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Mgr. Martin Bartuška

Dynamika půdní organické hmoty na rekultivovaných a nerekultivovaných výsypkách

Soil organic matter dynamics at reclaimed and unreclaimed post-mining sites

Disertační práce

Vedoucí závěrečné práce: Prof. Ing. Mgr. Jan Frouz, CSc.

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General introduction

Introduction

Mining provides raw materials essential for the development of modern industrial society. At the same time, it severely disrupts ecosystems. Restoration of ecosystems after mining is an essential precondition of future economic development in areas affected by mining. Soil provides ecosystem services crucial for life. It contributes to biodiversity, provides a habitat for organisms, acts as a water filter, and sequestrates carbon dioxide (CO_2) and nutrients. These functions are important for solving global problems such as low water quality, soil erosion and rising atmospheric levels of CO_2. The amount and quality of soil organic matter (SOM) is essential for all these issues. The subject of this thesis is to explore soil organic matter sequestration in post-mining soils and its relation to other ecosystem properties.

Mining soils and their development

Surface mining severely impacts ecosystems (Fisher and Fisher, 2006) by removing vegetation, soil and overburden, changing the elevation of the landscape, altering surface and subsurface geological structures, and disrupting the hydrological regime.

Reclaimed mining soils (RMS) are sometimes referred to as spoils or anthropogenic soils (Sencindiver and Ammons, 2000). They are often characterized by heterogeneous mixtures of rock fragments and sediment materials. In addition, RMS formed by surface coal mining, often contain varying quantities of coal particles distributed throughout the soil profile (Ussiri and Lal, 2008) and can also contain significant amounts of fossil organic matter (Kribek et al., 1998). These soils are composed of unweathered and unoxidized material often highly compacted by heavy equipment used to haul, convey and grade spoil material. They

also suffer from drastic loss of soil organic carbon (SOC, >70%) and nitrogen (N, >65%) caused by oxygen exposure, dilution of carbon by mixing with the subsoil horizon and reduced primary production during mining (Akala and Lal, 2001; Ganjegunte et al., 2009).

Despite these adverse conditions, mining soil offers some new opportunities. Probably the most important is the considerable ability of reclaimed mine lands to accumulate soil organic carbon (Lal et al., 1998; Follet et al., 2000; Ingram et al., 2008). The reasons for this high carbon accumulation in post-mining soil are not fully understood, but it may correspond with the already mentioned low organic carbon content of reclaimed mine soil, which are therefore far from saturated by organic matter (Follet et al., 2000; Lal, 2003). This is consistent with the findings of Vinduskova and Frouz (2013), who found that the rate of carbon storage in post-mining soil rapidly decreases with plot age; in other words, young carbon-poor soils accumulate carbon faster than older soils with higher carbon content. However, the stability of disturbed ecosystems – a function of SOM accumulation, transformation, and soil water and gas exchange processes – may require a period of at least 30 years to attain an equilibrium state (Sopper, 1992).

Studying post-mining soil development has immense potential for practical applications (Bradshaw, 1983, 2000). At the same time, post-mining soil represents an excellent model for studying *de novo* soil development. Moreover, reclaimed mining soils (RMS) represent unique models for the chronosequence approach because mining creates similar sites shaped using the same technology on the same substrates at different points in long time periods that are easy to date (Frouz et al., 2001; 2008). This results in frequent use of chronosequences for studying temporal changes at post-mining sites (Frouz et al., 2001; Sourkova et al., 2005; Frouz et al. 2008, 2009). Chronosequences are a very useful and powerful tool for studying processes and changes of soil over time, which is impossible to obtain in real time because these processes take place over decades or centuries. On the other

hand, this approach has the disadvantage that sites under study must have similar initial conditions and that it does not enable researchers to explore the variability in trajectories of individual sites (Vinduskova and Frouz, 2013). The first limitation is a particular concern for studies of SOM sequestration at sites disturbed by coal mining, because similar sites can apparently differ in fossil organic matter content (Rumpel et al., 1998; Vinduskova and Frouz, 2013), as already mentioned. This problem can be solved by studying one or several sites for an extended period. However, successional ecosystem changes often require many decades or centuries of observation, which is not feasible. This problem can possibly be overcome by studying a large number of chronosequence sites over a prolonged period of time. By taking this approach, one can get a better idea about the variation in ecosystem changes among sites in a manageable time frame. When studying SOM, this approach also eliminates the problem associated with variation in fossil SOM among sites, as we can observe real changes in SOM by comparing two time points at the same site.

Techniques used in post-mining soil reclamation

Soil recovery in post-mining landscapes is essential for the reconstruction of functional ecosystems. Various techniques have been applied to speed up post-mining soil recovery. One of the widely used methods of soil restoration is levelling of earth by heavy machinery. Earth levelling creates sites that are more homogeneous in chemical and physical properties. It is often accompanied by slope modelling to reduce erosion. These practices make future vegetation development more even and sites easier to reach with heavy machinery. Subsequent management is thus easier, including top soil application, fertilization with manure or organic matter, planting seedlings and seed drilling. However, earth levelling may increase soil compaction, which restricts root growth, especially of tree roots.

Consequently, levelling favours the development of grass ecosystems and suppresses the growth of trees. Loss of surface heterogeneity reduces the probability of airborne tree and shrub propagules establishing, as anemochorous species often prefer initial establishment in specific microhabitats that are removed by levelling. The benefits of levelling are likely to prevail should topsoil be applied, especially at sites dedicated to agricultural production or the restoration of grassland-dominated ecosystems (Frouz, 2013).

Introduction of seeds or seedlings is another basic land restoration operation. It speeds up succession and soil development. Litter input from planted vegetation affects soil development depending on litter qualities such as the C/N ratio. Easily decomposable litter promotes higher carbon storage and faster A horizon formation within 15–20 years (Frouz et al., 2008). Trees, however, affect site development over the long term. It takes several decades for differences between reclaimed and unreclaimed sites to diminish; older sites may even be difficult to discern (Frouz, 2007).

Application of topsoil, topsoil substitutes or complex amendments is irreplaceable for restoring agricultural land and reclaiming extremely polluted areas. Topsoil application is a very complex reclamation approach that instantly improves soil conditions and allows rapid establishment of a productive cover comparable to an ecosystem 20 years older. On the other hand, this method is extraordinarily expensive and has potential drawbacks. It may lead to soil compaction, nutrient release or loss of plant diversity. Productivity can also be influenced by the way topsoil is applied. Soil can, for example, be transferred in the form of undisturbed blocks with associated vegetation, or topsoil can be removed, stockpiled and then spread out (Frouz, 2013).

Soil organic matter

SOM is one of the most important reservoirs of carbon; it contains about three times more carbon than the atmosphere (Guo and Gifford, 2002). At the same time, SOM is a very dynamic pool. To illustrate, the amount of CO_2 released by soil respiration into the atmosphere is several times greater than the amount of CO_2 released by burning of fossil fuels (Raich and Schlesinger, 1992; Davidson and Janssens, 2006). Soil can either be a source or a sink for atmospheric CO_2, depending on land use and the management of soil and vegetation. Proper management can promote carbon sequestration, and bad management can cause large releases of CO_2 into the atmosphere (Lal, 2005). Soil organic matter also represents a source of nutrients and energy for soil organisms, so it is essential for nutrient cycling (Carter, 2002). Moreover, SOM affects many physical-chemical soil properties such as sorption capacity, water holding capacity, infiltration, sensitivity to erosion, pH and many others (Brady and Weil, 2007). It is therefore important to understand SOM dynamics as well as the role of SOM in ecosystems and the global carbon cycle.

SOM is comprises plant, animal and microbial residues in various stages of decomposition. During decomposition, the most readily decomposable parts of organic matter decompose first, and the remaining parts become less and less decomposable. Recent research indicates, however, that organic matter persists not because of intrinsic properties of organic matter itself, but due to physicochemical and biological factors of the surrounding environment that reduce the probability and rate of decomposition (Schmidt et al., 2011). Hence the turnover rate of these SOM residues varies due to interactions among biological, chemical and physical processes in soil. For example, many organic compounds are associated with mineral soil particles or physically protected in soil aggregates (Six, 2002). The amount of organic carbon stored in soil reflects a dynamic equilibrium between the input of organic carbon and the rate of SOM mineralization. Soil organic matter can be

conveniently partitioned into different fractions; these, however, do not represent static end products. Instead, they represent pools whose inputs and outputs are determined by environmental conditions. In a soil ecosystem, the rate of decomposition and accumulation of soil organic matter is determined by soil properties such as texture, pH, temperature, moisture, aeration, clay content, mineralogy and biological activity. Conversely, of course, soil organic matter influences or modifies many of these soil properties (Elliott, 1997; Lal, 2002).

Soil organic matter fractions

Although the soil environment is very heterogeneous, and even though SOC dynamics depend on many factors, most studies only consider the bulk soil and total carbon. Some studies explore differences among individual soil horizons or between the rhizosphere and bulk soil (Baldrian et al., 2008; Elhottova et al., 2006), though variation among microhabitats in soil is seldom measured (Mummey and Stahl, 2004; Mummey et al., 2006; Frouz et al., 2010). Techniques for fractionating carbon on the basis of lability have recently been developed. Recognizing subpools of soil carbon may have a greater effect on soil physical stability, and these subpools may be more sensitive indicators than total carbon values of soil carbon (Lefroy, 1993; Blair, 1995; Blair and Crocker, 2000). Soil organic matter can be conveniently partitioned into different fractions. The light fraction (LF) consists of particulate plant and animals tissues in different stages of decomposition. The LF is freely deposited in soil (not bound in complexes with mineral matter), but a certain part of this fraction can be incorporated in macroaggregates as intra-aggregate particulate carbon (Cambarella and Elliot, 1993), and thus become physically stabilized (bound). The turnover of this labile fraction is very fast and can show seasonal fluctuations due to changes in litter input (Boone, 1994).

Soil organic carbon (SOC) transformed by bacteria and stabilized in organomineral complexes with clay and silt is the so-called heavy fraction (HF). This is where the majority of SOC is found. The highest concentrations are associated with < 5μm mineral particles. However, clay-sized organomineral complexes show greater accumulation and subsequently more rapid loss rates than silt-sized particles, indicating higher stability of silt-SOC (Christensen, 1996). Turnover time of the HF is on the order of decades. The transfer of carbon pools from the LF to the HF, while it amounts to only a small portion, is driven by microbial biomass, so the rate is influenced by factors like soil moisture and temperature. Most models of SOC turnover postulate a passive pool (old or stable) of carbon with a turnover of 1,500–3,500 years (Jenkinson, 1990).

Aggregation

Carbon sequestration is a process of transforming atmospheric carbon into biomass and incorporating it into soil as organic matter. The balance between carbon added by decomposition of living photosynthetic plants (or other organisms) and losses from microbial respiration determines the amount of carbon accumulated in soil. Increased protection of carbon released into soil by microbial decomposition supports carbon sequestration. Soil aggregates are needed for long-term sequestration because they protect carbon from decomposition, resulting in much longer residence time for carbon (Shrestha, 2006). This link between SOC accumulation and soil aggregation dynamics is well known. Van Veen and Pauli (1981) point out that the equilibrium level of soil organic carbon is more dependent on the extent of protection than on the composition of plant residues added to the soil. Soil structure is a result of rearrangement, flocculation and cementation of soil particles mediated by the soil fauna, microorganisms, roots, inorganic binding agents and environmental

variables. Aggregates not only protect SOM (Tisdall and Oades, 1982), enhancing carbon accumulation, but also influence the structure of the microbial community (Hattori 1988), limit oxygen diffusion (Sexstone et al., 1985), regulate water flow (Prove et al., 1990), determine nutrient adsorption and desorption (Wang et al., 2001), and reduce run-off and erosion (Barthes and Roose, 2002). The following factors play major roles in aggregation and stabilization of soil: 1) soil fauna, 2) soil microorganisms, 3) roots, 4) inorganic binding agents and 5) environmental variables. By the nature of aggregates, carbon storage can be short-term in macroaggregates, long-term in microaggregates and stable in silt-clay fractions (Six et al., 2002). This is why, in order to understand SOM dynamics, we have to consider the roles of the soil matrix, the soil biota and their various interactions (Six et al, 2004) as well as the distribution of carbon pools.

Effect of management on SOM

The carbon pool is strongly affected by landscape management. The conversion of native ecosystems (e.g. forests, grasslands and wetlands) to agricultural uses, together with continuous harvesting of plant materials, has led to significant losses of plant biomass and carbon (Davidson and Ackerman, 1993). Practices such as tillage, fertilization, drainage, irrigation or biomass burning substantially increase carbon loss from soil; changes in tree species composition or conversion of forests into pastures also alter carbon storage in rather complex ways (Davidson and Ackerman, 1993; Guo and Gifford, 2002). To achieve long-term sustainability of human-exploited ecosystems, SOM dynamics need to be better understood and managed. Suitable agricultural and forestry practices can reduce carbon loss from soil and even promote carbon storage in soil (Davidson and Ackerman, 1993; Guo and Gifford, 2002). Further soil carbon loss is caused by the rising global average temperature.

Increased respiration supports CO_2 release, causing a positive feedback to global warming. Sourková (2005) and Frouz (2009) claim that disturbed and degraded areas under proper management can promote soil carbon accumulation and thus mitigate the increase in atmospheric CO_2. Moreover, as mentioned above, increased carbon stock in soil also has other beneficial effects. Organic matter contributes to plant growth through its effect on physical, chemical, and biological properties of soil. It serves as a source of nitrogen and phosphorus for plants, affects the biological functions and activities of the microflora and microfauna, and alters physical and chemical substrate properties (soil structure, aeration, retention of water and increased buffer and exchange capacity). This may have important repercussions in agriculture and forest production. Recovery of the soil carbon stock plays a crucial role in ecosystem restoration following severe disturbances.

Major questions and hypotheses

Questions

Previous research indicates that post-mining soils near Sokolov and also in other mining areas contain considerable amounts of fossil organic matter.Q1 Is the amount of fossil organic matter content constant over time, or is it reduced by microbial decomposition?

Q2 How can we improve the estimation of the SOM accumulation rate from chronosequence data; namely, how is it possible to overcome variation in fossil SOM content among sites?

Q3 How do the dynamics of individual SOM fractions vary over time, and how do they contribute to the overall pattern of SOM accumulation?

Q4 How do microbial communities vary among individual fractions, and how do these fractions contribute to the overall microbial community in bulk soil?

Hypotheses

H1 Fossil organic matter in overburden can be subject to microbial breakdown, which may cause its reduction over time. Thus during development of post-mining soils, changes in SOM content result from a combination of fossil SOM mineralization output and recent SOM accumulation.

H2 A long-term study of sites scattered along a chronosequence can bring about a better understanding of SOM accumulation, as this approach overcomes variation in fossil SOM content among sites. This approach also allows us to explore variation among SOM accumulation trajectories of individual sites and provides better insight into changes of SOM accumulation trajectories over the course of succession.

H3 Soil organic matter consists of several pools that have their own dynamics during pedogenesis.

H4 Each of these pools also contains a specific microflora that has its own dynamics during succession.

Major findings

The results of this study are summarized in four manuscripts, which have either been published or submitted for publication in international journals.

Paper 1 Frouz, J.; Cajthaml T., Kříbek, B.; Schaeffer, P.; & Bartuška, M. et al. (2011) Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter. Folia microbiol 56:389–396

Paper 2 Bartuška, M.; Frouz, J. (2013) Carbon accumulation and changes in soil chemistry in reclaimed open- cast coal mining heaps near Sokolov using repeated measurement of chronosequence sites. Eur J Soil Sci (submitted)

Paper 3 Bartuška, M., Frouz, J. (2010) Methods for measuring carbon dynamics in soil. Environmentalica 24:1–2,109–122

Paper 4 Bartuška, M., Pawlett, M., Frouz, J. (2013) Particulate organic carbon in reclaimed and unreclaimed post-mining soils and its microbial community composition. Catena (submitted)

Paper 1 deals with question 1 and the corresponding hypothesis. The subsurface microbial community and its influence on early stages of pedogenesis were studied on freshly mined-out overburden not inoculated by microorganisms from the surrounding environment. Measurements of respiration revealed that the deep microflora community in subsurface layers may substantially contribute to the microbial community in soil heaps, especially during early stages of succession. In this period, microscopic observations also indicated massive breakdown of the sediment implying decomposition of fossil organic matter (Paper 1).

Paper 2 deals with question 2 and the related hypothesis, Accumulation of soil organic matter and associated changes in soil chemistry are important drivers and indicators of ecosystem recovery at post-mining sites. Chronosequences, or sets of plots of known different age, are most useful for studies of interactions between chemical and biological soil properties over time. The method is limited by site heterogeneity, but this can be amended by repeated measurement after an extended period, which allows us to observe actual changes in soil chemistry, the carbon stock and other soil properties. Soil development is the most rapid within the first 50–60 years, after which it slows down. Chronosequences in combination with repeated measurements are therefore a valuable tool for monitoring pedogenesis and interactions among soil, vegetation and the biota. Two separate studies were conducted at post-mining sites near the town of Sokolov in the Czech Republic (1999 and 2010).

An alder chronosequence was studied in 1999 and again using the same method eleven years later at the same localities. The sites were 4 to 45 years old in 1999 and 15 to 56 years old during the second sampling in 2010. Soil pH decreased with site age, the decrease being more substantial in the upper soil layer. These changes over the 11 years were negatively correlated with the initial pH at the sites. At alkaline sites, the pH decreased. At acidic sites, by contrast, the pH increased. The amount of soil carbon rose with site age. On the contrary, with site age, the rate of carbon accumulation declined (based on differences between the first and second sampling). The average increases in the carbon stock corresponded well to values determined by the chronosequence and real-time approaches. Changes in soil nitrogen content exhibited a similar pattern as changes in soil carbon content. Phosphorus content did not differ significantly among sites, but tended to be lower at older than at younger sites.

Papers 3 and 4 deal with the role of SOM fractions in SOM accumulation and development of the soil microbial community (questions 3 and 4 and associated hypotheses). The results of recovering post-mining soil also depend on organic matter and associated microbial biomass, which is a basic precondition for successful restoration. Two chronosequences with different vegetation were studied. The aim was to compare the dynamics of soil carbon fractions and those of the microbial communities associated with these fractions. Soil properties such as soil carbon, pH, bulk density and the light fraction of particulate organic carbon (POC), be it free or bound in soil aggregates, were studied in two chronosequences at post-mining sites, both covering sites 10 to 50 years old. The first reclaimed chronosequence was vegetated by an alder plantation with a low C/N litter ratio (*Alnus glutinosa*); the second was unreclaimed and overgrown by natural regrowth with a high C/N litter ratio (*Salix caprea*, *Populus tremula* and *Betula pendula*). Using bulk soil and POC fractions, the microbial community was examined for intermediate and late succession stages by analysing phospholipid fatty acids (PLFAs). Soil carbon content in both

chronosequences increased and pH decreased with plot age. These changes were more pronounced at reclaimed sites. The amount of POC fractions (light, bound) increased with plot age. The light fraction was always greater than the bound fraction in both chronosequences. The higher amount and larger increase in both fractions was more pronounced at reclaimed than at unreclaimed sites. The carbon content in both fractions increased with succession age, having a higher carbon content at reclaimed sites. POC fractions affected the microbial community more than plot age. The microbial community of bulk soil in reclaimed plots was more similar to the community of the bound POC fraction. In unreclaimed plots, by contrast, the community was more similar to the light fraction. Observed differences in POC, and thus also the microbial community, correspond with the higher level of bioturbation at reclaimed sites, which promotes faster POC accumulation and brings the microbial community closer to bound POC.

Conclusion

Disturbed and post-mining sites represent a good model for studying SOM accumulation, changes in soil chemistry and soil biological properties during pedogenesis. This unique environment is suitable for complex studies of pedogenesis, the microorganism community, ecosystems and plant succession under different tree cover and landscape management. Our set of similar sites of known age provides a good collection of chronosequences which allows us to study decades of successional changes. It also enables us to compare historical development among different types of management, which reveals differences or similarities among various reclamation techniques. We can thus gain an overall view of post-mining processes and sufficient data for modelling future soil evolution and predicting the course of ecosystem development. To enhance the information value of chronosequence data, it is necessary to combine them with real-time datasets (known temporal

changes at different sites with similar management). This paints a detailed picture of SOM concentrations and other soil properties in different stages of ecosystems development. The dynamics and accumulation of SOM should not be reduced only to total carbon. Emphasis must be placed on the distribution of carbon in carbon fractions. Combining real-time data with the chronosequence approach and knowledge of SOM accumulation, SOM fraction distribution and soil properties can significantly improve the accuracy of predictive models.

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Paper 1

Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter

Jan Frouz & Tomas Cajthaml & Bohdan Kříbek & Philipe Schaeffer & Martin Bartuška & Renata Galertová & Petr Rojík & Vaclav Krištůfek

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J. Frouz (*): M. Bartuška : V. Krištůfek
Institute of Soil Biology BC ASCR,
Na Sádkách 7,
CZ37005 České Budějovice

T. Cajthaml
Institute of Microbiology AS CR, Videnska 1083
14220 Prague 4, Czech Republic

B. Kříbek
Czech Geological Survey, Klárov 3
CZ 11000 Prague 1, Czech Republic

P. Schaeffer
Biogeochemistry Group,
Université de Strasbourg, UMR 7177 du CNRS, ECPM,
25 rue Becquerel,
67200 Strasbourg, France

M. Bartuška：R. Galertová
Institute for Environmental Studies, Charles University, Benátská 2,
CZ12800 Prague 2, Czech Republic

P. Rojík Příbramská 381
Rotava,Czech Republic

Abstract

Three types of Miocene claystones (amorphous, lamellar, and transitional) were aseptically sampled from depths of 30 m and 150 m below the soil surface. Respiration of these sediments was measured under conditions that prevented inoculation by other microorganisms not indigenous to the claystones in situ. Microbial respiration was higher in lamellar than amorphous clay-stones and was not affected by sampling depth. During cultivation, microbial biomass (as indicated by PLFA) significantly increased. Microbial biomass after cultivation was significantly higher in sediments from 30 m than from 150 m depth. Both microbial respiration and biomass increased after glucose addition.

