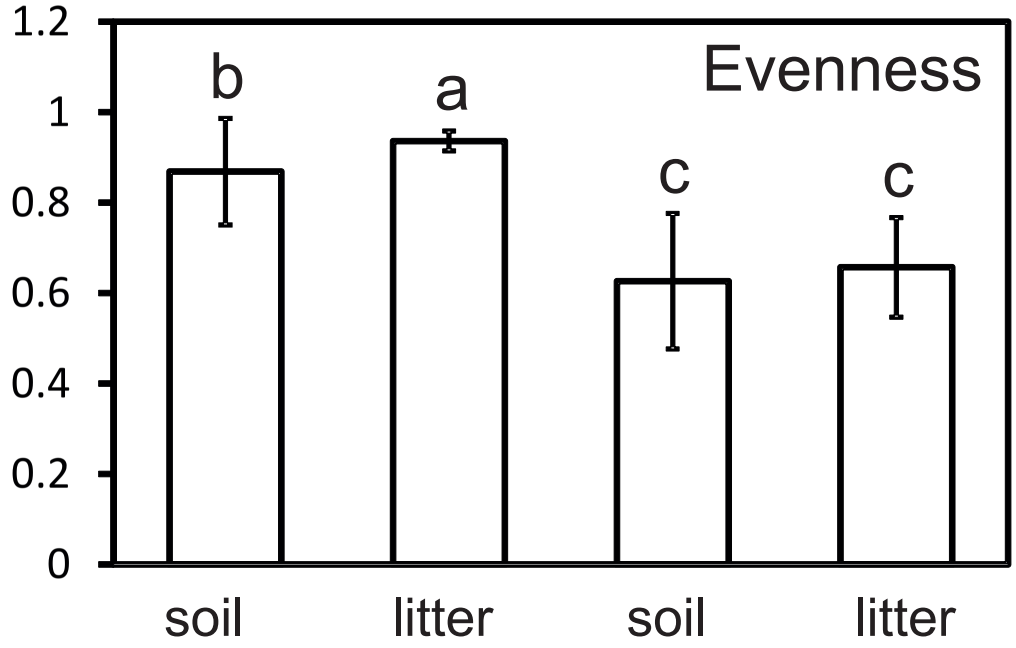
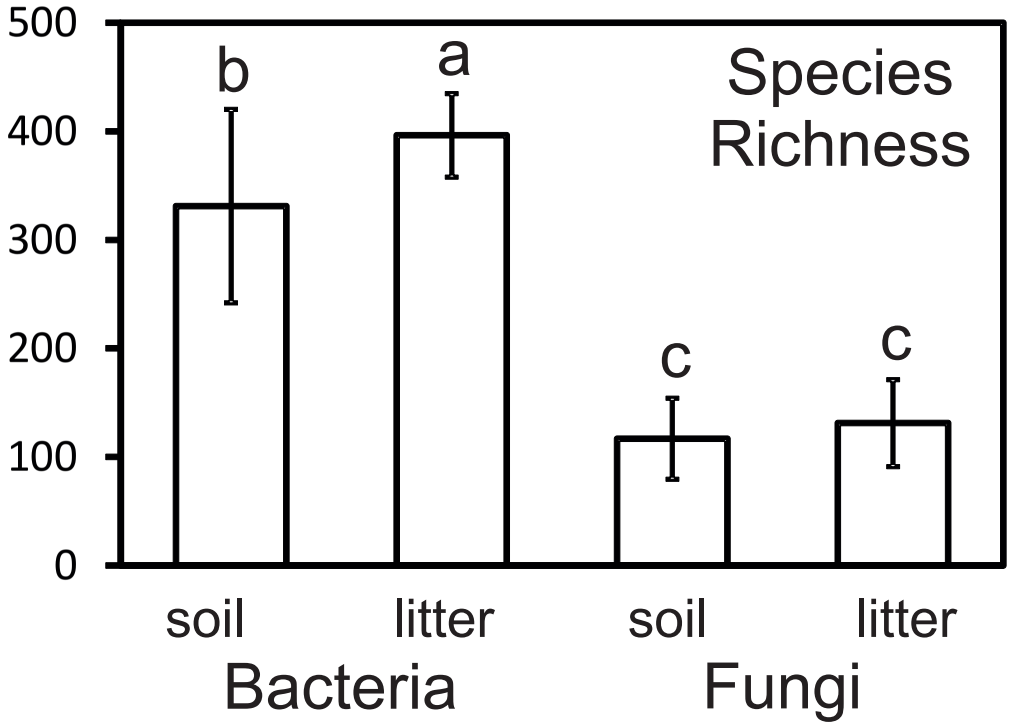

