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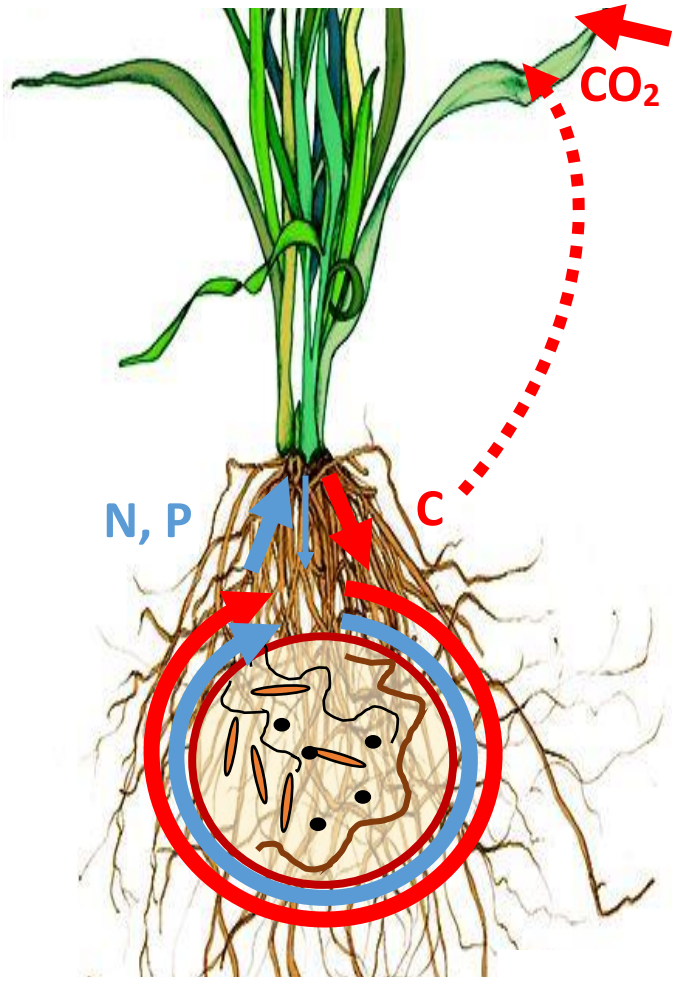
Plant–microbial–soil interactions as drivers of ecosystem C and N cycling

Habilitation thesis



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Plant–microbial–soil interactions play a central role in biogeochemical carbon (C) and nitrogen (N) cycles (Wardle et al. 2004; van der Heijden et al. 2008). Plant photosynthetic C fixation represents the main C input into terrestrial ecosystems that sustains the functioning of both the "green and brown" parts of the systems. Through the amounts and chemistry of C inputs into the soil, plants modify soil physico-chemical characteristics and regulate and feed diverse communities composed of symbiotic, root-associated and free-living organisms involved in the soil food web (Wardle et al. 2004; Fornara et al. 2011; Doornbos et al. 2012; Bardgett et al. 2014). The soil communities decompose the plant inputs and transform them into stable soil organic matter (SOM) (Kogel-Knabner 2000; Miltner et al. 2012 and others; Kallenbach et al. 2016) in processes during which CO_2 is evolved and nutrients strongly limiting plant productivity are released in available forms (Chen et al. 2014). In this way, heterotrophic microbial activity feeds back on plant nutrient acquisition and, consequently, influences selection of plant traits, plant diversity and production of the system (van der Heijden et al. 2008; Friesen et al. 2011; Lau & Lennon 2011).

The key role of linkages between plants and soil microorganisms and microbially mediated soil processes in functioning of terrestrial ecosystems under present conditions and in the response to ongoing environmental changes has been increasingly recognized (Bardgett et al. 2009; Chapin et al. 2009; Freschet et al. 2013). Understanding impacts of human-induced environmental changes on the functioning of terrestrial ecosystems requires a combined aboveground–belowground approach. At the same time, the aboveground–belowground relationships should to be studied at different spatial and temporal scales (Bardgett et al. 2005). Small spatial scale studies working with individual plants and their associated soil microorganisms enable to explore the role of root C efflux and its quality in mediating plant–microbial–soil interactions, while studies at larger scale are needed to link plant productivity and vegetation diversity (representing C input to soil and its quality, respectively) to ecosystem C and nutrient fluxes. The aboveground–belowground relationships operate over hierarchy of temporal scales, ranging from days to seasons, to millenia, with different key drivers and mediators of the interactions, different consequences for ecosystem structure and functions and different methods required for the studies (Bardgett et al. 2005).