Introduction

The occurrence of microflora in deep, subsurface layers is well documented (Frederikson et al. 1991; Lehman et al. 2001; Inagaki et al. 2002; Borscik et al. 2003; Pfiffner et al. 2006; Elhottová et al. 2006; Fredrickson and Balkwill 2006; Mauclaire et al. 2007). These microorganisms contribute to many important geological processes (Pedersen 1993). Despite this, microflora from deep clay sediments has seldom been studied (Elhottová et al. 2006, Mauclaire et al. 2007). Massive layers of these Miocene sediments have been excavated and deposited in large heaps that cover thousands of hectares before coal is extracted during opencast mining (Frouz and Nováková 2005; Helingerova et al. 2010). In the current study, we determine the potential contri-bution of deep, subsurface microflora to microbial activity in freshly deposited spoil substrates. To do so, we studied the activity and biomass of microflora in samples of Miocene claystones incubated under laboratory conditions that prevented colonization by non-indigenous microorgan-isms. We also studied how the activity and biomass are

affected by moisture fluctuation, freezing, or glucose addition. Moisture fluctuation and freezing are known to cause mechanical breakdown of the sediment, which can increase availability of fossil organic matter. Addition of glucose mimics input of easy decomposable substrates from litter or root exudates, which can trigger priming effect and accelerate decomposition of fossil organic matter. Finally, we studied the nature of the fossil organic matter, which is only carbon source in these deep subsurface layers.

Methods

Sampling

Samples were collected in the "Družba" open-mine pit near Sokolov at 30 m depth (in 2009 and 2010; 50°12′27.150″N, 12°42′10.125"E) and at 150 m depth (in 2010; 50°12′33.391″N, 12°42′21.128″E). The sampled material represented Miocene clay sediment that was deposited in meromictic lakes 21.3–16.5 Ma BP (Rojik 2004). Both layers were below the line of weathering, which is 15 m below the surface. Sampling methods closely followed those used by Elhottová et al. (2006). An intact solid block of claystone (ca. 0.5×0.3×0.3 m) was taken from the freshly opened mine front. Three types of substrates were sampled: a brown, lamellar sediment; a grayish-blue amorphous sediment; and a transitional layer between these two. Two blocks from each sediment type were sampled on each sampling occasion, but the transitional layer was sampled only in 2010. The lamellar sediment was formed under anoxic conditions at the bottom of a deep tertiary lake, while the amorphous sediment was formed when the lake was shallower and sediment was well aerated (Rojik 2004). Each block was wrapped in sterile foil and transported in a sterile plastic bag to the laboratory, where they were exposed to UV radiation twice for 1 h and were subsequently seared with a flame burner in a microbiological flow box (Lamin Air, Holten, Denmark). A

30-mm surface layer then was removed, and the block was seared again with a burner. The block was then aseptically cut open, and three mixed samples (each 50 g) were obtained from the core of each block.

Microbial analysis

There were three respiration experiments. In experiment 1, which was conducted in 2009, respiration was measured in lamellar and amorphous sediment that had been collected at 30 m depth. Respiration was measured after 1 week and then at 2-week intervals until the experiment was terminated after 91 days. Bottles were weighed after each titration, and if substantial reduction in weight was detected as a result of desiccation, an appropriate volume of sterilized tap water was added to compensate for water loss.

In experiment 2, which was conducted in 2010, samples of lamellar, amorphous, and transitional sediment from 30 m depth and 100 m depth were preincubated at 20°C for 2 weeks before respiration was measured for 2 weeks as described earlier.

In experiment 3 which was parallel to experiment 2 and had similar design, the following five treatments were applied to the transitional sediment from both depths at the end of the preincubation period. In the "water" treatment, 5 ml of water was added at the start of the 2 weeks when respiration was measured. In the "glucose" treatment, 5 ml,of a 2% glucose solution was added at the start of the 2 weeks when respiration was measured. In the "freezing" treatment, samples were frozen at −18°C for 24 h and then thawed at 20°C for 24 h at the start of the 2 weeks when respiration was measured. The last two treatments were combinations of freezing followed by water addition ("freezing and water") or by glucose addition ("freezing and glucose") in which freezing and additions of water or glucose were performed as described above.

For measurement of microbial respiration, 10 g of sediment (fresh mass) was placed in a 100-ml airtight bottle. There were six bottles from each treatment always tree from one sediment block. The CO2 that evolved from the sediment was trapped in 3 mL of 500 mmol/L NaOH and then quantified by titration with 50 mmol/L HCl after addition of BaCl2. Control bottles, without sample, were used to assess CO2 trapped during handling. Bottles were incubated in the dark at 20°C. Bottles were manipulated under aseptic conditions, and all tools and chemicals were sterilized to prevent contamination of deep, subsurface sediment by non-indigenous microorganisms.

Before and after experiment 1 and after experiment 2, and in selected treatment after experiment 3, 2 g of sediment was sampled from three replicates in each treatment. The microbial biomass in these samples was determined based on analysis of phospholipid fatty acids (PLFAs). The samples were extracted with a chloroform–methanol–phosphate buffer (Bligh and Dyer 1959), separated sing solid-phase extraction cartridges (LiChrolut Si 60, Merck), and subjected to mild alkaline methanolysis as described by Šnajdr et al. (2008). The free methyl esters of the PLFAs were analyzed by gas chromatography–mass spectrometry (Varian 3400; ITS-40, Finnigan). The GC instrument was equipped with split/splitless injector, and a DB-5MS column was used for separation (60 m, 0.25 mm i.d., 0.25 mm film thickness). The temperature program started at 60°C and was held for 1 min in splitless mode. Then, the splitter was opened, and the oven was heated to 160°C at 25 K/min. The second temperature ramp was up to 280°C at 2.5 K/min, and this temperature was maintained for 10 min. The solvent delay time was 8 min. The transfer line temperature was 280°C. Mass spectra were recorded at one scan per second under electron impact at 70 eV and mass range of 50–350 Da. Methylated PLFAs were identified according to their mass spectra and by comparison with a mixture of chemical standards obtained from Sigma. Šnajdr et al. (2008) was used to identify major groups of microflora based on fatty acid characteristics. Total

content of all PLFA molecules was used as a measure of total microbial biomass. Small samples (1.5×1.5× 1.5 mm) collected at the end of experiment 2 were frozen in liquid nitrogen and immediately observed using Cryo high-resolution with a JEOL JSM-7401F cryo-scanning electron microscope.

Sediment characteristic

In 2010, the chemical characteristics were determined for the three sediment layers. Three samples were collected from each of the three layers. Total C and N were determined using a Carlo Erba CNH elemental analyzer. Lamellar sediment from 30 m depth was used for more detailed analysis. Liquid chromatography and the separation of extracts into individual fractions were carried out as described by Landais et al. (1991).

The aliphatic fraction of the extracted organic material was analyzed using a Varian 3400 GC/MS system. A Restek RTX-5MS capillary column was used (length, 300 m; phase thickness, 0.1 μm). The temperature program was: 80°C to 150°C (10 K/min.), 150°C to 300°C (3 K/min), and isothermal program at 300°C was applied thereafter. Results were expressed in the form of the total ion chromatograms. 13C CP/MAS NMR spectra were measured using the Bruker MSL 200 spectrometer, working at a frequency of 50.32 MHz for 13 C in a 4.7-T static magnetic field. The number of accumulation was 4,096–10,000 recording, and the rate of sample rotation was 5 kHz.

Statistical analysis

Microbial respiration, biomass, and C and N content among depths and sediment types or treatments were compared using two-way ANOVAs. When interactions between depth and other factors were significant, oneway ANOVAs were used for each depth. Statistica 6.0 was

used for statistical analysis. The effect of cultivation on changes in PLFA composition was explored using principal component analysis (PCA), which was computed by CANOCO 4.0.

Results

In experiment 1, in which respiration in amorphous and lamellar substrates from 30 m depth was studied for 91 days, respiration was higher after 1 week than after 3 weeks of cultivation (Fig. 1). After the third week, water was added to compensate for desiccation, and respiration slightly increased. Respiration was higher in the lamellar than in the amorphous sediment (t test, $p<0.01$). As indicated by PLFAs in experiment 1, total microbial biomass, and the biomass of fungi, total anaerobes, G+ bacteria, and G− bacteria had significantly increased by the end of the experiment (Table 1). According to two-way ANOVAs, microbial biomass was not significantly affected by sediment type or the interaction between sediment type and cultivation. The composition of the microbial community before cultivation as indicated by specific PLFAs was similar in both sediments. After cultivation, the lamellar sediment was enriched by actinobacteria (indicated by PFLA 10Me17), while the microbial composition of the amorphous sediment became more variable (as indicated by substantial spread in PCA diagram (Fig. 2). In experiment 2, which included the three sediments from 30 and 150 m depths, microbial respiration after weeks of incubation was not significantly affected by sampling depth but was significantly affected by sediment (Fig. 3a) and the interaction between sediment and depth. Respiration was higher in the lamellar than in the amorphous or transitional sediment, and respiration was higher in the transitional sediment from 30 m than from 150 m, but the opposite was true for the lamellar sediment ($p<0.05$, least significant differences (LSD) post hoc test). Total microbial biomass after cultivation was significantly higher in samples from 30 m than from 150 m depth. The same was true for anaerobes, G+ bacteria, and G− bacteria (Table 2). In the experiment 3, microbial respiration

in the transitional sediment from 30 m depth but not from 150 m depth was significantly increased by addition of glucose, by freezing, and by the combinations of freezing and water or glucose ($p<0.05$, LSD post hoc test; Fig. 3b). Addition of glucose also increased microbial biomass in sediment from 150 m depth (Table 3). After incubation in experiment 2, untreated sediment was examined by cryo-high-resolution scanning electron microscopy. Traces of microorganisms were locally abundant (Fig. 4). Filamentous bacteria or actinomycetes were the most common. Individual filaments were less than 1 μm wide and more than 50 μm long. A greater branching and a higher density of filaments occurred in zones with visible breakdown of the sediment (Fig. 4). The amount of C in the sediments ranged from 2.8 to 10.5, the content of C and N was higher in 30 m than in 150 m depth, and in both cases, transitional sediment had the highest C and N content (Table 4). 13C NMR spectrum of the organic matter from the lamellar sediment from 30 m depth (Fig. 5) shows predominantly aliphatic character of the organic matter. As documented by the presence of a broad band between 0 and 105 ppm which is assigned to the aliphatic carbon, the greater part of the band (0–53 ppm) indicates the presence of a methyl group C, methylene group C in saturated chains, and methylene bridge and bridgehead C of naphtalenes. The smaller part of the band (53–105 ppm) indicates the presence of Osubstitued aliphatic C in ethers, alcohols, and methoxy groups. Band intensity is much lower for the aromatic carbon (104–164 ppm). The amount of bitumen (organic matter extractable from the rock with dichloromethane) ranged from 15 to 25 mg/g TOC. The bitumen fraction of typical claystone contained an average of 70% polar substances (asphaltenes and maltenes); the amount of aromatic hydrocarbons varied between 3% and 26% and aliphatic hydrocarbons, 22% and 26%. The latter are dominated by n-alkanes in the C16–C27 range (n-C17 predominant), pristane, phytane, and cyclic terpenoids (Fig. 6) mainly comprising phyllocladane and various pentacyclic

triterpenoids of bacterial (hopanes; com pounds 8, 9, 15, 16 on Fig. 6) and higher plant (compounds 10–14) origin.

Discussion

The levels of microbial respiration recorded in both experiments were lower than levels previously recorded in the organic horizon of surface soils (Helingerova et al. 2010), but the levels were comparable with microbial respiration observed in recently (0–5y) deposited spoil at post-mining sites (Frouz and Nováková 2005). The respiration of deep, subsurface microflora recorded in the current study was also within an order of magnitude of the respiration in the mineral layer (50–100 mm depth) of forest at post-mining sites (Helingerova et al. 2010). The respiration reported here was also comparable to that observed in other deep, subsurface sediments (Kieft and Rosacker 1991). In addition, total microbial biomass after cultivation in the current study was similar to that recorded in a young (4-year-old) post-mining site created by excavation and dumping of sediment from the same mine (Baldrian et al. 2008). These data support the idea that the microflora in deep, subsurface layers may substantially contribute to the microbial activity in the spoil heaps particularly in the early stages. The constant, relatively long-term microbial activity in both types of sediment in experiment 1 indicates that deep, subsurface microorganisms can decompose fossil organic matter (Petsch et al. 2001). In agreement with previous studies, we have found that fossil organic matter in Miocene sediment mainly contains aliphatic components (Kříbek et al. 1998) so this is most likely to be decomposed. However, we have calculated that during the 91 days of incubation in the first experiment, only up to 1% of the total organic matter was mineralized. Hence, we cannot exclude the possibility that the microorganisms used some rare part of the fossil organic matter that is easily degraded. On the other hand, microscopic observation indicated massive breakdown of the sediment (Fig. 4), which is consistent with decomposition of major components of the fossil organic matter. Although lamellar sediment

is thought to have been deposited under strongly anaerobic conditions (Kříbek et al. 1998), it did not contain a greater abundance of anaerobic microflora than the other sediments according to PLFA data in the current study. This suggests that the microbial community may be more affected by deep burrowing and recent sediment conditions than by conditions during sedimentation.

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Appendices

Table 1 Concentration (micrograms per gram) of total PLFA and PLFA markers (means±SD, n=3) characteristic of various groups of microorganisms in two types of Miocene sediment (amorphous and lamellar; excavated from 30 m depth from opencast mine) before and after 91 days of aseptic cultivation (experiment 1)

Sediment type and before or after incubation	Fungi	Bacteria	Actinobacteria	G+	G−	Anaerobes	Total
Amorphous before	0.6±0.4	101±110	22±32	6±7	8±2	63±71	135±134
Lamellar before	0.8±0.5	54±30	20±25	2±0	8±1	23±10	83±35
Amorphous after	2.6±0.9	368±150	28±21	28±13	84±81	231±109	1,769±1,191
Lamellar after	1.4±0.4	297±11	41±26	23±8	24±1	195±27	591±74
Cultivation	0.004	0.003	ns	0.001	<0.001	0.003	<0.001
Type	ns	ns	ns	ns	ns	ns	ns
Interaction	ns	ns	ns	ns	ns	ns	ns

The last three rows indicate p values for two-way ANOVAs on the effects of sediment type and depth ns not significant

Table 2 Concentration (micrograms per gram) of total PLFA and PLFA markers (means±SD, n=3) characteristic of various groups of microorganisms in three types of Miocene sediment (amorphous, lamellar, and transition; excavated from 30 m and 150 m depth from opencast mine) before and after 2 weeks of aseptic cultivation

Sediment and depth	Fungi	Bacteria	Actinobacteria	G+	G−	Anaerobes	Total
Amorphous, 30 m	2.0±0.5	701±115	24±2	54±1	126±83	554±137	901±98
Transition, 30 m	2.6±0.6	1,014±125	58±21	163±136	667±40	444±114	2,098±637
Lamellar, 30 m	2.5±1.1	2,306±1,125	35±23	55±22	1,017±992	1,959±911	2,906±1,616
Amorphous, 150 m	1.4±0.7	61±6	18±15	3±1	8±1	32±8	95±1
Transition, 150 m	1.0±1.0	230±83	17±11	57±38	37±3	130±25	286±99
Lamellar, 150 m	1.9±0.3	312±247	193±175	12±8	6±1	95±60	385±253
Depth	ns	0.002	ns	0.024	0.009	<0.001	0.001
Sediment	ns	ns	ns	ns	ns	ns	ns
Interaction	ns	ns	ns	ns	ns	ns	ns

The last three rows indicate p values for two-way ANOVAs on the effects of sediment type and depth ns not significant

Table 3 Concentration of total PLFA (micrograms per gram) and PLFA markers characteristic of various groups of microorganisms in transitional type of Miocene sediment excavated from 30 m and 150 m depths in opencast mine as affected by freezing (F) or by freezing and addition of glucose (F+G) and untreated control (C)

Treatment and sediment depth	Fungi	Bacteria	Actinobacteria	G+	G−	Anaerobes	Total
C, 30 m	2.6±0.6	1,014±124	58±20	162±135	666±40	443±113	2,098±636
F, 30 m	2.3±0.4	1,316±304	65±36	156±5	146±1,060	1,003±308	1,478±249
F+G, 30 m	13.4±12.0	1,746±108	568±544	253±10	379±90	667±331	2,145±47
C, 150 m	1.0±1.0	230±83a	17±11a	56±38	37±3b	130±25a	285±98a
F, 150 m	1.7±0.1	305±13a	22±3a	59±42	9±3a	209±60a	363±29a
F+G, 150 m	11.8±8.4	4,758±1,395b	1,132±393b	737±49	75±3b	2,767±935b	4,955±1,409b
Depth	ns	0.011	ns	ns	0.001	ns	0.005
Treatment	ns	<0.001	0.044	ns	0.011	0.008	0.001
Interaction	ns	0.002	ns	ns	ns	0.008	0.003

Values in the first six rows are means±SD of three replications. The last three rows indicate p values for two-way ANOVAs on the effects of treatment and sediment depth. At each depth, means followed by the same letter are not significantly different (one-way ANOVA, LSD post hoc test $p<0.05$); if no letter were preset no significant difference was found ns not significant

Table 4 Carbon and nitrogen content and C/N ratio for sediments and depths (means±SD, n=3)

Sediment and depth	C%	N%	C/N
Amorphous, 30 m	10.45±0.01a	0.35±0.01a	30.31±0.90c
Transitional, 30 m	11.15±0.30c	0.58±0.03b	19.43±1.41b
Lamellar, 30 m	5.22±0.06b	0.36±0.01a	14.56±0.37a
Amorphous, 150 m	2.97±0.01a	0.11±0.01	28.68±3.82
Transitional, 150 m	3.14±0.01c	0.11±0.01	27.69±0.41
Lamellar, 150 m	2.80±0.01b	0.09±0.01	31.19±0.53
Sediment type	<0.001	<0.001	<0.001
Depth	<0.001	<0.001	<0.001
Interaction	<0.001	<0.001	<0.001

The last three rows indicate p values for two-way ANOVAs on the effects of sediment and depth. At each depth, values followed by the same letter are not statistically different (one-way ANOVA, LSD post hoc test $p<0.05$); if no letter were preset, no significant difference was found

Fig. 1 Respiration in amorphous and lamellar Miocene sediments collected 30 m below the surface in opencast mine. Values are means± SD, n=6.

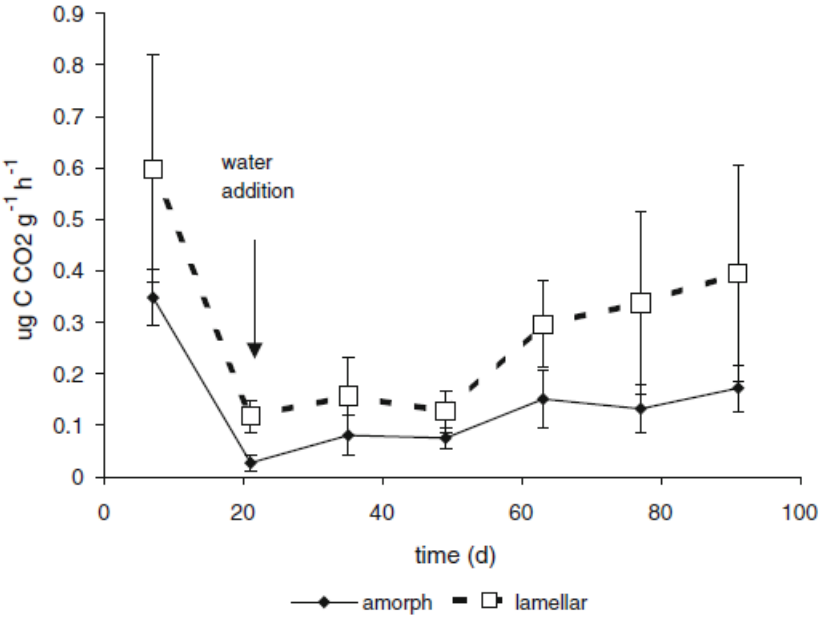


Fig. 2 Principal component analysis of PLFA in amorphous and lamellar sediment at the start (s) and end (e) of experiment 1. Arrows show individual PLFAs, and circles indicate the position of individual sediments at the start and end of the experiment. The first ordination axis explains 80.5 and the second explains 15.0% of data variability.