PC2 : 13.79%
PC1 : 18.75%
Candida
Cortinarius
Camarophyllopsis
Eladia
Ophiocordyceps
Armillaria
Chytridium
Jahnula
Monoblepharis
F/B
Sagenomella
Scutellospora
Rhizophydium
PLFAF
Cadophora
PLFAT
Archaeorhizomyces
Cox
Cryptococcus
Tomentella
Acaulospora
N
Schizangiella
PLFAB
Fusidium
Entophlyctis
Cyphellophora
Oidiodendron
Mortierella
Basidiobolus
Ambispora
Rhodotorula
Rhizomucor
P
pH
Blastobotrys
Plectosphaerella
Hygrocybe
Tetracladium
Glomus
Exophiala
Sporobolomyces
Leptodontidium
Penicillium
Geomyces
Neonectria
Sebacina
Russula
Umbelopsis
Phialocephala
Leohumicola
Capronia
Thysanophora

Figure 5


Shannon-Wiener Index
b
a
c
c
0
1
2
3
4
5
6
7
soil
litter
soil
litter
Bacteria
Fungi


Evenness
b
a
c
c
0
0.2
0.4
0.6
0.8
1
1.2
soil
litter
soil
litter
Bacteria
Fungi


Species Richness
b
a
c
c
0
100
200
300
400
500
soil
litter
soil
litter
Bacteria
Fungi

