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TRANSFORMATION OF ALIPHATIC AND AROMATIC COMPONENTS OF LITTER DURING DECOMPOSITION AND ITS RELEVANCE TO SOIL ENVIRONMENT AND SOIL BIOTA

Ph.D. Thesis

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Annotation

Transformation of litter chemical components during litter decomposition was investigated. The study focuses mainly on changes in quantity and quality of aliphatic and aromatic components of organic matter as identified potential precursors of stabilized soil organic matter. The effect of soil biota and other environmental factors were taken into consideration because of their high impact on the decomposition process.

Declaration [in Czech]

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List of papers and author's contribution

The thesis is based on following papers. They are referred in the text by the numerals:

(i) **Cepáková, Š.**, Frouz, J., 2015. Changes in chemical composition of litter during decomposition: a review of published ^{13}C NMR spectra. Journal of Soil Science and Plant Nutrition 15 (3), 805–815. (IF = 1.600)
Šárka Angst collected and analysed data and wrote the paper.

(ii) **Angst, Š.**, Mueller, C.W., Cajthaml, T., Angst, G., Lhotáková, Z., Bartuška, M., Špaldoňová, A., Frouz, J., 2017. Stabilization of soil organic matter by earthworms is connected with physical protection rather than with chemical changes of organic matter. Geoderma, 289, 29–35. (IF = 2.855)
Šárka Angst participated in the chemical analyses (^{13}C CPMAS NMR spectroscopy and thermochemolysis GC-MS) and wrote the paper.

(iii) **Angst, Š.**, Cajthaml, T., Angst, G., Šimáčková, H., Brus, J., Frouz, J. The effect of dead standing biomass on the litter decomposition process – manuscript
Šárka Angst prepared samples, participated in the chemical analyses (thermochemolysis GC-MS) and wrote the paper.

(iv) **Cepáková, Š.**, Tošner, Z., Frouz, J., 2016. The effect of tree species on seasonal fluctuations in water-soluble and hot water-extractable organic matter at post-mining sites. Geoderma, 275, 19–27. (IF = 2.855)
Šárka Angst collected and prepared samples, ran analyses and wrote the paper.

Content

Abbreviations

C	Carbon
CPMAS	Cross polarization magic angle spinning
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
G	Guaiacyl-type lignin
H	P-hydroxyphenyl-type lignin
HWC	Hot water-extractable C
N	Nitrogen
NMR	Nuclear magnetic resonance
OM	Organic matter
Py GC/MS	Pyrolysis gas chromatography/mass spectrometry
S	Syringyl-type lignin
SOM	Soil organic matter
UV-B	Ultraviolet-B
WEOC	Water-extractable OC
WEOM	Water-extractable OM
WSC	Water-soluble C

1 Introduction

1.1 Soil as a part of the global carbon cycle

The global carbon (C) budget is connected through fluxes between biota and soil, the atmosphere and the oceans (Woodwell et al., 1978). The soil pool comprises approximately three times more C than the atmosphere (Houghton, 2005; Oelkers and Cole, 2008). With an increasing number of studies focusing on evidences and impacts of global climate changes, this great C pool has come into spotlight as a potential sink of anthropogenic emissions of C (e.g. Post et al., 1982; Melillo et al., 1993; Lal, 2004).

According to estimations, soil organic matter (SOM) itself comprises around 1370 Gt C and litter 150 Gt C (as achieved from a balance between litterfall and decomposition; Schlesinger, 1977; Foley, 1995). The mentioned C budgets (SOM and litter) account for organic C that is part of organic matter (OM) in different decomposition stages; from fresh litter, up to stabilized SOM. Decomposition rate and consequently the fate of organic C entering the soil system is dependent on a complex of various factors that often interact (Berg and McClaugherty, 2003). Ecosystem type (including factors such as climate, topography, parent material) and land use (e.g., deforestation, cultivation techniques) determine the net primary production (C captured through photosynthesis) (DeGryze et al., 2004) and widely affect decomposition processes. Ecosystem factors influencing litter decomposition include climatic conditions, e.g., temperature and humidity (e.g. Hobbie, 1996), the abundance of litter decomposers (e.g. Standen, 1978; Frouz et al., 2013) and quality of litter (e.g. Quideau et al., 2001). These controls determine, how much C is mineralized and released back to the atmosphere, how much C becomes a part of the food chain and how much C is incorporated into soil and stabilized there.

Therefore, a better understanding of mechanisms of litter decomposition as a crucial process leading to SOM formation and consequently to soil C sequestration may improve our knowledge about the global C cycle.

1.2 Stabilization of soil organic matter

The term SOM includes a wide spectrum of dead organic materials in soil or directly on the soil (Sollins et al., 1996). These include plant litter, root exudates, and metabolites and debris of soil organisms in various stages of decomposition. Some authors (e.g. Oades, 1988) also include living organisms in the SOM definition. Dead OM in soil or OM entering the soil, e.g. leaf litter, is subject to decomposition. Most of this OM is utilized by microorganisms as a source of C and nutrients. Part of the OM, usually a minor amount of the initial OM, becomes stabilized and thus protected against decomposition. As a result, C, as component of SOM, is sequestrated in soil. Retention and thus stabilization of SOM in soil is driven by several stabilization mechanisms. These mechanisms may prolong the residence time of C in soil from less than few weeks in case of labile OM (Wu et al., 2012) up to thousands of years (Post et al., 1982). Apart from ecosystem dependent factors, such as climate, soil acidity and redox state, water availability and presence of potential degraders (Schmidt et al., 2011), the following stabilization mechanisms are commonly regarded as important for SOM sequestration: (1) chemical recalcitrance of individual components of OM, (2) physical protection of OM through occlusion within aggregates and (3) association of OM with soil mineral surfaces (Lützow et al., 2006).

(1) Recalcitrance refers to the intrinsic molecular-level characteristics of OM entering the soil. It comprises the elemental composition of OM, the presence of certain functional groups in molecules, molecular conformation and bonding between molecules (e.g. cross linkages) (Sollins et al., 1996), making some biomolecules more resistant to decomposition than others. Recalcitrance, thus a selective preservation of certain compounds of OM, has been earlier assumed as a decisive mechanism for SOM stabilization. Especially lignin has been regarded as a main precursor of stabilized SOM because of its aromatic structure and inter-unit linkages (Hudson, 1986; Hammel, 1997). However, the relevance of recalcitrance for SOM stability has been questioned due to the implementation of new techniques in soil science, such as ^{13}C cross polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy (Wilson, 1987; Preston, 1996; Kögel-Knabner, 1997) and molecular extraction methods (e.g., Klotzbücher et al., 2011; Carrington et al., 2012). It seems that any OM of natural origin entering the soil can be decomposed in the long-term (Lützow et al, 2006) and recalcitrance plays a role only in early stages of decomposition (Marschner et al., 2008). SOM stabilization is thus rather a consequence of

ecosystem dependent factors (see above) and the interaction of OM with soil mineral surfaces, or its occlusion in aggregates (Schmidt et al., 2011).

(2) Aggregation protects SOM from decomposition by forming physical barriers between OM and microbes (Six et al., 2002). Aggregates are mostly created through activity of soil biota that produces cementing agents (e.g. secretions, mucus and root exudates) (Lützow et al., 2006), encrusting OM with soil particles, which then becomes the nucleus for relatively stable aggregates (Oades, 1984; Six et al., 2004). Particularly earthworms can have a substantial influence on aggregation: they form stable casts and exert pressure on the walls of their burrows, thereby compacting the soil (Bronick and Lal, 2005). The barrier between decomposers and OM is only spatial, however, often very stable.

(3) The probably most emphasized stabilization mechanism is the interaction of SOM with soil mineral surfaces by chemical or physicochemical binding with silt and clay particles (Edwards and Bremner, 1967; Six et al., 2002). Among others, this process is important for dissolved OM (DOM) stabilization (Kalbitz et al., 2005) and it has been shown that SOM constituents, such as lignin or lipids, may be so strongly bound to the mineral phase that they are non-extractable by currently available extraction methods (e.g., Hernes et al., 2013; Lin and Simpson, 2016). Adsorption of SOM to mineral particles thus decreases biodegradation rates of SOM as it decreases the availability of substrate to decomposers.

1.3 The decomposition process

Litter decomposition is a complex of processes comprising not only decay of organic substrate, but also transformation and synthesis of new chemical compounds. In early stages of decomposition, readily decomposable material, e.g., primarily carbohydrates, is utilized first. The decomposition rate is thus fast in the beginning and decreases with time as the less decomposable compounds, primarily aliphatic and aromatic compounds, prevail (Berg and McClaugherty, 2003). The following chapters focus on these compounds in more detail. The description of litter components is organized according to their assumed intrinsic recalcitrance. For illustration, the components are depicted in a NMR spectrum (Figure 1).

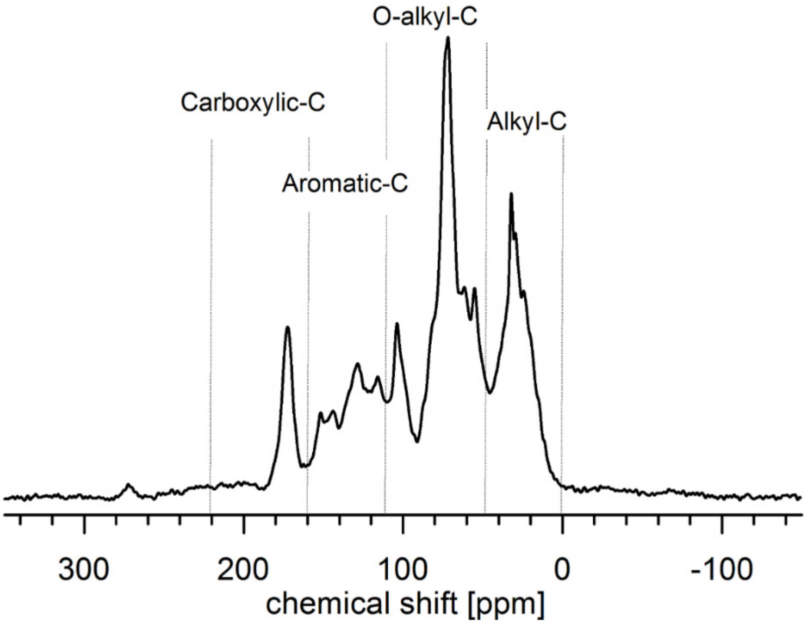

Figure 1. ^{13}C NMR spectrum of fresh alder leaf litter. Integration regions are determined according to Kögel-Knabner et al. (1992). NMR spectroscopy is a useful tool to get an overview of the overall chemical composition of organic material and has often been applied in soil science. This technique is able to provide information about the chemical composition of a whole solid sample without the need of a special preparation of that sample prior to measurement. It is possible to obtain resonance signals of all C in the analysed matrices, which are characterized by a specific chemical shift relative to a standard compound (usually tetramethylsilane).

1.3.1 Carbohydrates

Carbohydrates are the most abundant component of plant litter. This is also illustrated in the NMR spectrum (Figure 1), where the O-alkyl-C integration area, mostly represented by carbohydrates, is the largest one. A major part of carbohydrates consists of the polymers cellulose and hemicellulose (Berg and McClaugherty, 2003).

Carbohydrates are generally regarded as a source of easily available C and energy for microorganisms and therefore readily decomposed (Berg and McClaugherty, 2003). This is especially valid for carbohydrates forming outer structures of OM material. Further, high concentrations of carbohydrates are leached from litter, which is why they represent a major part of DOM. The solubility of carbohydrates even increases during litter decomposition (Balaria and Johnson, 2013). Leached carbohydrates can be subsequently stabilized on mineral particles in deeper soil horizons and become part of stabilized subsoil OM (Guggenberger et al., 1994). Carbohydrates also form complexes with aliphatic or aromatic compounds and become thus protected, e.g. lipopolysaccharides and lignocellulose complexes. After the exhaustion of readily utilizable C, microorganisms (predominantly fungi) also decompose these complexes in order to gain energy (Baldock and Skjemstad, 2000). Therefore, the decomposition of cellulose, as a part of lignocellulose complexes, starts usually later (Berg et al., 1982) and is relatively slow as compared to non-protected cellulose or e.g. arabinans and galactans that are part of hemicellulose (Berg and McClaugherty, 2003). However, a broad spectrum of decomposer organisms (fungi, eubacteria) are able to decompose cellulose and eventually only traces of this biopolymer are present in mineral soils (Kögel-Knabner, 2002).

1.3.2 Aromatic components

Aromatic compounds (having their characteristic peaks in the aryl-C region of an NMR spectrum (Figure 1)) were for a long time regarded as inherently recalcitrant. Especially lignin-derived aromatics (characteristic peaks at 119, 130, 150, and 56 ppm; Kögel-Knabner, 1992), i.e. the second major part of plant biomass, were regarded as main source of stabilized SOM because of their disordered polymeric structure. The recalcitrance of lignin has been partly challenged when its relatively good decomposability through the activity of especially white-rot fungi was demonstrated (Baldock et al., 1997). Nowadays, it seems lignin recalcitrance plays a role only in early stages of decomposition. After the decrease of O-alkyl-C, lignin decomposition increases (Baldock and Skjemstad, 2000) and can be even higher than the overall decomposition. Eventually, only a relatively minor part of lignin is reported to be preserved in soil, but rather as a result of interaction with fine soil fractions than inherent recalcitrance (Marschner et al., 2008).

Lignin is created from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (Vanholme et al., 2010) which are connected through inter-unit linkages (Talbot et al., 2012; Figure 3). Determination of these lignin units brings insights into potential litter decomposability or the stage of decomposition (see below). However, the determination of lignin is complicated because of its heterogenic structure (Kögel, 1986). Several different methods for lignin determination have been developed that, however, may simultaneously extract also other chemical substances. The latter statement is particularly valid for the earlier often used 'Klason' lignin method that additionally extracts acid-insoluble components.

Besides lignin, other aromatic compounds, especially tannins, play a role as potential precursors of stabilized SOM. Tannins are plant-derived polyphenolic compounds whose contents in plants increase with environmental stresses (Lorenz et al., 2000). Polyphenols reduce digestibility of plant tissues and thus defend plants against herbivory (Schultz et al., 1992). A reduced biodegradability of OM connected with a higher content of aromatics is not only valid for the solid state, but also for soluble OM (Marschner et al., 2008).

1.3.3 Aliphatic components

Aliphatic components (i.e. alkyl-C, Figure 1) comprise low-molecular-weight substances, such as short-chain fatty acids, as well as high-molecular-weight substances such as lipids. The carboxyl-C integration region in an NMR spectrum (Figure 1) is mostly associated with the COOH groups of short- and long-chain fatty acids (whose aliphatic part appears in the alkyl-C region).

Lipids are abundant as structural components of plants and microorganisms. They are part of membranes, plant cuticula and cell walls. Further, they serve as energy storage and protective agents (Lorenz et al., 2007). A contribution of soil fauna to the alkyl content in soil is rather of minor role (Kögel-Knabner, 2002). Only a small fraction of lipids is soluble in water and leached as DOM, and the solubility of lipids further decreases with progressing litter decomposition (Balaria and Johnson, 2013). In soil, most of the lipids are thus decomposed in situ and are mainly translocated by bioturbation or in particulate form by percolating water. Prominent representatives of lipids are the biopolyesters cutin and suberin (Kolattukudy, 1980; Figure 2) that are used as biomarkers for above- and below-ground sources of OM. Cutin occurs in aerial (above-ground) parts of plants whilst suberin is associated with below-ground parts, i.e., roots. The resistance of cutin and suberin to decomposition, especially in soil, is still not completely understood (Angst et al., 2016). These lipids are often reported as being preserved in soil (Lorenz et al., 2007; Carrington et al., 2012). On the other hand, Angst et al. (2016) observed that cutin and suberin are readily decomposed in the forest floor at early stages of decay. Kögel-Knabner (2002) also concluded that cutin and suberin should be easily decomposable. However, mineral association likely increases the stability of cutin- and suberin-derived OM in soils (Lin and Simpson, 2016).

Aliphatic biopolymers, such as lipids, have been identified as the major part of stabilized SOM, earlier also referred to as humin (Almendros and Sanz, 1992). Baldock et al. (1997) supposed that the alkyl-C content set in relation to the O-alkyl-C content in SOM can serve well as a sensitive index evaluating the extent of OM decomposition (described in more detail below).

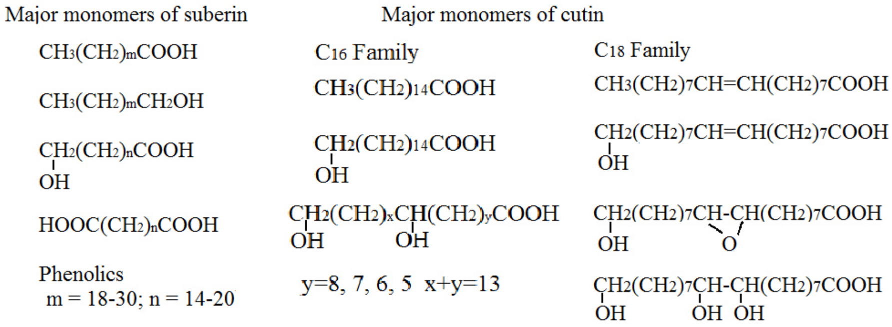

Figure 2. Structure of the monomers of suberin and cutin as suggested by Kolattukudy (1980).

1.3.4 Interactions between litter components

As mentioned earlier, litter components influence themselves mutually and thus even labile compounds of OM can be protected from decay. The mechanisms through which the litter components interact are still not completely understood (Talbot et al., 2012). Lignin is reported to be linked with polysaccharides and nitrogen (N) within hemicellulose-protein matrices in the cell wall of plants (Hammel, 1997). Kuiters (1990) described insoluble polyphenol-protein complexes that are formed after degradation of the cell structure of fallen leaves. These complexes are hardly biodegradable and can protect more than 60 % of N of fallen litter (Kuiters, 1990).

1.4 Evaluation of organic matter decomposition

The evaluation of litter decomposition is difficult because of its complexity. To facilitate the investigation of decomposition, two approaches are possible: micro- and mesocosm experiments in the laboratory or the field. In the field, one of the most common approaches is the use of litterbags. Mesh bags are filled with fresh litter, placed into soil and collected after some time of incubation. Subsequently, the chemical changes in the OM are determined. The loss of undecomposed OM is affected by the mesh–size of litterbags, which determines access of fauna to the bags but also the loss of solid OM fragments. Further, the method takes into consideration only the insoluble residues of OM. Newly shed litter, however, is comprised of substantial amounts of water-soluble compounds that can be leached from litterbags, transported down the soil profile and be stabilized on mineral surfaces as actually undecomposed OM. If not accounted for, part of the OM thus escapes the subsequent analyses. This is especially valid for some deciduous leaf litter where the amount of DOM may reach a substantial portion of the whole litter mass (Berg and McClaugherty, 2003). For example, Uselman at al. (2007) observed that 8.2% of leaf litter C account for dissolved organic C (DOC). To study decomposition of DOM, lysimeters are usually used (e.g. Kaiser et al., 2002).

Further, decomposition of both solid and dissolved parts of SOM can be studied using mesocosm experiments. Although mesocosms simplify environmental conditions, they are a useful tool to study the fate of a certain part of SOM during decomposition, such as stabilization of dissolved or solid SOM in different soil compartments. The incubated bulk soil in mesocosms is usually separated into different SOM fractions at the end of the experiment. These fractions are chosen to distinguish functional SOM pools that are stabilized by different mechanisms and thus have different turnover rates (Lützow et al., 2007). The methods of fractionation include physical separation (e.g. according to aggregate stability, particle size and density), chemical separation (e.g. based on solubility, hydrolysability, resistance to oxidation) and combinations of both (Lützow et al., 2007). The pools are often defined according to their turnover rates as active (easily decomposable), slow (stabilized by physico-chemical mechanisms) and passive pool (biochemically recalcitrant) (Zimmerman et al., 2007). Due to a need for a site-specific adaption of the fractionation protocol, there is still no universal standard procedure for the separation of specific fractions.

Because of the complexity of litter decay, various indicators or indexes have been developed in order to evaluate litter decomposition and decomposability.

- *Litter mass loss*

Litter mass loss is a gravimetrical method commonly measured using litterbags. It observes mass loss from fresh litter over time. This method, however, has several limitations as mentioned above. As Cotrufo et al. (2009) concluded, this method provides only a rough model of litter decomposition and does not provide any information about where the OM is lost to.

- *Respiration activity*

Another often used method is the measurement of respired CO_2 from systems. The measured CO_2 corresponds to the amount of mineralized OM.

- *Initial C:N ratio and initial lignin:N ratio*

Initial lignin concentrations (Meentemeyer, 1977), alternatively the initial ratio between lignin and N concentrations (Mellilo et al., 1982), appeared to be good predictors of litter decay. According to Taylor et al. (1989), the initial C:N ratio substantially better predicts litter mass loss than the lignin:N ratio, and Karberg et al. (2008) concluded that the C:N ratio is the best predictor for litter mass loss. These ratios, however, reflect well only early stages of decomposition. Later, their explanatory power decreases.

- *Lignin chemistry*

Not only the amount of lignin but also the chemical composition of lignin plays a role in litter decomposition (Talbot et al., 2012). Distinguishing between unit types, which lignin consists of (guaiacyl- (G), syringyl- (S) and p-hydroxyphenyl (H) lignins) provides more information about susceptibility of the entire lignin biopolymer to decomposition. Revealing the chemical structure of lignin may explain variations in species specific litter decay rates. According to Talbot et al. (2012), the effects of lignin on total litter decay rates are highest in cases where plant lignin mainly consisted of aldehyde units. Similarly, whilst p-hydroxyphenyl units of lignin were preferentially lost during litter decay, guaiacyl units relatively increased. The distinct lignin monomers determine what kind of bonds the lignin polymer is constituted of (Vanholme et al., 2010). This, consequently, influences the cell wall properties and thus the overall litter decomposability. Therefore, it seems that rather lignin monomeric composition than lignin quantity affects the decomposition process and C loss (Frouz et al., 2015).