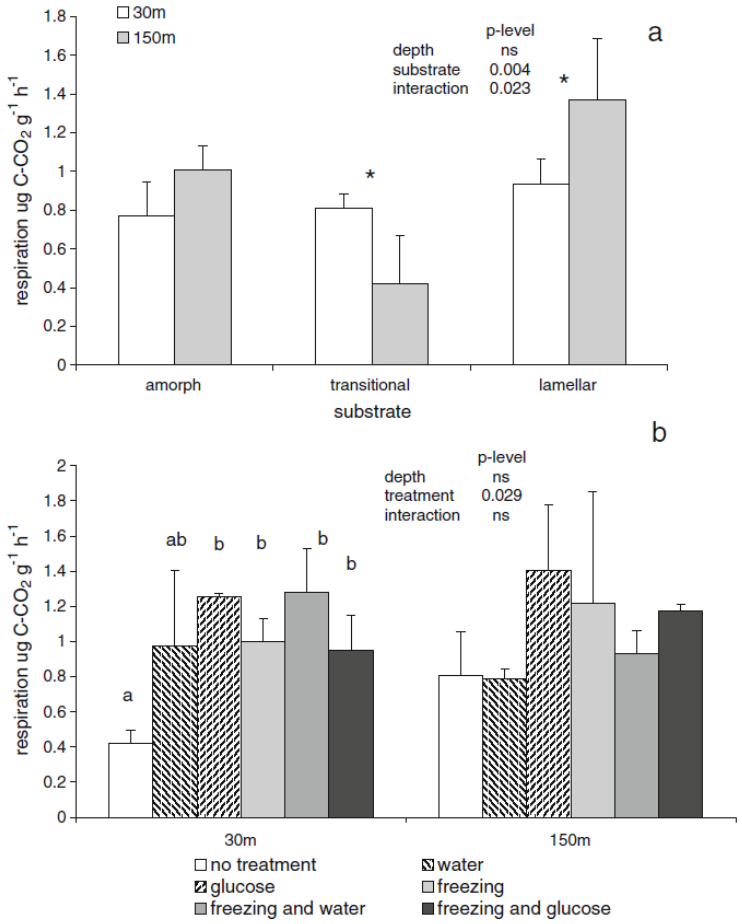


Fig. 3 Respiration of Miocene sediment collected at 30 m and 150 m depths in the opencast mine. A) Respiration as affected by sediment type and depth without any treatment. B) Respiration in transitional sediment as affected by depth and the following before incubation treatments: addition of water, addition of glucose, freezing, freezing and water, and freezing and glucose. Inserted tables indicate p values for the two-way ANOVA for sediment type and depth in a) or for treatment and depth in b). Within each sediment type in a, asterisk indicates a significant difference between depths (t test). Within each depth in b, values with the same letters are not significantly different (one-way ANOVA); the absence of letters indicates that differences were not significant.

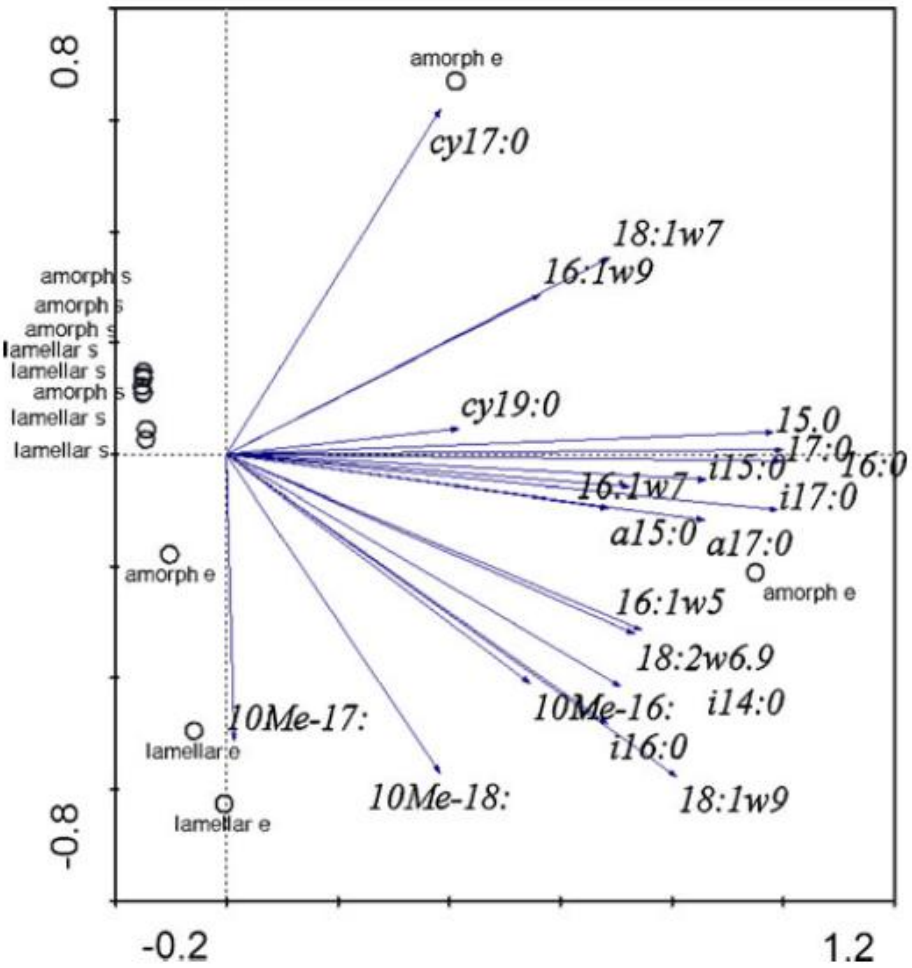


Fig. 4 Cryo-scanning electron micrograph of microorganisms in lamellar sediment after incubation in experiment 2. Microorganisms are indicated by arrows.

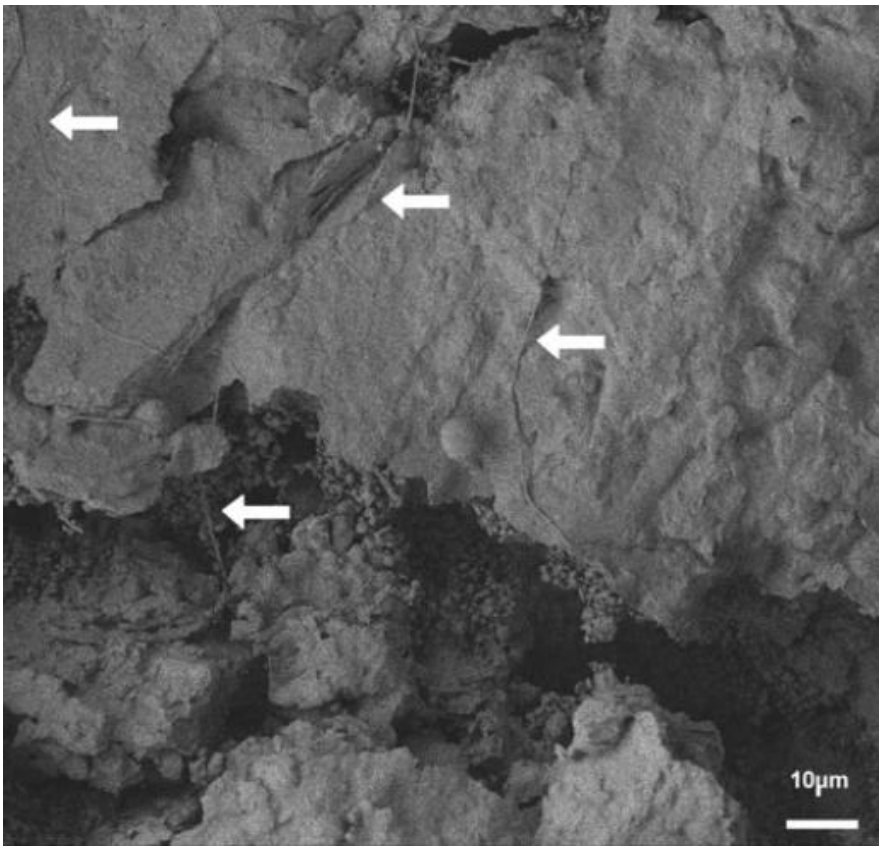


Fig. 5 Characteristic ^{13}C NMR spectrum of the organic matter from the lacustrine claystone from the lamellar sediment, 30 m depth.

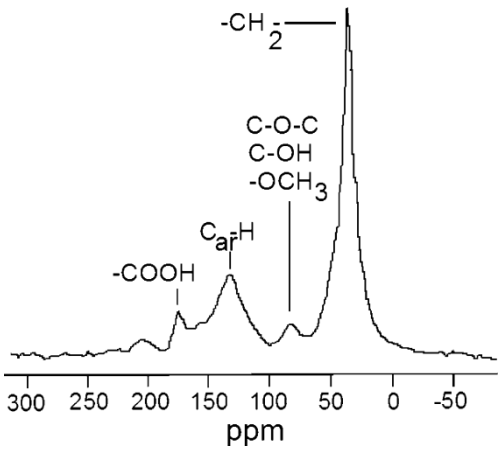
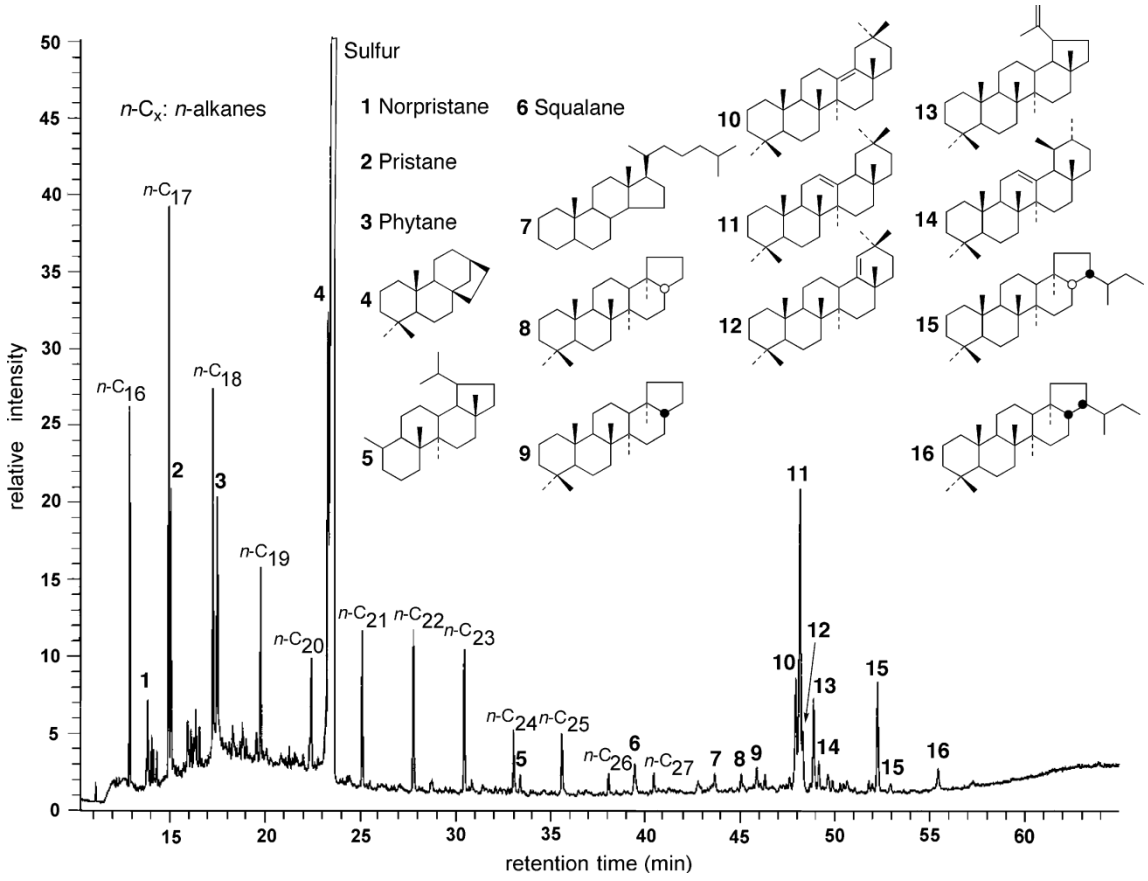


Fig. 6 A total ion chromatogram (TIC, the MS detector signal vs. time) of the aliphatic fraction of the extractable organic matter from the lamellar sediment, 30 m depth.



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Paper 2

Carbon accumulation and changes in soil chemistry in reclaimed open-cast coal mining heaps near Sokolov using repeated measurement of chronosequence sites.

Submitted manuscript

Martin Bartuska[1,2], Jan Frouz[2]

1-Institute of soil bioloy BC ASCR, Na Sádkách 7, CZ 37005 České Budějovice, Czech Republic

2-Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, CZ12800, Praha, Czech Republic

***Corresponding Author:** Martin Bartuska (bartuska@email.cz)

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Abstract

Accumulation of soil organic matter and associated changes in soil chemistry are important drivers and indicators of ecosystem recovery at post-mining sites. To elucidate the temporal changes in the ecosystem properties of these sites, researchers typically use a chronosequence approach, i.e., at one time they compare similar sites of various ages. Although useful, this approach has an important limitation, which is site variability. In this study, we amended chronosequence approach by repeated measurements of sites after extended period. We used post-mining sites near Sokolov where the soil C stock and soil chemistry variables had been measured in 1999, 11 years before the current study. In 2010, we used the same methods to repeat these measurements on the same sites, allowing us to assess real-time shifts in the investigated variables in individual plots. All sites had been reclaimed by the planting of alder in the graded overburden without topsoil application; the overburden consisted of alkaline clay shales. Sites were 4 to 45 years old in 1999 and 15 to 56 years old in 2010.

Soil pH gradually deceased with site age; this decrease was more pronounced in the upper soil layer. Changes in pH over the 11 years between 1999 and 2010 were negatively correlated with the initial pH; as a consequence, pH decreased in alkaline sites and increased in acidic sites. Soil C increased with site age; based on the difference between the first and second sampling time, the rate of increase declined with site age. The average increases in C stock were similar as determined by the chronosequence and real-time approaches. Changes in soil N content were similar to changes in soil C content. P content did not differ significantly among sites but tended to be lesser in older than younger sites.

Key words: soil chemistry, carbon stock, pedogenesis, reclamation, alder, *Alnus glutinosa*

Introduction

Soils of post-mining areas can be used to study the rate of elementary processes of soil formation (Schafer et al., 1979; Frouz et al., 2001; Sourkova et al, 2005; Frouz et al. 2008, 2009). The material overlaying the mined resource is removed from the deep subsurface (e.g., from as deep as 200 m in the Sokolov area) and deposited in heaps. This material, which is called overburden, typically differs substantially from natural soil (Bradshaw, 1997). Overburden texture is often either extremely sandy or clayey and its pH is often either extremely extremely high or low. Overburden typically lacks organic carbon derived from recent plant material but may contain various quantities of fossil C (Rumpel et al., 1998; Vinduskova and Frouz, 2013). The overburden, which is also referred to as spoil material, usually has very low biological activity (Schafer et al., 1979; Frouz et al., 2001; Frouz and Novakova, 2005) and thus represents an excellent model for studying *de novo* soil development. Moreover post-mining sites are well suited for a chronosequence approach because mining repeatedly creates new sites over a long period of time. These sites are usually formed from the same substrate and by the same technology and can be easily dated. As a result, many similar sites of various ages are available. This has resulted in the frequent use of chronosequences to study temporal changes in post-mining sites (Frouz et al., 2001; Sourkova et al., 2005, Frouz et al. 2008, 2009) as well as for studies of vegetation succession or succession changes of other ecosystem properties (Frouz et al. 2008, Frouz 2014).

The chronosequence approach is a powerful tool because it enables researchers to investigate processes that are difficult or impossible to investigate in real time because they occur over decades or centuries. The chronosequence approach however requires that the sites used in the chronosequence have similar initial conditions, which may not always be the case. The latter limitation is a particular concern for studies of soil organic matter (SOM) sequestration because apparently similar sites can substantially differ in fossil organic matter

content (Rumpel et al., 1998; Vinduskova and Frouz, 2013). Here comparison of the same sites in two time steps may help, because assuming that final content in certain time is given by initial content + changes then if you compare the same site in two different times, then you will get changes over time which are not directly affected by initial conditions.

The rate of soil formation is quite variable and is influenced by the composition of parent materials (Androchanov et al. 2000, 2004; Reintam et al. 2002; Machonina 2003), climatic conditions (Archegova 2009), vegetation developments (Nierop and Buurman1998; Klaas et al. 2001; Abakumov 2008; Abakumov and Frouz 2009), and land-use management practices, i.e., reclamation vs. spontaneous restoration (Goleusov 2000; Abakumov and Gagarina 2006; Cerli et al. 2006, 2008). A main process in soil formation is the accumulation and transformation of organic material. The accumulation of organic C results in changes in physical and chemical soil properties, such as water-holding and sorption capacity, nutrient content and availability, and soil bulk density (Allison, 1973; Leeper and Uren, 1993; Begon et al. 1996; Herric and Wander, 1998).

The objective of our study was to combine a chronosequence approach with repeated measurements of whole chronosequence to study of SOM sequestration. By using published data describing SOM sequestration in a chronosequence of post-mining sites near Sokolov (Sourkova et al., 2005) and by repeating the measurements at the same sites 11 years later, we were able to identify the soil chemistry changes after this time period on sites of various age. Comparison of C content on the same place in two different times should give us carbon accumulation rates unaffected by content of geogenic carbon and it can give us also better insight in variability of accumulation rates between individual sites. This is essential because recent SOM sequestration in developing post mining soils affect most of the other key soil parameters, such as water holding capacity sorption pH (Brady and Weil, 2002), that

determine soil ecosystem functions and plant growth. To show relationships between SOM and other soil parameters we apply similar approach also to some other soil parameters.

Materials and methods

Description of study sites

This study was done on reclaimed heaps produced by open-cast coal mining near Sokolov (Czech Republic) (50°14'31"N, 12°41'13"E). The average altitude of the study sites ranges from about 500 to 700 m a.s.l. The mean annual precipitation is 650 mm, and the mean annual temperature is 6.8 °C. The heaps are formed by clay shells that have an alkaline pH and that contain 2–10% fossil carbon that consists mainly of algal kerogen (Frouz et at., 2011). For more details about the study sites, see Frouz et al. (2001, 2011), Sourkova et al. (2005), and Helingerova et al. (2010).

This study compared the C stock N P content and pH in 13 of 19 sites that had been reclaimed by the planting of alder (*Alnus glutinosa*) and that had been studied 11 years earlier as part of a chronosequence (Sourkova et al., 2005); the same methods used by Sourkova et al. (2005) were used in the present study. Only 13 of the original 19 sites were used because six of the sites had been damaged. These sites were located by using field maps from the previous study using GPS coordinates measured by Garmin Colorado 300. In most cases, the site locations were also verified by field labels left from the previous research. At 10 sites, we found field labels marking the middle of the sampling location, and we sampled in the same location, i.e., the location of the new sampling area was within 5 m of the original. At three sites, we could not find the field labels and depended on GPS coordinates provided by the previous study; at these sites, the location of the new sampling areas were within 20 m of the original. Although six of the original sites could not be included, the study encompassed the

entire time scale of the chronosequence, although it was advanced by 11 years in the current study. The sites were from 4 to 65 years old in the original study and from 15 to 76 years old in the current study. Site age is counted from site leveling by earth moving machinery and tree planting which typically happen in the same year. Alder (mainly *Alnus glutinosa* with some specimens of *Alnus incana*) seedlings are planted in 1m distance (10 000 seedlings ha^{-1}) and thinned after 10 years. Canopy closures in about 15 year sites development of tree height is visible on Fig 1a. Understory is dominated by *Calamagrostis epigeios* (see Mudrak et al., 2010 for more extensive species list) which is also dominant part of understory litter. Litter input increase in young sites and peaked soon after canopy closure, latter it can be highly variable (Fig 1b).

Sampling and analysis

At each of the 13 sites, soil samples with an area of 10 cm^2 were taken from 0-5 and 5-10 cm depth. For analysis, four individual samples from corresponding layers were mixed to form one composite sample, and three composite samples were taken from each layer and site.

Samples were air dried, weighed, crushed, and homogenized. Part of each sample was milled and passed through a 0.01-mm screen to determine total C, N, and P.

Bulk density was determined as the weight of the dry composite sample divided by its volume (200 cm^3). C and N were analysed with a Carlo Erba elemental analyser. The total P content of soil was determined in composite samples by mineralization with perchloric acid (Sommers and Nelson, 1972), and orthophosphate ions were quantified by the ascorbic acid and ammonium molybdate method (Murphy and Riley, 1962; Watanabe and Olsen, 1965).

Part of each composite sample was used for pH determination with a 1:5 (w/v) soil:water suspension and a WTW 526/538 pH meter (Germany) with a combination electrode.

Calculation and data processing

For visualize trend of the changes in individual variables among sites of different ages, the data pooled from both sampling occasions were fit to linear, logarithmic, or second-order polynomial equations. The equations with the best fit were used, only equations that were significant ($p<0.05$) are presented, df of these regression was 22 for all parameters studies. Individual soil parameters on individual sites are result of their initial value and changes over time. Consequently these trends are affected by variation in initial conditions among sites which we do not know.

To see how the individual chemical parameters change over time annual changes were calculated as the values obtained in this study for a specific site minus the values in Sourkova et al. (2005) for the same site divided by 11, i.e. for each site (2010 value – 1999 value)/11. In next step we test by t test if mean annual changes calculated from annual changes of all individual sites differ from 0 to see if there is any general trend of the changes across the sites. Then we calculate regression these of annual changes with mean age of each plot calculated as age in 2010 + age in 1999 divided by two. This was done to explore if the rate of changes in given parameter have some trend with plot age. To calculate regressions we always try linear, exponential, logarithmic and second order polynomial regression and choose those with the best fit (highest R^2). Df for t test and regression calculated from site annual changes was 12 for all parameters studies. All computation was done in Statistica 5.0.