This habilitation covers a part of my work focused on understanding of plant–microbial–soil interactions, which operate at day to seasonal scale, and their implications for C and N cycling in non-forest temperate systems. The thesis consists of two parts. The first part is focused on rhizodeposition as a key mediator linking plants and microbes. I specifically address my research contribution to the quantification and characterization of rhizodeposition flux as an important part of plant C budget and a significant soil C input, with some methodological aspects of isotope labeling approach used for such purpose. The second part summarizes my research in grasslands and peatlands. It has increased the knowledge on how is the variation in plant–microbial–soil interactions related to different plant traits and how changing environmental conditions impact ecosystem functioning with the main focus on biogeochemical C and N cycling and soil C sequestration.

PLANT INPUTS TO THE SOIL

Plant-derived C inputs to the soil, which supply heterotrophic microbial activity and represent a source for SOM formation, consist of root and shoot litter and root rhizodeposits. The two sources represent qualitatively different fluxes, which enter the soil at different times of the year, select for different soil microbial communities and vary in their impacts on soil C and N cycling (Knops et al. 2002; De Deyn et al. 2008; Orwin et al. 2010; Metcalfe et al. 2011; Grigulis et al. 2013a).

Litter originating from aboveground and belowground plant tissues is predominantly composed of structural C-rich compounds, dominated by cellulose and hemicelluloses complexed with lignin structures and covered with protective waxes (Gleixner et al. 1999). It enters the soil mostly at the end of the vegetation season when plants become dormant and a part of their biomass senesces. Litter feeds the "winter microbial community" dominated by fungi (Lipson & Schmidt 2004; Bardgett et al. 2005; Schmidt et al. 2007), which are able to cleave the complex compounds to low-molecular weight forms due to the production of wide spectrum of extracellular enzymes. The winter community immobilizes nutrients, especially N, in its biomass having slow turnover during the time when there is no plant nutrient demand and thus plays an important role in N retention in the ecosystem (Schmidt & Lipson 2004; Schmidt et al. 2007). Litter at different levels of decay retain in the soil for relatively long time (months to years) and form a large portion of SOM.

"Rhizodeposits" collectively refer to organic compounds, which are continually released to the soil by living roots during the vegetation season in the process called rhizodeposition (Kuzyakov & Domanski 2000; Jones et al. 2009; Pausch & Kuzyakov 2018). Differently from the rather uniformly composed litter, rhizodeposits consist of a mixture of highly available low molecular-weight root exudates, polymeric mucilage, lysates from the turnover of root hairs and epidermis, enzymes, siderophores, vitamins, hormones, and even whole cells sloughed-off from the edges of root caps (Jones et al. 2004; Neumann et al. 2009; Dennis et al. 2010). Through this flux, plants regulate a wide range of soil properties in the vicinity of their roots such as pH, water availability, nutrient mobilization (Hutsch et al. 2002) or aggregate formation (Six et al. 2004). Rhizodeposits are inputs that supply C and energy (and to some extent N and P) to microbes thereby influencing microbial structure along a continuum from symbionts and root-associated microorganisms to classical heterotrophs, which co-metabolize original or transformed root-released compounds together with stable SOM (Hogberg & Read 2006; Paterson et al. 2007). Through the root-derived C inputs and specifically through rhizodeposition, plants form a unique and highly spatio-temporaly dynamic environment in the vicinity of their roots, which is called the rhizosphere (Jones et al. 2004; Kuzyakov & Xu 2013; Kuzyakov & Blagodatskaya 2015) [1].

[1] The role of roots and their interactions with soil microorganisms through rhizodeposition was shortly summarized for Czech readers in the popular science book of Hana Šantrůčková et al. (2018): Ekologie půdy. Episteme, 259 p. ISBN 978-80-7394-695-1

THE ROLE OF PLANT C INPUTS IN SOIL PROCESSES AND C SEQUESTRATION

Historically, the amounts and quality of aboveground plant litter inputs were mainly addressed in relation to soil C sequestration. However, many long-term residue management studies held in arable systems showed that aboveground litter has only a limited impact on soil C levels (Campbell et al. 1991; Stewart et al. 2007; Heitkamp et al. 2012) as compared to that of root systems (Rasse et al. 2005; Freschet et al. 2013). Similarly, our data obtained during experiments held in semi-natural grasslands suggested that management-induced changes in plant C partitioning below ground rather than variation in the aboveground production affected soil C levels, microbial biomass and its activity in the soil (e.g. **Uhlirova et al. 2005** comparing cutted, mulched and unmanaged grasslands; **Kotas et al. 2017** comparing grasslands under various combinations of long-term fertilization and cutting).