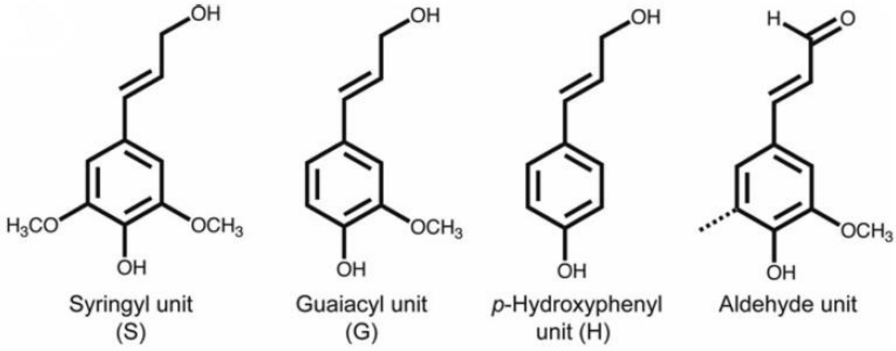

Figure 3. Phenylpropanoid precursors of lignin. Alcohol subunits (monolignols) create syringyl lignins, guaiacyl lignins, p-hydroxyphenyl lignins; aldehyde subunits create aldehyde-rich lignins. Taken from Talbot et al. (2012).

- *Alkyl-C to O-alkyl-C (A/O-A) ratio*

The ratio between the alkyl-C and O-alkyl-C integration regions of an NMR spectrum provides, according to Baldock et al. (1997), a sensitive index of the extent of the decomposition process. The ratio is based on the finding, that usually more stable aliphatic compounds (alkyl-C) are more resistant to decomposition and consequently accumulate during decomposition relative to the more easily degradable carbohydrates found in the O-alkyl-C region.

- *Ratio according to Bonanomi*

More recently, Bonanomi et al. (2011) have established an index based on the ratio of the chemical shift regions of 70–75 to 52–57 ppm of an NMR spectrum, which relates the C2, C3, and C5 of carbohydrates to the methoxyl-C of lignin. They found that this index positively correlated with decomposition rates of litter during all observed phases, in contrast to the other used indices in that study (C:N and lignin:N ratio) (Bonanomi et al., 2013).
Recently, this index has widely been applied (e.g. Gentsch et al., 2015; Angst et al., 2016).

1.5 Factors influencing litter decomposition

Apart from the previously discussed chemical composition of the substrate (see chapter 1.3), litter decomposition is additionally influenced by various other factors that mutually affect each other. These include climatic factors (like temperature, precipitation but also exposition to sunlight), structure and activity of soil microorganisms or soil fauna and soil texture.

1.5.1 Climatic factors

According to Prescott (2010), a direct influence of temperature and precipitation on litter decomposition is only small. However, together with oxygen availability, these factors highly affect the activity of microorganisms, which are important decomposers of litter (Hammel, 1997). While temperature and precipitation have been in the focus of many studies (e.g. Coteaux et al., 1995; Hobbie, 1996), the role of solar radiation has not been long recognized. Yet, Austin and Vivanco (2006) showed that solar radiation has a determining effect on OM decomposition in semi-arid ecosystems. Since the study of Austin and Vivanco (2006), solar radiation, especially the ultraviolet-B (UV-B) part of the spectrum, has been studied as a possibly factor affecting litter decomposition (e.g. Brandt et al., 2007; Henry et al., 2008; Song et al., 2012). Most of the conducted studies so far have focused mainly on the previously mentioned arid and semi-arid ecosystems (e.g. Gallo et al., 2006; Uselman et al., 2011). Only a few studies have also been conducted in temperate ecosystems (Rozema et al., 1997; Frouz et al., 2011). The mentioned studies brought partly contradictive results concerning the impact of solar radiation on litter decomposition. While some of the studies found a positive impact on litter decomposition, some studies reported no impact at all (Gallo et al., 2006), and some even found a retarded litter decomposition after exposure to solar radiation (Uselman et al., 2011). Litter quality is probably the key factor determining if solar radiation will affect litter decomposition. Uselman et al. (2011) suggested that high UV-B radiation may result in a decrease of microbial activity. This can especially affect the decomposition of high quality litter, which is readily decomposed and where microbial activity plays a decisive role. Decomposition of such a type of litter is thus rather retarded by solar radiation. Another situation is set concerning low quality litter. In a study of Brandt et al. (2010), an increased decomposition of low quality litter under UV-B exposure has been observed. A suggested reason is a possible disruption of the chemical structure in litter, that is exposed to radiation (King et al., 2012). This would lead to a facilitation of OM decomposition in later stages. The concept was later referred to as photofacilitation (Yanni et al., 2015).

The underlying mechanisms of photodegradation causing an acceleration of the changes in chemical composition of decaying litter are still not fully understood. Brandt et al. (2007) observed a decrease of holocellulose but no effect on the lignin fraction of litter. Other studies observed an impact of solar radiation on lignin loss or changes in its chemical composition (Henry et al., 2008; Austin and Ballaré, 2010). Together with other phenolic compounds, lignin is considered to be photoreactive. As lignin and cellulose create lignocellulose complexes, a faster loss or a chemical alteration of lignin can also result in an increased decomposition of cellulose. The degradation of lignin may lead to a release of labile carbon compounds or increase their accessibility to decomposers (Austin and Ballaré, 2010). According to King et al. (2012), the role of photodegradation is more pronounced with increasing C:N ratio of OM but not with an increasing lignin content.

Photodegradation may be a possible explanation why some plants keep standing dead biomass. This may provide more favourable conditions for insolation that may lead to photodegradation of recalcitrant structures and later to facilitation of litter decomposition when the litter finally reaches soil.

1.5.2 Fauna

Dominant decomposers of SOM are microorganisms, encompassing both fungi and bacteria (Berg and McClaugherty, 2003). However, also soil fauna plays an important role in litter decomposition. The presence of litter-feeding macrofauna leads to more extensive removal of litter from the litter layer and its transport to underlying soil horizons (Frouz et al., 2007; Scullion and Malik, 2000). One of the most important species for the translocation of litter fragments is earthworm. Earthworms are able to largely modify their soil environment. Their bioturbation activity and OM fragmentation contributes to the formation of stabilized OM and the development of a thicker organo-mineral layer (Frouz et al., 2013). Earthworms affect the decomposition process also indirectly: they create large pores and enable a higher water infiltration and gaseous exchange in soil (Bossuyt et al., 2005). Earthworms selectively feed on high quality litter. But their activity is important for low quality litter decomposition, too. According to Lorenz et al. (2000) who focused on tannin decomposition, earthworms indirectly contribute to the decomposition of these phenolic compounds by affecting the associated microbial community. Also Bi et al. (2016) suggested that earthworms accelerate degradation of phenolic acids. Besides an effect on phenolic components, Scullion and Malik (2000) reported an increase in the carbohydrate content of earthworm-processed OM.

Despite an often reported accelerating effect of earthworms on litter decomposition, they are supposed to increase stabilization of SOM in mineral soil (Zhang et al., 2013; Frouz et al., 2014). However, the mechanisms underlying this process are still not well understood (Vidal et al., 2016). Many authors assume that the effect of earthworms on SOM sequestration is based mainly on physical protection of OM within casts (Lavelle and Martin, 1992; Bossuyt et al., 2005), where OM is better mixed with clay than outside of casts.

1.5.3 Tree species effects on litter decomposition and soil C dynamics

The susceptibility of litter to decomposition differs depending on the nature of the plant species. The rate of litter decomposition is driven by the litter quality of the respective species, especially in early stages of decomposition. Litter type predestines biological (abundance and diversity of microbial community and soil fauna) and physico-chemical processes in soils and consequently drives mineral soil and forest floor dynamics (Hobbie et al., 2006). Litter with a low C:N ratio (‘high’ quality litter) supports the development of a thick organo-mineral topsoil horizon and only a small or an even non-existent organic layer. In the presence of litter with a high C:N ratio (‘low’ quality litter), however, a thin organo-mineral horizon and a thick organic layer are characteristic (Frouz et al., 2013). According to Hobbie et al. (2006), tree species affected microbial mediated litter decomposition mainly via different lignin and calcium contents. However, in a later study, they did not observe any influence of variable lignin contents of tree leaves on SOM dynamics (Hobbie et al., 2007). In more recent studies, Mueller et al. (2012 and 2015) found that tree species effects on soil acidity (e.g., via root exudation or leaf litter chemistry) resulted in substantially different mineral soil C stocks and that lignin contents of leaf litter controlled the amount of C lost from the soil as CO_2. These partly inconclusive results highlight that the effect of tree species on litter decomposition and soil organic C pools is still not well understood (Vesterdal et al., 2013).

2 Aims of the study

The overall aim of this thesis was to evaluate the transformation of OM during litter decomposition with emphasis on aliphatic and aromatic components. Primary recalcitrance of these components largely affects early stages of litter decomposition. As an integral part of the decomposition process, this phase has consequently a substantial impact on the formation of stabilized SOM.

Litter decomposition comprises a number of different processes that mutually influence each other. The objectives of the thesis were thus projected in order to cover this topic from different angles to answer some of the unresolved questions of litter decomposition using several approaches divided into four studies. The focus of each study is illustrated in Figure 4. To get an overview, the first aim was to summarize *general patterns* (Figure 4(i)) of litter decomposition across plant species and climate regions. For this purpose, a revaluation of NMR data from different peer-reviewed journals was made. The second aim was to investigate the impact of *soil macrofauna* (Figure 4(ii)), in our case the earthworm *L. rubellus*, on litter decomposition. Even though earthworms belong to well-studied soil organisms, there are still knowledge gaps especially concerning their impact on SOM chemical composition and stabilization. The third aim was to investigate *abiotic factors* affecting litter decomposition (Figure 4(iii)). For this purpose, the effect of climatic factors on decomposition of dead standing litter and their impact on later decomposition in soil was studied. The study of this phenomenon has received little attention so far in temperate climate regions. The forth aim was focused on the *labile part* (Figure 4(iv)) of SOM that is usually readily utilized by microorganisms or leached into deeper soil horizons. This part of SOM is affected by interaction of both, biotic and abiotic factors. A common garden experiment at Sokolov post-mining sites, with similar site characteristics and only differing in tree species, allowed to evaluate the intrinsic impact of trees vs. abiotic factors (i.e. seasonality) affecting the labile part of SOM.

These specific research aims were investigated in individual studies:

(i) Changes in chemical composition of litter during decomposition: A review of published ^{13}C NMR spectra:

This study was based on the re-valuation of a large dataset of published ^{13}C NMR spectra from peer-reviewed articles. These articles covered decomposition of litter from various plant species under different climatic conditions. As a basis for the subsequent studies, the first study describes patterns in chemical changes of OM during the early stages of litter decomposition (Figure 4 (i)).

Research questions: Does a general pattern of litter decomposition across plant species and climate regions exist? What are the similarities in litter decomposition that related plant species have and what are the similarities in litter decomposition under similar climatic conditions?

(ii) Stabilization of soil organic matter by earthworms is connected with physical protection rather than with chemical changes of organic matter:

In the second study, a mesocosm experiment was conducted. Mesocosms with and without presence of earthworms, one of the most important soil ecosystem engineers affecting stabilization of SOM, were studied. This study was designed to reveal whether earthworms contribute to stabilization of SOM by induction of chemical changes in OM composition, a topic that yielded contradictory findings so far (Figure 4 (ii)).

Research questions: How do earthworms affect C loss and thus C stabilization in soil? Does their feeding activity change the chemical composition of SOM? Does mechanical mixing and mixing mediated by earthworms lead to substantial differences in chemical composition of SOM? Does the effect of earthworms vary according to soil (clay x sand) and plant (alder x willow) system?

(iii) The effect of dead standing biomass on the litter decomposition process:

Some plant species, such as oak or specific grasses, keep their dead biomass standing for a considerable amount of time (a phenomenon called marcescence). The reason for this phenomenon is still unexplored. The third study thus focused on a possible link between marcescence and a facilitation of litter decomposition in soil after it had been exposed to climate conditions (Figure 4 (iii)).

Research questions: Does exposure to sunlight and other climatic conditions change the chemical composition of marcescent litter? May holding of marcescent litter facilitate later litter decomposition in soil?

(iv) The effect of tree species on seasonal fluctuations in water-soluble and hot water-extractable organic matter at post-mining sites:

The forth study focused on the most mobile and labile fraction of SOM, extractable with water. This fraction originates from decomposing litter, root exudates and products of microbial activity. This part of OM is not considered in most decomposition studies as they mainly focused on the insoluble part of OM (Figure 4 (iv)).

Research questions: Are quality and quantity of hot water-extractable C (HWC) and water-soluble C (WSC) more dependent on tree species or on season? Do annual WSC and HWC quantity and composition show similar patterns at sites that only differ in dominant tree species?

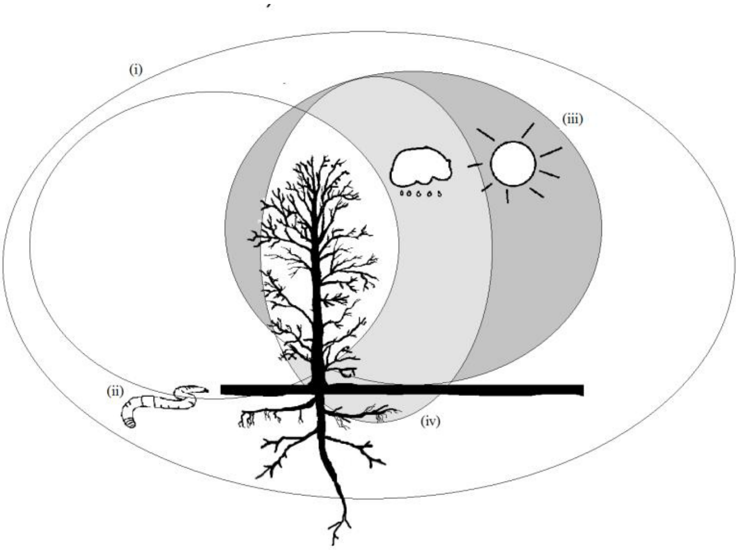

Figure 4. Summary of conducted studies. Individual studies are marked with circles that encompass the different factors and entities investigated in the respective study: (i) ***General patterns*** *of litter decomposition across plant species and climate regions. (ii) Effect of* ***earthworms*** *on chemical composition of SOM. (iii) Effect of* ***climatic conditions*** *on decomposition of marcescent leaf litter. (iv) Quality and composition of water-extractable OM (****WEOM)*** *in relation to dominant tree species and season.*

3 Description of study sites

All soil samplings and field experiments were conducted at post-mining sites near Sokolov, Czech Republic.

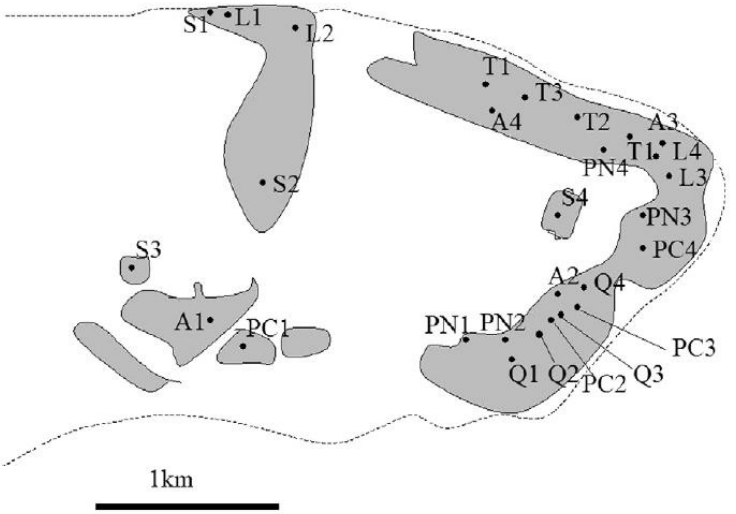

Figure 5. Sokolov post-mining area with marked study sites. The sites are formed by monoculture stands. For the purpose of this thesis, the following sites were used: PC = spruce (Picea omorica), Q= oak (Quercus robur), A = alder (Alnus glutinosa), S = succession with predominance of willow (Salix caprea). (Figure taken from Frouz et al., 2013).

The Sokolov post-mining district (50°14′04″N, 12°41′04″E, 450–520 m a.s.l.) was formed during the last 40 years by the piling of overburden originating from brown-coal mines. The spoil material consists mainly of tertiary clay with predominance of illite and kaolinite (Frouz et al., 2013). The pH of the soil is about 8 (Helingerová et al., 2010). The mean annual precipitation is 650 mm, and the mean annual temperature is 6.8 °C.

The study area is covered by a mosaic of reclaimed forest stands with various dominating tree species (Figure 5). Several parts of the post-mining area were unreclaimed and had experienced spontaneous revegetation with predominance of the grass *Calamagrostis epigeios* and willow shrubs.

4 Results and conclusions

The first part of this thesis (study **(i)**) focused on general patterns of changes in litter composition during proceeding decomposition. A dataset of about 130 ^{13}C NMR spectra provided an overview of early litter decomposition under different climate conditions and various types of plant species. The plant species were divided into groups as follows: conifers, broadleaf trees, herbs and evergreen shrubs (more in Table 1). Four climate regions were determined: tropical, Mediterranean-subtropical, temperate and boreo-alpine.

Concerning variation according to climate zone, boreo-alpine litter contained significantly more aliphatic compounds, whilst temperate litter tended to contain higher amounts of aromatic compounds. Mediterranean-subtropical litter was characterized by higher amounts of carbohydrates.
Independent of climate zone or species, analysis of the whole dataset showed a consistent trend of a relative decrease of carbohydrates as they are utilized during litter decomposition, and a relative increase of aliphatic and aromatic components. These findings imply a probable basic mechanistic role of the latter components for the formation of stabilized SOM. The described patterns led us to focus especially on the transformation of recalcitrant components of OM in the subsequent case studies.

Table 1. Individual characteristics of litter divided into groups according to plant species type.




<table>
<tr><th>Conifers</th><th>Broadleaf</th><th>Herbs</th><th>Evergreen shrubs</th></tr>
<tr><td colspan="4"><i>Initial litter</i></td></tr>
<tr><td></td><td colspan="3">Largest portion of initial leaves is constituted by carbohydrates</td></tr>
<tr><td></td><td></td><td>Contain the lowest portion of aromatic and aliphatic components</td><td>Contain the highest portion of aromatic and aliphatic components</td></tr>
<tr><td colspan="4"><i>Early stage of decomposition</i></td></tr>
<tr><td></td><td>Significant relative increase in aliphatic components</td><td>Significant relative increase in aromatic components</td><td></td></tr>
<tr><td></td><td colspan="2">Significant relative decrease in carbohydrates</td><td></td></tr>
<tr><td colspan="4"><i>Late stage of decomposition</i></td></tr>
<tr><td colspan="2">Significant relative decrease in carbohydrates</td><td></td><td rowspan="2">Significant relative decrease in carbohydrates</td></tr>
<tr><td>Significant relative increase in aromatic components</td><td>Significant relative increase in aliphatic components</td><td></td></tr>
</table>

Study **(ii),** focusing on transformation of aromatic compounds, emerged from previous findings that earthworms may increase the amount of stabilized SOM (Frouz et al., 2014; Zhang et al., 2003). We hypothesized that this increase can be induced through chemical changes in OM composition, especially regarding aromatics. This assumption arose from a study (Frouz et al., 2015) where the passage of OM through the gut of the macrofauna *Bibio marci* changed the composition of OM: ^{13}C NMR spectroscopy revealed a relative increase in aliphatic components and pyrolysis chromatography/mass spectrometry (Py GC/MS) revealed changes in lignin chemistry of OM after passage through the gut. For the purpose of study (ii), laboratory microcosms were established. In order to keep the conditions in the microcosms most similar to a real-life system, we used a combination of soil and litter naturally occurring at the sampling sites (clay + alder and sand + willow) in two types of treatments: treatments where OM was fragmented and mixed by earthworms (*Lumbricus rubellus*) and treatments in which OM was fragmented and mixed mechanically without the presence of earthworms. These treatments were incubated for 126 weeks. Thereafter, soil was fractionated according to Zimmermann et al. (2007) in order to gain soil functional pools as defined in the Rothamsted model.

Even though treatments with *L. rubellus* showed lower C loss from the system (results published in Frouz et al., 2014) at the end of the experiment, we did not observe any significant differences between earthworm and mechanically mixed treatments in C, N and phenol contents, composition of major chemical groups of litter studied by solid-state ^{13}C NMR spectroscopy, and composition of aromatic components studied by Py GC/MS. The observed lack of differences in chemical composition between the studied treatments suggests that lower C loss and thus greater SOM sequestration in the earthworm treatment is likely related with physical protection of SOM inside cast aggregates rather than with chemical changes in SOM mediated by earthworms. However, ^{13}C NMR spectroscopy revealed slightly different trends of changes in SOM composition in the slow pool at the end of the experiment. The spectra implied that litter decomposition proceeded differently in earthworm and mechanically mixed microcosms. This finding suggests that a two years' experiment is not long enough and differences in SOM chemical composition may occur even later.

In study **(iii)**, we focused on the possible relevance of holding dead biomass standing for the future decomposition of that biomass in soil. The phenomenon of holding dead biomass, called marcescence, is characteristic for several plant species in various extents. To our knowledge, a possible linkage between marcescence and a facilitation of later litter decomposition in soil has not been described. We hypothesized that climatic factors (photodegradation, but also leaching of DOM) may facilitate decomposition of marcescent litter later in soil. For the purpose of the study, we chose three types of plant species according to the extent to which they hold their dead biomass: alder *Alnus glutinosa* (where all litter falls to the ground during senescence), oak *Quercus robur* (which keeps some dead leaves on the tree) and the grass *Calamagrostis epigeios* (which keeps most of the dead biomass standing). All litters were exposed to climate conditions for one year in the field and subsequently placed into soil together with 'control' litter of the same species, which was previously kept in a dark, dry place. We were thus able to observe whether the exposure to climate conditions facilitated subsequent decomposition in soil or not.