Soil carbon stock in the 0–10 cm layer was calculated based on the mass of the layer in t ha^{-1} multiplied by the percentage of C in the soil divided by 100. The weight of the layer was calculated as 500 m^3 (the volume of a 5-cm-thick layer covering 1 ha) multiplied by bulk density in t m^{-3}. After this was done for both the 0-5 cm and 5-10 cm layer, the values from both layers were pooled. Carbon stock changes over time were processed by the same way as describe above for other parameters.

Results

Based on pooled data from the current study and from Sourkova et al. (2005), soil pH decreased with site age (Fig. 2a). The decrease was significant at both 0-5 and 5-10 cm depths but was more pronounced in the upper layer. Although the annual rate of pH change was positively correlated with site age, the pH declined as younger sites aged but increased as older sites aged (Fig. 2b). Changes in pH after 11 years (2010-1999) were negatively correlated with initial pH in 1999 (Fig. 3), i.e., the pH decreased with age in sites that were initially alkaline and increased with age in sites that were initially acidic. This is true for both soil layers.

Soil C content increased with site age (Fig. 4a). When data from both sampling times were pooled, however, this increase was significant for the 0-5 cm layer but not for the 5-10 cm layer. Changes in C content between the first and second sampling time indicate a relatively constant annual increase in C content in the 0-5 cm layer irrespective of site age, with some small decrease in the oldest sites that was only marginally significant (Fig. 4b). The mean increase in soil C content in the 0-5 cm layer was 1.52 ± 2.0% during the 11 years (the mean annual increase was 0.14 ± 0.19%), which was significantly higher than 0 (*t* test, p=0.03). In the 5-10 cm layer, the increase in C content over the 11 years did not significantly differ from 0.

Soil N content increased with site age, but when data from both sampling times were pooled, this relationship was significant only for the 0-5 cm layer (Fig. 5a). The N content increased between the first and second sampling time, and the annual change in N content in the 0-5 cm layer was higher in 20- to 30-year-old sites than in younger and older sites (Fig. 4b). In the 5-10 cm layer, the annual increase in N content between the first and second sampling time was relatively constant irrespective of site age, with some small decrease in oldest sites that was only marginally significant. The mean increase in soil N content in the 0-

5 cm layer was 0.13 ± 0.14% during 11 years (the mean annual increase was 0.012 ±0.013%), which was significantly higher than 0 (*t* test, p=0.01). In the 5-10 cm layer, the increase in N content over 11 years did not significantly differ from 0 (*t* test).

Based on pooled data from both sampling times, the content of total phosphorus in soil was not statistically related to site age in either soil layer (Fig. 6a). The annual changes were negative in 20- to 30-year-old sites but were close to zero in younger and older sites (Fig. 6b). In the 0-5 cm layer, the mean change in total soil P content was -213 ± 173 mg kg^{-1} during the 11 years (the mean annual decrease was 19 ± 16 mg kg^{-1}), which was significantly lower than 0 (t test, p=0.002). In the 5-10 cm layer, the change in P content over the 11 years did not significantly differ from 0 (t test).

Based on pooled data from both sampling times, soil bulk density significantly decreased with site age in the 0-5 cm layer but was unrelated to site age in the 5-10 cm layer (Fig. 7a). In the 5-10 cm layer, the mean change in bulk density was -0.11±0.15 g cm^{-3} during 11 years (the annual decrease was 0.010 ±0.014 g cm^{-3}), which was significantly less than 0 (*t* test, p=0.018) (Fig. 7b). In the 0-5 cm layer, the mean change during the 11 years did not significantly differ from 0 (Fig. 7b).

Pooled data from both sampling times indicated that the soil C stock at 0-10 cm depth significantly increased with site age (Fig. 8a). Changes in C stock in 0-10 cm layer between the first and second sampling time were positive, with significant decrease over time (Fig. 8b). The mean increase in the soil C stock at 0-10 cm depth was 10.1 ± 17.1 t ha^{-1} during 11 years (the annual increase was 0.92±1.57 t ha^{-1}), which was only marginally significant (*t* test, p=0.054).

Discussion

In agreement with other studies, soil C content increased with time in post-mining soils, indicating that these soils can be a significant sink for C (Vinduskova and Frouz, 2013). The rate of C sequestration agreed observed in our study 0.9 t C ha^{-1} $year^{-1}$ with values reported in previous studies (Jonas, 1972; Insam and Domsch, 1988; Dageforde et al., 2000; Vinduskova and Frouz, 2013). Namely Vinduskova and Frouz (2013) who revived available literature data from post mining soils give mean value for 0 – 30 year old plot 2.1 t C ha^{-1} $year^{-1}$. However they also mentioned that C sequestration value decreased with plot age if we calculate expected C sequestration from equation of Vinduskova and Frouz (2013) for 28 year plot, (which is mean age of our plots) the value would be 1.2 t C ha^{-1} $year^{-1}$ which is very good agreement with value obtained in this study. For comparison soil under modern agricultural use represent rather source then sink of C (Paustian et al., 1997).

Comparison of the two data sets, i.e., the data collected in 1999 by Sourkova et al. (2005) and the data collected in 2010 as part of the current study, revealed that the rate of carbon storage in post-mining soils decrease with plot age (Fig 8b). This decrease in the rate of soil C accumulation agrees with the meta-analysis of Vinduskova and Frouz (2013) and also agrees with the preliminary observations of Sourkova et al. (2005). This slowing in the rate of C sequestration is consistent with the concept of soil C saturation, i.e., with the concept that soil with a given input of C can retain only some defined level of C. The mechanism of C saturation is not completely understood but involves soil depth, clay content, and the ability of clay minerals to bind organic matter (Gulde et al., 2008). Even though C storage slowed as the post-mining sites aged, the C contents of the post-mining sites are higher than in similar forests in the surrounding landscape outside of the mining area. However it is not clear if this suggests that post mining soils are C saturated. Fig 1b shows that litter input increased in young sites and peaked after canopy closure on older sites littre input stagnate or decrease.

Consequently observed slowing in the rate of C storage with site age (fig 8b) correspond more likely with decrease of litter input rather than indicate reaching C saturation level.

Moreover microscopic observation and study of soil texture even in the oldest sites has indicated that, although a substantial proportion of the clay shales that form the original overburden is mechanically broken into small pieces, only a small proportion is transformed into clay particles (Frouz et al., 2007; Kuraz et al., 2011). Future weathering of this material may release more clay particles, which may increase overall C storage. Moreover, our study was limited to upper 10 cm, which may not be problem as the current results show that most of the C storage occurs in the surface soil horizon, i.e., at 0-5 cm soil depth. Previous study showed that bioturbation mainly due to soil mixing by earthworms substantially contributes to soil C storage in these sites (Frouz et al., 2009), where most earthworms were determined to be epigeic and endogeic species (Frouz et al., 2001). It is possible that future colonization of the sites by anectic earthworm species may increase the depth of the A horizon and hence increase overall C storage.

A substantial proportion of the total C stock in reclaimed mining sites is represented by fossil organic matter (Kribek et al., 1998; Frouz et al., 2011. Consequently, when we substract fossil C the amount of recent organic matter even in the oldest reclaimed site is lower than in the semi-natural forest in the surrounding landscape. Although the fossil organic matter may gradually decompose and thereby affect the overall C content, we have little knowledge about how interactions between fossil and recent organic matter affect C saturation.

In agreement with the previous study (Sourkova et al., 2005), N content and C content showed a similar pattern of increase with site age, indicating a gradual accumulation of soil organic matter. However differences between sites show that the pattern of N content over time is complex and is apparently affected by both the gradual built up of N content due to N

fixation and soil organic matter accumulation and the loss of soil N by leaching, denitrification, and assimilation into plant biomass.

In contrast to C and N, soil P content did not increase with site age, which agrees with the findings of Sourkova et al. (2005). Moreover, comparison of data from 1999 and 2010 indicated a decrease in P content in the studied soils. This can be explained by the fact that, unlike C and N, which are fixed from the atmosphere by photosynthesis and nitrification, P originates from the parent soil material and is gradually lost by leaching.

Soil pH gradually decreased with site age, which was also in agreement with Sourkova et al. (2005). The comparison of data from 1999 and 2010 indicated, however, that pH changes are negatively correlated with the original pH, which means that pH decreases in alkaline soils and increases in acidic soil (Fig 3). This pattern reflects the establishment of an active buffering system based on the balance between basic cations and organic matter (Brady and Weil, 2002).

Our study shows that repeated measurement of post-mining sites may help to solve the problem of original heterogeneity of soil conditions which is particularly crucial to study accumulation in post mining sites. For example content of fossil C content in overburden in our sites vary from 2 to 10% (Frouz et al., 2011). This study show that C content increase is about 1.5% in 11 years. Hence initial variation in C content may substantially contribute to the pattern produced by comparison of sites of different age.

By repeated measurements of the same time one can get real change in observed parameter over certain time period, unaffected by initial conditions. When this is done along chronosequence we get also information how these changes vary with succession age.

Conclusion

Chronosequence approach combined by repeated measures are good method to solves the problem caused by the initial heterogeneity in fossil carbon content among sites, which is an important limitation of the chronosequence approach in post mining sites. This approach show that the rate of carbon storage decreased with site age, most likely due to reduction of litter input. The changes in soil N content with site age were similar to those for soil C content. C and N accumulation with site age evidently results in the tendency of soil pH to increase in acidic soils and decrease in alkaline soils. Soil P content decreased with site age, presumably because of leaching and plant uptake. Finally, the results show that the repeated measurement of sites over time.

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Appendices

Fig 1. Height of alder tree and canopy closure in individual sites during the 1st sampling (1999) and 2nd sampling (2010) based on data of Sourkova et al., 2005 and Dvorščík (pers. com) (a) and litter input in selected studied sites during investigated period (between 1999 and 2010) based on Frouz et al. (2014) (b). Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natral sites are missing as we have no data for them.

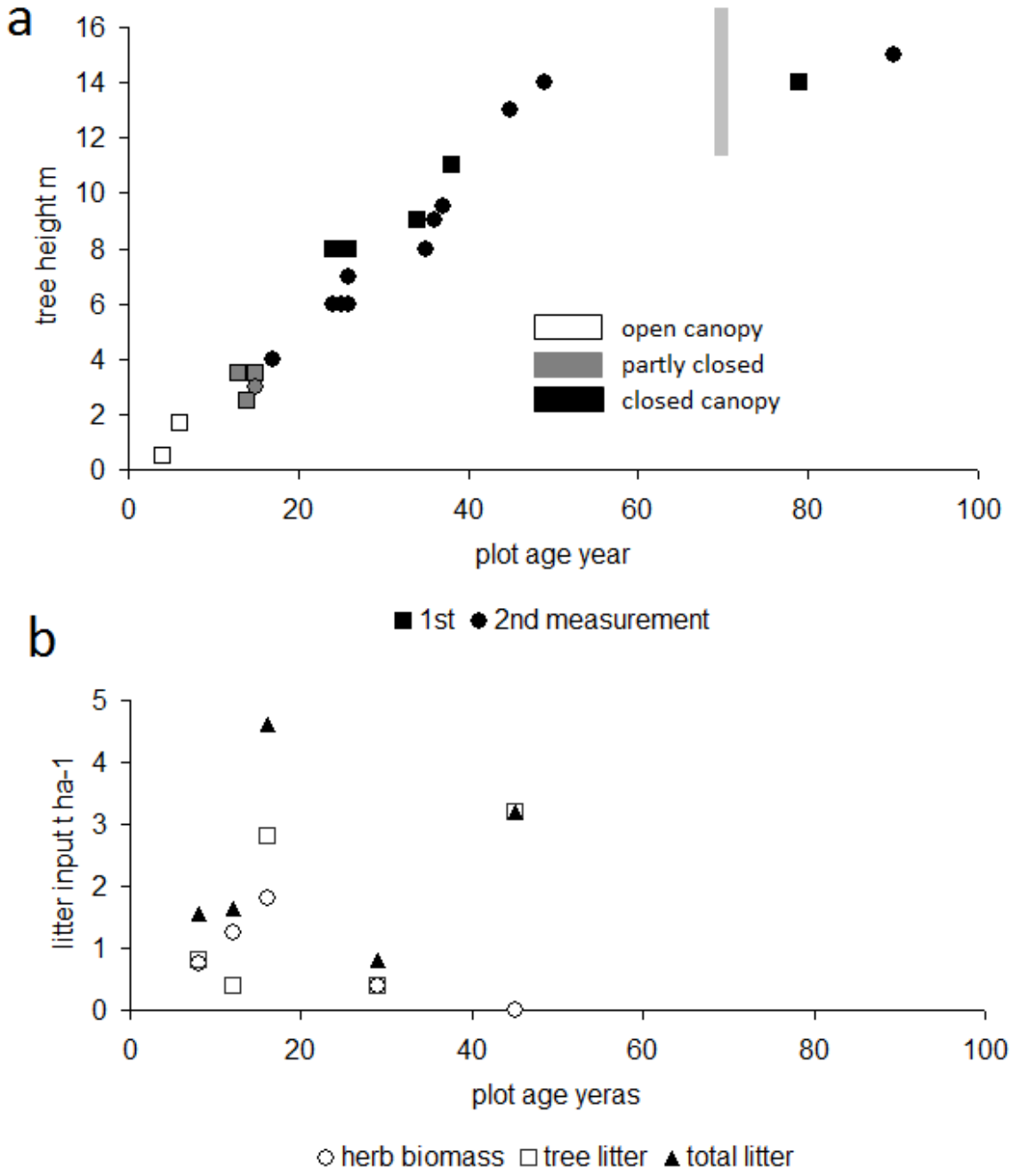


Fig. 2. Soil pH of post-mining sites determined in the 1st sampling (1999) and 2nd sampling (2010) as a function of site age and soil depth (a) and annual rate of change over 11 years at the same sites (2nd sampling values minus 1st sampling values divided by 11) as a function of mean site age (age at the 1st plus 2nd sampling divided by 2) and depth (b). Regressions for 0–5 cm depth and 5–10 cm depth are indicated by solid and dashed lines, respectively. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natral sites are missing.

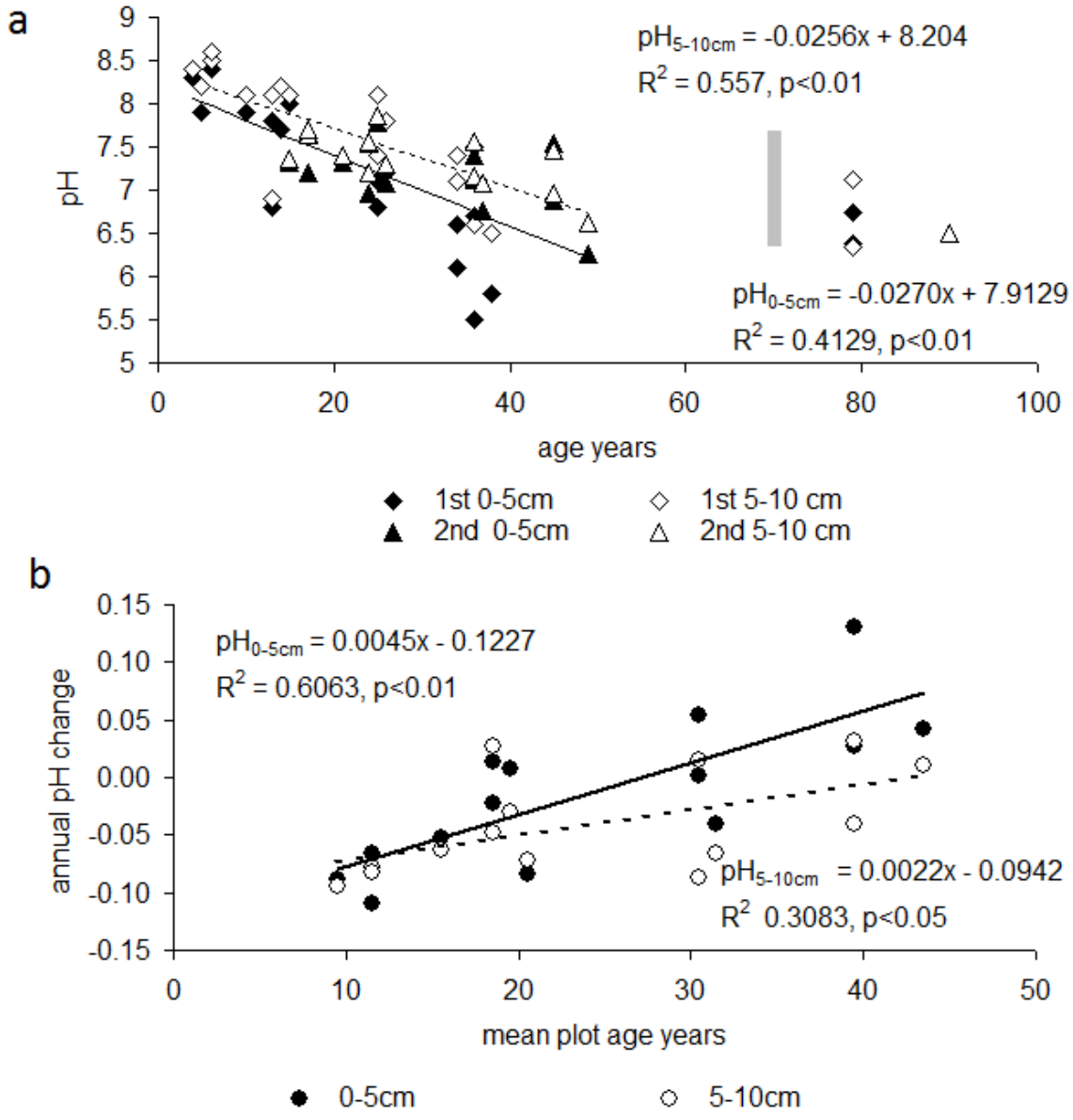


Fig. 3. Annual rate of change in soil pH over 11 years (values in 2nd sampling minus values in 1st sampling divided by 11) as related to pH at 1st sampling.

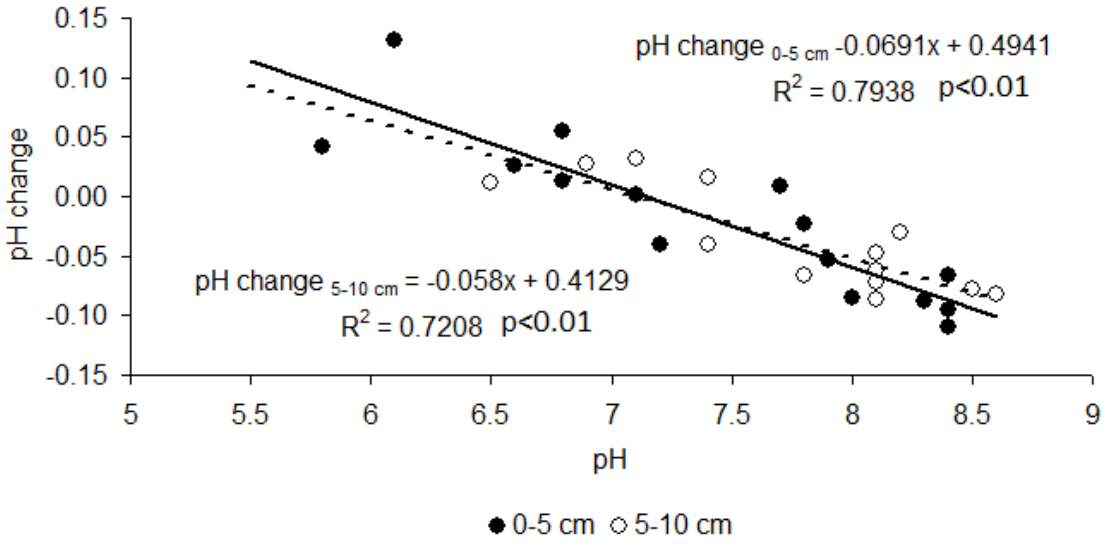


Fig. 4. C content (% w:w) of post-mining sites in the 1st (1999) and 2nd (2010) sampling as a function of site age and soil depth (a) and annual rate of change in C content (2nd sampling value minus 1st sampling value divided by 11) over 11 years in the same sites as a function of mean site age (age at the 1st plus 2nd sampling divided by 2) and soil depth (b). Relationships between site age and C value (a) and relationships between mean site age and C changes. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natural sites are missing.