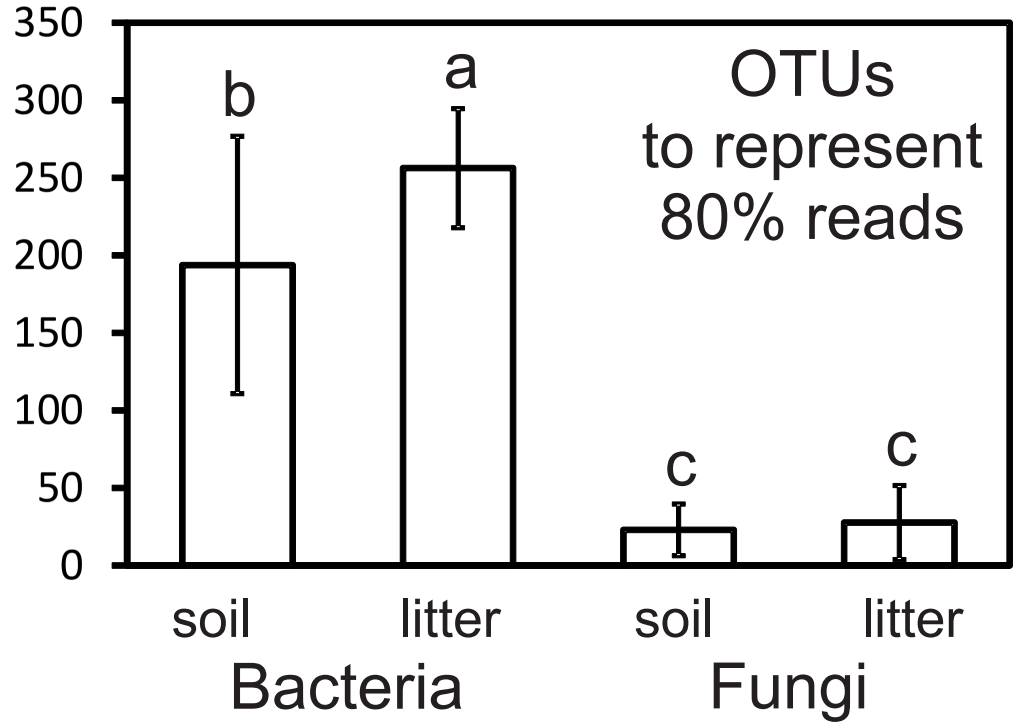
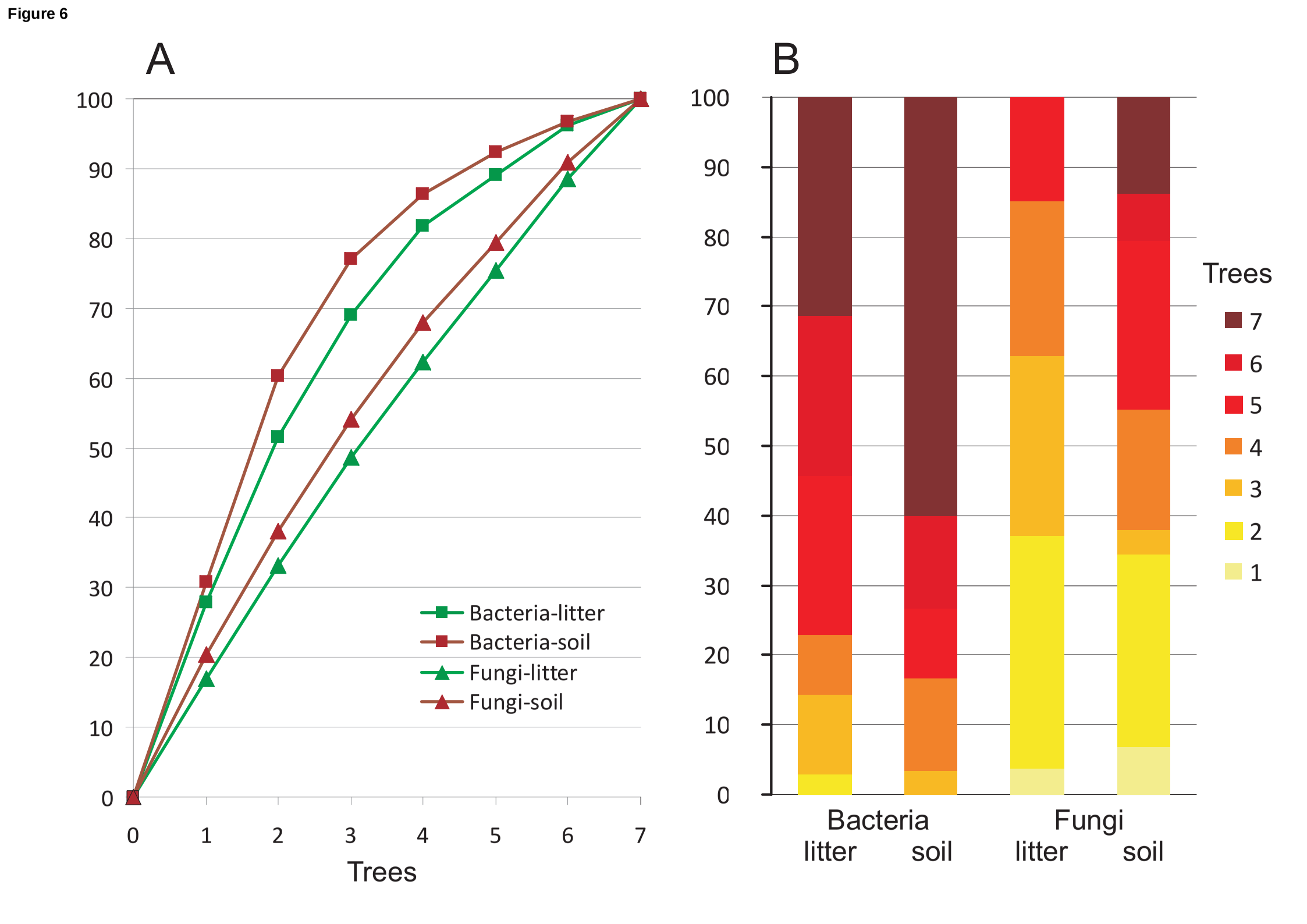

OTUs to represent 80% reads
b
a
c
c
0
50
100
150
200
250
300
350
soil
litter
soil
litter
Bacteria
Fungi

Figure 6


A
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Trees
Bacteria-litter
Bacteria-soil
Fungi-litter
Fungi-soil
B
100
90
80
70
60
50
40
30
20
10
0
Bacteria
litter
soil
Fungi
litter
soil
Trees
7
6
5
4
3
2
1