From the observed species, only *C. epigeios* showed significant differences in decomposition between initial litter and litter that has previously been exposed to climatic conditions. The exposed litter decomposed about 30% faster. The exposure to climate conditions decreased the content of aromatic components in litter of *C. epigeios* and changed its chemical composition. The other two species did not show substantial differences in litter decomposition between initial litter and litter that has previously been exposed. These results suggest that the litter of *C. epigeios* may have specific properties that are prone to an accelerated decomposition, when the litter is previously exposed to climatic conditions. This may also be the reason why some types of species are marcescent, however, for proving this statement, a larger experiment design with more species would be necessary.

As our previous studies focused solely on the solid part of OM, eventually, we focused also on the part of OM that is easily leached from litter or is excreted as root exudates or as metabolites of microbial activity (Kalbitz et al., 2000). Because of its lability, this OM is either fast utilized by microorganisms or may be translocated in the soil profile and stabilized on mineral particles. In study **(iv)**, we investigated seasonal changes in the contents and compositions of water-extractable OM (WEOM) at four different tree species stands (an alder, oak, and a spruce stand, and a stand that was overgrown by a spontaneous succession dominated by willow). These stands differed only in the type of tree species. Water-extractable organic C (WEOC) was separated into two pools according to the efficiency of water extraction: HWC and WSC. HWC is regarded as a pool of mineralizable SOM (Ghani et al., 2003), which is partly derived from soil microbial C (Sparling et al., 1998) and represents a readily available source of nitrogen (N) in soils (Keeney and Bremner, 1966). WSC is a substantially smaller fraction of the soluble C pool than HWC. WSC represents that portion of C that can be very rapidly utilized and is considered to be an indicator of the availability of respiratory C substrate (Uchida et al., 2012).

Trends in WEOC concentrations were remarkably similar among tree species. The more labile pool of WEOC (WSC) peaked in early summer and another peak was detected in late autumn in the mineral horizons. These peaks were more pronounced at deciduous tree sites than at sites with spruce. Concerning the composition of WEOM, liquid state ^{1}H NMR spectroscopy showed a higher relative content of aliphatic components in summer and a higher relative content of carbohydrates in winter. These results suggest that the peaks had different sources of C (probably microbial activity and root exudates in summer and leaching of fresh OM from litter in winter). The composition of WEOM was influenced by both tree species and seasonality. However, species was the most important factor. The study implies that tree species is the dominant factor determining quality of WEOM, meanwhile seasonality largely determines concentrations of WEOM throughout the year.

5 Future prospects

This work focused on litter decomposition from different perspectives. As the content and transformation of aliphatic and aromatic components has a major impact especially on early stages of decomposition, this is an important topic in soil science. Our studies demonstrated how complex factors affecting the transformation of these components are. Future research should be directed especially to the fate of these components in soil in later stages of decomposition. Further, a key research question will be the evaluation of the importance of aliphatic and aromatic compounds in stabilized SOM pools because their relevance to these pools it is still under debate.

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7 Research articles

PAPER I

Changes in chemical composition of litter during decomposition: a review of published ^{13}C NMR spectra

Cepáková, Š., Frouz, J., 2015

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Abstract

The aim of the study was to evaluate changes in chemical composition of litter during early stages of decomposition based on a comparative analysis of published ^{13}C NMR spectra. We collected over 130 ^{13}C NMR spectra from peer-reviewed articles that contained spectra for undecomposed litter and for litter in at least one stage of decomposition. We measured the areas of peaks representing individual chemical components and interpreted the proportions of those peak areas as proportions of respective chemical components. Results indicate that fresh herb litter tends to contain more carbohydrates and less phenolic compounds and to have lower remaining mass, indicating faster decomposition. Carbohydrates consistently decrease during decomposition, while the proportions of aromatic and aliphatic compounds increase. Changes in the proportions of individual chemical components are negatively correlated with their initial content. The proportion of aromatic components correlates positively with the C/N ratio and negatively with the decomposition constant.

1. Introduction

Soil is a major part of the global carbon cycle (Schlesinger, 1990). Litter is considered to be a key organic source of carbon input into the soil, so litter decomposition plays a crucial role in the Earth's carbon budget. Litter decomposition influences soil biogeochemistry of plant stands and affects the formation of soil organic matter (SOM). Due to its decomposability litter affects organic carbon accumulation in the soil and, consequently, stabilization of SOM (Lv and Liang, 2012). Differences across ecosystems are the result of different climates, varying litter input into the soil (Aranda and Comino, 2014), and changing availability of litter carbon for decomposition and potential carbon stabilization in soil (Six *et al.*, 2002). SOM, in turn, determines important soil properties, including its fertility. The mechanisms of carbon stabilization in soils are still not well understood, although they have received much attention recently (Lützow *et al.*, 2006). The stabilization of SOM entails physical protection and chemical changes. Physical protection of soil organic matter comprises binding of SOM into soil aggregates and coating by clay minerals (Oades, 1984). Chemical changes include the formation of humic substances, which are either aromatic (humic and fulvic acids) or aliphatic (humin) in character (Aiken, 1985; Song *et al.*, 2011; Abakumov *et al.*, 2013). There is still some uncertainty, however, which compounds in litter have greater relative stability in the soil and which compounds contribute most to the formation and stabilization of SOM (Lorenz *et al.*, 2007).

Litter quality is considered to be one of the most important drivers of litter decomposition (Kara *et al.*, 2014). Especially critical for organic matter dynamics are the C/N ratio and the content of nitrogen and lignin (Swift *et al.*, 1979; Bonanomi *et al.*, 2013).

Litter quality is widely studied through relationships between litter stoichiometry and the rate of decomposition (e.g. Hobbie and Vitousek, 2000). By contrast, much less attention has been paid to chemical compositional changes of organic carbon in litter during decomposition processes in the soil. The technique of solid state ^{13}C NMR spectroscopy allows the non-destructive collection of data about individual components of litter material. This powerful method is useful for observing the chemical behaviour of litter material and evaluating the quality of organic matter. Small sample amounts provide structural information about the material studied without the need to extract it. We focused our research on aliphatic, aromatic and carbohydrate components. The objective of this study is to evaluate the patterns of changes in chemical composition of litter during the process of decomposition. We especially focused on aliphatic and aromatic compounds, which have been considered as the fundamental components of stable organic matter.

2. Methods

Using the keywords "litter decomposition", "litter decay" and "^{13}C NMR spectroscopy", we searched the Web of Science (http://apps.webofknowledge.com) for papers which published original ^{13}C NMR spectra. There are more related, especially early, NMR literature which was not possible to include in this study (e.g. Hempfling *et al.*, 1987; Preston *et al.*, 2000; Baldock *et al.*, 1992). The articles either do not contain useable NMR spectra or approach litter decomposition in different way. For that reason, it would not be possible subsequent evaluation of those data. Overall, it was extracted over 130 NMR spectra covering almost fifty various cases of decaying litter from twelve different articles (Table 1). These span various types of litter decomposing under various climatic conditions. For each study, at least two spectra, representing initial plant material and less decomposed (<1y) litter, were available. For many of the studies, spectra representing more decomposed litter (>1y) were also available. These spectra were used to quantify the basic carbon fractions according to Wilson (1987) based on the area of corresponding peaks relative to the total area of all peaks in the spectra using WebPlotDigitizer software (http://arohatgi.info/WebPlotDigitizer). The work is focused on chemical shift regions representative of three types of carbon components: aliphatic (10–45 ppm), carbohydrate (60–90 O-alkyl and 90–110 acetal and ketal groups) and aromatic (110–160 ppm). The content of individual chemical components of litter was quantified for each plant species. For the purpose of this study, the plant species were divided into several groups: conifers, broadleaf trees, herbs and evergreen shrubs.

In addition, the C/N ratio and information about climate conditions given in the articles were used for purpose of this study. The decomposition constant (*k* value) was calculated from remaining mass data available in the articles. Missing climate data were replaced (mean annual temperature, mean annual precipitation) by values obtained from www.weatherbase.com using geographic coordinates or described locations. Climatic conditions were categorized as follows: tropical, Mediterranean-subtropical, temperate and boreo-alpine.

Table 1. Peer-reviewed journals used as sources of NMR spectra for undecomposed litter and litter in at least one stage of decomposition.

Reference	**Collected NMR spectra (n)**	**Reference**	**Collected NMR spectra (n)**
Almendros et al. 2000	24	Mathers et al. 2007	16
Carvalho et al. 2009	15	Ono et al. 2009	8
De Marco et al. 2012	10	Ono et al. 2011	8
Frouz unpublished data	3	Ono et al. 2013	14
Lorenz et al. 2000	6	Quideau et al. 2005	9
Lorenz et al. 2004	8	Webster et al. 2000	10

Because there are no replications for the individual spectra, a classical meta-analysis was not performed, but individual NMR spectra were treated as single data points. Each spectrum was therefore considered as an individual statistical entry. The shifts of individual carbon fractions were compared between initial litter and decomposed litter using a pair t test. The difference was expressed as the percentage in decomposed litter minus the percentage in original litter. This was calculated for all available entries as well as for entries divided according to the duration of decomposition (less than one year and more than one year) and according to the type of plant (conifer, broadleaf tree, herb, evergreen shrub). Using a one-way ANOVA, differences in the proportions of the same chemical categories among various types of litter were tested. Linear correlation coefficients were calculated between initial percentages of the given chemical components and the shifts in corresponding group percentages during decomposition. Similarly, correlation coefficients were calculated between initial percentages of the given chemical categories and the C/N ratio of the litter, and between the same percentages and the decomposition constant *k*. All the mentioned calculations were carried out by Statistica 10.0.

Principal component analysis (PCA) was performed by Canoco 4.0 to visualize the variation in the proportions of individual chemical components (based on NMR spectra) among litter types considered in individual studies in relation to the type of plant, climatic zone, litter C/N ratio and decomposition constant.

3. Results

3.1. Initial litter quality

The ^{13}C NMR litter spectra show that carbohydrates constitute the largest proportion in initial litter (Table 2). This proportion, expressed as percentages of the total peak area of carbon in O-alkyl and acetal and ketal groups, ranges from 33.18 and 10.70 to 44.92 and 11.06 %, respectively, depending on the litter type group. Fresh litter also contains a large proportion of aliphatic components (16.43 to 25.96 %). Aromatic components are less variable in fresh litter (11.37 to 17.52 %). The relative content of carbon types expressed as the percentage of the total spectral area is shown in Table 2.

Despite certain tendencies apparent in some litter types, there is no significant difference in the chemical composition of fresh litter among the litter types under consideration except for aromatic compounds from herbs. Litter of evergreen shrubs and conifers tends to be higher in recalcitrant (aliphatic and aromatic) compounds. Herb litter is characterized by higher content of easily degradable compounds (carbohydrates).

With regard to the climate, boreo-alpine litter contain significantly more aliphatic compounds. Litter in temperate areas tends to contain higher amounts of aromatic compounds, and Mediterranean-subtropical litter is characterized by higher amounts of carbohydrates. The C/N ratio positively correlates with the content of aromatic components (see Table 3). Temperate litter has a significantly higher C/N ratio than tropical litter (Figure 1). Evergreen and boreo-alpine litter has higher C/N ratios, too. These tendencies are also apparent in the PCA ordination

Table 2. Percentages of individual chemical components (means± SEM) in individual types of litter. Statistically homogeneous groups of litter types in the same stages of decomposition are marked by the same letters (ANOVA, LSD post hoc test $p<0.05$). Asterisks mark significant differences between initial conditions and decomposed litter based on a pair t test (initial vs decomposed litter, $p<0.05$). If decomposed litter was collected repeatedly, several pairs were tested, so the n in each t-test corresponds to the n for the given decomposed litter.

	Chemical shift region [ppm]						
	10–45	45–60	60–90	90–110	110–160	160–200	n
Corresponding chemical component	Aliphatic		Carbohydrates		Aromatic		
Initial							
Conifer	21.36 ±1.35	7.74 ±0.43	38.43 ±1.69	10.9 ±0.50	16.16 ±0.56a	5.42 ±0.53a	12
Broadleaf tree	19.38 ±0.93	8.34 ±0.33	39.02 ±1.18	11.26 ±0.38	15.55 ±0.40a	6.45 ±0.41a	22
Herb	16.43 ±1.28	7.45 ±0.53	44.92 ±1.78	11.06 ±0.60	11.37 ±0.66b	8.75 ±0.66b	9
Evergreen shrub	25.96 ±1.85	5.19 ±0.84	33.18 ±2.53	10.7 ±0.89	17.52 ±0.70a	7.45 ±1.05a	4
Less than one year							
Conifer	21.85 ±1.36	8.85 ±0.62ab	33.51 ±1.89	10.4 ±0.57	18.82 ±0.92a	6.57 ±0.43	6
Broadleaf tree	23.26 ±1.20*	9.35 ±0.30b	32.21 ±1.39*	9.79 ±0.44*	17.47 ±0.62ab	7.93 ±0.53*	15
Herb	17.31 ±1.07	7.2 ±0.73a	40.41 ±6.41*	10.64 ±1.00	15.11 ±5.13b*	9.33 ±2.54	22
Evergreen shrub	27.03 ±3.39	7.99 ±0.92ab	30.89 ±1.91	11.54 ±0.98	17.32 ±2.27a	5.23 ±0.60	3
More than one year							
Conifer	21.93 ±0.92	8.99 ±0.39	33.17 ±1.23*	10.38 ±0.39	19.02 ±0.59*	6.51 ±0.29*	14
Broadleaf tree	23.81 ±1.58*	9.03 ±0.33*	34.52 ±1.29*	9.54 ±0.40*	14.79 ±0.56	8.31 ±0.44*	14
Herb	14.92 ±1.67	6.76 ±0.47	35.25 ±2.46	10.91 ±0.43	20.53 ±1.04	11.64 ±0.93	4
Evergreen shrub	25.05 ±0.52	7.49 ±0.02*	25.89 ±0.25*	9.98 ±0.24	19.72 ±0.28	11.87 ±0.81	2

diagram. Its first ordination axis is related to the gradient between carbohydrates and aliphatic components whereas the second component is related to the presence of aromatic compounds. Herbs ($p<0.05$) correlate with the first ordination axis (0.285). It is characterized by correlation with the content of carbohydrates. The second ordination axis correlates with differences between temperate and tropical plants, which significantly positively or negatively correlate with the second axis (0.348 and -0.361). The second axis also positively correlates with the C/N ratio.

The initial C/N ratio is negatively correlated with the decomposition constant *k* (-0.3547). Initial content of aromatic components is negatively correlated with *k*. The decomposition constant is positively correlated with initial concentrations of carbohydrates, but there is no correlation between *k* and initial aromaticity/aliphaticity ratios.

Table 3. Correlation coefficients between the initial percentages of given functional groups and shifts in the percentages of those groups during decomposition. Separate computations were made for periods shorter and longer than one year and for both of these periods together. The two bottom rows contain correlation coefficients between the initial percentage of the given functional group and the C/N ratio and k, respectively. Only significant r values are presented.

	Chemical shift regions [ppm]					
	10−45	45−60	60−90	90−110	110−160	160−200
Corresponding chemical component	Aliphatic		Carbohydrate		Aromatic	
Early <1y		-0.79242				
Late >1y	-0.48828	-0.67056	-0.44315	-0.62977	-0.7259	-0.35184
Both	-0.17173	-0.7305		-0.45493	-0.46738	
Correlation with C/N					0.419237	
Correlation with *k*			0.36481		-0.41919	

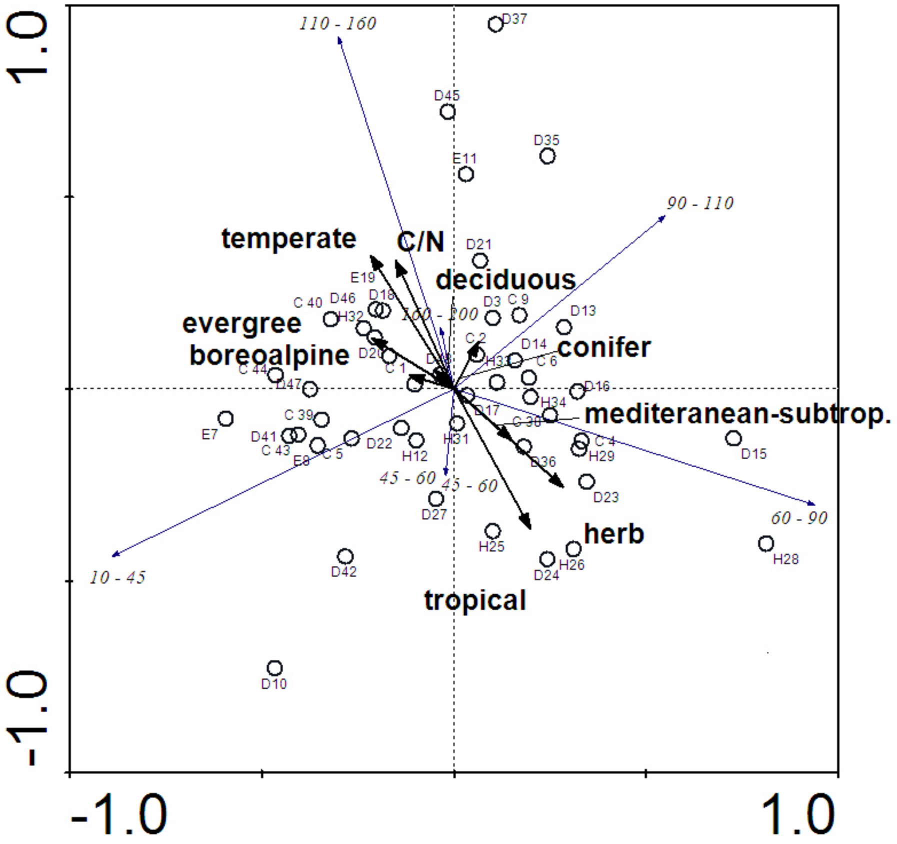

Figure 1. Principal component analysis (PCA) visualizing variation in the proportion of individual components among individual NMR spectra in relation to plant type, climatic zone and litter C/N ratio. Positions of individual components are marked by arrows. Bold arrows indicate the influence of plant type, climatic zone and C/N ratio.

3.2. Changes of litter during litter decomposition

Results obtained using all spectra indicate that aromatic and aliphatic compounds increase significantly during decomposition whereas the proportion of carbohydrates significantly drops (Figure 2).

The NMR spectra of decomposed material were divided into categories representing two stages of decomposition: (1) lasting less than one year and (2) lasting more than one year (Table 2). Aliphatic components significantly increase only in deciduous litter during litter decomposition. A significant decrease in carbohydrates during the early stage of decomposition was found only in litter of deciduous trees and herbs. However, carbohydrates significantly decrease in all litter types during the late stage of decomposition except for herb litter. Aromatic components significantly increase in herb litter during the early stage of decomposition and in conifer litter during the late stage of decomposition (Table 2).

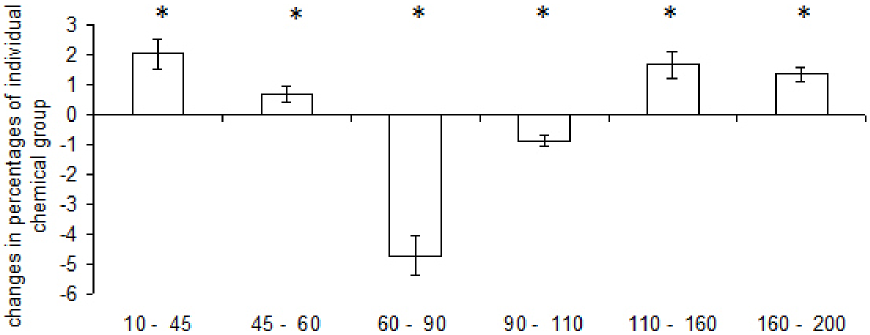

Figure 2. Changes in percentages of individual chemical components between the initial and the final stage of litter decomposition. Values are means ±SEM. Changes marked by asterisks are statistically significant.

The patterns of changes in chemical composition during decomposition vary the most in the early stage of litter decomposition. Decomposition patterns in the late stage are highly similar among litter types.

We found changes in individual chemical components to be negatively correlated with the original content of the same components. Substances that are more abundant in initial litter tend to decrease more during decomposition (Table 3). The C/N ratio decreases during litter decomposition. The decrease depends more on the value of the C/N ratio at the start of the decomposition (0.605, $p<0.01$) than on the decomposition period (0.262, non-significant).

4. Discussion

The collected data demonstrate a significant increase in the proportion of both aliphatic and aromatic compounds during litter decomposition, although the proportions do not necessarily increase in all cases.

Aromatic compounds have been widely assumed to be highly resistant to decomposition and have therefore been thought to control litter decomposition (De Marco *et al.*, 2012), which is in agreement with our observation. A signal indicating aromatic substance comes mainly from lignin and tannins. Aromatic compounds have been regarded as the main contributors to the formation of humic substances. This is supported also by classical theory that aromatic components form substantial portion of humic substances: fulvic and humic acids. On the other hand, the accumulation of aromatic compounds is not necessarily the dominant process taking place during humification (Lorenz *et al.*, 2000; Ono *et al.*, 2000), and aromatic compounds may not necessarily be the building blocks of humic substances (Almendros *et al.*, 2000). Data from a recent study (Abakumov *et al.*, 2013) show that decomposition of litter with a higher content of lignin and a higher lignin/N ratio leads to a higher proportion of low-molecular fulvic acids and that litter with a lower lignin/N ratio leads to humic acids with higher aromaticity.