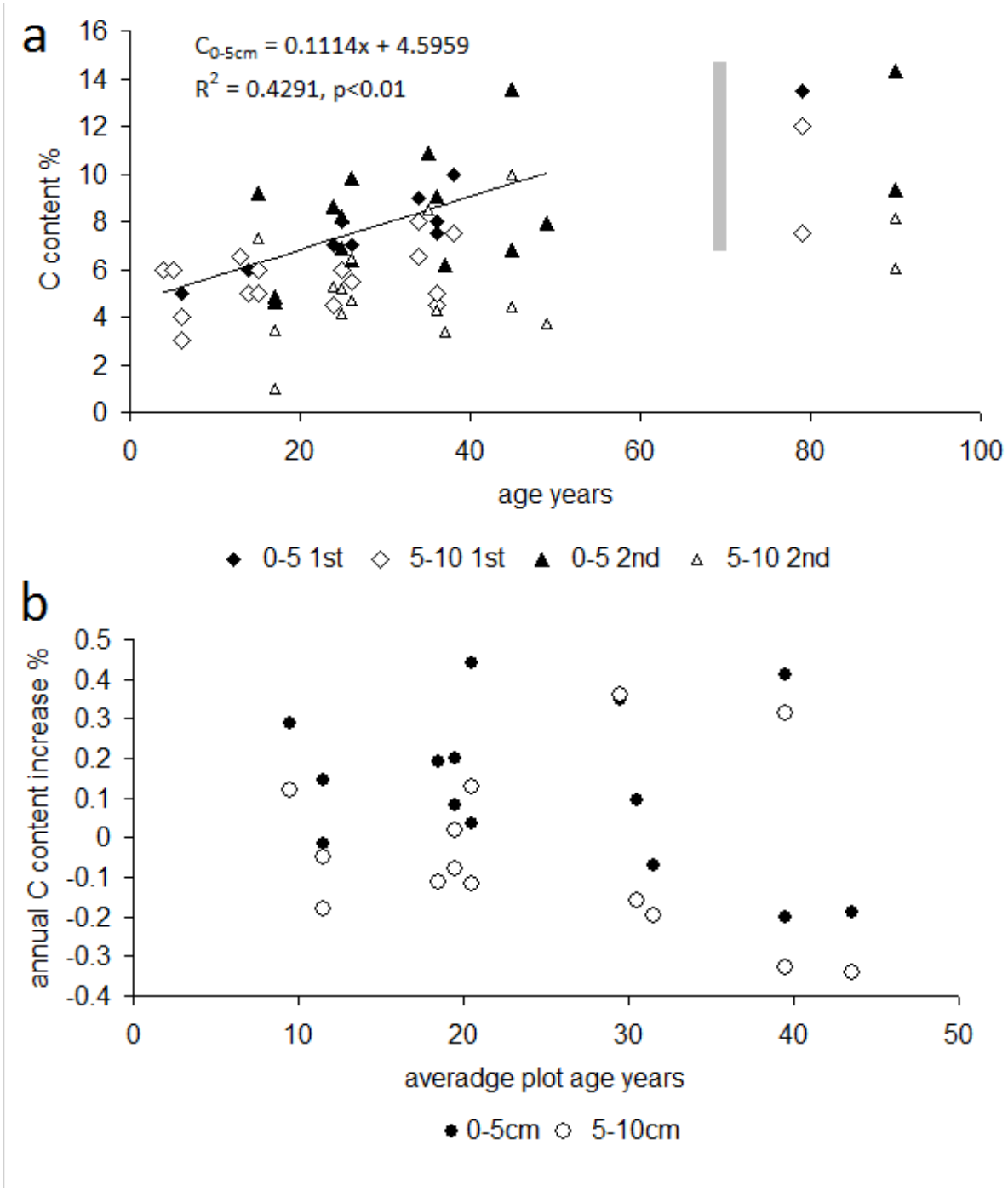


Fig. 5. N content (% w:w) of post-mining sites in the 1st (1999) and 2nd (2010) sampling as a function of site age and soil depth (a) and changes in N content (2nd sampling value minus 1st sampling value divided by 11) over 11 years in the same sites as related to mean site age (age at the 1st plus 2nd sampling divided by 2) and soil depth (b). Relationships between site age and N value (a) and relationships between mean site age and N changes. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natural sites are missing.

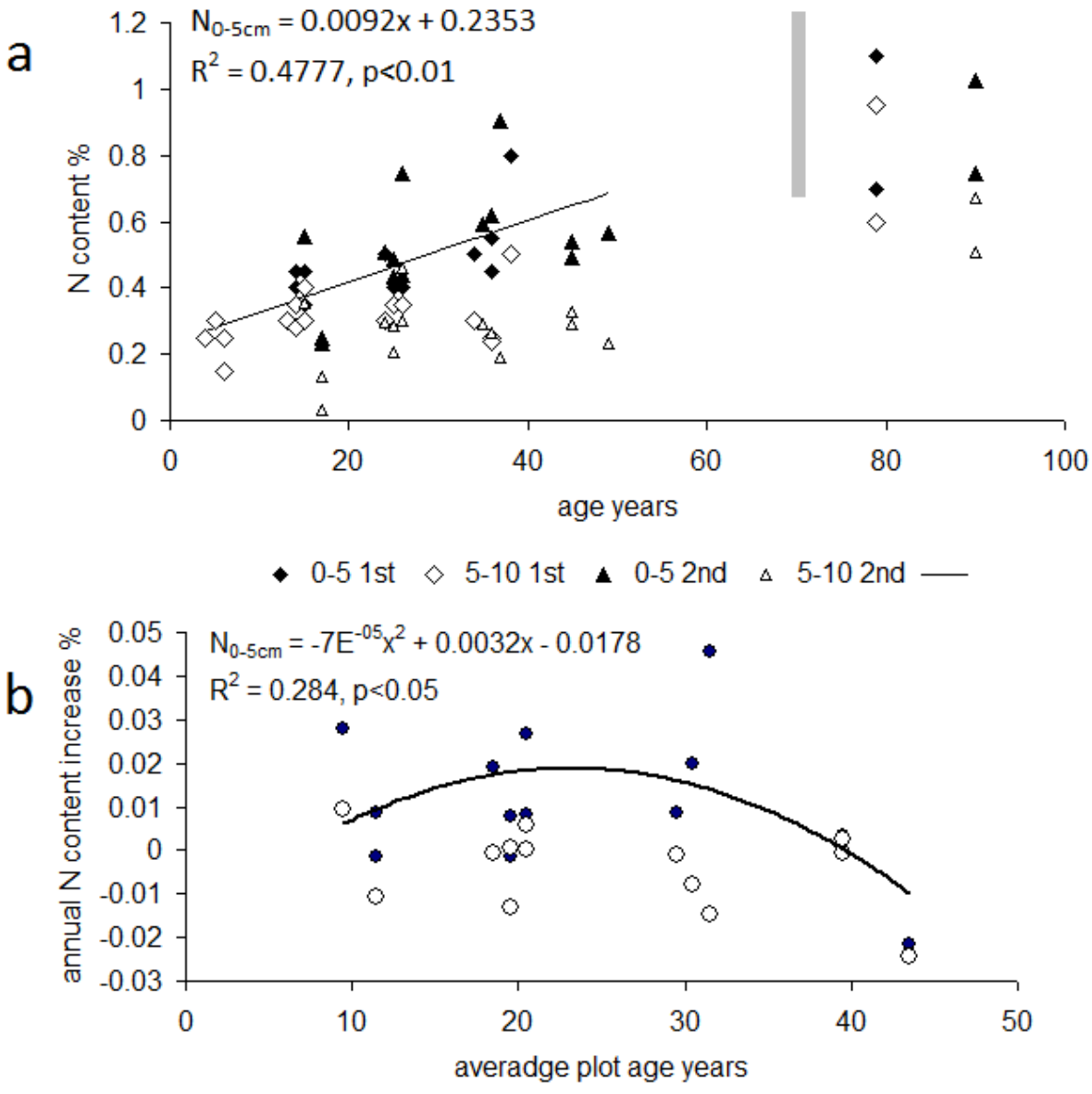


Fig. 6. P content of post-mining sites in the 1st (1999) and 2nd (2010) sampling as a function of site age and soil depth (a) and changes in P content (2nd sampling value minus 1st sampling value divided by 11) over 11 years in the same sites as related to mean site age (age at the 1st plus 2nd sampling divided by 2) and soil depth (b). Relationships between site age and P value (a) and relationships between mean site age and P changes. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natural sites are missing.

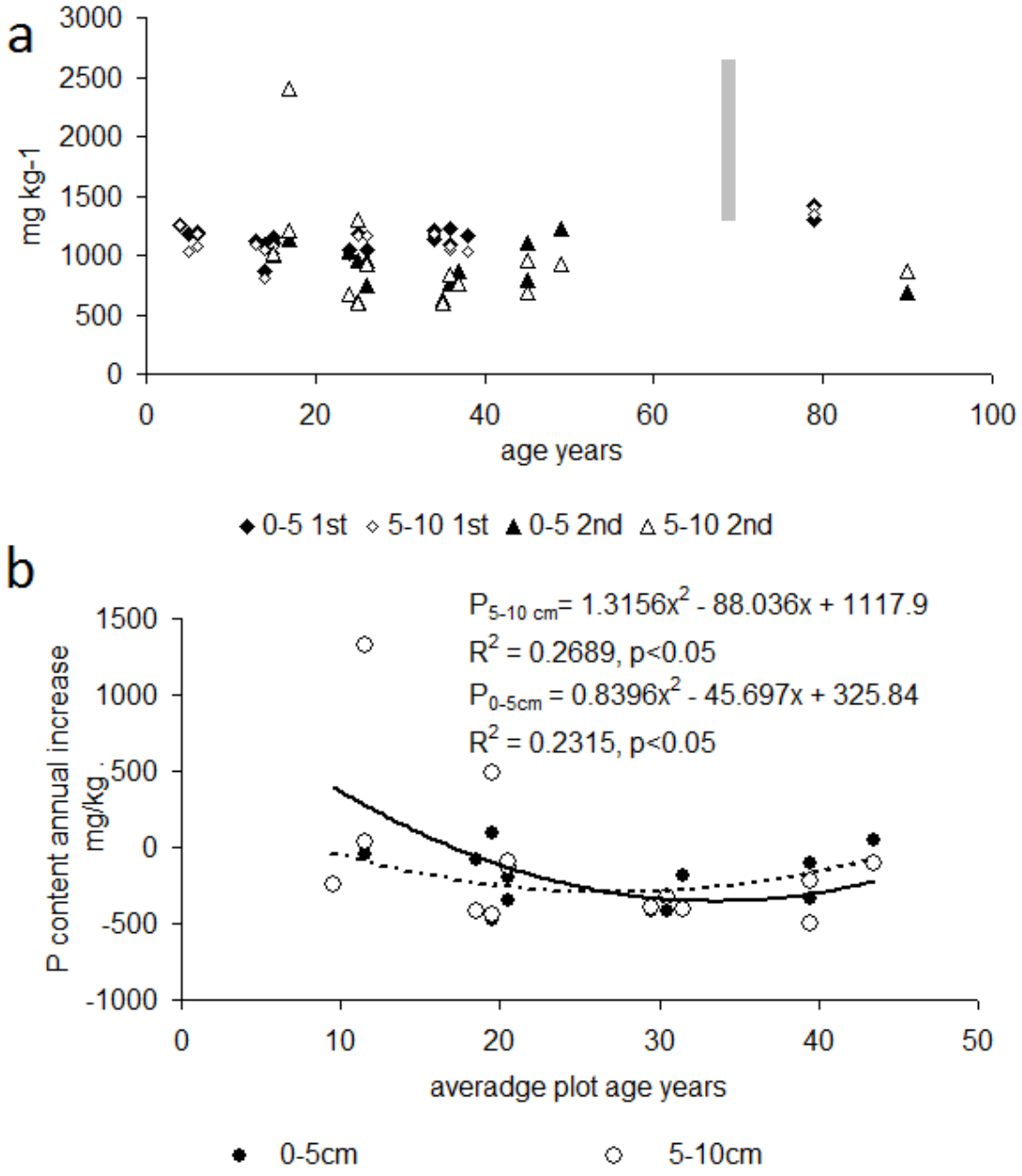


Fig. 7. Bulk density of post-mining sites in the 1st (1999) and 2nd (2010) sampling as a function of site age and soil depth (a) and changes in bulk density (2nd sampling value minus 1st sampling value divided by 11) over 11 years at the same sites as related to mean site age (age at the 1st plus 2nd sampling divided by 2) and soil depth (b). Relationships between site age and bulk density (a) and relationships between mean site age and bulk density changes. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natural sites are missing.

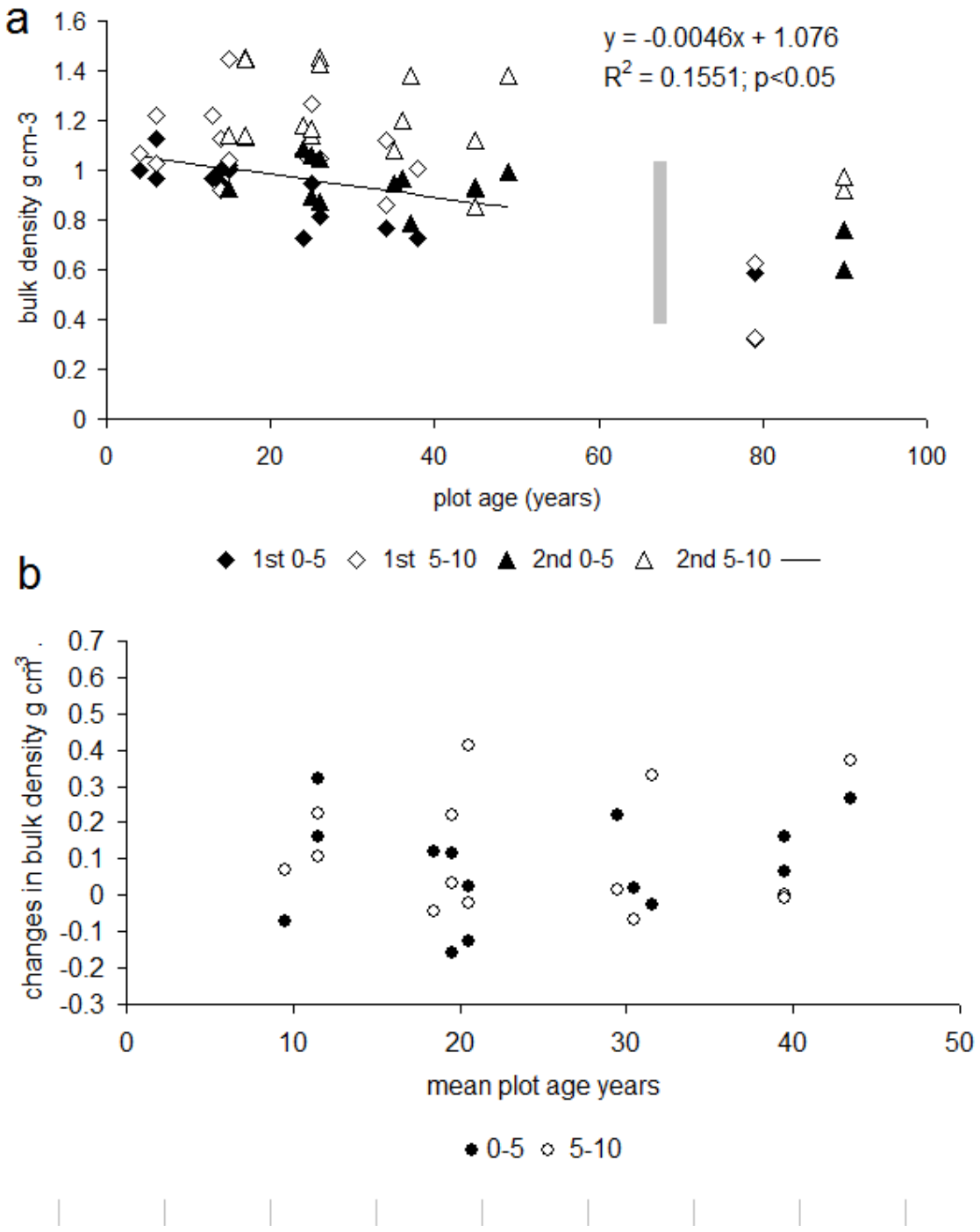
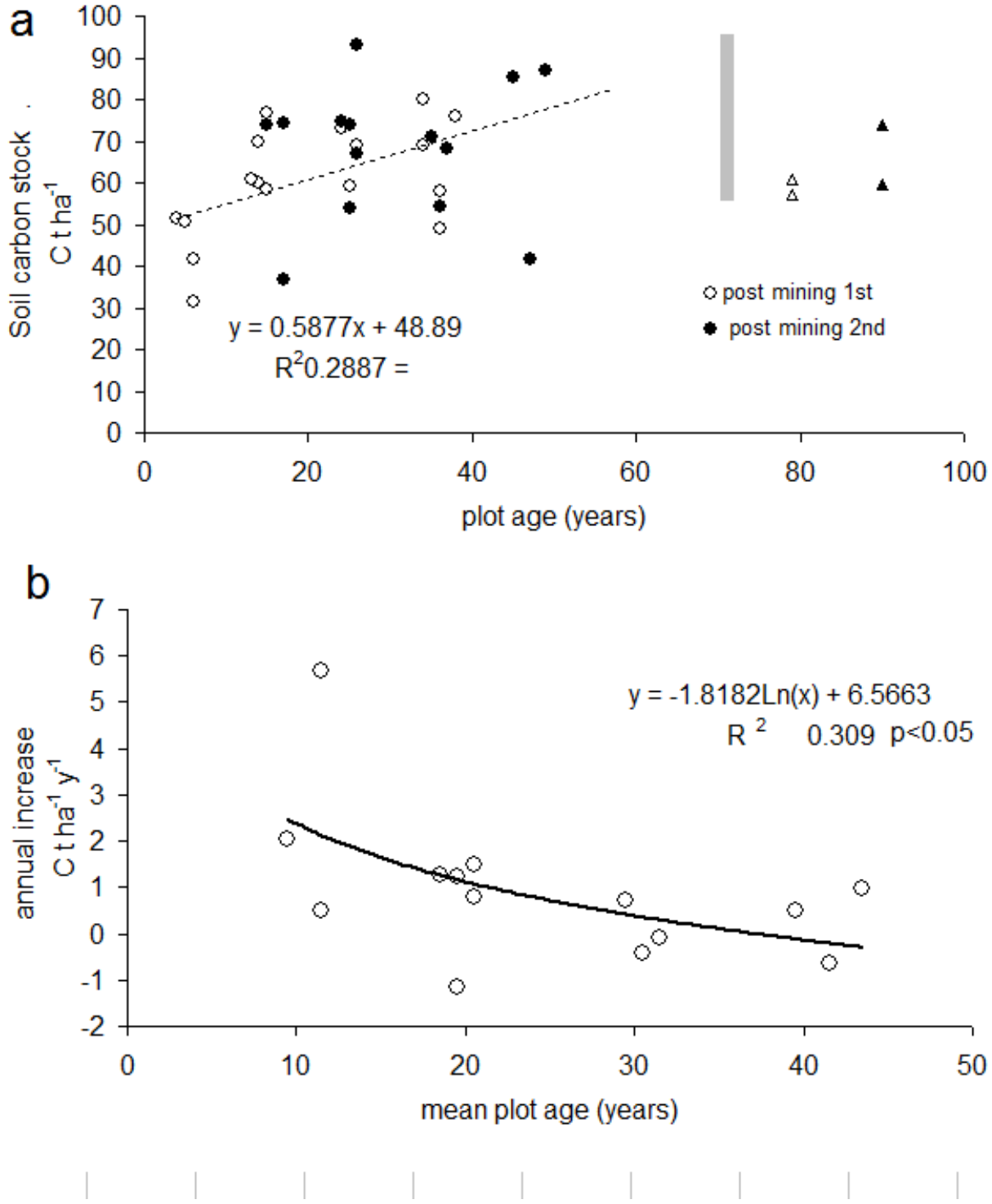


Fig. 8. C stock of post-mining sites in the 1st (1999) and 2nd (2010) sampling as a function of site age and soil depth (a) and the annual changes in C stock (2nd sampling value minus 1st sampling value divided by 11) over 11 years at the same sites as related to mean site age (age at the 1st plus 2nd sampling divided by 2) (b). In (a), natural both 1st and natural 2nd refer to values obtained from adjacent forest areas that were not mined between site age and C stock value (a) and relationships between mean site age and C stock changes. Grey line in fig a separate reclaimed sites on the left from natural site o the right, in fig b natural sites are missing



Paper 3

Methods for measuring carbon dynamics in soil

Review

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MARTIN BARTUŠKA [1, 2], JAN FROUZ [1, 2]

[1] Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Benátská 2, CZ-128 01 Prague 2, Czech Republic; frouz@natur.cuni.cz

[2] Institute of Soil Biology, Biology Centre of the Academy of Sciences of the Czech Republic, Na Sádkách 7, CZ-370 05 České Budějovice, Czech Republic

Abstract

This paper compares and evaluates the approaches and methods used to estimate soil organic carbon dynamics, i.e., the change in the carbon stock over time. A direct approach is to sample the soil carbon pool over time. This includes determining the depth distribution of bulk density, the proportion of fine soil, and the content of carbon from fine soil. Because changes in the carbon pool are generally slow, repeated measurement over several decades is required. The time required for determination can be greatly reduced by the use of natural chronosequences or radiocarbon dating. An indirect approach is to budget carbon input and output from the soil. Inputs include surface litter, dead roots, and root exudates. Quantification of belowground input from decaying roots and exudates is difficult. The major output of carbon from the soil is soil respiration, although substantial carbon also may be emitted from soil as CH_4 or may be leached from soil as dissolved organic carbon in some ecosystems. The partitioning of soil respiration into autotrophic (root) and heterotrophic (microbial) respiration is essential but difficult. A promising approach is to both partition the soil carbon stock (by determining the age of individual fractions) and to measure soil respiration and the age of the produced CO_2. This combined approach provides estimates of the amount of carbon in soil and its contribution to C loss from the soil.

Keywords

Soil organic matter, carbon stock, bulk density, physical fractionation, chemical fractionation, SOM stabilization, dry oxidation, wet oxidation, root exudates, soil respiration, radiocarbon technique

Introduction

Soil organic matter (SOM) globally represents one of the most important reservoirs of carbon and contains about three times more carbon than the atmosphere (Guo and Gifford 2002). At the same time, soil carbon system is very dynamic; for example, the quantity of global carbon released from the soil into the atmosphere is many times greater than all the carbon released by the burning of fossil fuels by humans (Raich and Schlesinger 1992; Davidson and Janssens 2006). Hence, if the carbon exchange between soil and atmosphere is globally changed such that carbon storage in soil is increased by several percent, it would have a similar impact on the global carbon budget as the reduction of the emissions agreed upon in the Kyoto protocol.

The soil carbon pool is strongly affected by agricultural and forestry practices. Tillage, fertilization, drainage, irrigation, biomass burning, and changes in tree species composition may substantially increase the carbon loss from soil (Davidson and Ackerman 1993; Guo and Gifford 2002). The loss is also increased by rising temperature, which causes potentially dangerous positive feedback with global warming (Davidson and Janssens 2006). On the other hand, suitable agriculture and forestry practices can reduce carbon loss from the soil and even promote carbon storage in soil (Guo and Gifford 2002; Davidson and Ackerman 1993). In particular, proper management of disturbed and degraded areas has a large potential to promote soil carbon sequestration (Šourková et al 2005; Frouz et al 2009). This can mitigate the increases in atmospheric CO_2 concentration and therefore reduce global warming. Moreover, increased carbon storage in soil may have other beneficial effects. For example, it may increase the ability of soil to hold water, which may help plants adapt to climate change. According to many climate predictions, water shortages will become more common in many regions. Thus, the proper management of the soil carbon can both mitigate global climatic change and help plants and other organisms adapt to climate change.

Soil carbon, however, is generally ignored by politicians who deal with global climatic change in part because of the methodological difficulties in accurately estimating soil organic carbon stock and soil carbon dynamics. The purpose of this review is to summarize, compare, and evaluate the approaches and methods that have been used for estimating soil organic carbon dynamics in terms of changes in carbon stocks over time. To reduce the complexity of the topic, we focus mainly on mineral soils and do not consider peat soils and other water-saturated soils. In particular, methods are evaluated for their suitability as potential tools for monitoring the dynamics of soil carbon pool. Such monitoring would presumably be used by agencies to promote soil carbon storage to thereby mitigate rising atmospheric CO_2 concentrations.

The main carbon flows influencing SOM dynamics are illustrated in Fig. 1. Carbon fixed by photosynthesis is relocated into the soil as litterfall and roots exudates. As these carbon inputs are decomposed, CO_2 is released to the atmosphere. The decomposition rate of organic matter depends on the chemical composition of the organic materials, the binding of the organic materials to mineral particles (for example soil aggregates), and environmental conditions. During decomposition, easy decomposed substances are rapidly removed, leaving more resistant substances. Moreover, after various transformation processes these resistant substances decrease the decomposability of remaining OM and lead to SOM stabilization. Other processes, such as methane formation, may be important in water-flooded soils but are not subject of this review. Similarly, substantial carbon can be leached from soil by rain and irrigation (Richey et al 1990; Hope et al 1994) but this review does not concern leaching as a pathway of C flux.

Approaches for measuring carbon stock changes over time can be considered as direct or indirect. In the direct approach, carbon stock is measured at different times. In the indirect approach, carbon stock is estimated based on soil carbon inputs and outputs.

Carbon stock in soil

Evaluating the soil carbon stock requires information on the carbon concentration and soil bulk density in soil horizons throughout the entire soil profile. If the carbon was established in the fine fraction of soil (without gravel), information on the quantity of fine fractions is also needed.

Soil organic carbon (SOC) varies with depth and usually decreases with depth. The distribution of SOC is also greatly affected by root distribution, amount of plant litter, and the soil biota (Lal and Kimble 2001). SOC content and soil density may also vary horizontally, especially in complex landscapes with several soil types, and may also be influenced by landscape and soil management as well as wind and water erosion (Lal et al 1998; Follett et al 2000; Lal and Kimble 2001). The substantial number of samples needed for carbon stock estimation can be reduced by using composite samples or habitat stratification (Šourková et al 2005).

Soil bulk density (SBD) is defined as the ratio of dry soil mass to bulk soil volume (including pores) and is expressed as megagrams per cubic meter (Mg m^{-3}), which is numerically equivalent to grams per cubic centimeter (Lal and Kimble 2001). SBD is affected by aggregation and aggregate stability, clay content, and the nature of clay minerals. High activity clays (2:1 minerals) tend to have higher bulk densities than low activity clays (1:1 minerals) (Lal and Kimble 2001). SBD changes through the year with changes in moisture and temperature. In particular, cycles of freezing and thawing and wetting and drying alter SBD and the related parameters of soil aggregation, porosity, and pore size distribution. These changes are greater in the surface than the sub-surface horizons. Because SBD can change with time, sampling should be done in a short time period (Lal and Kimble 2001).

Several methods are commonly used to determine SBD. In the core method, a cylindrical core of known volume is driven into the soil to a desired depth. The intact core is removed, dried in an oven at 105 °C, and weighed. In the excavation method, a hole is excavated to the desired depth, lined with plastic, and filled with a measured volume of water; the excavated soil is then dried and weighed. In the clod method, a large soil aggregate is weighed before it is coated with paraffin or another water-repellent substance and its volume is measured in a graduated cylinder. In the radiation method, the radiation transmitted or scattered by soil is measured; this method requires that the soil water content is known and also requires a calibration curve derived from soils with a range of known bulk densities (Lal and Kimble 2001). Although SBD is important for assessing SOC, reliable measurement of SBD remains a challenge (Lal and Kimble 2001).

SOC and SOM are quantified by wet or dry combustion methods. Wet oxidation uses an acid potassium dichromate solution. The advantage of acid oxidation is that it removes carbonate C, which is an inorganic C form, from the measurement. This method, however, suffers from many interferences and low recoveries. It requires the use of correction factors,

which vary among soils or even horizons and SOM fractions (Gillman 1986; Rosell 2001). Dry oxidation is done in one of two main ways: combustion followed by "weight loss of ignition measurement" (WLOI), and dry oxidation of SOC with collection and determination of evolved CO_2. In WLOI, selection of combustion temperature is critical to minimize errors (Gallardo 1987). Temperatures under 500 ºC may cause incomplete oxidation of SOM. On the other hand, temperatures higher than 500 ºC may cause the release of CO_2 from carbonates and hydroscopic water from clay minerals, decomposition of hydrated salts, and oxidation of Fe^{2+} (Shulte and Hopkins 1996; Cambardella and Gajda 2001). WLOI may be reasonably accurate and economical for estimating SOM if precautions with respect to hygroscopicity and hydrated salt content are taken into account (Cambardella and Gajda 2001).

Dry oxidation of SOC with collection and determination of evolved CO_2 uses a stream of O_2 with temperatures over 900 ºC. Catalysts (MnO_2 and CuO) convert the released CO to CO_2 (Tiessen et al 1981; Cambardella and Gajda 2001). Because dry oxidation uses high temperatures, release of CO_2 from soil carbonates can result in the overestimation of the amount of SOC.

For estimation of SOM dynamics or sequestration rate, changes in the carbon stock over time must be estimated. Measurements over time can be obtained by repeated sampling of one place at different times or by the use of a chronosequence, i.e., the sampling at one time of similar areas that differ in age. Another approach is to use SOM dating either with a labeled substrate or ^{14}C dating. ^{14}C dating is very useful for dating SOM that was created relatively recently because the world-wide increase in ^{14}C content resulting from thermonuclear bomb testing during the 20th century provides a convenient reference point.

Fractionating of soil organic matter

The methods for measuring SOC dynamics discussed in the previous paragraphs are insufficiently sensitive to identify changes over small periods of time (1 year) that occur in response to changes in management practices or land use. This is a problem because even small differences in C content can mean significant changes in C stock. SOM consists of a series of fractions or pools that differ in rate of biochemical and microbial degradation, and management practices can influence the distribution of organic C among these fractions (Cambardella and Elliott 1994; Golchin et al 1994). Information on the individual fractions allows researchers to improve their simulations of C dynamics (Vonlutzow et al 2007).

SOM can be fractionated physically or chemically or by a combination of physical and chemical methods (Zimmermann et al 2007). In physical fractionation, SOM is separated based on density or particle size with subsequent determination of C content for each density or particle size (Cambardella and Elliott 1993; Golchin et al 1994; Swanston et al 2002; Dubeux et al 2006). Density fractionation is based on the observation that, during humification, some components of SOM become more associated with mineral particles and thus occur in particles of higher density. From a density perspective, SOM can be divided into (i) a light fraction consisting of mineral-free organic matter composed of partly-decomposed plant debris, and (ii) a heavy fraction consisting of organic matter adsorbed or deposited by microorganisms on aggregate surfaces and sequestered within organomineral aggregates (Strickland and Sollins 1987). Among the physical fractionation methods, floatation with dense solutions of sodium polytungstate (SPT) (e.g., Golchin et al 1994; Golchin et al 1997; John et al 2005; Sollins et al 2006; Crow et al 2007) and colloidal silica (Ludox) (Hassink 1995; Meijboom et al 1995) is widely used. SPT has several advantages over other high-density liquids. It is less toxic than organic liquids or solutions of $ZnBr_2$ or NaI, has a lower viscosity at high concentrations than other inorganic solutions, and can be used to produce a wide range of densities (1.0-3.1 g cm^{-3}). SPT, however, was more inhibitor than Ludox in mineralization studies of isolated density fractions (Magid et al 1996). The maximum density achievable with Ludox is 1.4 g cm^{-3} (e.g., Cambardella and Elliott 1994; Golchin et al 1994; Meijboom et al 1995; Six et al 1999).

Density methods have advantages. They are less disruptive than chemical methods and provide a separation of SOM fractions that is more sensitive to soil cultivation than are total C measurements (Cambardella and Elliott 1994; Six et al 2000). The identified fractions are also thought to represent meaningful functional pools that relate to essential soil processes such as SOM mineralization and aggregate formation (Janzen et al 1992; Christensen 2001). SPT solutions are also reusable. The disadvantages are that used SPT is contaminated with carbon, and thus requires cleaning before reuse. Cleaning is especially important if the SPT is used for stable isotope measurements on the isolated fractions because small amounts of carbon could be exchanged between the soil and the SPT solution during fractionation. Another problem with SPT is that, when the Na^+ ion disassociates from the polytungstate in aqueous solution during the fractionation procedure, other cations in the soil (especially Ca^{2+}) can associate with the polytungstate and form insoluble species that precipitate even when the SPT solution is recycled and concentrated (Six et al 1999).

In addition to understanding how SOM is fractionated, it is also important to understand the relationship between SOM and soil aggregation. Aggregates not only physically protect SOM (Tisdall and Oades 1982) but also influence microbial community structure (e.g., Hattori 1988), limit oxygen diffusion (Sexstone et al 1985), regulate water flow (e.g., Prove et al 1990), determine nutrient adsorption and desorption (e.g., Linquist et al 1997; Wang et al 2001), and reduce run-off and erosion (Barthes and Roose 2002). All of these processes have profound effects on SOM dynamics and nutrient cycling. Tisdall and Oades (1982) developed a conceptual model that describes how SOM attaches to soil aggregates via temporary, transient, and persistent cementing or binding agents. Persistent binding agents consist of degraded, aromatic humic materials associated with polyvalent metals strongly sorbed to clays; persistent binding agents are responsible for the construction of microaggregates (0.053-0.250 mm). In contrast, temporary and transient binding agents such as polysacharides released from roots and from fungal hyphae are responsible for the stabilization of microaggregates into macroaggregates (>0.25 mm). Because of the relatively labile nature of temporary and transient binding agents, soil management has a greater effect on macroaggregates and the associated SOM than on microaggregates, which have more stabilized and humified organic matter (Six and Jastrow 2002). Macroaggregates may form around particulate organic matter (POM). POM is composed of partially decomposed plant and animal residues (Christensen 2001), and as POM is decomposed and microbial exudates are released, the macroaggregate becomes more stable, the C:N ratio decreases, and microaggregates form inside (Oades 1984). The method most commonly used to distinguish aggregate types is sieving. There are two types: dry sieving and wet sieving.

Dry sieving is less disruptive than wet sieving to the physical habitat of the soil microbial community (Schutter and Dick 2002; Gartzia-Bengoetxea 2009). Thus, dry sieving is preferred for the determination of potential habitat influences on soil microorganisms. In dry sieving, the sieves are mechanically shaken to separate soil into the following aggregate-size classes: >20, 20-10, 10-5, and 5-2 mm (large macroaggregates or megaaggregates); 2.0-0.25 mm (macroaggregates); 0.25-0.053 mm (microaggregates); and <0.053 mm (silt and clay fraction).

In wet sieving, air-dried soil on a 2-mm sieve is submerged in deionized water, resulting in slaking of the soil. Slaking disrupts aggregates due to the built up of internal air pressure during the rapid wetting of the soil (Kemper et al 1985; Six et al 1999). The soil is then

washed through a series of three sieves (2, 0.25, and 0.053 mm) (Elliott 1986). Bound carbon in aggregates is released by sonification for analysis.

Measurements of carbon in different size aggregates, in conjunction with density fractionation methods that physically separate soil organic matter into light and heavy fractions, can provide considerable insight into how SOM responds to ecosystem management (Elliott 1986; Strickland and Sollins 1987; Six et al 1998).

Another method used to determine availability of organic carbon in soil is chemical fractionation based on the solubility and affinity of SOC compounds in different solvents or extracting solutions (Swift 1996). Humic substances (HS) contained in soil are formed by the biochemical transformation of plant and animal debris, and consist of various types of transformed molecules with complex structure. With aqueous solutions, HS fractions can be separated into humic acids, fulvic acids, and humin. These fractions differ in chemical composition, acidity, degree of hydrophobicity, and self-association of molecules.

Humic acids (HAs) are defined as the fraction that is water insoluble under acid conditions (pH < 2). HAs coagulate as a dark brown to black oozy material at low pH but become soluble at higher pH values. The molecular weight of HAs range from 1500 to 5000 Da in streams and from 50,000 to 500,000 Da in soils. Unlike HAs, fulvic acids (FAs) are soluble under all pH conditions and have molecular weights ranging from 600 to 1000 Da in streams and from 1000 to 5000 Da in soils. Humins arse insoluble in water (Malcolm 1990; McDonald 2004; Magdaleno and Coichev 2005).

The method most commonly used for extraction of humic substances is solubility-based fractionation: alkali extraction (Stevenson 1994). It uses a 0.1 to 0.5 M NaOH and Na_2CO_3 solution with a soil to extractant ratio of from 1:2 to 1:5 (g ml^{-1}). This method leads to recovery of approximately two-thirds of SOM. The disadvantages of this method are the dissolving of silica from mineral matter, the contamination of the organic fractions separated from extract, the dissolving of protoplasmatic and structural components from fresh organic tissues, the condensation between amino acids and C=O groups (Mailard reaction), and the auto-oxidation of organic particles in contact with air. This latter oxidation should be minimized by performing all steps in N_2 or another inert atmosphere.

The solid substance resulting from the extraction of humic substances described in the previous paragraph is considered to be the insoluble humin fraction. The supernatant is filtered to remove suspended plant material and is treated with 1 M HCl to pH >2. The

precipitated material is the HA fraction, which is separated from the FA fraction by centrifugation. Subsequently, hymatomelanic acid can be separated from the HA fraction by alcohol extraction. The rest of the HA fraction is redissolved in base and electrolyte is added, which yields the last two parts of the HA fraction: dissolved brown humic acids and precipitated grey humid acids.

After the supernatant described in the previous paragraph has been centrifuged to remove HA, the supernatant is filtered through XAD-8 (macroreticular nonionic resin with binding properties for humic substances in acid condition) to absorb pigments. The FA fraction is obtained by subsequent elution with bases and desalination (Stevenson 1994).

After the different fractions of total C content have been physically and chemically separated, they are measured analytically with specialized techniques.

Budget of soil carbon

Another way to determine SOC dynamics is to quantify carbon input and output rates (Zerva et al 2005). Carbon enters soil from both aboveground and belowground sources. The aboveground carbon input is relatively easy to measure. For grasses and forbs, the aboveground biomass of the herb layer is harvested and weighed when biomass is maximal (Frouz et al 2009); this method assumes that all of the biomass of the herb layer will become litter during winter. For tree and shrubs, various litter traps can be used (Zerva et al 2005; Frouz et al 2009). Researchers using these approaches must cope with the heterogeneity of vegetation and with potential weight loss of litter before collection (i.e., loss of dead litter before herb harvest or decomposition of litter in litter traps).

The belowground C input, which consists mainly of C from dead roots and root exudates, is difficult to measure. Methods used to measure belowground C input include sequential root coring, the use of ingrowth cores and minirhizotrons, and the measurement of C fluxes (Vogt et al 1998). In sequential root coring, roots are extracted from soil samples collected several times during the season to determine changes in root biomass over time. For determination of root production, live and dead roots must be distinguished in each sampling. Dead roots increase due to root dieback and decrease due to root decomposition. Decomposition can be measured on paired samples in which two groups of samples are taken, roots are extracted from one group and quantified while the second group of samples is returned to the soil after the plants die (e.g., due to desiccation). Root decomposition can be estimated based on the

reduction in root mass between the first group and second group after the second group has been incubated in soil. Root production during a certain time interval can then be calculated as changes in live root biomass plus changes in dead root biomass plus amount of roots that decompose during this time interval. The latter can be calculated as amount of dead roots at the beginning of time interval multiplied by decomposition rate measured during the time interval (Vogt et al 1998). Ingrowth cores are based on the quantification of roots that grow into a volume of root-free soil; the latter is created by replacing a volume of soil containing roots with a volume of the same soil lacking roots (Vogt et al 1998). Sequential root coring and ingrowth cores provide direct estimates of root biomass production. In contrast, microrhizotrons or root windows provide indirect estimates of root production. Glass tubes or flat glasses are inserted into the soil, and the same spot is photographed at regular intervals. The length of new roots in contact with glass is determined (Vogt et al 1998). Another approach is based on the partitioning of root and microbial respiration and will be discussed later.

Measuring root exudates is extremely difficult. In the field, various labeling techniques are used to estimate the quantity of carbon transferred from belowground parts of plants to the soil. These techniques include pulse labeling, continuous labeling, and a method based on the difference in the natural abundance of ^{13}C in C3 and C4 plants (Kuzyakov and Domanski 2000). Pulse labeling and continuous labeling by ^{13}C-CO_2 allow researchers to study the fate of ^{13}C in soil. The technique based on the natural abundance of ^{13}C is similar but the plants are not labeled; in this case, the natural abundance of ^{13}C is measured when C4 plants are introduced into a soil that was previously used only by C3 plants or vice versa (Kuzyakov and Domanski 2000). Root exudates can also be indirectly estimated by partitioning autotrophic and heterotrophic soil respiration. Soil respiration is roughly three times higher than aboveground litterfall-C, which indicates that carbon input from root respiration and microbial decomposition of dead roots and root exudates combined is roughly double the annual aboveground litterfall (Davidson et al 2002).

The decomposition rate can be measured experimentally or estimated from soil respiration. An experimental and classical way to directly measure soil respiration is with litterbags (Irmer 1995). In litterbag experiments, litter or some another organic material such as filter paper is placed in nylon mesh bags. The bags are placed on the soil or in the soil and are periodically recovered. The decomposition rate can be determined based on the decrease in litter mass over time. Various mesh sizes can be used to partition the effects of soil organisms based on

organism size. For example, two mesh sizes can be used in the same experiment: a mesh with very small openings will exclude all soil organisms except the microflora and a mesh with large openings permits both soil microflora and soil fauna to enter the bag and contribute to decomposition. The difference between decomposition measured in the bags with the large and small openings is assumed to reflect the contribution of the soil fauna to overall decomposition (Irmer 1995). Experiments with litterbags either accessible or non-accessible to macrofauna indicated that decomposition was 5-40 % higher in the presence of macrofauna (Scheu and Wolters 1991; Irmer 1995). Litter bags indicate the decomposition rate only for the particular location (on the litter layer, on the soil surface, in the soil) and environment. Mass loss in litterbags may be caused by various processes resulting in a different final stage of organic C. For example, mass loss may result from mineralization and the release of CO_2, leaching of water-soluble substances, or fragmentation of the litter and its deposition in the soil outside of the litterbag in the form of soil faunal excrement. In treatments not accessible to macrofauna, most of the litter C is probably lost by release of CO_2 and by leaching. In litterbags accessible to macrofauna, on the other hand, most of the lost litter was deposited in the mineral soil layer outside of the litterbag (Wachendorf et al 1997). To clarify which processes contribute to litter mass loss, a method was developed that used microcosms with litterfall and mineral layers (Frouz 2002; 2008; Frouz et al 2006; 2008). With this method, researchers can measure the mixing of organic matter in the surface layer of soil in ecosystems in which the soils are mainly mixed by epigeic earthworms (Frouz et al 2006).

The decomposition rate can also be indirectly determined based on heterotrophic soil respiration. As mentioned earlier, however, heterotrophic respiration (the respiration of the decomposers) is accompanied by root (autotrophic) respiration, and the two kinds of respiration are difficult to separate. The classical approach for measuring microbial respiration in the laboratory is to measure respiration in a volume of soil from which the roots have been removed (Hanson et al 2000; Helingerová 2010). During the laboratory manipulations, however, soil is substantially altered by mixing, and thus extrapolation of respiration measured in the laboratory to the field is problematic. In addition to the large spatial variability (Katayama et al 2009), the key problem in field measurements of soil respiration is to distinguish autotrophic and heterotrophic respiration, i.e., respiration from living roots vs respiration from the decomposition food chain (Hanson et al 2000). Root and rhizosphere respiration can represent from 10 to 90 % of the total soil respiration (Hanson et al 2000), with mean values of 46 % in forest soils and 60 % in nonforest soils (Hanson et al 2000).