Our results also show increase in aliphatic alkyl structures that accumulate during litter decomposition which is in agreement with other studies (Baldock *et. al.*, 1992; Almendros *et al.*, 2000). Most studies have revealed a relative increase in alkyl intensity over the duration of the decomposition process (Lorenz *et al.*, 2000; Lorenz *et al.*, 2004; Quideau *et al.*, 2005; Carvalho *et al.*, 2009; De Marco *et al.*, 2012; Bonanomi *et al.*, 2013). The alkyl intensity peak is assumed to come mainly from recalcitrant surface waxes, cutin, suberin, lipids and amino acids. The increase of aliphatic carbon compounds in litter of different types could also be caused by microbial synthesis (Hopkins *et al.*, 1997). Aliphatic carbon compounds could increase due to cross-linking of long-chain alkyl compounds during humification (Kögel-Knabner *et al.*, 1992). Recently, several studies (e.g. Almendros *et al.*, 2000; Mathers *et al.*, 2007) have emphasized the role of aliphatic compounds as a resistant fraction of litter, which is in agreement with our results. Our results also corroborate the recent observation that humin, the most recalcitrant organic soil fraction, is composed mainly of aliphatic hydrocarbons (Simpson *et al.*, 2002; Song *et al.*, 2011), and hence relative accumulation of the alkyl fraction during decomposition may play an important role in the stabilization of soil organic matter. Our observation (Figure 2) that aliphatic components of litter increase during decomposition supports this idea. We therefore assume that the proportion of aliphatic compounds in litter could play a part in stabilizing soil organic matter including aromatic components of litter.

The collected data clearly demonstrate a progressive decrease in carbohydrate-derived carbon. This trend is obvious in all of the studies. According to Almendros *et al.* (2000), carbohydrates do not systematically decrease in all species. However, our results show a decrease of carbohydrates in all groups considered. Carbohydrates are easily degradable compounds. Their decay, however, can sometimes be slowed down by the ligninocellulose complex. This happens because saccharides bind to lignin and thus evade decomposition.

The negative correlation between the initial C/N ratio and decomposition constant *k* is in agreement with most studies (Bonanomi *et al.*, 2013); a low initial C/N ratio of litter indicates subsequent higher loss of plant biomass during the decomposition, and conversely, a higher initial C/N ratio indicates slower decay of litter.

Litter decomposition constants differ from one species to another. Litter properties affect decomposition constants differently among litter types (litter from conifers, broadleaf trees, herbs and evergreen shrubs) and among climatic categories of litter (tropical, Mediterranean-subtropical, temperate and boreo-alpine). In general, mass loss diminishes gradually along a gradient from herb litter, through broadleaf tree and coniferous litter, to evergreen shrub litter (Preston *et al.*, 2000). Our data also support this pattern and show that it is mostly caused by chemical composition. Even climatic groups are characterized largely by their chemical composition. For example, Mediterranean shrubs are often characterized by a thick waxy cuticle, which is important in decomposition (Quideau *et al.*, 2005). In our study, we found a strong gradient in litter quality between temperate and tropical plants. This finding has to be taken with caution because the plants whose litter was considered in the available studies may not be representative of the vegetation in the given regions. On the other hand, our results, which show that temperate plants are more associated with higher C/N ratios and higher lignin content, are in agreement with the findings of Aerts (1997) that temperate plants have significantly higher C/N ratios than tropical ones.

5. Conclusion

As already mentioned in the introduction, both stoichiometry and chemical composition of organic matter play important roles in litter decomposition. Our results support this; there is a significant correlation between the decomposition constant *k* and initial content of carbohydrates and aromatic compounds. Our results, however, also show that there is a strong correlation between the C/N ratio and initial content of aromatic components, which means that stoichiometry and organic matter composition are at least partly linked.

There is an obvious increase in the content of aromatic and aliphatic compounds during litter decomposition. We therefore expect these components to play relevant roles in humification processes and, consequently, SOM stabilization.

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PAPER II

Stabilization of soil organic matter by earthworms is connected with physical protection rather than with chemical changes of organic matter

Angst, Š., Mueller, C.W., Cajthaml, T., Angst, G., Lhotáková, Z., Bartuška, M., Špaldoňová, A., Frouz, J., 2017
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Abstract

Earthworms are important drivers for the formation of soil structure and play a key role in soil organic matter (SOM) dynamics. Our previous long-term (126 weeks) laboratory experiment showed that carbon (C) loss declined through time in soil when litter was mixed and consumed by earthworms (*Lumbricus rubellus*). Eventually, the C loss was lower than in treatments where litter was mechanically mixed into soil with exclusion of earthworms. However, it is not clear if the solely physical manipulation of soil or biological activity of earthworms lead to different SOM quality, which would result in a distinction in C loss and consequently C sequestration. Thus, we differentiated between physical (mechanical mixing) and earthworm effects on SOM composition. Two types of soil were used in the experiment: clay and sand, and these were incubated with alder (*Alnus glutinosa*) and willow (*Salix caprea*) litter, respectively. The combination of soils and litter types corresponds to the natural combinations at the sampling sites.

To explain underlying mechanisms of a lower C loss in the earthworm vs. mechanically mixed treatment, we separated SOM fractions in order to gain pools defined in the Rothamsted model. Chemical differences between initial litter and the active and slow pool of SOM obtained by fractionation were studied. No significant differences between the earthworm and mechanically mixed treatment were found in C, nitrogen (N), and phenol contents, composition of major chemical groups of litter studied by solid-state ^{13}C NMR spectroscopy, and composition of aromatic components of SOM studied by analytical pyrolysis (Py GC/MS). This lack of differences in chemical composition suggests that greater SOM sequestration in the earthworm treatment is likely to be connected with physical protection of SOM inside cast aggregates rather than with chemical changes in SOM mediated by earthworms.

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Corrigendum

Stabilization of soil organic matter by earthworms is connected with physical protection rather than with chemical changes of organic matter

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ABSTRACT

Earthworms are important drivers for the formation of soil structure and play a key role in soil organic matter (SOM) dynamics. Our previous long-term (126 weeks) laboratory experiment showed that carbon (C) loss declined through time in soil when litter was mixed and consumed by earthworms (*Lumbricus rubellus*). Eventually, the C loss was lower than in treatments where litter was mechanically mixed into soil with exclusion of earthworms. However, it is not clear if the solely physical manipulation of soil or biological activity of earthworms lead to different SOM quality, which would result in a distinction in C loss and consequently C sequestration. Thus, we differentiated between physical (mechanical mixing) and earthworm effects on SOM composition. Two types of soil were used in the experiment: clay and sand, and these were incubated with alder (*Alnus glutinosa*) and willow (*Salix caprea*) litter, respectively. The combination of soils and litter types corresponds to the natural combinations at the sampling sites.

To explain underlying mechanisms of a lower C loss in the earthworm vs. mechanically mixed treatment, we separated SOM fractions in order to gain pools defined in the Rothamsted model. Chemical differences between initial litter and the active and slow pool of SOM obtained by fractionation were studied. No significant differences between the earthworm and mechanically mixed treatment were found in C, nitrogen (N), and phenol contents, composition of major chemical groups of litter studied by solid-state ^{13}C NMR spectroscopy, and composition of aromatic components of SOM studied by analytical pyrolysis (Py GC/MS). This lack of differences in chemical composition suggests that greater SOM sequestration in the earthworm treatment is likely to be connected with physical protection of SOM inside cast aggregates rather than with chemical changes in SOM mediated by earthworms.

1. Introduction

Litter decomposition represents a crucial process leading to soil organic matter (SOM) formation and plays therefore an essential role in soil carbon (C) sequestration. Litter decomposition is driven by a complex interaction of various factors. Besides initial litter quality, climate and soil physical and chemical properties, the presence of soil fauna and various management practices play an important role in decomposition processes.

Earthworms as soil ecosystem engineers profoundly affect soil environment and thus litter decomposition (García-Palacios et al., 2014). They influence the storage of soil C and nitrogen (N) (Frouz et al., 2014; Ketterings et al., 1997; Lavelle and Martin, 1992; Zhang et al., 2003) through their high leaf litter consumption rates connected with the burrowing of plant material. Earthworms also positively influence soil physical properties such as aeration or water retention and infiltration (Bossuyt et al., 2005; Kladivko et al., 1997). An increase in earthworm density may therefore support an improvement of soil quality and consequently lead to a higher amount of sequestered C in soil.

The passage of soil and organic matter (OM) through the earthworm gut creates casts formed from differently sized aggregates. During aging, the casts become stable and SOM is highly protected inside these structures (Lavelle and Martin, 1992). Although earthworms belong to the most studied group of soil fauna (e.g. Edwards and Bohlen, 1995), the mechanism of SOM protection inside their casts is still not completely understood (Vidal et al., 2016).

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Many studies link the stabilization of SOM to physical protection within cast aggregates (e.g. Bossuyt et al., 2005; Lavelle and Martin, 1992; Six et al., 2004; Zhang et al., 2003). The pore diameters of cast aggregates are smaller (Görres et al., 2001) than in bulk soil and thus prevent microbial access to SOM within them (Bossuyt et al., 2005). Also, chemical changes of SOM composition inside casts were described (Guggenberger et al., 1996; Parle, 1963; Zhang et al., 2003). Earthworm casts are reported to be enriched in polysaccharides and lignin. Carbohydrates serve as cementing agents (Scullion and Malik, 2000), supporting enhanced stabilization of OM inside casts. Compared to initial litter, the relative content of carbohydrates may decrease and the relative contents of aliphatic and resistant lignin components may increase during the passage of OM through the guts of macrofauna as it was observed in fresh macrofauna casts (Frouz et al., 2015). Thus, it seems that the physico-chemical stabilization processes of SOM also play a role within casts.

In the present study, we investigate if earthworms influence the soil system mainly through mechanical activity or the earthworm impact comprises also chemical changes in OM that would alter C stabilization in soil. Our study benefited from a long-term (126 weeks) laboratory experiment (Frouz et al., 2014) comparing soil systems with and without presence of earthworms (*Lumbricus rubellus*). The same amount of litter was either consumed and mixed with soil by earthworms or was simply mixed and fragmented mechanically. The study by Frouz et al. (2014) showed that earthworms increased C mineralization shortly after they had been introduced into the system as compared to no-earthworm treatments. However, over a longer period of time, C mineralization decreased and consequently, C loss in soil was lower in earthworm treatments. In the present study, we especially focused on differences in chemical composition of SOM in the described treatments possibly being responsible for a lower C loss and a greater C sequestration in microcosms where OM was mixed into the soil by earthworm activity.

Two soil and leaf litter combinations were used in the experiment: clay with alder (*Alnus glutinosa*) and sand with willow (*Salix caprea*). These two combinations were used as they represent the most common combination of plant species and soil substrate in post-mining sites near Sokolov, where sampling was done. The post-mining sites provided the advantage of no earthworm occurrence and the soil substrates contained negligibly small contents of recent organic C. Thus, the impact of the earthworms could directly be detected. Incubation of two types of soil allowed us to observe whether soil particle size played a role in C loss and sequestration. Soil was fractionated according to Zimmermann et al. (2007) in order to obtain soil functional pools corresponding to those defined in the Rothamsted model. An active and a slow pool of SOM were used for chemical analysis. Investigating the mechanisms of how the C is sequestered in these treatments may substantially help to improve our understanding of C sequestration in post-mining sites and the role of earthworms in C sequestration in general.

2. Materials and methods

2.1. Collection of materials

The material used for the laboratory experiment was collected at post-mining sites near Sokolov, Czech Republic. The soil originated from two approximately 10 years old sites (i.e., the soil had been deposited by mining operations 10 years earlier as dumped material on the heaps) with little vegetation cover. One site consisted of sand and the other site contained tertiary clay with a predominance of kaolinite and illite (Frouz et al., 2013). Although small patches of toxic material exist in the mining area, the Cypris clays, which have been used in this experiment and which form the majority of overburden, do not have any toxicity issues; the same applies for the sand used (Frouz et al., 2005). Soil was collected from the top 10 cm following removal of the vegetation cover (i.e. of plants and litter). Sand material was passed through a 2-mm screen, and clay material was passed through a 5-mm screen. The C content was 4 g C kg^{-1} in sand and 24 g C kg^{-1} in clay material. Further soil substrate characterizations are given in Table 1.

Leaf litter used for the laboratory experiment corresponded to the dominant trees at Sokolov post-mining sites: alder (*Alnus glutinosa*) and willow (*Salix caprea*). Leaf litter was collected using litter traps (0.5 × 0.5 m frame consisting of a nylon mesh located 0.5 m above the ground) at the time of litter fall (October 2009). The collected litter was subsequently sorted to remove woody debris and small branches. Leaves were cut into pieces ca. 1 cm × 3 cm and air dried. The C content was 444 g C kg^{-1} in alder and 421 g C kg^{-1} in willow litter. Alder is a typical tree used for the controlled restoration of clay soils at Sokolov post-mining sites (Frouz et al., 2001), and willow is a characteristic colonizer of sandy soils at post-mining sites (Mudrák and Frouz, 2012). Based on these aspects, alder litter was incubated with the clayey soil and the willow litter with the sandy soil. The earthworms (*Lumbricus rubellus*) were collected at the same locations as the litter. The differentiation of *L. rubellus* from other earthworm species present at the sites was made based on general morphology on living specimen.

2.2. Experimental design and analysis

The experiment was designed as four different treatments. Two combinations of soil and litter (clay + alder; sand + willow) were treated either by mechanical mixing of litter into the soil, or by earthworm activity where the litter was left on the surface of incubated soil. Every combination had six replicates. In the experimental design, we also considered a treatment with neither earthworm activity nor mechanical mixing. However, because the litter biomass in this ‘control’ treatment was decomposed only in a relatively small area on the soil surface and likely some leaching of DOM, this treatment was inappropriate for the observation of differences in SOM.

Laboratory microcosms were 250-ml glass bottles filled with soil (sand or clay) and leaf litter (willow or alder). The mineral layer consisted of 100 g dry weight equivalent soil, which was moistened to field capacity determined on bulk soil (Kuráž et al., 2012). The leaf litters for the mechanical mixing with the two soils were homogenized using a 1-mm screen. The size of OM fragments was chosen according to previous findings (Frouz et al., 2011) where the size of OM fragments inside aggregates of earthworm origin were similar to that used in the present study. The leaf litters introduced into the earthworm treatments were pieces ca. 1 cm × 3 cm in size. Before adding the plant material to the microcosms, the leaf litter was moistened in plastic bags to 70% water content (g of water/100 g dry litter). This procedure minimized the leaching of soluble OM before addition to the microcosms.

Leaf litter was either added to the soil surface or mixed into the soil as homogenized pieces. In the earthworm treatments with the litter on top of the soil surface, two earthworms were added to each microcosm. In the mechanically mixed treatments, OM fragments were divided into four equal parts. The first part was spread uniformly on the surface and mixed into the soil using a spate for 5 min. Then, the next portion was added and the whole procedure was repeated until all litter was mixed into the soil. The litter was added to all treatments four times in total: once at the beginning and three times thereafter, always

Table 1
Chemical properties of the soil substrates used in the experiment. Chemical properties are from studies of Kuráž et al. (2012) and Frouz et al. (2016). The particle size distribution in the soil was determined using the FAO method.

Soil type	C (%)	N (%)	pH	Conductivity (μS cm^{-1})	Clay (%)
Clay	2.4	0.14	8.75	780	63
Sand	0.4	0.02	6.65	104.5	3

three weeks after the earthworms had completely incorporated the previously added litter into the soil in microcosms with earthworms. Each additional litter weighed 4 g of dry equivalent. The earthworm microcosms were controlled weekly for the activity and vitality of the earthworms. In some cases, dead earthworms were observed, which were then removed and replaced by another earthworm of the same size. The microcosms were kept in the dark at 16 °C for 126 weeks. The lids of the microcosms kept the moisture at the same level (as checked by weighting of bottles) and simultaneously enabled air exchange with the surrounding atmosphere.

The earthworms were removed from the microcosms at the end of the incubation. Most of the soil in the earthworm treatments was formed from casts. The mineral layers were dried and then fractionated in order to receive active, passive and slow OM pools according to Zimmermann et al. (2007). A combination of physical and chemical methods (sonification, density fractionation and acid oxidation) resulted in pools that corresponded to the Rothamsted model. The initial litter used for the microcosms experiment was ground.

The initial litter, the active pool and the willow slow pool were analysed for C and N contents with a NC 2100 soil analyser (ThermoQuest Italia S.p.A.) and subjected to ^{13}C cross polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy. The alder slow pool was not analysed because of an assumed contribution of fossil C in samples originating from the clayey post-mining site soil. The NMR spectra were measured with a Bruker Avance^{III} 200 spectrometer (Karlsruhe, Germany). Three replicates of each sample were spun in a zircon-oxide rotor at 5.0 kHz with a recycle delay time of 0.4 s for soil and 1 s for litter. The spectra were processed with a line broadening of 50 Hz, phase adjusted and baseline corrected. The following chemical shift regions were considered: 0–45 ppm (alkyl-C, i.e., aliphatic components), 45–110 ppm (O/N-alkyl-C, i.e., carbohydrates), 110–160 ppm (aryl-C, i.e. aromatic components) and 160–220 ppm (carboxylic-C). Additionally, the alkyl-C/O/N-alkyl-C ratio (Baldock et al., 1997) and the 70–75/52–57 ppm ratio (Bonanomi et al., 2013) were calculated.

The determination of lignin chemistry and measurements of total soluble phenols were performed on the active pool because the main differences among treatments were expected in that pool. Thermochemolysis-GC–MS (Sampedro et al., 2009) was used for determination of lignin-related structures: guaiacyl (G), syringyl (S) and hydroxymethyl (H). The initial litter and the active pool in four replicates were treated with an excess of tetramethylammonium hydroxide (TMAH, 25% aqueous solution), placed on wolfram wire spirals and dried in a desiccator. Pyrolysis was performed with a PYR-01 pyrolyzer (Labio, Czech Republic) directly in the injector of a GC–MS (Varian 3400/Finnigan ITS 40 ion trap detector). The precise description of TMAH-Py-GC–MS instrumentation is presented in Frouz et al. (2015). The identification of pyrolysis products was done both by interpreting the fragmentation patterns and comparing mass spectra with the NIST02 library. The percentages of pyrolysis products were calculated from the relative areas of the peaks after recalculation according to the exact weight of samples. The final values were the means of triplicate runs.

Total soluble phenols were extracted from the initial litter and active pool using 80% methanol and spectrophotometrically determined. Phenols in the extracts were quantified using Foline–Ciocalteu reagent (Singleton et al., 1998).

2.3. Statistical analysis

One-way ANOVA followed by LSD post hoc tests was used to compare individual chemical characteristics (C and N content, C:N ratio, content of phenolic compounds and chemical shift regions) of individual treatments for each particular combination of litter and soil. Computations were made using the statistical program Statistica 12.

Data from TMAH-Py-GC–MS and ^{13}C NMR spectroscopy were subjected to principal component analysis (PCA). Computations were done using the statistical program Canoco 4.5.

3. Results

The C content and C stock of bulk soil did not differ in the treatment with earthworms vs. the treatment with mechanical mixing in sandy soil and willow litter (Table 2). In clayey soil and alder litter, the earthworm treatment showed a higher C content and also a higher C stock than the treatment with mechanical mixing (Table 2). However, the C and N content of individual pools did not differ in the treatment with earthworms vs. the treatment with mechanical mixing in both soil and litter types (Table 3). The C:N ratio differed only in the slow pool, where it was significantly higher in microcosms with earthworms (Table 3). Solid state ^{13}C CPMAS NMR spectroscopy did not show any differences in chemical composition between the treatment with earthworms vs. the treatment with mechanical mixing independent of species and soil type (Table 4 and Fig. 1). The content of phenolic compounds as well as the composition of major chemical groups of aromatic compounds of SOM based on pyrolysis also did not show any significant differences among treatments in the active pool (Table 3 and Fig. 2b).

In the microcosms with willow litter and earthworms in sandy soil, SOM from the slow pool had a significantly higher content of aromatic components (aryl-C) and a lower content of carbohydrates (O/N-alkyl-C) than initial litter (Table 4). SOM from the slow pool in microcosms, where willow litter was mechanically mixed, had a significantly higher content of aliphatic components (alkyl-C) than initial litter. SOM from this pool also showed a significantly higher alkyl-C/O/N-alkyl-C ratio than initial litter. The 70–75/52–57 ratio, as an indicator of the state of decay of organic substances, was significantly lower in the active pool in microcosms where alder litter was mechanically mixed than in initial litter. This ratio was lower in the slow pool from both treatments with willow litter and sandy soil, too (Table 4).

The PCA diagram indicated a positive correlation between initial litter and carbohydrates (Fig. 2a). The slow pool negatively correlated with carbohydrates and positively correlated with aliphatic and aromatic components. It also showed a positive correlation with the alkyl-C/O/N-alkyl-C ratio (Fig. 2a).

4. Discussion

4.1. Differences in chemical composition of earthworm vs. mechanically mixed SOM

The long-term laboratory experiment indicated that after two years, earthworms supported a higher C stock and thus promoted C sequestration in microcosms with clay and alder litter in comparison to mechanically mixed microcosms (Table 2). This was not observed

Table 2
Dry mass of soil, C content in mineral soil layer, and C stock in whole microcosms at the end of the experiment from the following treatments: litter was added to the soil surface with earthworms (Earthworm) or was fragmented and mechanically mixed into the soil (Mixed). Input of C introduced to the system at the beginning with soil was 0.4 g bottle^{-1} for sand and 2.4 g bottle^{-1} for clay, then in total 6.7 and 7.0 g C bottle^{-1} was introduced during the experiment with willow or alder litter for sand and clay, respectively. Values presented in the table are means and SEM. Statistically homogeneous groups in the same column are marked by the same letter (ANOVA, LSD post hoc test. $p < 0.05$)*.

Litter type, soil	Treatment	Dry mass (g)	C content ($g\ C\ kg^{-1}$)	C stock ($g\ C\ bottle^{-1}$)
Alder, clay	Earthworm	105.0 ± 0.34a	53.5 ± 2.6a	5.65 ± 0.16a
	Mixed	104.6 ± 0.81a	30.2 ± 8.5b	3.15 ± 0.87b
Willow, sand	Earthworm	102.7 ± 0.75a	28.9 ± 0.1b	2.99 ± 0.03b
	Mixed	102.6 ± 0.20a	30.8 ± 1.7b	3.16 ± 0.17b

*According to Frouz et al. (2014).

Table 3
C and N content, C:N ratio and content of total soluble phenols in initial leaves litter (alder *Alnus glutinosa* and willow *Salix caprea*) and in active or slow pool of SOM originated from the following treatments: litter was added to the soil surface with earthworms (Earthworm) or was fragmented and mechanically mixed into the soil (Mixed). Values presented in the table are means and SEM. Statistically homogeneous group in the same column are marked by the same letter (ANOVA, LSD post hoc test, $p < 0.05$).

Litter type, soil	Treatment	C content ($g\ C\ kg^{-1}$)	N content ($g\ kg^{-1}$)	C:N ratio	Phenols ($mg\ kg^{-1}$)
Initial litter					
Alder		444 ± 12a	28 ± 0a	16.0 ± 0.2c	18.1 ± 0.3a
Willow		421 ± 12a	22 ± 1b	19.2 ± 0.5bc	10.9 ± 0.1c
Active pool					
Alder, clay	Earthworm	282 ± 2b	15 ± 0c	18.6 ± 0.2bc	11.3 ± 1.2bc
	Mixed	316 ± 52b	17 ± 2c	18.3 ± 0.7bc	13.2 ± 0.4b
Willow, sand	Earthworm	288 ± 5b	11 ± 1d	26.3 ± 2.6a	11.6 ± 0.1bc
	Mixed	269 ± 14b	11 ± 2d	23.8 ± 2.5ab	10.1 ± 0.5c
Slow pool					
Willow, sand	Earthworm	42 ± 7c	2 ± 1e	28.4 ± 5.1a	– –
	Mixed	42 ± 1c	3 ± 1e	14.0 ± 2.8c	– –

in microcosms with sand and willow even though the previous experiment by Frouz et al. (2014) showed that earthworms reduced C loss in both observed soil-plant systems, as the systems age. In contrast, at earlier stages treatments influenced by earthworms showed an enhanced mineralization as compared to the mechanically mixed microcosms, after 20 weeks both, the earthworm and mechanically mixed treatments had similar C mineralization rates, and after 70 weeks the patterns were reversed and the earthworm treatments had the lowest C mineralization (Frouz et al., 2014). Remarkably, this course was valid for both used soil-plant systems, demonstrating a possible common mechanism through which the earthworms influence C loss and C sequestration in soils independent of soil and plant type. These patterns are in line with a review of Zhang et al. (2013) who described a decrease of earthworm-induced CO_2 with time and concluded that earthworms facilitate C sequestration in the long-term. In this regard, it has to be noted that the duration of the experiments has a crucial impact on the effect of earthworms on C sequestration. Most studies conducted so far did not exceed 200 days and mostly found a rather negative or no effect of earthworms on C sequestration (Lubbers et al., 2013). We therefore especially aimed at investigating differences in the chemical composition of SOM between the studied microcosms at later stages (after 70 weeks, i.e., 280 days) that have been underrepresented in the literature so far. We were thus able to reveal a possible influence of earthworms that would explain the reduced C respiration rates observed by our previous and few other studies (Frouz et al., 2014; Zhang et al., 2013) at later stages of incubation.

Our results did not show any differences in the C and N content and the C:N ratio between the earthworm treatment vs. the treatment with mechanical mixing in active soil pools for both soil-plant systems. As also the solid-state ^{13}C CPMAS NMR spectroscopy did not show any differences in chemical composition between the examined treatments, we focused on the composition of aromatic components in the active pools. We did so because the differences in C loss may be rather affected by distinct lignin decomposition in the treatments than overall lignin quantity (Frouz et al., 2015). Lignin can be preserved relatively undecomposed in the earthworm casts (Hong et al., 2011), which could result in different lignin chemistry in the observed treatments. Also phenols could be influenced by the presence of earthworms. Coulis et al. (2009) reported an almost complete disappearance of tannins during the passage of litter through soil animal guts. However, even the content of phenols and the composition of aromatic compounds of SOM did not show any significant differences among treatments in the active pool. These findings indicate that other stabilization mechanisms of SOM than the slowdown of decomposition mediated by chemical changes in OM composition play a decisive role in earthworm casts.

4.2. Changes in chemical composition during litter decomposition

The NMR data revealed that the decomposition of initial litter in the microcosms was associated with a relative decrease of O/N-alkyl-C content. Contrarily, alkyl-C and aryl-C increased gradually from active to slow pool. Both aliphatic and aromatic components are considered to relatively accumulate in SOM during litter decomposition due to their relative resistance to decomposition. These typical patterns of changes in chemical composition of OM during decomposition (Fig. 2a, Table 4), have been well described in a number of previous studies (e.g. Baldock et al., 1997; Bonanomi et al., 2013; Cepáková and Frouz, 2015).

Slightly different trends of chemical changes in OM composition from initial litter to the slow pool were observed in earthworm vs.

Table 4
Relative contributions of peak areas (percent of total area) for different chemical shift regions (ppm) and ratios between specific spectral ranges determined by solid state ^{13}C CPMAS NMR spectroscopy in initial leaves litter (alder *Alnus glutinosa* and willow *Salix caprea*) and in active or slow pool of SOM originating from the following treatments: litter was added to the soil surface with earthworms (Earthworm) or was fragmented and mechanically mixed into the soil (Mixed). Values presented in the table are means and SEM. Statistically homogeneous groups in the same column are marked by the same letter (ANOVA, LSD post hoc test. $p < 0.05$).

Litter type, soil	Treatment	Chemical shift regions				Ratios	
		Alkyl-C	O/N-Alkyl-C	Aryl-C	Carboxyl-C	Alkyl-C/O/N-alkyl-C	70–75/52–57
Initial litter							
Alder		23.9 ± 1.5ab	50.1 ± 0.4a	18.8 ± 0.6b	6.7 ± 1.0b	0.48 ± 0.03b	2.18 ± 0.15a
Willow		22.9 ± 0.4b	49.0 ± 0.3ab	18.3 ± 0.2b	9.6 ± 0.3ab	0.47 ± 0.01b	2.10 ± 0.05a
Active pool							
Alder, clay	Earthworm	27.5 ± 0.4ab	44.7 ± 3.4ab	20.9 ± 1.5ab	6.2 ± 1.3ab	0.62 ± 0.06b	1.80 ± 0.02abc
	Mixed	27.9 ± 1.9ab	44.7 ± 4.0a	21.2 ± 3.1ab	5.3 ± 1.8b	0.63 ± 0.05b	1.42 ± 0.34bc
Willow, sand	Earthworm	20.5 ± 1.3b	44.1 ± 1.3ab	24.2 ± 0.7ab	10.4 ± 0.2a	0.47 ± 0.04b	1.93 ± 0.07ab
	Mixed	22.3 ± 0.9b	44.1 ± 0.3ab	23.1 ± 0.7ab	9.7 ± 0.8ab	0.51 ± 0.02b	1.91 ± 0.07abc
Slow pool							
Willow, sand	Earthworm	26.1 ± 3.3ab	34.0 ± 3.1c	28.0 ± 2.3a	10.8 ± 3.3a	0.77 ± 0.03ab	1.28 ± 0.04c
	Mixed	36.3 ± 8.6a	35.3 ± 1.0bc	22.6 ± 3.9ab	4.6 ± 5.5b	1.02 ± 0.21a	1.24 ± 0.18bc

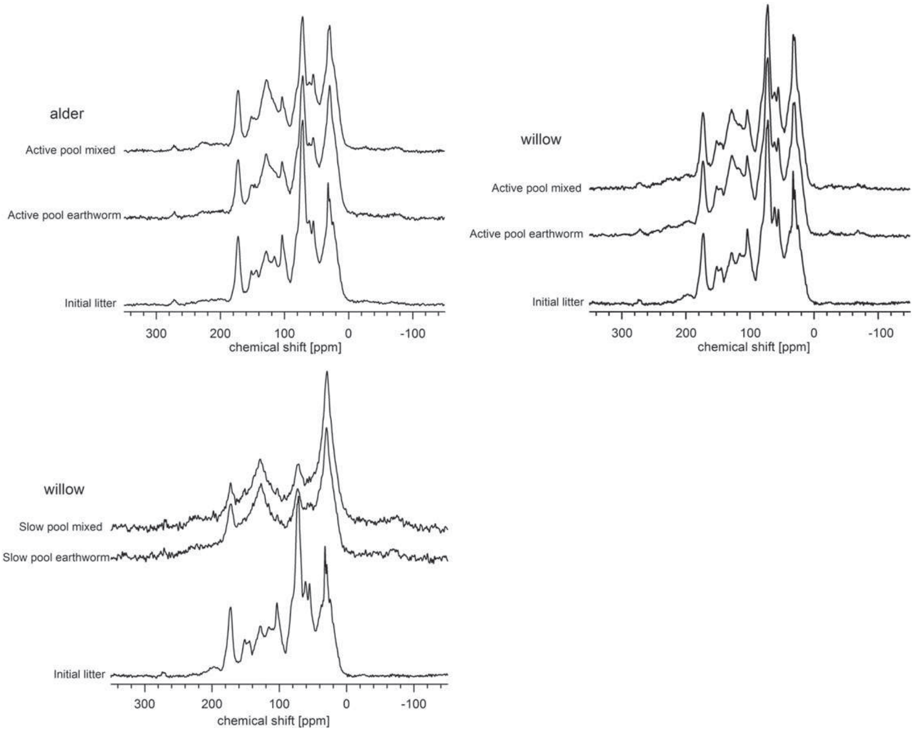

Fig. 1. Solid-state ^{13}C NMR spectra of initial leaf litter (alder *Alnus glutinosa* and willow *Salix caprea*) and active or slow pool of SOM originating from the treatments: litter was added to the soil surface in treatments with earthworms (earthworms) or was fragmented and mechanically mixed into the soil (mixed). The spectra are means of replicated measurements ($n = 3$).

mechanically mixed treatments in the microcosms with sand and willow (Table 4). A significant increase in aryl C with respect to the initial litter could be observed in the slow pool of earthworm-mixed microcosms. This finding is in accordance with Guggenberger et al. (1996), who suggested that high amounts of intact lignin are associated with sand-sized separates of earthworm casts. The observed relative enrichment in aromatic components in the earthworm-mixed microcosms can be related to the passage of litter through the earthworm guts that negatively affects the number of fungi in casts (Schönholzer et al., 1999). Because fungi are the dominant decomposers of lignin, a decrease in their abundance is likely to be decisive for an inhibited decomposition and a relative enrichment of aryl-C in the slow pool of the earthworm-mixed sand microcosms. A stabilization of lignin compounds by organo-mineral association or occlusion within aggregates

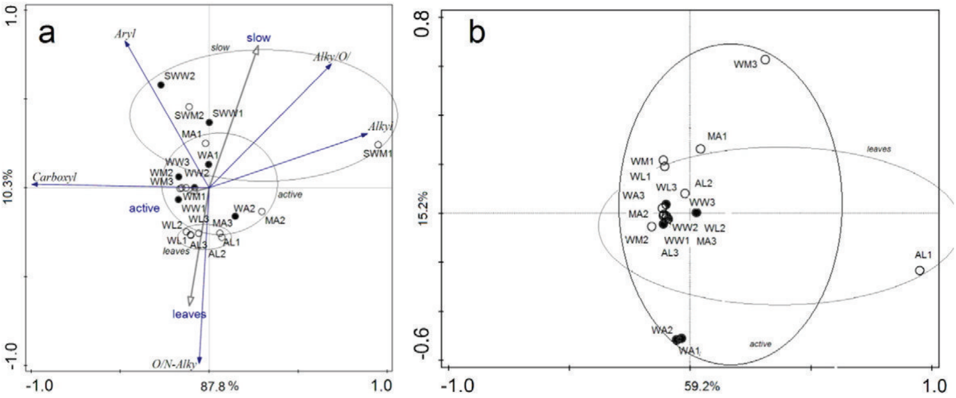

Fig. 2. Principal component analysis (PCA) ordination diagram based on (a) relative contribution of peak areas (percent of total area) for different chemical shift regions (Alkyl, O/N alkyl, Aryl and Carboxyl-C) and alkyl-C/O/N alkyl-C ratio (Alkyl/O/) determined by solid state ^{13}C CPMAS NMR spectroscopy in initial leaf litter (alder *Alnus glutinosa* and willow *Salix caprea*) and in active or slow pool of SOM originating from the following treatments: litter was added to the soil surface in the treatments with earthworms (W) or was fragmented and mechanically mixed into the soil (M). (b) Composition of major chemical groups of aromatic compounds of SOM determined by TMAH-Py-GC–MS in initial leaf litter and the active pool of SOM. Treatments are marked with circles. Initial litter: AL = alder; WL = willow. Active pool: WW = willow from W treatment; WM = willow from M treatment; WA alder from W treatment; MA alder from M treatment. Slow pool: SWW = willow from W treatment; SWM = willow from M treatment.

was likely of minor importance due to the dominance of sand-sized mineral material.

Furthermore, there was a trend of lower amounts of aliphatic components in the slow pool of the earthworm-mixed microcosms compared to the mechanically mixed microcosms. This observation may be connected with a higher degree of fragmentation of OM during the passage through the earthworm guts and an increased number of bacteria in the earthworm guts compared to soil (Schönholzer et al., 1999). The fragmentation results in a higher surface area for microbial attack and an increased number of microorganisms in the earthworm gut may decompose commonly more resistant aliphatic compounds (Lorenz et al., 2007) more effectively.

The used types of initial litter did not differ in the evaluated parameters of chemical composition except for phenol content (Table 3). Interestingly, in the active pool the phenol content was almost aligned both between litter type and treatments at the end of the experiment. Bi et al. (2016) suggested that earthworms avoid feeding on phenolic compounds; yet adding leaves to the substrate can induce their decomposition. Hence, earthworm activity can accelerate phenol degradation (Bi et al., 2016). Our results however did not indicated a higher decrease of phenolic compounds in earthworm treatments and thus it seems that when phenolic compounds are part of the food resource (added litter), they are decomposed both by microbial and earthworm activity.

4.3. Physical stabilization of SOM

Ultimately, it seems that earthworms helped sequestering SOC rather through a reduced bioavailability inside aggregates of earthworm origin than through chemical changes in SOM. We infer this suggestion from the fact that earthworm microcosms were predominantly formed by casts. Mechanical disturbance probably destabilized soil aggregates whereas the earthworms incorporated organic debris into soil and produced new aggregates (Shipitalo and Protz, 1989). The origin of aggregates is often related to the interaction of clay minerals and OM (e.g. Frouz et al., 2011; Lavelle and Martin, 1992; Schrader and Zhang, 1997; Shipitalo and Protz, 1989). This may explain why the C stock of the earthworm vs. the mechanically mixed treatment was significantly higher in clay microcosms, but not in sand microcosms (Table 2). As no distinctions in the examined chemical parameters were observed, it seems that the higher C stock in earthworm treatments was solely driven by occlusion of SOM in soil aggregates. According to Scullion and Malik (2000), OM is better mixed with clays in the presence of earthworms than in soil where earthworms are missing. This is mainly due to the mutual passage of OM and mineral soil particles through the earthworm guts connected with organo-mineral complexation of OM with clay (Frouz et al., 2015; Lavelle, 1988).

4.4. Implications of the study for natural systems

Through simplifying the complexity of the natural environment, microcosms can increase the understanding of natural processes (Fraser and Keddy, 1997). Microcosms represent a useful approach to gain valuable insights into otherwise hardly observable processes in soil. However, the possibility to manipulate the parameters of the experiment, to investigate various treatments, and to observe otherwise difficultly monitored earthworm activities (Shipitalo et al., 1988) entails also limitations. Concerning litter decomposition, the microcosms used in the present study with a negligible amount of recent C reflected situations in uncultivated soils after anthropogenic disturbance (post-mining lands). In this regard, our study is representative for such sites and probably also for initial soil development. However, the finding that earthworms helped to stabilize C in the long-term mainly due to interactions with the mineral soil matrix and probably not by chemical changes of SOM, may also be applicable to a broader set of ecosystems. We have to be aware, though, that more factors infer with the role of earthworms in natural systems (e.g. content of recent SOC, more developed and different micro-, meso- and macrofauna communities), which may alter their effect on C sequestration observed in the present study.

5. Conclusion

Although the passage through earthworm guts initially accelerates the decomposition process of OM by mineralization, fragmentation and subsequent increase of the microbial activity, this process was not substantially reflected in the resulting SOM chemical composition as compared to systems without the presence of earthworms, where leaf litter was mechanically mixed into the soil. Thus, higher C storage in treatments with earthworms at later stages seems to be connected solely with the sequestration of SOM in stable earthworm casts after their aging. However, different trends in SOM composition in the slow pool showed that litter decomposition proceeded differently in earthworm and mechanically mixed microcosms. This finding suggests that a two years experiment is still not long enough and differences in SOM chemical composition may occur later.

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PAPER III

The effect of dead standing biomass on the litter decomposition process

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manuscript

Abstract

Some plants keep large amounts of their dead biomass standing for significant periods of time before the litter reaches the soil. During that period, dead standing biomass is exposed to sunlight and other climate conditions which may cause photodegradation, leaching and further processes leading to a facilitation of litter decomposition and changes in biomass chemical composition.

The objective of this study was to explore the effects of simulated exposition of dead standing biomass to sunlight and other climate conditions on litter decomposition of plants that naturally keep dead standing biomass in various extents. We collected freshly senesced biomass of *Calamagrostis epigeios* (which keeps most of dead biomass standing), *Quercus robur* (which keeps some dead leaves on tree) and *Alnus glutinosa* (where all litter falls to the ground after senescence). These litter types were either exposed to environmental climate conditions for a year or kept in a dry dark place. After a year of exposure, both litter treatments were placed in soil for another year. We monitored the mass loss of the plant materials, and chemical changes during decomposition using thermochemolysis-GC-MS and solid-state ^{13}C CP/MAS NMR spectroscopy.

Only *C. epigeios* showed a significant difference in decomposition between initial litter and litter that has previously been exposed. The exposed litter decomposed about 30% faster. In contrary to the other two species, the exposure to climate conditions decreased the content of aromatic components in litter of *C. epigeios* and changed their chemical composition. This result suggests that plants keeping a substantial amount of dead standing biomass after senescence may have specific properties of litter that are prone to photodegradation. This process facilitates further litter decomposition.

1. Introduction

Leaves of most of non-evergreen plant species senesce and litter completely falls every autumn. However, some species hold marcescent leaves in various extents. Marcescence is characteristic for trees such as beech and oak but to date, the ecological consequence of holding dead biomass aboveground is not well understood. Otto and Nilsson (1981) suggested that retention of dead leaves could close a nutrient cycle for the trees. The retention of part of leaves may prolong time for release of nutrients when leaves are shed gradually and trees have thus more time for taking the nutrient up. The theory was, nonetheless, challenged by e.g., Abadía et al., (1996) and Dunberg (1982). Abadía et al. (1996) proposed that marcescent leaves result from long photosynthetically active leaves that suddenly die off at the very end of the season. Other theories were related to the ecological advantages of marcescence such as a protection against herbivores (Mingo and Oesterheld, 2009).

We assume that marcescence may have a relevant influence on litter decomposition in soil after the leaves have finally fallen off the tree. This assumption emerges mainly from the fact that the factors operating in early decomposition stages of marcescent litter and litter that has senesced and fallen on the ground markedly differ. Soil biota is excluded from the decomposition of dead standing biomass and abiotic factors such as photodegradation and leaching play a major role. This may then influence the subsequent decomposition of marcescent litter in soil. We thus hypothesize that the strategy of holding dead biomass may facilitate subsequent decomposition in soil. In this regard, the impact of litter exposure to climate conditions on its decomposition would be related to the extensiveness of marcescence that differs between species. The strategy of holding dead biomass may be relevant especially for litter with a low quality containing high amounts of lignin. Lignin is reported as a photochemically reactive compound (e.g. Austin and Ballaré, 2010) and thus, exposure to higher levels of UV radiation may help to decay this complex polymer.

To test our hypotheses, we established a field experiment using litters of plants that hold dead biomass in various extents: alder *Alnus glutinosa* (where all litter falls to the ground during senescence), oak *Quercus robur* (which keeps some dead leaves on the tree) and the grass *Calamagrostis epigeios* (which keeps most of dead biomass standing). All litters were exposed to the same climate conditions in the field and subsequently buried in soil together with litter of the same species, which was previously kept in a dark, dry place. Such an experimental design provided the possibility to observe whether the exposure to climate conditions, especially UV radiation, facilitates subsequent decomposition in soil or not.

2. Material and methods

2.1. *Study area and study sites*

The field decomposition experiment was carried out on a spoil heap in the Sokolov brown-coal mining district, Czech Republic. The spoil heaps were formed during the last 40 years by the piling of tertiary clay overburden originating from brown-coal mines. The pH of the soil was about 8 (Helingerová et al., 2010). The mean annual precipitation was 650 mm, and the mean annual temperature was 6.8 °C. The study area was covered by a mosaic of reclaimed forest sites with various dominating tree species of the same age of 40 years (Frouz et al., 2013). For this study, oak (*Quercus robur*) and alder (*Alnus glutinosa*) forest sites were chosen. Several parts of the post-mining area were unreclaimed and were colonized by spontaneous revegetation with predominance of the grass *Calamagrostis epigeios*. This grass was also examined in this study.

2.2. *Experiment and measurements*

For the decomposition experiment, senescent litter were picked during the time of litter fall. Leave litter was collected using litter traps made with 0.5 mm nylon mesh on an iron frame (0.5 x 0.5 m) that were located 0.5 m above soil surface. The traps were in three replications. The grass *C. epigeios* was collected as standing dead biomass. The biomass was dried, cut if necessary (*C. epigeios*) and mixed. Part of the litter material was placed in litterbags and the rest of the material was stored in a dark, dry place at laboratory conditions. The nylon litterbags were 10 x 15 cm large with 0.2 mm mesh size. The mesh was chosen because of previous measurement (Alvarez and Lipp-Symonowicz, 2003) showed that the same type of material was translucent for UV radiation. The litterbags were hung on a rope about 1 m above the soil surface for one year at the study area. After that year, 3 g of both types of litter, i.e. stored and exposed litter, were placed in litterbags and buried in the soil about 4 cm under the surface. Both, stored and exposed litter of all studied species, were buried in three replications. The litterbags were collected from the soil after another year. The litter was dried and weighed. Eventually, four types of litter were analysed: initial litter, litter exposed for one year to climate conditions (Exposed), exposed litter buried for one year in soil (Exposed decomposed) and initial litter buried for one year in soil (Decomposed).