Because these are rough approximations and respiration rates can vary greatly among soils, the real values should be determined for the particular soil being studied (Raich 1998; Hanson et al 2000; Bond-Lamberty et al 2004; Trumbore 2006; Litton et al 2007). Autotrophic and heterotrophic respiration can be partitioned by measuring the respiration of fresh roots, or by comparing respiration for soils with and without roots (Hanson et al 2000; Yi et al 2007; Marsden et al 2008). In both cases, however, the soil is disturbed due to root removal, and this can influence respiration of the whole system. Another approach is to measure soil respiration in spots that differ in the natural abundance of roots, to regress respiration on root mass, and finally to estimate heterotrophic soil respiration from that point in the regression when root mass is zero (Jia et al 2006). Interestingly, the estimate of heterotrophic respiration obtained with this approach did not correspond with the measurement obtained with root-free soil (Jia et al 2006).

A promising approach for the separation of autotrophic and heterotrophic respiration is the use of radiocarbon techniques to determine the age of C in the CO_2 produced by soil respiration (Hanson et al 2000; Ryan et al 2005; Trumbore 2000; 2006). These techniques can be very accurate for carbon that has recently been fixed in a plant tissue due to the artificial increase in ^{14}C concentration resulting from the detonation of thermonuclear bombs during the last century. By comparing the age of soil carbon and the carbon in CO_2 produced by soil respiration, researchers can estimate the proportion of the respired carbon that was recently derived from plants vs that derived from older soil carbon (Trumbore 2000). However, the age of soil carbon is not equal. SOM consists of carbon pools of various ages, from litter that contains very fresh carbon to humic acids that consist of very old carbon (Tate et al 1993; Gaudinski et al 2000; Trumbore 2000; 2006; Ryan et al 2005). Radiocarbon measurements indicate that recent soil organic matter, such as litter and fine organic debris, contribute greatly to heterotrophic soil respiration while older, humified organic matter contributes much less, even though the older organic matter may represent a larger portion of total SOM than the younger pool (Gaudinski et al 2000).

Another method that can be used to study soil carbon dynamics is the observation of whole ecosystem carbon balance by the eddy covariance technique (Baldocchi 2003). This technique allows researchers to estimate the overall sink or source of C in the whole ecosystem (Curtis et al 2002; Baldocchi 2003; Chen et al 2003; Gough et al 2008). It is then possible to estimate plant biomass change and to estimate the amount of carbon in soil. Although eddy covariance is increasingly being used for estimation of soil C sequestration, in various ecosystems, their

use for carbon storage estimate has been limited because it requires the measurement of plant production on the site (Curtis et al 2002; Chen et al 2003; Gough et al 2008).

Conclusions

The soil carbon stock represents an important global pool of carbon. Moreover, more carbon enters the atmosphere from soil than from the burning of fossil fuels. To use soil carbon as a powerful tool in the mitigation of rising concentrations of atmospheric CO_2, simple and accurate methods for estimating soil carbon dynamics are needed. Such dynamics can be estimated with many different techniques. In one group of techniques, changes in the carbon stock are measured over time, either by repeatedly sampling the same site over decades or by sampling a chronosequence at one time. Chronosequences can be especially useful but are not always available. In another group of techniques, carbon flows in and out of soil are measured; although these measurements provide real-time estimates of carbon dynamics, the measurements are technically demanding and have significant sources of errors. It follows that the measurement of carbon input and output is not currently practical for large-scale monitoring. In a new promising technique, the combination of SOM fractionation and carbon dating is used. The soil carbon stock is partitioned and the age of individual fractions is determined. At the same time, soil respiration as well as of respired CO_2 age are determined. This approach can be used to estimate the amount of carbon in the soil and C loss from the soil. Although this approach is promising, its use in large-scale monitoring is probably limited by the cost of radiocarbon dating.

Because of the difficulties in monitoring soil carbon, management decisions may not be based solely on monitoring. Instead, governments should encourage stakeholders to use technologies that are known to promote carbon storage in soil.

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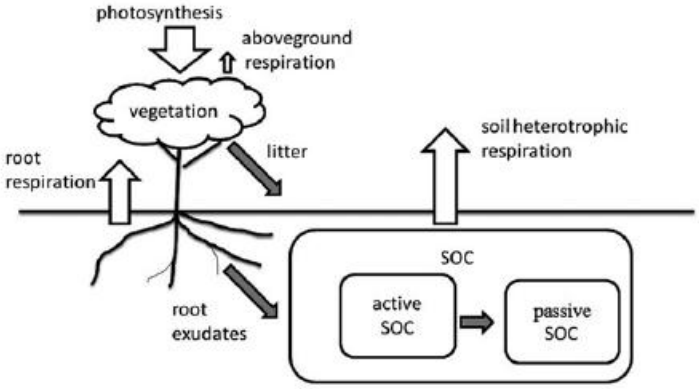
Appendices

Fig. 1. Soil organic carbon (SOC) transformation in soil. White arrows represent carbon in CO_2, and grey arrows represent carbon in organic matter. Carbon lost by leaching or by production of CH_4 is not represented.



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Paper 4

Particulate organic carbon in reclaimed and unreclaimed post-mining soils and its microbial community composition

Submitted to publish

Martin Bartuška[1, 2], Mark Pawlett[3], Jan Frouz[1, 2]

[1]Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Benátská 2, CZ-128 01 Prague 2, Czech Republic; frouz@natur.cuni.cz

[2]Institute of Soil Biology, Biology Centre of the Academy of Sciences of the Czech Republic, v. v. i., Na Sádkách 7, CZ-370 05 České Budějovice, Czech Republic

[3] School of Applied Science, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

Corresponding author: Martin Bartuška, bartuska@email.cz, tel:+420777978679, Institute of Soil Biology, Biology Centre of the Academy of Sciences of the Czech Republic, v. v. i., Na Sádkách 7, CZ-370 05 České Budějovice, Czech Republic

Abstract

Objective

Recovery of soil organic matter and associated microbial biomass is basic precondition for successful restoration of post mining soil. The aim is to compare the dynamics of soil C fractions and of the microbial communities associated with these fractions in two chronosequences of post-mining sites with different plant communities.

Methods

Soil carbon, pH, bulk density, and light fraction of particulate organic carbon (POC), either free or bounded in soil aggregates were studied on two chrosequences of post mining sites, both covering age from 10 to 50 year. Reclaimed chronosequence was overgrown by alder plantation, unreclaimed plots were vegetated by natural re-growth (*Salix caprea*, *Populus tremula* and *Betula pendula*). In intermediate and late succession stages microbial community in bulk soil and POC fractions were studied using PLFA.

Results

Soil C content increased and pH decreased with plot age, these trends were more pronounced in reclaimed sites. Light and bounded POC fraction increased with age, higher values and higher increase were found in reclaimed sites.

In both chronosequences light fraction was in order of magnitude higher than bound fraction. C content in both fractions increased with succession age, having higher C content in reclaimed sites. Microbial community was more affected by POC fraction than plot age. Bulk soil of reclaimed sites was more similar to bounded POC while bulk soil of unreclaimed soils was similar to light POC fraction.

Conclusions

Observed differences in POC, thus microbial community, correspond with higher level of bioturbation in reclaimed sites which promote faster POC accumulation and microbial community to be more close to bounded POC.

Practice

These results are important for restoration of post mining regions and economic and ecologic landscape services.

Implication

Carbon accumulation understanding is important for improvement of models and soil carbon dynamic predictions.

Keywords: soil organic matter, PLFA, chronosequence, soil carbon dynamic, pedogenesis

Introduction

Soil formation and the reestablishment of soil biological functions are among the basic preconditions for ecosystem development during primary succession or on new surfaces or locations where soil has been destroyed (Bradshaw 1997). Soil microorganisms are essential for ecosystem processes, such as organic matter decomposition and mineralization (Coleman et al. 2004). The quantity and activity of soil microorganisms are sensitive indicators of soil quality (Powlson et al. 1987). Consequently, there are many studies covering the development of soil microbial communities during succession and during various stages of pedogenesis (Frouz and Novakova, 2005; Helingerova et al., 2010; Sourkova et al., 2005). Although only the bulk soil was investigated in most of these studies, soil is very heterogeneous. Some studies appreciate this by exploring differences between individual soil horizons or between

rhizosphere and bulk soil (Baldrian et al., 2008; Elhottova et al., 2006) but variation among individual microhabitats in soil is seldom measured (Frouz et al., 2010; Mummey et al., 2006; Mummey and Stahl, 2004).

In this study, we explored how microbial communities differ between soil microhabitats, how the occurrence of microbial microhabitats changes during succession, and how that variation corresponds with the microbial community in bulk soil. To define individual microbial microhabitats, we used individual fractions of soil organic matter (SOM). SOM was divided into fractions with a rapid and slow turnover rate (Parton et al., 1987; Molina et al.,1983; Van Veen and Pauli, 1981; Van Veen et al., 1984; Verberne et al., 1990). To describe C changes in SOM quality, we physically fractionated SOM based on density (Golchin et al., 1994). Physical fractionation by density yields a light and a heavy SOM fraction. The light fraction consists largely of non- or partially decomposed plant residues that are not associated with soil minerals (Sollins et al., 1984; Spycher et al., 1983) and that have a rapid turnover rate; the C pool in the light fraction is assumed to play a dominant role in soil nutrient dynamics. The heavy fraction is more closely associated with minerals and represents a more recalcitrant and stable part of SOM (Spycher et al., 1983; Sollins et al., 1984).

The aim of this study was to compare the dynamics of soil C fractions and of the microbial communities associated with these fractions in two chronosequences of post-mining sites overgrown with different plant communities. One chronosequence consisted of unreclaimed sites re-vegetated by natural regrowth that produced litter with a high C/N ratio. The other chronosequence consisted of sites that were reclaimed by the planting of alder trees that produced litter with low C/N ratio. This enabled us to evaluate the effect of litter quality and the effect of reclamation practice. We used post-mining soils because studies of such soils are relevant to their restoration and also because post-mining soils are well suited for chronosequence analysis in that the mining operation repeatedly creates new sites over a long

period of time. These sites are usually formed from the same substrate and with the same technology and can be easily dated. The study was conducted near Sokolov in one of the major open-cast coal mining areas in the Czech Republic. Considerable information about soil development and microbial communities has been collected for the soils in this area, where more than 6000 ha have been affected by mining (Baldrian et al., 2008; Frouz and Novakova, 2005; Helingerova et al., 2010; Sourkova et al., 2005).

Methods

The study was conducted at the post-mining sites near the town of Sokolov in the Czech Republic (50°14'29"N, 12°40'14"E). The sites have an elevation of 500–600 m a.s.l., a mean annual precipitation of 650 mm, and a mean annual temperature of 6.5°C (Frouz et al. 2001). The spoil heaps in the Sokolov region originated from open-cast brown-coal mining and consist mainly of tertiary clays that are well supplied with mineral nutrients (Sourkova et al., 2005; Stys, 1981). The spoil material also contained significant amounts of fossil organic matter. This organic matter is not related to coal but is mostly represented by type II Kerogen derived from dead algae; the algae had fallen to the bottom of a tertiary lake that overlaid the coal layers in geological time (Kríbek et al., 1998).

Two chronosequences, both covering sites from 0 to 50 years old, were chosen. One chronosequence consisted of eight sites reclaimed by the planting of a mixture of alder species (*Alnus glutinosa* and *Alnus incana*). The other chronosequence consisted of six unreclaimed sites spontaneously colonized by vegetation (dominated by *Salix caprea*, *Betula pendula*, and *Populus tremula*). Site ages and soil characteristics are provided in Table 1.

Sampling

For each site, three composite samples for each of two layers (0–5 and 5–10 cm below the litter layer) were prepared by mixing two samples per composite taken with a cylindrical corer (5.1 cm diameter, 5 cm long). The samples were transported to the laboratory, weighed, homogenized, and passed through a 2-mm sieve.

Soil analysis

The procedure for density fractionation of particulate soil organic carbon (POC) was based on Hassink et al. (1995). A 15-g quantity of soil (dry soil equivalent) per composite soil sample was fractionated in a silica suspension with a density of 1.37 g/cm^3 to determine the amounts of light fraction of particulate organic matter (LF). After the first density fractionation, the residual mineral fraction was ultrasonicated for 2 min at 1,200 Hz, and then the fractionation was repeated. The POM released by ultrasonic treatment was called bound POM (B). Both fractions were rinsed with deionised water and weighed. A portion of each fraction was frozen at -70 °C and subsequently subjected to PLFA analysis. The rest was dried at 60 °C for 5 h, weighed, and analyzed for organic C. Total organic soil C and C in LF and B fractions were determined by wet acidified dichromate oxidation (Jackson 1958). The pH of composite samples was determined with a WTW 526/538 pH meter (Germany) with a combination electrode in a water suspension (1/5, w/v, soil/water).

Phospholipid fatty acid analysis

The microbial community structure in the middle-aged and old-aged sites was determined by phospholipid fatty acid (PLFA) analysis using a method based on Frostegård et al. (1993). PLFAs were extracted from approximately 7 g of fresh soil and from the LF and B

fractions using chloroform, methanol, and citrate buffer at a ratio of 1:2:0.8 (v/v/v). The PLFA was fractionated by solid phase extraction and then derivatized by mild alkaline methanolysis.. The resultant fatty acid methyl esters (FAMEs) were analysed by gas chromatography (6890 N Agilent Technologies) using G2070 ChemStation for G.C. systems software. FAMEs were separated using an HP-5 (Agilent Technologies) capillary column (30 m long, 0.32 mm ID, and 0.25 μm film), which contained 5% phenylmethyl siloxane. The temperature program started at 50 °C (1 min), increased at 25 °C/min to 160 °C, at 2 °C/min to 240 °C, and at 25 °C/min to 310 °C (10 min). The injector temperature was set at 310 °C, the flame ionisation detector was set at 320 °C, and the flow was set at 1 ml/min. PLFAs were identified by comparing sample retention time to a standard qualitative bacterial acid methylester mix (Supelco) and by using gas chromatography coupled with mass spectroscopy (Agilent Technologies). Indicator fatty acids included 18:2ω6,9 for ectomycorhizal fungi (Frostegård and Bååth 1996; Kaiser 2010) and the sum of i15:0, ai15:0, 15:0, 16:1, i16:0, 16:1ω9, 16:1ω7 t, i17:0, ai17:0, cyc-17:0, 17:0 and cyc-19:0 for total bacteria (Frostegård and Bååth 1996). The fungal/bacterial ratio was calculated using the fungal biomarker (18:2ω6) divided by the summed mol% of bacterial fatty acids (Frostegård and Bååth 1996).

Statistical analysis

The general linear model (GLM) was used to assess the effect of reclamation and site age on the investigated parameters, with reclamation as a categorical predictor and age as a continual predictor. GLM computation was done by Statistica 10.0. Principal component analysis (PCA) was used to visualize relationships between the composition of the microbial community as indicated by PLFA and environmental variables. Redundancy analysis (RDA) and Monte Carlo permutation tests were used to test for significant relationships between

environmental variables and microbial community variables. Both PCA and RDA were computed using CANOCO 4.0.

Results

Soil pH was alkaline (ranging from 8.9 to 8.0) early in both chronosequences but decreased significantly with age (Table 1). This decrease was more pronounced in the 0-5 cm layer than in 5-10 cm layer (Table 1), and the decrease was significantly faster in the reclaimed than in the unreclaimed chronosequence (Table 1).

Total C was greater in the 0-5 cm layer than in the 5-10 cm layer and increased significantly with age in both layers. This increase occurred significantly faster in the reclaimed than in the unreclaimed chronosequence (Table 1). Bulk density did not change with age and did not differ between reclaimed and unreclaimed chronosequence (Table 1).

The amounts of LF and B fractions significantly increased with age in both layers (Table 2). In the 0-5 cm layer for both fractions, this increase was significantly greater in the reclaimed than in the unreclaimed chronosequence (Table 2). In the 5-10 cm layer, the increase did not differ between the reclaimed and unreclaimed chronosequences.

The content of C in both the LF and B fraction was significantly affected by site age (Table 3) in the 0-5 cm layer. In the of 5-10 cm layer, the difference in C content was significant only for the B fraction (Table 3). C content was significantly higher in both the LF and B fraction of both layers in reclaimed than in unreclaimed chronosequences (Table 3). Consequently, C content in both fractions increased with site age and was higher in reclaimed than in unreclaimed sites (Fig. 1). The quantity of C was an order of magnitude higher in the LF than in the B fraction.

The total PLFA content was significantly higher in reclaimed than in unreclaimed sites but differences between bulk soil and individual POC fractions did not show any clear pattern (Fig. 2).

The fungal/bacterial ratio, as indicated by the ratio of fungal- to bacterial-specific PLFAs, was significantly higher in the unreclaimed than in the reclaimed chronosequence (Fig. 3). In all sites, the fungal/bacterial ratio was highest in the LF fraction, lowest in the bulk soil, and intermediate in the B fraction. The difference in the fungal/bacterial ratio between the bulk soil and the LF fraction was statistically significant, but the ratio in the B fraction did not differ significantly from that in the bulk soil or LF fraction (Fig. 3).

The two major PCA axes together explained 69% of the data variability. The first axis explained 50.6% of the variability and corresponded to difference between reclaimed and unreclaimed sites and between B and LF fractions. The LF fraction was associated with unreclaimed sites while the B fraction was associated with reclaimed sites. The second axis explained 18.5% of the data variability and corresponded to site age (Fig. 4). The difference between PLFAs of the bulk soil and those of individual fractions was substantial. According to RDA (ordination diagram not shown), reclamation and the LF fraction were the only significant variables indicated by forward selection. The statistical significance of these two variables was confirmed by a Monte Carlo permutation test ($p=0.002$) and explained 52% of the data variability.

Discussion

A comparison of Table 2 and Table 1 indicates that POC represents only a small proportion of the total C. However, a substantial amount of C in these sites consists of fossil organic matter (Kribek et al., 1998), namely type II kerogen. Kerogen can account for 5–6% of soil C in post-mining sites (Frouz et al., 2011). If we subtract this value from the C content

in Table 1, we can infer that most of the C sequestered in the studied post-mining sites is in form of POC and is mainly the LF fraction. This is in agreement with Abakumov at al. (2013), who studied the same sites and reported that humic acids increase with site age and that humic acids, which are usually associated with clay minerals in the heavy fraction of organic matter, represent only a small percentage of the total SOM. This observation suggests that most of organic matter incorporated in the soil at these sites is POC that lies between soil aggregates or is loosely bound in macroagregates and that only a small percentage of the POC is bound in microaggregates within macroaggregates. Our inference is also supported by direct microscopic observation of SOM in thin soil sections and by X-ray tomography of soil aggregates (Frouz et al., 2007, 2010) and is consistent with Poll et al. (2003), who found that the coarse soil fraction, which is likely to contain relatively fresh and labile organic matter, is more affected than the fine fraction by the long-term addition of farm manure.

Some studies (Sculion and Malik, 2000; Six et al., 2004) emphasise the role of earthworms and other soil macrofauna in incorporating SOM into microaggregates inside soil aggregates. In our studies of post-mining sites, however, soil macrofauna in reclaimed sites and in older unreclaimed sites (Frouz et al., 2001, 2007, 2008b) substantially promote the incorporation of the LF fraction, i.e., that fraction of organic matter that lies between aggregates or is only loosely bound inside macroaggregates. In these sites, the macrofauna is dominated by epigeic and endogeic earthworms and litter-feeding macroarthropods (millipedes, isopods and dipteran larvae), which is typical for moder humus (Ponge, 2003), which is the type of humus that develops on these sites (Frouz et al., 2007). These litter-feeding macroarthropods fragment litter and transform it into fecal pellets that form the fermentation (Oe) layer, which can be several mm to several cm thick in our sites (Frouz et al., 2007). Earthworms can incorporate these pellets into the mineral soil by consuming them and incorporating them into their own faeces. In addition, by maintaining a porous interface

between the Oe layer and the top mineral A layer, endogeic earthworms facilitate the downward movement of litter fragments by percolating water.

In contrast to the moder type of humus that develops in reclaimed post-mining sites and in older unreclaimed post-mining sites, a mor type of humus (Ponge, 2003) develops in young and medium-aged unreclaimed post-mining sites. Examination of thin soil sections from the latter sites indicates that litter fragments produced by mechanical breakdown of litter and by soil fauna (which are dominated by mesofauna rather than by megafauna) remain in the Oe layer and do not enter in the mineral soil. This is explained by the high abundance of fungi (Baldrian et al. 2008), which form a dense network of mycelia (Frouz et al., 2007) that prevents fine organic fragments from being carried down into the mineral soil by percolating water. The situation changes as these unreclaimed sites age (Table 2, Fig. 1) and are colonized by endogeic earthworms (Frouz et al., 2007, 2008a). As noted in the previous paragraph, endogeic earthworms form a loose and permeable interface between the Oe and A layers and thereby facilitate the downward movement of POC.