Because of some penetration of soil into the litterbags, about 1.5 g of buried material from each litterbag was used for litter mass loss after ignition at 550°C for 6 h. The values were then used for calculating the total mass loss of organic matter from litter during the experiment. The rest of the material and initial litter was milled to a fine powder and used for total C and N contents determination with an EA 1108 Elementar Analyser (Carlo Erba Instruments, Italy).

Thermochemolysis-GC-MS was used for determination of lignin-related structures: guaiacyl (G), syringyl (S) and hydroxymethyl (H) (Sampedro et al., 2009). The initial litter and both types of litter that were buried in soil were treated with an excess of tetramethylammonium hydroxide (TMAH, 25% aqueous solution) in four replicates, placed on wolfram wire spirals and dried in a desiccator at room temperature. Pyrolysis was performed with a PYR-01 pyrolyzer (Labio, Czech Republic) directly in the injector of a GC-MS (Varian 3400/Finnigan ITS 40 ion trap detector). The precise description of TMAH-Py-GC-MS instrumentation is presented in Frouz et al. (2011). Pyrolysis products were identified both by interpreting the fragmentation patterns and comparing mass spectra with the NIST02 library. The percentages of pyrolysis products were calculated from the relative areas of the peaks after recalculation according to the exact weight of samples. The final values were the means of triplicate runs.

Aliquots of each three replicate samples were pooled to yield one replication of every treatment and subjected to ^{13}C cross polarization magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy. The NMR spectra were measured with a Bruker Avance III HD 500 WB/US NMR spectrometer (Karlsruhe, Germany, 2013) in a 4-mm ZrO2 rotor. The magic angle spinning (MAS) speed was 9-11 kHz in all cases, with a notation frequency of B_1 (^{1}H) and B_1 (^{13}C) fields fields for cross-polarization $\omega_1/2\pi = 62.5$ kHz. Repetition delay and number of scans was 4 s and 1024, respectively. TPPM (two-pulse phase-modulated) decoupling was applied during evolution and both detection periods. The phase modulation angle was 15°, and the flip-pulse length was 4.8–4.9 µs. The applied notation frequency of the B_1 (^{1}H) field was $\omega_1/2\pi = 89.3$ kHz. The ^{13}C scale was calibrated using glycine as external standard (176.03 ppm; low-field carbonyl signal).

The ^{13}C CP/MAS NMR spectra were quantified according to Wilson (1987) based on the area of the appropriate peak relative to the total area. Additionally, the alkyl-C/O/N-alkyl-C ratio (Baldock et al., 1997) was calculated.

2.3. *Statistical analyses*

One-way ANOVA (analysis of variance), followed by LSD *post hoc* tests, was used to compare individual characteristics (C and N content, C:N ratio) of individual treatments. The t-test was used to compare decomposition between decomposed (unexposed) and exposed decomposed (exposed) litter. Computations were made using the statistical program Statistica 13. Data from TMAH-Py-GC-MS were subjected to principal component analysis (PCA). Computations were done using the statistical program Canoco 4.5 (Šmilauer and Lepš, 2014).

3. Results

After one year of litter decomposition in soil, the treatments which were previously exposed to climate conditions and the treatments without prior exposition did not differ in litter mass loss, except for the grass *C. epigeios*, where the previously exposed litter decomposed faster (Fig. 1).

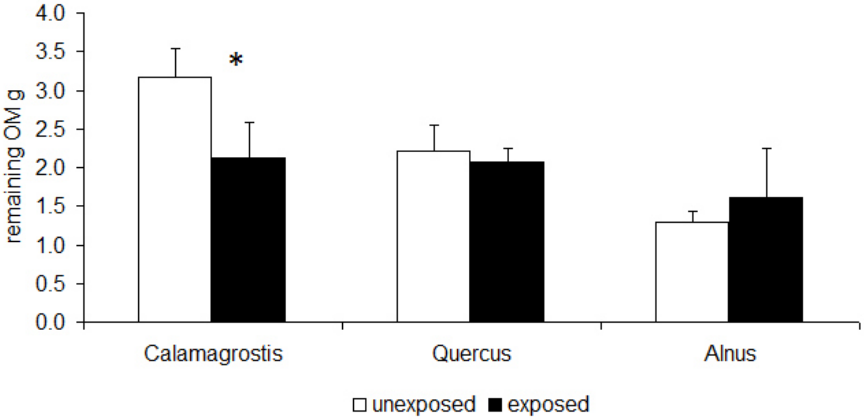

Figure 1. Amounts of remaining OM (g) of C. epigeios, Q. robur and A. glutinosa after 1 year of decomposition in soil (unexposed) or after 1 year of aerial exposure + 1 year of decomposition in soil (exposed). Bars marked by the asterisks are statistically different (t-test, P < 0.05).

The C and N contents and the C:N ratio of initial litter did not change after one year of exposition except for a decrease in the C:N ratio of *C. epigeios* (Table 1). The C content and the C:N ratio decreased after one year of decomposition in soil for *C. epigeios* and *Q. robur* , but these characteristics did not differ between litter which was previously exposed to climate conditions and that without previous exposition (Table 1). The N content decreased only for *A. glutinosa* litter after one year of decomposition in soil for both treatments (exposed and unexposed). This was also reflected in the C:N ratio of decomposed *A. glutinosa* litter. Whilst the C content decreased after one year of decomposition in soil, the C:N ratio did not change for that period of time (Table 1). This observation was valid for both examined treatments.

Table 1. The C and N contents and C:N ratio in leaves of C. epigeios, Q. robur and A. glutinosa in initial litter (Initial) and following treatments: after 1 year of aerial exposure (Exposed), after 1 year of decomposition in soil (Decomposed) and after 1 year of aerial exposure + 1 year of decomposition in soil (Exp. dec.). Values are the means ± SEM. Values in a column followed by the same letter are not statistically different (one-way ANOVA, LSD post hoc test P < 0.05).

	C (%)	N (%)	C:N
C. epigeios			
Initial	43.5 ±0.4a	0.61 ±0.08a	72.4 ±9.5a
Exposed	42.8 ±0.5a	0.74 ±0.05a	58.1 ±3.3b
Decomposed	17.3 ±2.9b	0.61 ±0.06a	28.3 ±3.5c
Exp. dec.	18.2 ±1.5b	0.69 ±0.08a	26.6 ±1.9c
Q. robur			
Initial	46.5 ±0.4a	0.78 ±0.01a	59.9 ±1.1a
Exposed	47.1 ±0.2a	0.87 ±0.08a	54.4 ±5.2a
Decomposed	21.2 ±1.0b	0.87 ±0.05a	24.3 ±0.8b
Exp. dec.	22.7 ±2.1b	0.87 ±0.04a	26.2 ±1.8b
A. glutinosa			
Initial	48.4 ±0.9a	3.04 ±0.30a	16.1 ±1.7a
Exposed	48.6 ±0.1a	3.38 ±0.21a	14.4 ±0.9a
Decomposed	12.5 ±0.9b	0.77 ±0.04b	16.1 ±0.6a
Exp. dec.	13.6 ±2.0b	0.82 ±0.12b	16.6 ±0.4a

Changes in chemical composition of litter varied between the tree species. *Q. robur* showed a relative increase in aliphatic components but a relative decrease in aromatic components after exposition to climate conditions (Table 2). In case of *A. glutinosa*, aliphatic and aromatic components relatively increased and carbohydrates decreased after one year of exposition to climate conditions. However, the differences in composition of previously exposed and unexposed litter were negligible for both species after decomposition in soil (Table 2).

Table 2. Relative contributions of peak areas (percent of total area) for different chemical shift regions (ppm) and ratio between specific spectral ranges determined by solid state ^{13}C CP/MAS NMR spectroscopy in leaves of C. epigeios, Q. robur and A. glutinosa in initial litter (Initial) and following treatments: after 1 year of aerial exposure (Exposed), after 1 year of decomposition in soil (Dec.) and after 1 year of aerial exposure + 1 year of decomposition in soil (Exp. dec.).

Litter type, treatment	Chemical shift regions				
	Alkyl-C	O/N Alkyl-C	Aryl-C	Carboxyl-C	Alkyl-C/O/N-alkyl-C
C. epigeios					
Initial	7.4	81.5	9.2	2.0	0.09
Exposed	9.1	82.0	7.4	1.6	0.11
Dec.	11.0	74.4	11.7	3.0	0.14
Exp. dec.	15.6	69.0	12.2	2.9	0.23
Q. robur					
Initial	18,0	64,7	13.4	3.8	0.28
Exposed	23.0	65.0	9.1	2.9	0.35
Dec.	20.3	60.9	15.0	3.8	0.33
Exp. dec.	21	59.4	15.5	4.2	0.35
A. glutinosa					
Initial	26.0	56.1	12.7	5.1	0.46
Exposed	29.0	49.8	15.3	5.9	0.58
Dec.	28.7	49.3	17.3	4.8	0.58
Exp. dec.	28.4	47.4	18.7	5.4	0.60

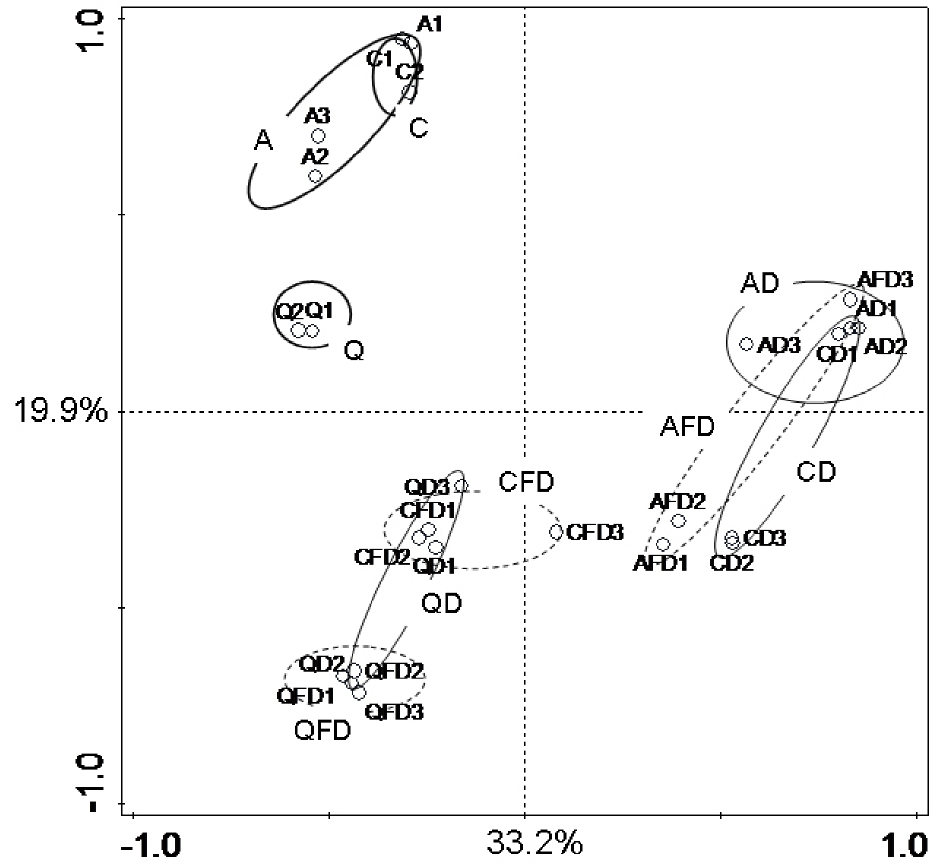

Figure 2. Principal component analysis (PCA) ordination diagram based on composition of major chemical groups of aromatic compounds in litter determined by TMAH-Py-GC-MS. Treatments are marked with circles. Initial leaves litter: C = C. epigeios; Q = Q. robur; A = A. glutinosa. 1 year of decomposition in soil: CD = C. epigeios; QD = Q. robur; AD = A. glutinosa. 1 year of aerial exposure + 1 year of decomposition in soil: CFD = C. epigeios; QFD = Q. robur; AFD = A. glutinosa.

After one year of exposition of *C. epigeios* litter to climate condition, aliphatic components relatively increased and aromatic components decreased (Table 2). *C. epigeios* showed, as only species, differences in chemical composition of previously exposed and unexposed litter after a year of decomposition in soil. For both treatments, aliphatic and aromatic components relatively increased and carbohydrates decreased during decomposition. In exposed litter, however, the aliphatic components showed relatively higher abundances and carbohydrates dropped more rapidly than in unexposed litter (Table 2). This result was also projected in the alkyl-C/O/N-alkyl-C ratio of *C. epigeios,* where the previously exposed litter showed values that were almost twice as high (Table 2).

The principal component analysis (PCA) ordination diagram, based on the composition of major chemical groups of aromatic compounds in litter, revealed that differences in litter decomposition of previously exposed and unexposed litter occurred only in case of *C. epigeios* (Fig. 2).

4. Discussion

From the three observed species, only the decomposition of *C. epigeios* litter in soil was influenced by prior exposure to climate conditions. This was manifested in higher litter mass loss (Fig. 1) and a higher alkyl-C/O/N-alkyl-C ratio (Table 2) as compared to the unexposed litter. We assume that climate factors such as leaching of water soluble organic compounds from litter (Michalzik et al., 2001) or photodegradation due to solar radiation may be crucial for the observed faster litter decomposition. The effect of solar radiation, particularly the ultraviolet (UV) spectrum, has come into spotlight in recent years (e.g. Austin and Vivanco, 2006; Gehrke et al., 1995; Rozema et al., 1997) with the assumption, that UV radiation may cause photochemical degradation of litter, especially of phenolic compounds. Photodegradation should play an important role in dry environments of arid and semi-arid regions (Austin et al., 2009; Austin and Vivanco, 2006; Uselman et al., 2011), but studies that observed an enhanced decomposition of litter due to solar radiation have also been conducted in temperate ecosystems (Frouz et al., 2011; Rozema et al., 1997). Our results partly confirm the findings of these previous studies, but it seems the impact of photodegradation on litter decomposition varies depending on plant species.

After one year of exposition to climate conditions, *C. epigeios* litter had a significantly lower C:N ratio than initial litter (Table 1) and the relative contribution of aliphatic components increased, while the contribution of aromatic components decreased (Table 2). A relative decrease in aromatic components and an increase in aliphatic components were also observed in exposed litter of *Q. robur*. As was postulated in a previous study (Frouz et al., 2011), the photodegradation of photochemical reactive phenolic compounds, including lignin (e.g. Moorhead and Callaghan, 1994; Rozema et al., 1997), may have a decisive impact on the observed chemical changes in exposed litter that enhanced the later decomposition in soil. Although photodegradation was likely responsible for the relative decrease in aromatic compounds in exposed *Q. robur* litter as compared to the unexposed litter after the first year, the chemical composition and mass loss of both treatments after the subsequent year in soil were negligible. An explanation for this observed pattern may be provided by the NMR spectra of *C. epigeios* (Table 2). Initial litter did not have an outstanding content of aromatic components in comparison to the other investigated species, but a relatively high content of carbohydrates. Therefore, we assume that the observed facilitation of subsequent decomposition of previously exposed litter in soil may be based on lignin photodegradation that disintegrated the lignocellulose complex. Because of a relatively high contribution of carbohydrates in *C. epigeios* litter, this can be a crucial process leading to faster microbial uptake of carbohydrates later in soil. In this respect, rather the amount of carbohydrates associated with lignin might be crucial for decomposition than the sole quantity of lignin (Angst et al., 2016). If most of the lignin present in the litter of *C. epigeios* was associated with carbohydrates, photodegradation should have highly affected the subsequent litter decomposition in soil through the previous release of carbohydrates. In *Q. robur*, however, most of the lignin that was degraded by photodegradation after the first year was probably not associated with carbohydrates. This inference is supported by a substantially higher content of aromatic compounds and a lower amount of carbohydrates in initial litter of *Q. robur* as compared to the grass species. It follows that decomposition in soil after exposure to climate conditions was not necessarily faster than that of the unexposed litter because less lignocelluloses were disintegrated by photodegradation. This suggests that the impact of photodegradation on subsequent litter decomposition in soil is to some extent dependent on the chemical properties of the respective litter material.

A further factor that supports the abovementioned indications and might explain the observed variation in the impact of photodegradation on litter decomposition is the C:N ratio of the litter. The C:N ratio may also explain why some plant species hold marcescent litter. The species examined in the current study varied in the amount of marcescent litter and also in its quality. Whilst *A. glutinosa* produces litter with a low C:N ratio that completely falls in autumn, *Q. robur* with a substantially higher C:N ratio holds part of its litter as marcescent. The grass *C. epigeios* holds most of its dead biomass marcescent for a substantial amount of time. Its litter had by far the highest C:N ratio and, as described above, the difference in mass loss of exposed and unexposed litter was highest from all studied species (Fig. 1). Thus, abiotic factors (i.e. solar radiation) may play a more important role in litter with high C:N ratio, which is generally more resistant to microbial decay. As photodegradation may lead to the breakdown of phenolic macromolecules and to disintegration of lignocellulose complexes, litter biomass becomes more susceptible and available to microbial utilization. Such a process has previously been described as photo-facilitation (Frouz et al., 2011, Yanni et al., 2015). This implies, that marcescence can be a way how species with poorly decomposable leaves indirectly decrease the C:N ratio and thus the stoichiometric differences to the soil microbial biomass.

Contrary to *C. epigeios*, the litter of *A. glutinosa* has sufficient amounts of nitrogen (i.e. low C:N ratio) and is thus well utilizable by microbes. This may also explain the non-existence of naturally marcescent leaves of this species. From the ^{13}C NMR spectra, we assume that litter of *A. glutinosa* was not influenced by photodegradation because the relative amount of aromatic components did not decrease. Only carbohydrates decreased after exposition to climate conditions. Because carbohydrates have not been recognized as photochemically reactive (Austin and Ballaré, 2010), their decrease resulted most probably from leaching. Leaching of soluble compounds from litter represents an important process during litter decomposition. Together with nutrients, labile organic compounds, mostly carbohydrates (Cepáková et al., 2016; Kaiser et al., 2002), are commonly leached. Leaching may also be indirectly increased by photodegradation (Qualls and Richardson, 2002; Uselman et al., 2011). However, the difference between exposed and unexposed litter of *A. glutinosa* was balanced through subsequent decomposition in soil and the mass loss at the end of the experiment was even marginally higher for unexposed litter (Fig. 1). These results indicate that the possibly photodegradation induced accelerated leaching of carbohydrates did not have any impact on the subsequent decomposition in soil.

5. Conclusion

The reaction of litter on exposure to climate conditions differed between species. It facilitated subsequent litter decomposition in soil only for the grass species investigated in this study (*C. epigeios*), whereas the decomposition of litter from the investigated tree species (*Q. robur* and *A. glutinosa*) in soil did not show a distinct response to previous climate exposure. Our results indicate that exposure of litter to climate conditions seems to be most relevant for species with a long marcescence and favourable conditions for insolation, such as bare surfaces with minimal shading (e.g., grass species). Further, litter quality and the amount of easily degradable compounds associated with biomolecules that are susceptible to photodegradation (e.g., lignocellulose complexes) may also determine its impact.

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PAPER IV

The effect of tree species on seasonal fluctuations in water-soluble and hot water-extractable organic matter at post-mining sites

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Geoderma, 275, 19–27

Abstract

Seasonal changes in the contents of water-soluble carbon (WSC) and hot water-extractable carbon (HWC) were investigated in the organic and underlying mineral horizons of soils in a common garden experiment at 40 years old post-mining sites. The sites were planted with alder, oak, or spruce or overgrown by a spontaneous succession dominated by willow. Sampling was performed at 2-month intervals from April 2013 until March 2014. Two major peaks in WSC in the mineral horizons were evident: one in early summer and another in late autumn or winter. The peaks were more pronounced at sites with deciduous trees than with spruce. According to liquid-state ^{1}H NMR spectroscopy, hot-water extracts (HWE) from the mineral horizons had a higher relative content of aliphatic components in summer, while the winter samples had a larger relative abundance of carbohydrates. The results suggest that the peaks had different sources of carbon. RDA with variation partitioning showed that both tree species and season significantly affected the composition of HWE, but that tree species was most important, explaining 67.3% of data variability. However, the trends in temporal variation in concentrations and chemical composition were remarkably similar at the studied sites suggesting the changes were mainly induced by seasons.

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The effect of tree species on seasonal fluctuations in water-soluble and hot water-extractable organic matter at post-mining sites

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ABSTRACT

Seasonal changes in the contents of water-soluble carbon (WSC) and hot water-extractable carbon (HWC) were investigated in the organic and underlying mineral horizons of soils in a common garden experiment at 40 years old post-mining sites. The sites were planted with alder, oak, or spruce or overgrown by a spontaneous succession dominated by willow. Sampling was performed at 2-month intervals from April 2013 until March 2014. Two major peaks in WSC in the mineral horizons were evident: one in early summer and another in late autumn or winter. The peaks were more pronounced at sites with deciduous trees than with spruce. According to liquid-state ^{1}H NMR spectroscopy, hot-water extracts (HWE) from the mineral horizons had a higher relative content of aliphatic components in summer, while the winter samples had a larger relative abundance of carbohydrates. The results suggest that the peaks had different sources of carbon. RDA with variation partitioning showed that both tree species and season significantly affected the composition of HWE, but that tree species was most important, explaining 67.3% of data variability. However, the trends in temporal variation in concentrations and chemical composition were remarkably similar at the studied sites suggesting the changes were mainly induced by seasons.

1. Introduction

Water-extractable organic matter (WEOM) represents the most mobile fraction of soil organic matter (SOM). Most of these mobile organic compounds are regarded as labile in nature (Ghani et al., 2003), with turnover times from weeks to less than few years (Wu et al., 2012). This mobile fraction mostly consists of carbohydrates and proteins (Balaria and Johnson, 2013; McDowell, 1988) which can be assumed as easily decomposed fraction of organic matter, but also includes aliphatic and aromatic components (Kaiser et al., 2001). Particularly the aromatic components tend to be adsorbed by soil mineral particles (Kaiser and Guggenberger, 2000; Rasse et al., 2005) and stabilized on their surfaces (Guggenberger and Kaiser, 2003). Hence, WEOM can be both an important source of available carbon (C) for soil organisms and also a possible precursor for the formation of stabilized SOM.

WEOM is derived from belowground and aboveground sources. Belowground sources include root litter, root exudates, associated mycorrhizae, and soil microorganisms (Haynes and Francis, 1993; Melillo et al., 1989; Smith, 1969). The contribution of belowground sources increases in ecosystems with a well-developed fine root system. Uselman et al. (2007) also underline the particular importance of roots to the distribution of SOM in greater soil depths. The main aboveground source is leaf litter. Leaf litter is considered as the overall dominant source of WEOM (Kalbitz et al., 2000), however, microbial processes greatly influence the ultimate amount and composition of released organic matter (Kaiser et al., 2001).

Two pools of water-extractable organic C (WEOC) are distinguished based on the efficiency of water extraction: hot water-extractable C (HWC) and water-soluble C (WSC). HWC is regarded as a pool of mineralisable SOM (Ghani et al., 2003) which is partly derived from soil microbial C (Sparling et al., 1998) and that represents a readily available source of nitrogen (N) in soils (Keeney and Bremner, 1966). Changes in this labile pool are used to make inferences about changes in SOM content (Ghani et al., 2003). Tracking of HWC can therefore increase our understanding of belowground C dynamics (Uchida et al., 2012). Hot water extraction also results in the partial release of C that has been adsorbed onto the mineral soil particles (Kalbitz et al., 2005).

WSC is a substantially smaller fraction of the soluble C pool than HWC. WSC represents that portion of C that can be very rapidly utilized and is considered an indicator of the availability of respiratory C substrate (Uchida et al., 2012). WSC contains very little C derived from microbial cells (van Ginkel et al., 1994). WSC is considered to include dissolved organic C (DOC) that is usually collected in situ (Zsolnay,

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1996) and can be denominated as soil-derived DOC (Kalbitz et al., 2003).

Tracking both WEOM pools can lead to a better understanding of belowground dynamics of the most labile SOM in forest ecosystems. Most studies focused on dynamics in labile SOM by evaluating dissolved organic matter (DOM) in order to clarify seasonal changes (e.g. McDowell and Likens, 1988; Michalzik and Matzner, 1999). Less attention has been paid to differences in labile SOM mediated by different tree species (Don and Kalbitz, 2005). We hypothesize that a different origin and thus amount and quality of litter will have an important influence on the seasonal course of labile SOM.

The current study benefited from a well-studied (Frouz et al., 2009, 2013) common garden experiment where several tree species were planted on the same geological substrate on a spoil heap created by coal mining in the Czech Republic. Over a 1-year period, we evaluated the seasonal changes in WSC and HWC quantity and composition in two soil horizons at sites that differed only in the dominant tree species. The tree stands had the same age and were planted on a relatively small area in several replications. We could thus exclude environmental factors like differences in soil substrate, hydrology, climate and age in tree stands that would have great impact on the studied SOM pools (Zsolnay, 1996; Kaiser et al., 2001) and were able to solely investigate the influence of the respective tree species.

Four other hypotheses were tested. First, the seasonal changes in WSC and HWC concentrations will be greater at sites dominated by deciduous trees than by evergreen trees. We related the assumption mainly to the absence of seasonally driven senescence and higher recalcitrance of evergreen litter. Second, the changes in WSC and HWC concentrations during the course of the year will show similar patterns in the studied tree stands. This should be mainly driven by differences in the growing and dormant season connected with changes in microbial activity. Third, seasonal changes will be greater for WSC than for HWC, as the WSC pool is the most labile C pool. Finally, the composition of evaluated WEOM pools will differ depending on the season. These differences in composition of WEOM pools may also help to reveal major sources of these pools.

2. Materials and methods

2.1. Study area and study sites

The study was carried out on a spoil heap in the Sokolov brown-coal mining district, Czech Republic (50°14′04″N, 12°41′04″E, 450–520 m a.s.l.). The spoil heaps were formed during the last 40 years by the piling of overburden originating from brown-coal mines. The overburden material consisted mainly of tertiary clay with a predominance of illite and kaolinite (Frouz et al., 2013). The pH of the soil is about 8 (Helingerová et al., 2010). The mean annual precipitation is 650 mm, and the mean annual temperature is 6.8 °C. The distributions of the precipitation, mean temperature and water content of samples in each sampling month are described in Table 1. No snow cover was present during sampling.

The study area is covered by a mosaic of reclaimed forest stands with various dominating tree species. Forest stands of the same age of 40 years old (Frouz et al., 2013) were chosen for this study. Several parts of the post-mining area were unreclaimed and had experienced spontaneous revegetation. For this study, the following types of forest stands were chosen: unreclaimed stands dominated by willow (*Salix caprea*), and reclaimed stands dominated by oak (*Quercus robur*), alder (*Alnus glutinosa*), and spruce (*Picea omorica*). Each type of stand was represented by two separate, replicate sites formed by parallel strip of homogeneous vegetation 50–80 m wide and 400–800 m long. Along the longitudinal axis of each strip we selected three sampling points about 100 m apart. The sampling points in individual strips were typically much closer to the closest sampling point in a different treatment than to the closest sampling point in the same treatment. Because of that we assumed individual sampling points as an independent replicates. The soil surface at the two unreclaimed willow stands consisted of ridges and depressions as a result of the heaping performed by the mining machinery. Because conditions and especially water contents are likely to differ at the depressions vs. the ridges, we collected data separately at the depressions and ridges at each sampling point of the unreclaimed willow stands; for simplicity, these are referred to as “willow depression sites” and “willow ridge sites”. As a consequence, there were five kinds of sites: alder, oak, spruce, willow depression, and willow ridge. Ridges and depressions were absent at the reclaimed sites because the overburden had been levelled by earthmoving machinery before tree seedlings were planted.

Soil development varied between sites. In the alder and oak sites, intensive bioturbation occurred. This resulted in a faster formation of the A horizon, while under spruce and willow, bioturbation was much less intensive and the formation of the A horizon was slower resulting in the development of an Oe horizon. Consequently, the depth of the Oe horizon at spruce and willow sites was on average 1 and 2 cm while the Oe horizon was discontinuous and only temporarily occurring at the oak and alder sites (Frouz et al., 2013). The average depths of the A horizon were 9, 5, 3, and 3 cm at the alder, oak, willow and spruce sites, respectively (Frouz et al., 2013). More detailed information about the soil profile development can be found in Frouz et al. (2013) and Abakumov and Frouz (2009).

2.2. Sampling and measurements

Samples were collected every other month (April, June, August, October, December, and February) from April 2013 to February 2014. Three soil cores were collected from the organic (Oe) and mineral horizon of the soil at each site as described above. The Oe horizon represented that part of the profile with a predominance of partly decomposed litter. The Oe horizon was sometimes absent in the oak and alder sites, and the thickness of the Oe horizon varied during the year. The mineral horizon was sampled to a depth of 5 cm.

Replicate soil samples were separately passed through a 4-mm screen and stored at 4 °C until they were processed (within 1 week). At the time of processing, each replicate sample was divided into three separate subsamples, which were used for determination of moisture content, water-soluble extracts (WSE), and hot-water extracts (HWE). The moisture content of fresh samples was determined by oven drying at 105 °C for 24 h. WSE and HWE were determined by a modified method of Ghani et al. (2003) and Haynes and Francis (1993). Samples were extracted with water using a 1:10 ratio. HWE was extracted from soil in hot water (80 °C) for 16 h. After the 16 h extraction period, the HWE samples were shaken for 10 min to obtain a suspension. WSE samples were shaken in cold water (20 °C) for 1 h. The WSE and HWE were then passed through a 0.45 μm filter, and the filtrate was collected for subsequent analysis. All results are presented on an oven-dry basis.

Table 1
Sum of precipitation, means and SEM of air temperature and water content (WC) in samples at the studied sites for sampling months.

	April	June	August	October	December	February
Precipitation (mm)	36.4	130.4	69.8	38.6	38.8	22.2
Temperature (°C)	7.3 ± 1.0	15.0 ± 0.8	14.2 ± 0.6	8.3 ± 0.5	0.5 ± 0.6	1.2 ± 0.4
WC (g g^{-1})	0.32 ± 0.01	0.37 ± 0.03	0.22 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.36 ± 0.01

Total organic carbon (TOC) and total nitrogen (TN) of the extracts were determined with a TOC and TN analyser (Formacs, Skalar) using high-temperature combustion. The analyser measured three analytical replications for each sample. HWC after removal of readily soluble carbon was described (Ghani et al., 2003) to have a strong positive correlation with microbial biomass-C. We therefore additionally calculated the pool "microbial biomass correlated-C" as the difference between HWC and WSC (referred to as HWC–WSC).

To estimate aromaticity, soil filtrates were further analysed for UV absorbance at 254 nm ($SUVA_{254}$) and 280 nm ($SUVA_{280}$) using a spectrophotometer (Hach Lange, DR 6000 UV-VIS, Germany); the data were normalized according to extract concentrations (Chin et al., 1994; Potter and Wimsatt, 2003).

Liquid-state ^{1}H-nuclear magnetic resonance (liquid ^{1}H NMR) spectroscopy was used to determine the chemical composition of the HWE from the mineral horizon. Extracts from samples collected in June and December were freeze-dried and re-dissolved in D_2O (99.8% isotope enrichment, Sigma Aldrich). To obtain meaningful spectra, it was necessary to further suppress the residual water signal by excitation sculpting pulse sequence (Hwang and Shaka, 1995). The spectra were recorded on a Bruker Avance III spectrometer operating at 600 MHz proton frequency and equipped with a TCI cryoprobe. A total of 64 scans were accumulated following four dummy scans with total repetition delay of 5 s. June and December samples were used for this analysis because TOC concentrations were highest in these two months (see Results). The HWC pool rather than the WSC pool was used for the liquid ^{1}H NMR spectroscopy because HWC has higher C concentrations, and because WSC and HWC were expected to be closely related (Kalbitz et al., 2005; Schwesig et al., 1999). Sample replications were combined to obtain sufficient material for the analysis. The NMR spectra were evaluated according to reported signals in Kaiser et al. (2002).

2.3. Statistical analysis

Statistica 12 was used for statistical analysis. Main effects ANOVAs were used to compare individual characteristics among the six sampling months and among the five types of site using six replicates in each vegetation type. The LSD Fisher test was used for post hoc comparisons. Data were tested for normality and homogeneity before ANOVA. If necessary, data were log-transformed before ANOVAs were conducted. Relationships between individual characteristics measured in individual sampling points were evaluated using linear regression and correlation coefficient.

Redundancy analysis (RDA) with variation partitioning (Lepš and Šmilauer, 2003) performed with the Canoco statistical program was used to determine the percentage of variability in ^{1}H NMR data accounted for by site and season.

3. Results

3.1. Seasonal changes in WSC and HWC concentrations

WSC ranged from 0.3 to 1.3 g C kg^{-1} for mineral soils and from 0.8 to 2.9 g C kg^{-1} for the organic horizon if an organic horizon was present (Fig. 1a, c; Tables 2, 3). A developed organic horizon was present at willow depression and ridge sites and at spruce sites. An organic horizon was thin or absent at the alder and oak sites. The WSC concentration in the organic horizon peaked in June (Fig. 1a; Table 2). After June, WSC concentrations in the organic horizon decreased. This trend was not observed at the alder sites, where the concentration peaked in October. Annual mean WSC concentration was significantly higher at willow depression sites than at the other sites (Table 3).

WSC concentrations in the mineral soil horizon peaked in June at alder sites and at the willow depression sites (Fig. 1c). WSC concentrations in the mineral soil horizon declined in October at spruce, alder, and willow depression sites (Fig. 1c). WSC concentrations in mineral soils also peaked in December at alder and spruce sites and at the willow depression sites. The increases and decreases in WSC concentrations in the mineral soil horizon were more pronounced at the deciduous tree sites than at the spruce sites. WSC at oak sites and at willow ridge sites did not show clear seasonal changes in the mineral soil horizon. The annual mean concentration of WSC was higher at reclaimed sites than at unreclaimed sites (Table 3).

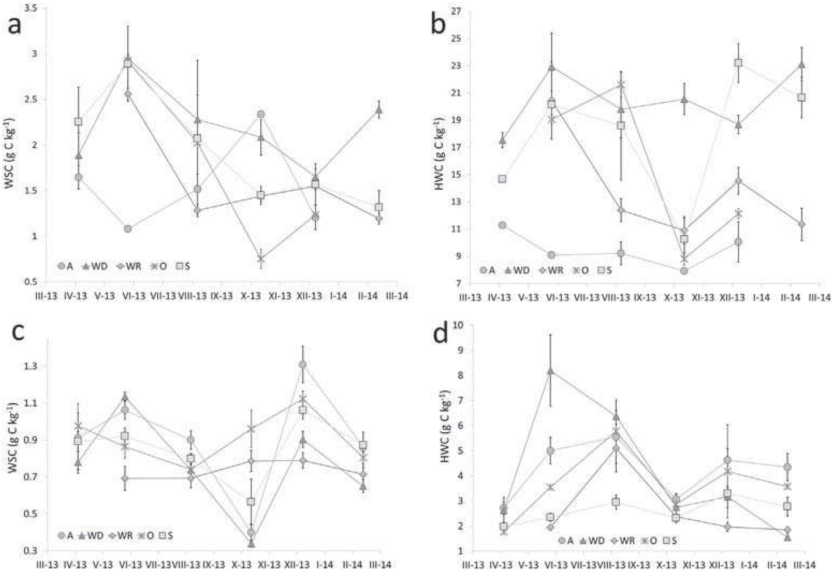

Fig. 1. Seasonal changes in the concentration of (ac) water-soluble carbon (WSC) and (bd) hot water-extractable carbon (HWC) in (ab) the organic horizon and in (cd) the mineral soil horizon. Site abbreviations: A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge. Along the X axis, Roman numerals indicate the months, and Arabic numerals indicate years.

Table 2

Course of the concentrations of water-soluble carbon (WSC) and hot water-extractable carbon (HWC), nitrogen (N) and SUVA in water-soluble extracts (WSE) and in hot-water extracts (HWE) in the organic and in the mineral horizon and relationship between WSC and HWC in the mineral horizon. Values are means and SEM. Means followed by different letters are significantly different based on ANOVAs that compared months (averaged across all sites).

Organic horizon

	WSC	HWC	N_{WSE}	N_{HWE}	$SUVA_{WSE}$	$SUVA_{HWE}$
	(g C kg^{-1})	(g C kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(l mg^{-1} m^{-1})	(l mg^{-1} m^{-1})
April	2.0 ± 0.2c	14.4 ± 1.1abc	91 ± 14c	1023 ± 74bc	1.10 ± 0.07bc	1.48 ± 0.11ab
June	2.5 ± 0.3c	19.4 ± 1.7c	94 ± 13c	1235 ± 121bc	1.60 ± 0.33c	1.44 ± 0.08ab
August	1.9 ± 0.3abc	16.3 ± 1.6b	108 ± 18c	923 ± 88b	1.15 ± 0.07bc	1.41 ± 0.06a
October	1.6 ± 0.1ab	12.3 ± 1.2a	40 ± 5ab	650 ± 68a	0.88 ± 0.09a	1.65 ± 0.05bc
December	1.5 ± 0.1a	15.3 ± 1.1bc	28 ± 2a	672 ± 56a	1.02 ± 0.06ab	1.33 ± 0.04a
February	1.7 ± 0.2c	18.4 ± 1.4b	48 ± 7b	868 ± 57b	1.12 ± 0.07bc	1.70 ± 0.06c

Mineral horizon

	WSC	HWC	N_{WSE}	N_{HWE}	$SUVA_{WSE}$	$SUVA_{HWE}$	HWC - WSC
	(g C kg^{-1})	(g C kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(l mg^{-1} m^{-1})	(l mg^{-1} m^{-1})	(g C kg^{-1})
April	0.95 ± 0.07bc	2.3 ± 0.2a	37 ± 6c	150 ± 15ab	1.92 ± 0.41c	1.58 ± 0.07bc	1.6 ± 0.2a
June	0.94 ± 0.04c	4.1 ± 0.5b	24 ± 3b	256 ± 35c	1.37 ± 0.26bc	1.52 ± 0.05bc	3.2 ± 0.5c
August	0.77 ± 0.02b	5.1 ± 0.4c	25 ± 3b	170 ± 17b	0.70 ± 0.06a	0.82 ± 0.10a	4.3 ± 0.4d
October	0.60 ± 0.05a	2.7 ± 0.1a	13 ± 1a	123 ± 14a	1.40 ± 0.19bc	1.43 ± 0.05bc	2.0 ± 0.1bc
December	1.03 ± 0.04c	3.5 ± 0.2b	15 ± 3a	151 ± 17ab	1.08 ± 0.19ab	1.40 ± 0.04b	2.4 ± 0.2bc
February	0.78 ± 0.03c	2.8 ± 0.2a	15 ± 2a	149 ± 21a	1.42 ± 0.18c	1.70 ± 0.07c	1.9 ± 0.2ab

HWC ranged from 1.5 to 8.2 g C kg^{-1} for the mineral horizon and from 7.9 to 23.2 g C kg^{-1} for the organic horizon, if present (Fig. 1b, d; Tables 2, 3). A clear seasonal pattern was not evident for HWC in the organic horizon (Fig. 1b). The annual mean concentration was highest at willow depression sites and lowest at alder sites (Table 3).

HWC concentrations in the mineral horizon increased from spring to summer and subsequently declined in autumn. This trend was more pronounced at the deciduous tree sites than at the spruce sites (Fig. 1d). The annual mean HWC concentrations were significantly lower at spruce sites and at willow ridge sites than at the other sites (Table 3).

3.2. Seasonal changes in dissolved total N (TN) concentration

TN in WSE ranged from 7 to 47 mg kg^{-1} for the mineral horizon and from 22 to 136 mg kg^{-1} for the organic horizon, if present (Fig. 2a, c; Tabless 2, 3). The pattern of seasonal changes in the TN concentrations in WSE was similar in the mineral and organic horizons. The concentrations were usually high in spring and summer and then decreased (Fig. 2a, c, Table 2). At the alder sites in the mineral horizon, however, TN decreased in October and subsequently increased (Fig. 2c). The annual mean TN concentration in the mineral horizon was highest at the alder sites and lowest at the willow ridge sites (Table 3).

TN in HWE ranged from 50 to 439 mg kg^{-1} in the mineral horizon and from 417 to 1536 mg kg^{-1} in the organic horizon, if present (Fig. 2b, d; Tables 2, 3). Concentrations in HWE in the organic horizon peaked in June at spruce and both willow sites (Fig. 2b). The annual mean TN concentration in the organic horizon was highest at willow depression sites (Table 3). Concentrations in the mineral horizon also peaked in June at alder and willow depression sites (Fig. 2d). The annual mean TN concentration in the mineral horizon was highest at the alder sites (Table 3).

3.3. Specific UV absorbance

Because $SUVA_{254}$ and $SUVA_{280}$ were strongly correlated ($r = 0.99$, $P < 0.01$), results are presented only for $SUVA_{254}$. $SUVA_{254}$ was mostly higher for mineral horizon extracts than for organic horizon extracts (Tables 2, 3). For the mineral horizon, spruce sites had the highest and willow ridge sites the lowest $SUVA_{254}$ in both WSE and HWE (Table 3).

$SUVA_{254}$ in WSE did not exhibit a clear pattern of seasonal change (Fig. 3a, c). Whilst $SUVA_{254}$ in HWE of organic horizons also did not

Table 3

Course of the concentrations of water-soluble carbon (WSC) and hot water-extractable carbon (HWC), nitrogen (N) and SUVA in water-soluble extracts (WSE) and in hot-water extracts (HWE) in the organic and in the mineral horizon and relationship between WSC and HWC in the mineral horizon. Values are means and SEM. Means followed by different letters are significantly different based on ANOVAs that compared sites (averaged across all months).