The pH decreased in both soil layers with increasing site age. The addition and sequestration of organic carbon in the soil leads to formation of organic acids, which reduce the pH. This decrease was previously observed on reclamation sites as well as during primary succession (Crocker and Dicson 1957; De Kovel et al., 2000; Sourkova et al., 2005; Wandernaar and Sevink, 1992). In the alder plantations growing on the reclaimed sites in the current study, the pH decrease might also result from the activity of the rhizosphere microbiota. The rhizosphere microflora of leguminous plants as well as alder trees release organic acids, decreasing pH and thus increasing nutrient availability in the surrounding soil.

As determined by PLFA analysis, the soil microbial community of the post-mining sites substantially differed between the bulk soil, light POC, and bound POC, which agrees with previous findings that microbial communities differ in different parts of soil aggregates

or in different fractions of POC (Frouz et al., 2010; Mummey and Stahl, 2004; Mummey et al., 2006). The LF POC has a higher proportion of fungi than the B fraction or the bulk soil (Fig. 3). This is in agreement with the previous observation that incorporation of organic matter into soil by soil macrofauna increases the fungal/bacterial ratio (Frouz et al., 2007). We postulate that this occurs because litter and fermentation layers have a high fungal/bacterial ratio (Frouz et al., in press) and maintain that ratio when relatively large pieces of POC are incorporated either between soil aggregates or inside microaggregates. Later, as POC is bound inside microaggregates inside aggregates and comes into closer contact with clay minerals, bacteria become more important. Similar results were reported by Kong et al. (2011), who found a larger fungal biomass in a coarser microaggregate fraction than in a finer silt and clay fraction. The differences in microbial communities among the different fractions may be partly caused by pH, which is likely to drop as organic particles become more closely associated with alkaline mineral soil. Factors in addition to pH, however, are likely to have affected the microbial communities.

Consideration of the total amount of PLFAs as well as of the microbial community composition (as indicated by PCA) suggests that the microbial community in bulk soil is not simply a combination of the microbial community in both POC fractions. This indicates that other components of the microbial community, presumably living in some other soil microhabitats (such as the mineral surfaces or soil solution) significantly contribute to the microbial community of the bulk soil. This raises the question as to whether the analysis of the bulk soil reasonably represents the average soil community. Perhaps some components of the microbial community are more easily extracted than others from the bulk soil than from particular soil fractions. If this is the case, data from the bulk soil would overestimate the abundance of some microorganisms and underestimate the abundance of others. This

possibility is supported by the data in our study and by the data in Mummey and Stahl (2004) and Mummey et al. (2006).

The soil microbial communities differed substantially in the reclaimed vs. unreclaimed post-mining sites. This can be due to differences in litter quality because litter has a lower C/N ratio in reclaimed sites than in unreclaimed sites (Frouz et al. in press). The differences in microbial communities may also be due to differences in the rate of pH decrease or in other soil properties. It is also possible that the contribution of individual soil microhabitats to the overall microbial community recorded in bulk soil differed between reclaimed and unreclaimed sites. The soil microbial community in reclaimed sites is more closely associated with the B fraction of POC while that of unreclaimed soil is more closely associated with the LF fraction of POC.

Appendices

Table 1. Total carbon (C), pH, and bulk density in 0-5 and 5-10 cm soil layers in reclaimed and unreclaimed sites of various ages on a post-mining heap near Sokolov. U indicates unreclaimed sites, R indicates reclaimed sites, and the following number indicates the site age in years. Values are means ± SE. The bottom two rows indicate the significance (p value) of site age (a continual predictor) and reclamation (reclaimed vs. unreclaimed) based on GLM.

	pH		C content (%)		Bulk density (g cm^{-3})	
Type of site and age (years)	0-5 cm	5-10 cm	0-5 cm	5-10 cm	0-5 cm	5-10 cm
U9	7.89±0.06	7.97±0.01	6.68±0.42	6.41±0.51	1.02±0.04	1.10±0.01
U19	7.92±0.06	8.02±0.02	5.78±0.65	4.44±0.23	1.00±0.01	1.15±0.01
U21	7.72±0.10	7.93±0.05	9.30±1.66	6.33±0.91	0.94±0.08	1.11±0.01
U24	7.54±0.07	7.62±0.12	10.42±0.39	7.82±1.04	0.74±0.08	0.81±0.01
U28	7.72±0.03	7.92±0.11	5.59±0.19	6.16±1.02	1.02±0.07	1.07±0.04
U50	7.31±0.36	7.83±0.07	8.44±2.17	5.14±0.34	1.03±0.04	1.18±0.06
R10	7.41±0.19	7.95±0.12	12.78±1.25	6.92±0.26	0.80±0.05	0.99±0.04
R10	7.94±0.01	8.00±0.02	8.13±0.10	7.42±0.20	0.99±0.08	1.05±0.06
R15	7.27±0.12	7.6±0.09	4.98±0.57	4.31±0.46	1.19±0.07	1.22±0.09
R21	7.86±0.15	8.11±0.01	9.21±3.03	5.46±0.48	1.05±0.14	1.14±0.02
R25	8.09±0.08	8.18±0.05	10.95±0.36	6.39±0.62	1.03±0.60	1.15±0.04
R32	6.95±0.07	7.78±0.11	18.17±1.25	9.29±1.91	0.69±0.05	0.96±0.01
R34	6.7±0.35	7.68±0.13	16.19±0.43	8.79±0.69	0.90±0.12	1.02±0.03
R49	6.34±0.08	6.64±0.08	8.27±2.29	4.32±2.80	1.00±0.22	1.38±0.23
Age	1.8253E-09	1.9805E-06	0.0014	0.0126	0.80	0.11
Reclaimed	0.0003	0.0928	0.0001	0.0190	0.7900	0.1900

Table 2. The percentage of light (LF) and bound (B) POC at depth 0-5 and 5-10 cm in reclaimed and unreclaimed sites of various ages on a post-mining heap near Sokolov. U indicates unreclaimed sites, R indicates reclaimed sites, and the following number indicates the site age in years. Values are means ± SE. Bottom rows indicate significance (p value) of site age (continual predictor) and reclamation (reclaimed vs. unreclaimed) using GLM.

	0–5 cm depth		5–10 cm depth	
Type of site and age (years)	LF (%)	B (%)	LF (%)	B (%)
U9	0.22±0.06	0.57±0.38	0.32±0.06	0.67±0.39
U19	0.93±0.35	0.34±0.09	0.55±0.22	0.18±0.09
U21	1.01±0.46	0.466±0.1	0.53±0.19	0.22±0.16
U24	3.92±1.76	0.62±0.08	1.53±1.16	0.32±0.12
U28	2.84±1.56	0.73±0.29	2.9±1.90	1.56±1.03
U50	1.9±0.60	0.38±0.14	1.76±0.56	0.33±0.11
R10	2.97±0.71	0.58±0.21	0.51±0.13	0.11±0.05
R10	0.31±0.21	0.69±0.10	0.51±0.08	0.49±0.18
R15	1.09±0.41	0.33±0.14	0.69±0.38	0.59±0.25
R21	0.90±0.18	0.35±0.11	0.65±0.10	0.21±0.05
R25	3.86±0.18	1.91±0.67	1.96±0.08	1.03±0.33
R32	5.31±0.81	1.06±0.38	1.25±0.20	0.33±0.12
R34	10.94±2.68	2.13±0.45	2.71±0.64	0.86±0.01
R49	20.55±18.98	3.55±0.68	8.96±7.68	2.12±1.01
Age	0.0002	0.00002	0.0001	0.0125
Reclaimed	0.0230	0.0004	0.1496	0.2747

Table 3. The percentage of carbon (C) in light (LF) and bound (B) POC at depth 0-5 and 5-10 cm in reclaimed and unreclaimed sites of various ages on a post-mining heap near Sokolov. U indicates unreclaimed sites, R reclaimed sites, and the following letter indicates the site age in years. Values are means ± SE. Bottom rows indicate significance (p value) of site age (continual predictor) and reclamation (reclaimed vs. unreclaimed) using GLM.

	0–5 cm depth		5–10 cm depth	
Type of site and age (years)	LF (%)	B (%)	LF (%)	B (%)
U9	11.96±0.81	9.28±0.84	12.45±0.39	9.65±0.57
U19	16.41±0.68	14.90±0.75	12.62±2.96	17.74±0.84
U21	27.37±0.50	20.13±0.97	16.07±0.41	17.65±0.81
U24	17.09±0.97	17.26±1.19	13.58±0.72	13.69±0.48
U28	16.49±1.21	9.25±1.27	17.37±0.54	9.46±0.88
U50	25.12±0.49	22.9±1.42	11.42±0.72	14.96±0.80
R10	32.38±1.27	25.87±0.94	34.16±0.20	28.13±0.50
R10	12.33±0.63	10.79±0.87	14.83±1.81	10.08±0.10
R15	24.42±1.06	12.77±0.32	14.86±0.05	10.29±0.64
R21	41.27±1.66	34.67±0.81	37.61±2.87	34.66±0.22
R25	41.51±1.35	26.67±0.24	42.53±4.93	24.17±0.56
R32	39.77±0.73	33.80±0.23	40.08±1.52	31.24±1.39
R34	36.42±3.01	31.39±1.14	30.69±1.33	28.86±0.85
R49	37.35±11.58	27.03±0.90	34.97±5.97	21.51±8.63
Age	0.0004	0.0002	0.0584	0.0348
Reclaimed	5.54E-07	0.00001	8.79E-09	0.0008

Fig. 1. Carbon content in LF (a) and in B fraction in soil aggregates (b) in reclaimed and unreclaimed post-mining sites of various ages. Bars represent SD. The inserted tables indicate the statistical effects of reclamation and site age on the carbon content in each fraction (GLM).

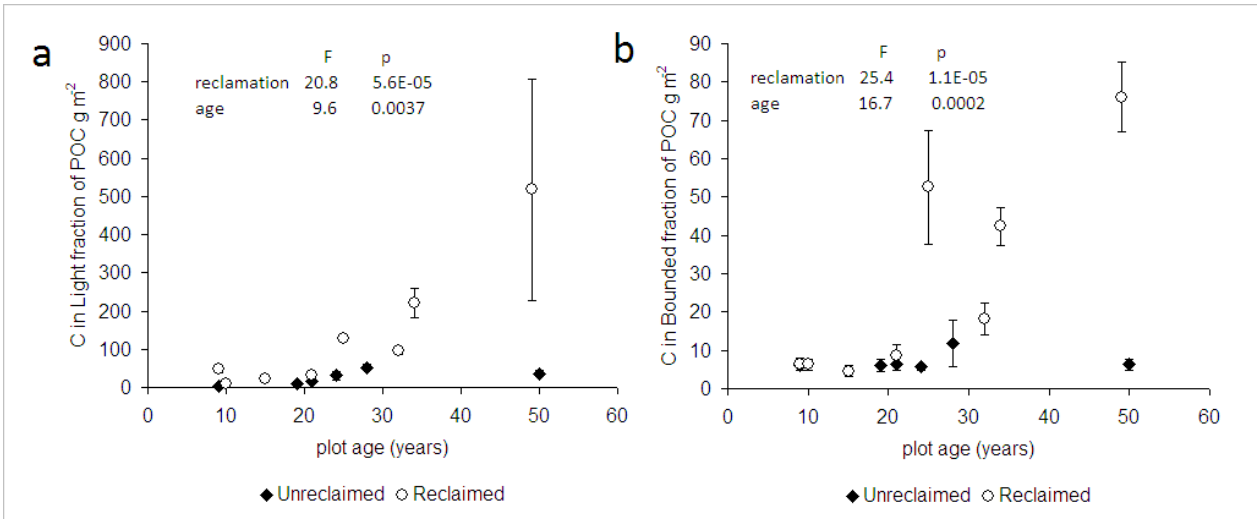


Fig. 2. Total content of PLFA in the bulk soil and in two POC fractions (LF and B) in reclaimed and unreclaimed post-mining sites of intermediate and older ages. LF refers to the light fraction, and B refers to the fraction bound in aggregates. The inserted table indicates the statistical effects of reclamation and site age on C stock in each fraction (GLM); superscripts indicate the results of mean comparisons.

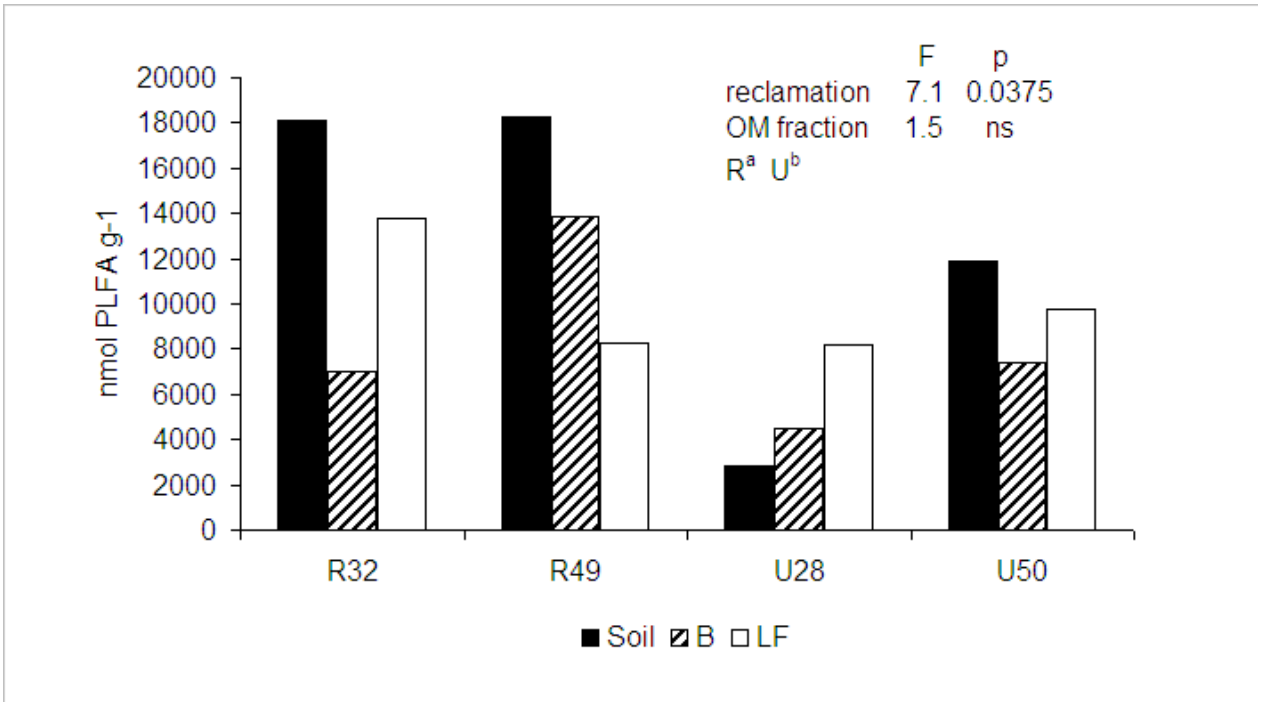


Fig. 3. Fungal/bacterial ratios based on the proportion of characteristic fungal and bacterial PLFAs in bulk soil and in two POC fractions (LF and B) in reclaimed and unreclaimed post-mining sites of various ages. LF refers to the light fraction, and B refers to the fraction bound in aggregates. The inserted table indicates the statistical effects of reclamation and site age on the C content in each fraction (GLM); superscripts indicate the results of mean comparisons.

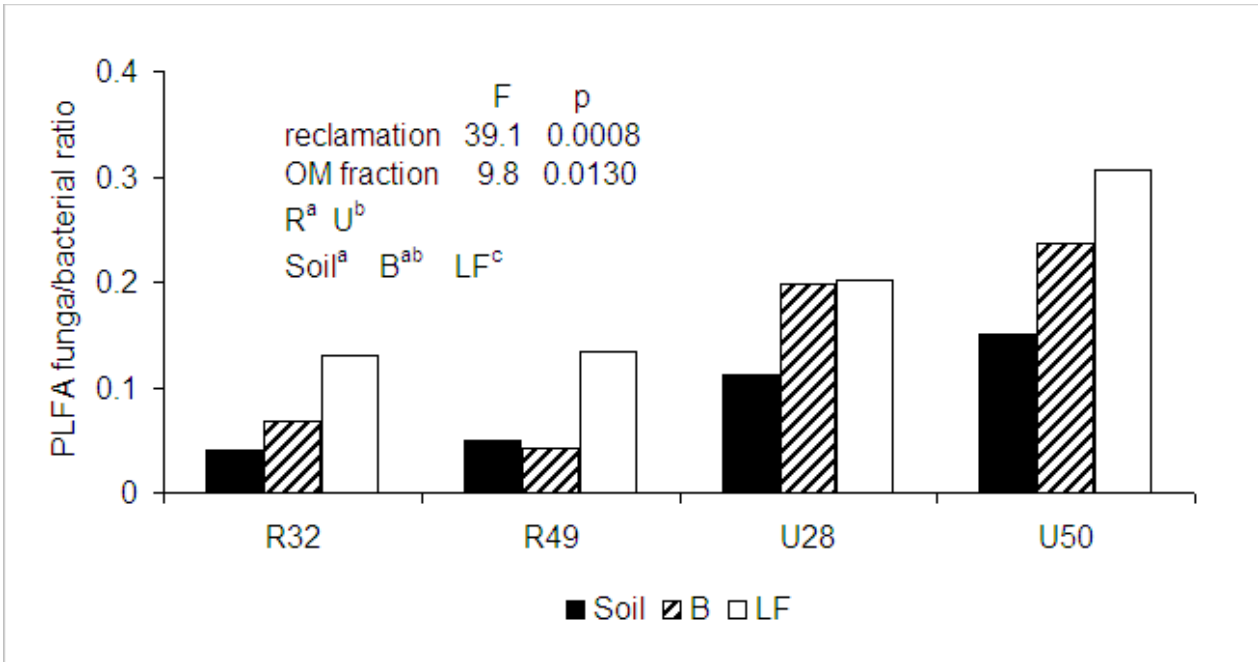
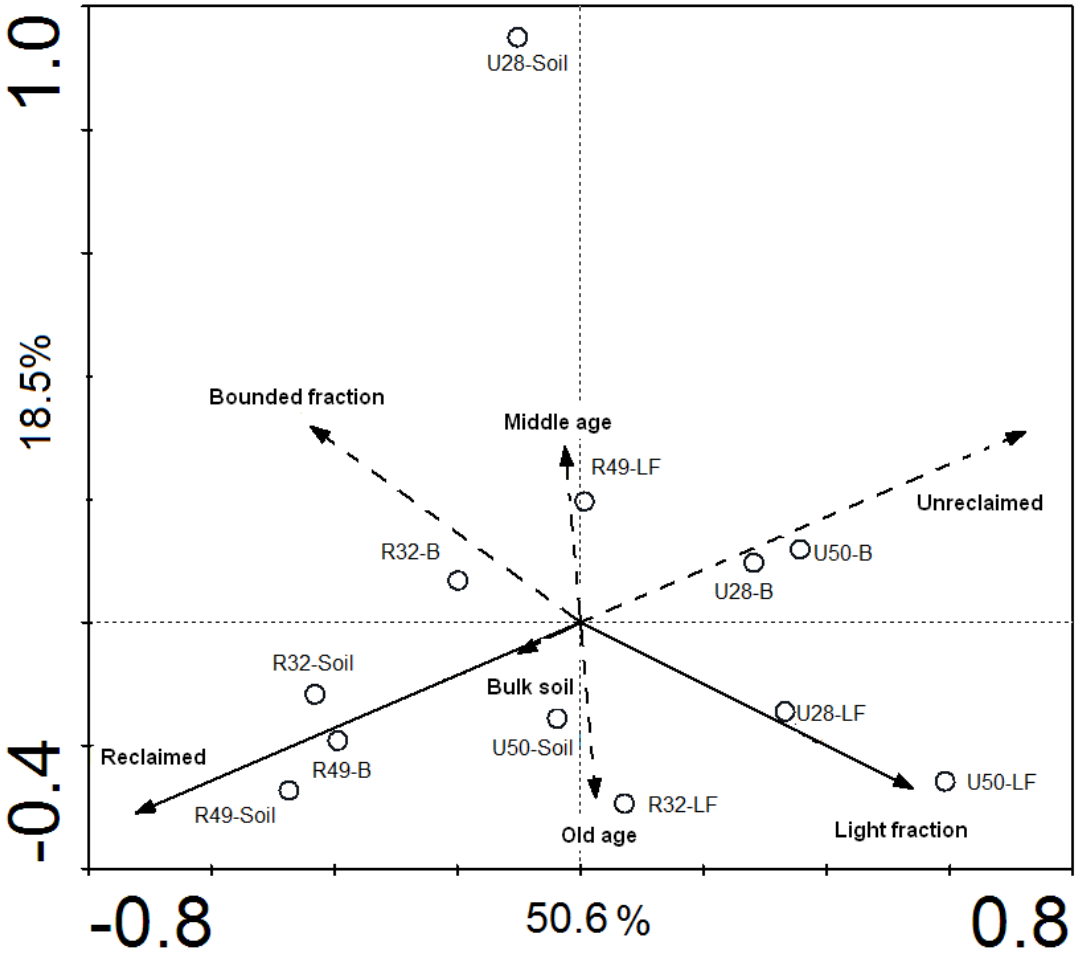


Fig. 4. PCA (principal component analysis) ordination diagram based on PLFAs in bulk soil and in two POC fractions (light fraction and bound fraction) in reclaimed and unreclaimed post-mining sites of various ages. Numbers near axes indicate the percentage of data variability explained by the given axis. Arrows represent environmental variables, and solid arrows represent variables found to be significant by forward selection and RDA.



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