Organic horizon

	WSC	HWC	N_{WSE}	N_{HWE}	$SUVA_{WSE}$	$SUVA_{HWE}$
	(g C kg^{-1})	(g C kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(l mg^{-1} m^{-1})	(l mg^{-1} m^{-1})
A	1.5 ± 0.1a	9.8 ± 0.7a	69 ± 11b	623 ± 59a	1.23 ± 0.09c	1.36 ± 0.06a
WD	2.2 ± 0.1b	20.4 ± 0.7d	81 ± 9b	1148 ± 57c	0.95 ± 0.04ab	1.59 ± 0.06b
WR	1.5 ± 0.1a	13.1 ± 0.8b	47 ± 7a	774 ± 60b	0.91 ± 0.12a	1.50 ± 0.07ab
O	1.4 ± 0.3a	13.6 ± 1.9b	44 ± 14a	651 ± 112a	1.23 ± 0.09bc	1.50 ± 0.06ab
S	1.9 ± 0.2a	17.7 ± 1.4c	55 ± 12a	724 ± 63ab	1.30 ± 0.12c	1.48 ± 0.04ab

Mineral horizon

	WSC	HWC	N_{WSE}	N_{HWE}	$SUVA_{WSE}$	$SUVA_{HWE}$	HWC - WSC
	(g C kg^{-1})	(g C kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(l mg^{-1} m^{-1})	(l (mg^{-1} m^{-1})	(g C kg^{-1})
A	0.91 ± 0.06bc	4.1 ± 0.3c	33 ± 4d	263 ± 20c	1.48 ± 0.20bc	1.47 ± 0.08bc	3.3 ± 0.3c
WD	0.72 ± 0.05a	3.8 ± 0.5b	17 ± 2b	134 ± 22a	1.10 ± 0.14b	1.40 ± 0.08b	3.0 ± 0.6b
WR	0.74 ± 0.02ab	2.8 ± 0.3a	11 ± 1a	120 ± 18b	0.59 ± 0.04a	1.13 ± 0.09a	2.0 ± 0.4a
O	0.90 ± 0.04c	3.8 ± 0.3bc	22 ± 3c	157 ± 11b	1.28 ± 0.22b	1.36 ± 0.07bc	3.0 ± 0.6bc
S	0.87 ± 0.05bc	2.6 ± 0.1a	18 ± 2bc	115 ± 7a	1.78 ± 0.24c	1.52 ± 0.05c	1.8 ± 0.3a

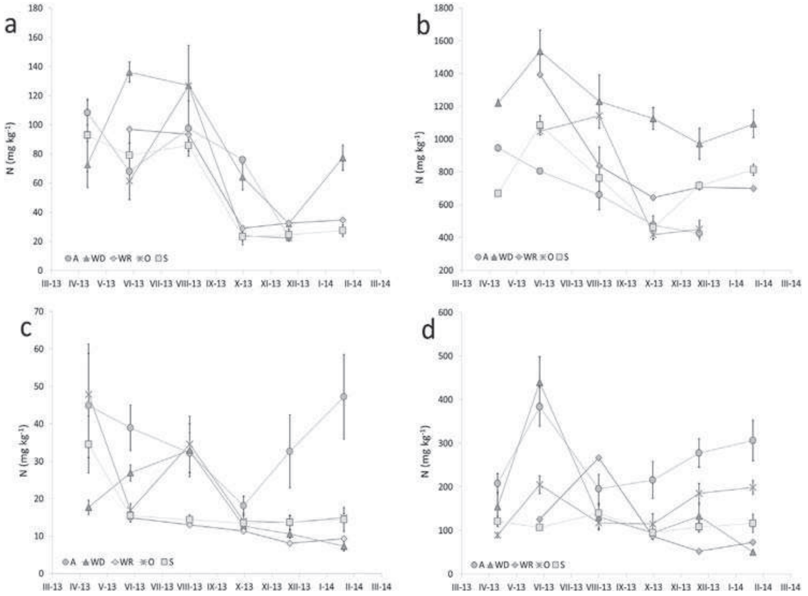

Fig. 2. Seasonal changes in concentration of nitrogen (N) in (ac) water-soluble extracts and in (bd) hot-water extracts in (ab) the organic horizon and in (cd) the mineral soil horizon. A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge. Along the X axis, the Roman numerals indicate months, and Arabic numerals indicate years.

exhibit a clear pattern of seasonal changes in the first part of the year, it showed a uniform increase in October, a decrease in December and again an increase in February (Fig. 3b). SUVA$_{254}$ in HWE of mineral horizons from sites with deciduous trees indicated a decrease in aromaticity in summer (Fig. 3d). In contrast, aromaticity at spruce sites remained relatively stable throughout the year.

3.4. The chemical composition of HWE as indicated by liquid ^{1}H NMR spectroscopy

Fig. 4 illustrates the composition of HWE from June and December for the five kinds of sites as determined by liquid ^{1}H NMR spectroscopy. We focused on the aliphatic region (0.8–3.0 ppm), the carbohydrate region (3.0–4.8 ppm), and the aromatic region (5.5–10.0 ppm) (Table 4). The differences between June and December peaks were evaluated using variation partitioning. The RDA analysis indicated that although HWE composition was affected by season, it was affected more by tree species; 29.5% of the variation between summer and winter was explained by season and 67.3% by site type (ordination diagram not shown). These effects explained 30.3% of the total variation. A higher relative abundance of carbohydrates was observed in December (Table 4). The aliphatic region had a higher relative abundance in June (Table 4). The aromatic region did not show major differences in relative abundance in June and December. Only oak sites showed a slightly

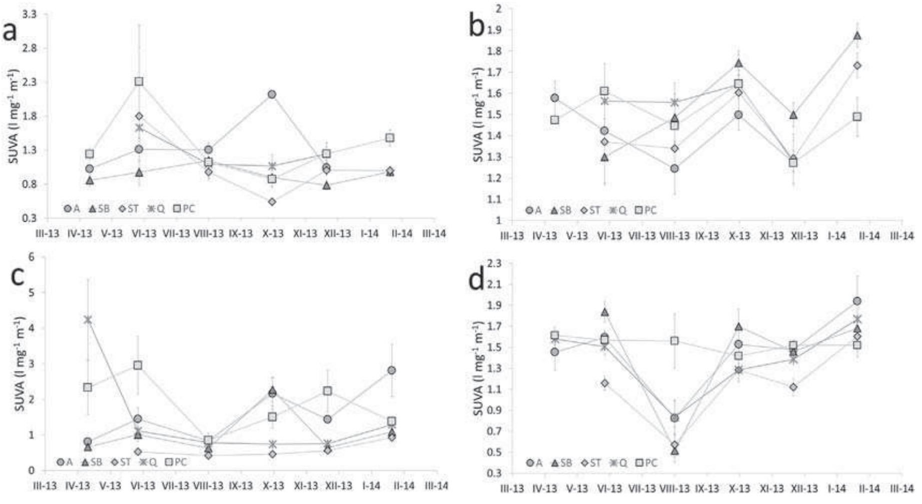

Fig. 3. Seasonal changes in the concentration of SUVA in (ac) water-soluble extracts and in (bd) hot-water extracts in (ab) the organic horizon and in (cd) the mineral soil horizon. A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge. Along the X axis, the Roman numerals indicate months, and Arabic numerals indicate years.

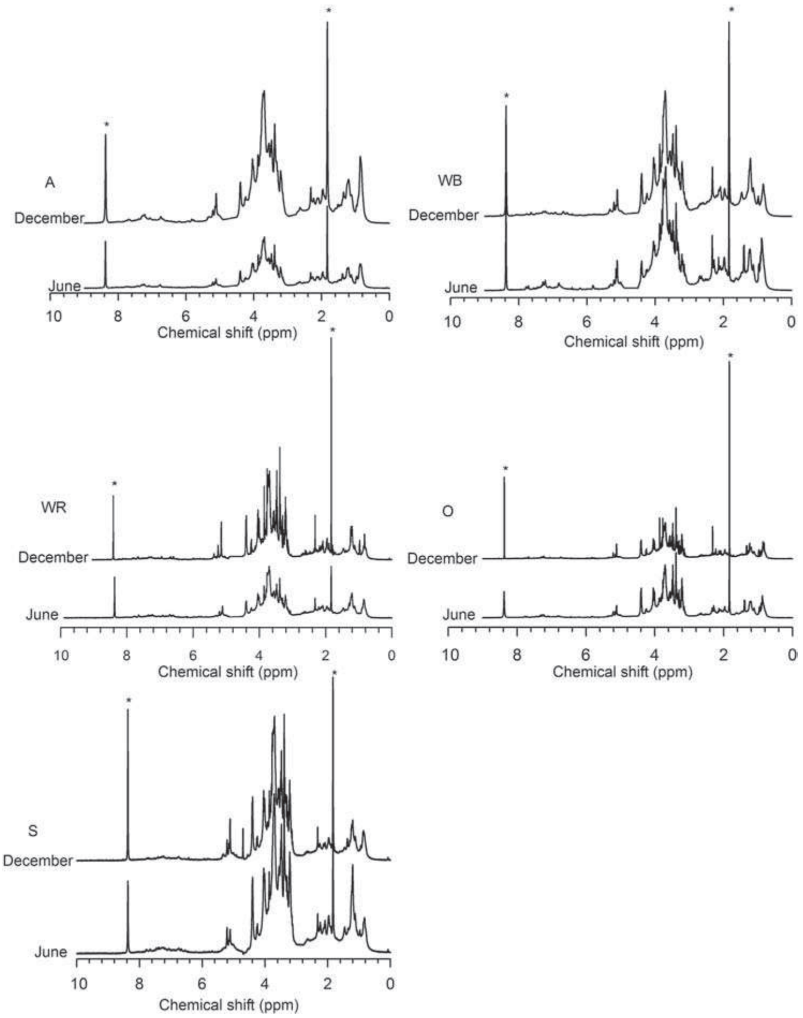

Fig. 4. Liquid-state ^{1}H NMR spectra of HWC extracts from the mineral horizon for June and December 2013. Extracts were from composite samples from replicates because the quantity of sample from one replicate was too small for the analysis. A = alder; O = oak; S = spruce; WB = willow depression; WR = willow ridge. Asterisks mark contamination.

greater aromaticity in December. The aromaticity at the other sites was equal or slightly lower (alder and willow ridge sites).

Table 4
Relative contribution of peak area accounted to carbohydrates, aliphatic and aromatic compounds proportional to area of all peaks (%) in HWC extracts from mineral horizon for June and December 2013. Extracts were from composite samples from replicates because the quantity of sample from one replicate was too small for the analysis. A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge.

Month	Compound	H moieties (%)				
		A	O	WD	WR	S
June	Aliphatic	35	30	35	35	32
	Aromatic	7	6	6	9	6
	Carbohydrates	54	60	55	52	59
Dec.	Aliphatic	33	33	34	30	26
	Aromatic	6	7	6	6	6
	Carbohydrates	56	56	56	61	64

3.5. Relationships between soil parameters

WSC was not correlated with HWC in the mineral and organic horizons at alder, willow ridge, oak, and spruce sites. A marginally significant correlation was detected at the willow depression sites, where $r = 0.38$ and $P = 0.051$. In mineral soils, the value of HWC minus WSC for oak and willow greatly increased from April to June, peaked in August, and then greatly decreased in October (Fig. 5). The alder sites did not peak in August and the concentration at the spruce sites varied only slightly.

For most sites, the water-extractable C was positively correlated with dissolved TN in both the WSE and HWE for both the mineral and organic horizon (Table 5). In WSE, the C:N ratio was higher in winter than in spring and summer for both the mineral and organic horizon, and the C:N ratio was highest at willow ridge sites and was lowest at the alder sites (data not shown). These trends were not evident in HWE (data not shown).

A negative correlation between the water-extractable C and $SUVA_{254}$ was observed mainly in HWE of the mineral horizon (Table 5).

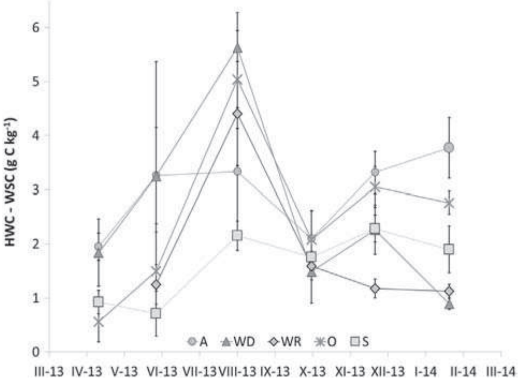

Fig. 5. Relationship between water-soluble carbon (WSC) and hot water-extractable carbon (HWC) in the mineral horizon of the soil as indicated by subtraction of WSC from HWC. A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge. Along the X axis, Roman numerals indicate months, and Arabic numerals indicate years.

WSC was not correlated with the soil water content ($P > 0.05$). Except for a slight decrease in August, the water content was stable at about 0.3 g g^{-1} throughout the year (Table 1).

4. Discussion

Over the year during which samples were collected on reclaimed and unreclaimed post-mining sites, WSC concentrations in the mineral horizon peaked in early summer, declined in autumn, and peaked again in early winter (Fig. 1c). High WSC concentrations in early summer that occurred in the mineral horizon also occurred in the organic horizon. However, the December peak that was detected in the mineral horizon was not detected in the organic horizon (Fig. 1a).

The organic horizon is considered to be the most important source of soluble C for the mineral horizon (Melillo et al., 1989; Qualls and Haines, 1991). Higher temperature and higher precipitation (Table 1) in early summer may result in increased microbial activity and production of labile C due to enhanced microbial breakdown of larger insoluble compounds to soluble ones (McDowell and Likens, 1988; Marschner and Kalbitz, 2003) in the organic horizon and their leaching to the lower horizons. The leached C from the organic horizon coincided with root growth and the associated release of root exudates, which commonly exceeds the demand of soil microorganisms during the start of growing season (Marschner and Kalbitz, 2003; Uchida et al., 2012) probably resulting in the observed summer peak. A similar increase in WSC concentrations from winter to summer was previously observed (Kaiser et al., 2001; Uchida et al., 2012). HWC concentrations also increased substantially in spring and peaked in summer in the mineral horizon (Fig. 1d). HWC positively correlates with soil microbial biomass-C (Sparling et al., 1998) and is also reported as easily available C pool for microbial utilisation (Balaria and Johnson, 2013; Ghani et al., 2003; Scheel et al., 2008). A high concentration of HWC in the growing season may therefore be anticipated. The seasonal changes in HWC–WSC concentration were even clearer and included a sharp peak in August (Fig. 5). This was probably due to an increase in microbial biomass that may follow the excess of available C in early summer. This pattern indicates that the water-extractable pool at the start of the growing season is mostly influenced by leaching of soluble plant residues, microbial metabolites and the release of root exudates. Our analysis of the composition of the June HWC peak showed that carbohydrates accounted for the largest part of the WEOM pool, which is in accordance with previous findings (Balaria and Johnson, 2013; Kaiser et al., 2002; Kalbitz et al., 2005). According to RDA and variation partitioning, aliphatic components also tended to be abundant in the summer peak in HWC. This increase in aliphatic components could result from an increased production of microbially oxidised compounds (Kaiser et al., 2001) and a release of soluble degradation products of cuticular lipids (Kaiser et al., 2002). The release of aliphatic components could be also connected with microbial degradation of carbohydrates. Due to the availability of an easily degradable microbial substrate, litter decay may be enhanced (Marschner et al., 2008) and more aliphatic components can be leached.

The high concentrations of available C in early summer are presumably followed by increased rates of microbial activity, which would in turn decrease the WSC concentration due to its enhanced biodegradation and mineralization which was observed in the current study, i.e., WSC concentrations declined in August to a minimum in October. The assumption that the decrease in WSC concentration was due to consumption by microorganisms is supported by Kaiser et al. (2001, 2002), who sampled leached C from organic forest floor horizons and thus excluded the consumption of C by soil microorganisms. In that case, the concentration of leached C remained high during the whole growing season until it declined in October, which is also in agreement with the observed trend in the current study; both WSC and HWC concentrations in the mineral horizon declined in October. We infer that this decline is caused by a decrease in microbial activity at the end of the growing season. The observed decline in N concentrations in WSE (Fig. 2a, c) supports the inference that the availability of C and N resources for soil microorganisms declines in late summer.

The abrupt increase in WSC concentration in the mineral horizon at the start of the dormant season (Fig. 1c) can be attributed to litter fall and the leaching of soluble material from fresh litter in combination with reduced rates of microbial activity and thus decomposition (Don and Kalbitz, 2005). This dormant season peak in WSC concentration

Table 5
Correlation between $SUVA_{254}$ (254), total organic carbon (C), total nitrogen (TN), and C:N ratio presented separately for water-soluble extract and for hot-water extract in the five sites throughout the year. A = alder; O = oak; S = spruce; WD = willow depression; WR = willow ridge. N indicates the number of measurements in the data set used for each correlation. Only significant *r* values ($P < 0.05$) are shown. Bold numbers are significant even after Bonferroni corrections apply.

		r values									
		Water-soluble extract					Hot-water extract				
Soil horizon and site		254-C	254-TN	C-TN	254-C:N	N	254-C	254-TN	C-TN	254-C:N	N
Organic	A	0.766				14					15
	WD			**0.830**	−0.466	30			**0.764**	0.393	23
	WR			**0.678**		22			**0.895**		22
	O			**0.678**		19			**0.963**		9
	S			**0.935**		31			**0.884**	−0.524	23
Mineral	A				−0.446	35	−0.478		**0.474**	**−0.555**	35
	WD	**−0.611**				30	−0.506		**0.766**	**−0.715**	30
	WR			−0.397		27	**−0.798**	**−0.777**	**0.904**		27
	O	0.410	0.526		−0.362	32	**−0.617**	0.516		**−0.863**	32
	S		0.469	**0.439**		33			**0.740**		33

was not matched by a peak in the organic horizon or in HWC concentration (Fig. 1a, d). This can be due to the low activity of microorganisms at that time of the year (if active, microorganisms could utilize and also retain the WSC) and the consequent rapid leaching of DOM into deeper layers. The liquid ^{1}H NMR spectroscopy of the HWE revealed that carbohydrates were abundant in the December peak. According to RDA and variation partitioning, carbohydrates tended to be more abundant in December than in June. The higher abundance of carbohydrates in December probably resulted from the leaching of fresh organic matter from litter accompanied by low microbial activity. This inference is consistent with Kaiser et al. (2001), who found that carbohydrate levels tended to be high in winter. Freezing and drying should help disrupt microbial and plant cells and thus help release carbohydrates into the soil solution. Snowmelt could also increase leaching of WEOM, but due to the small amount of snow fall and no snow cover during the experimental year, this effect was of negligible importance.

Similar trends in WSC and HWC concentration courses show that the seasonality has a great impact on the variability of available soluble OC. The individual trends of the tree species violated the described courses only in several exceptions. Unlike WSC levels at the willow depression sites in the mineral horizon, WSC levels at the willow ridge sites were relatively stable (Fig. 1c). Frouz et al. (2011) observed that biomass accumulation and levels of available Ca, K, and Na were greater in depressions than in ridges of post-mining sites. Water-soluble components are especially likely to accumulate in the depressions as a result of leaching. Consequently, the C:N ratio was highest at the willow ridge sites in the current study (data not shown). We assume that the heaping of spoil material creates very different microhabitats in relatively small areas and a slope off the ridge can result in a considerable depletion of labile organic matter in soil by leaching into the depression. The seasonal changes were more pronounced for HWC concentrations than for WSC concentrations at the willow ridge sites; even though the HWC concentration was lower at the willow ridge sites than at the other sites. This suggests that leaching indirectly affects HWC by depleting the most labile C components.

The HWC and TN levels in the mineral horizon were more stable at the spruce sites than at the other sites (Figs. 1d, 2d), i.e., the levels were more stable at sites with evergreen than with deciduous trees. Also HWC - WSC concentrations (Fig. 5) as indicator of microbial biomass were lowest and varied least at spruce sites. We thus suggest that the outstanding course of observed parameters in HWE at the spruce sites was due to lower microbial activity in the soils. Coniferous stands were previously characterized by lower microbial C and N than deciduous stands (e.g. Bauhus et al., 1998; Smolander and Kitunen, 2002).

Among the extracts from the five different sites, those from the alder sites had the highest N concentration and the greatest increase in N at the end of the growing season in the mineral horizon (Fig. 2c, d). This might be connected with the abundant growth of grasses at the alder sites (unpublished observation) that may transfer N to the soil when they die off in autumn. The high concentration of dissolved N may also help explain why the C:N ratio was lowest at the alder sites. According to Berg and McClaugherty (2008), a high N concentration can result in an increased production of DOM. This is also in accordance with an observed positive correlation between WSC or HWC and N concentrations (Table 5) at most of the sites for both the mineral and organic horizon.

Whilst seasonality seems to have great impact on the variability of WSC, HWC, and N concentrations, the quality of WEOM is rather influenced by tree species. The RDA analysis indicated that even the season affects the WEOM composition, the tree species is rather more important. This is also in accordance with a study of Embacher et al. (2007) who reported a marked seasonality in WEOM concentrations, but the quality was driven mainly by site.

We expected a high contribution of aromatic components in WEOM as indicated by the SUVA at spruce sites because of the high aromatic/phenolic content in conifer litter. Indeed, the aromaticity was higher for all extracts at the spruce sites than at the other sites with an exception for HWE in the organic horizon (Table 3). The differences in SUVA values between deciduous and evergreen sites can be also due to distinct biodegradability processes of deciduous and evergreen litter. Don and Kalbitz (2005) reported that the contribution of lignin-derived compounds to DOC increased during the decomposition of coniferous litter. The authors connected the increase of aromatic compounds with a decay of thick epidermic and hypodermic layers of needles at a certain decomposition stage.

The aromaticity of WSE was slightly lower for the organic horizon than for the mineral horizon. We assume that the lower aromaticity in the organic horizon followed from a higher contribution of less degraded OM to WEOM. This is related to the fact that UV absorbance is often well correlated with biodegradability (Marschner and Kalbitz, 2003).

Forest soils tend to higher values of WEOC concentrations than grassland or arable soils as reported in a review by Chantigny (2003). The WEOC concentrations at the studied post-mining sites were similar to those from natural forests observed by others (e.g. Kalbitz et al., 2003; Landgraf et al., 2006). The willow depression sites showed the highest amounts of WSC and HWC in the organic horizon followed by the spruce sites. This can be explained by the presence of a thick organic horizon and therefore an abundant source of WSC and HWC at these sites. Willow depression sites were probably in addition supplied with organic matter from the top of the ridge.

The mean WSC concentrations for the mineral horizon showed different magnitudes at the sites in the following order: alder > oak > spruce > willow. For HWC concentrations, the order was switched for the spruce and willow sites. It is remarkable that the sites with highest concentrations in the organic horizon had the lowest ones in the mineral horizon. A possible explanation may be that WSC and HWC are mineralized to a larger extent in well-developed organic horizons and therefore, less WEOM is leached into the horizons below. Another reason may be a lower leaching of WEOM that is retained in the thicker organic horizon. No substantial difference was found when comparing WSC concentrations at the spruce and deciduous sites which is in accordance with the results of others (e.g., Michalzik et al., 2001).

5. Conclusion

The concentration and chemical composition of WEOM in forest soils varied throughout the year. Our findings demonstrate that the variation in quality and quantity resulted from a combination of tree species and seasonality effects. The combination of seasonal changes in decomposition of both fresh and already decomposed litter, precipitation and temperature, and microbial activity mainly controlled changes in concentration of WEOM throughout the year. Also tree species affected the concentration of WEOM. However, the effect of species is distinct in the quality of WEOM.

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