In recent studies, root traits are recognized as significant players, which drive ecosystem processes (Freschet et al. 2013; Bardgett et al. 2014). The rooting depth, root size and density and physiological traits such as root respiration, rhizodeposition, nutrient uptake kinetics and biotic traits that involve interaction with symbionts determine the character and extent of the rhizosphere. A continual input of rhizodeposits makes the rhizosphere the most important hot spot of microbial activity in soil during vegetation season (Kuzyakov & Blagodatskaya 2015). The intensity and magnitude of processes running in the rhizosphere highly exceed its proportional volume, being only ca 5-25 % of the total soil volume (Finzi et al. 2015). This makes rhizosphere processes quantitatively important components of soil element cycling (Finzi et al. 2015) and important drivers of ecosystem functions like soil CO_2 efflux (Metcalfe et al. 2011) and soil C sequestration (De Deyn et al. 2008; Bardgett et al. 2009).

ISOTOPIC LABELING TECHNIQUES AS A TOOL FOR STUDYING RHIZODEPOSITION

Plant belowground C flux forms the "hidden" part of primary production and rhizodeposition remains the most uncertain piece of this flux and of soil C cycle. It is highly dynamic, rhizodeposits are rapidly redistributed among various soil C pools and CO_2 and all this runs on the background of the large soil C pool.

Exploring the dynamics of assimilate allocation in plant–soil system and subsequent separation of rhizodeposits from soil-derived C is possible using stable (^{13}C) and radioactive (^{14}C) isotope labeling techniques or ^{13}C natural abundance approaches. Practically, two main approaches in isotopic techniques can be distinguished: pulse and continuous (steady-state) labeling (Warembourg & Estelrich 2000; Studer et al. 2014).

During pulse labeling, plants are exposed to highly ^{13}C or ^{14}C enriched CO_2 over a short time period of a few hours. Assimilates synthesized during that period are massively labeled, which enables tracking their rapid distribution in the plant–soil system, including their release from roots – the exudation of recent assimilates. We advantageously used pulse labeling for assessing the transfer time of the recent assimilates from leaves to the

soil, for the quantification of their exudation flux and dynamics in small soil C pools with fast turnover: dissolved organic C and microbial biomass. In a semi-natural grassland under field conditions, we focused on ^{13}C partitioning among several soil C pools, on their turnover and on final relative ^{13}C distribution in the system (**Kastovska & Santruckova 2007**). In microcosms with individual plants grown in the soil from their original biotope, we tested the effect of increasing soil N availability on C fixation and assimilate distribution in the system with the focus on belowground C fluxes of two wetland graminoids – one more conservative and another more competitive in their nutritional strategies (**Kastovska et al. 2015; Kastovska et al. 2017**). In **Edwards et al.** (**2018**), we compared exudation fluxes of recent assimilates and their decomposability among three peatland species.

Continuous labeling, when plants assimilate labeled CO_2 (commonly cheap ^{13}C-depleted CO_2) over a longer time period of days to weeks (Schnyder et al. 2004), even years (Free Air CO_2 Enrichment (FACE) experiments), ensures the homogeneous labeling of whole plant biomass and, therefore, of all sources of root-derived C flux. Continuous labeling thus enables a reliable quantification of total net root C input over the labeling period, its contribution to different SOM pools and to soil CO_2 efflux (Thornton et al. 2004; Esperschutz et al. 2009). We started to use the continuous labeling technique in the frame of the running GA CZ project No. 16-21743S for studying plant-mediated rhizosphere effects of two graminoids (conservative and competitive) on soil C and N cycling, SOM formation and mineralization of preexisting soil C. Similar information as from continuous labeling can be obtained using the ^{13}C natural abundance approach, when C3 is changed for C4 vegetation and vice versa [2].

RECENT ASSIMILATES AS AN IMPORTANT COMPONENT OF RHIZODEPOSITION FLUX

Rhizodeposition is supplied by both recent assimilates and compounds derived from "older" root C: a continuous root turnover, secretions (e.g. enzymes and metallophores) or previously assimilated C mobilized either from senescing tissues or from stores (Thornton et al. 2004). The recent assimilates are transported in soluble forms to the non-mature, leaky root elongation zone, where from may be lost by diffusion. This flux of simple soluble compounds is called exudation. Differently, the chemical forms released from the older C are predominantly more complex organic compounds, reflecting their prior utilization in biochemical pathways within the plant. These compounds may be processed at different rates in soil, and most likely by different microorganisms than exudates (Jaeger et al. 1999; Thornton et al. 2004).

Recently assimilated C is an important source for rhizodeposition (Meharg & Killham 1988). It is evidenced by pulse-labeling studies showing that rhizodeposition is driven and regulated by plant photosynthetic activity (Kuzyakov & Cheng 2001; Kuzyakov & Cheng 2004). Transport of recent assimilates below ground is a fast process. Roots serve as a tubing system providing their rapid transport and release to the soil. Consequently, the

[2] Studying C flux in the plant–soil–atmosphere system using ^{13}C labeling was shortly summarized for Czech readers in the popular science book of Jiří Šantrůček, Hana Šantrůčková et al. (2018): Stabilní izotopy biogenních prvků. Použití v biologii a ekologii. Academia, 175 p. ISBN 978-80-200-2772-6

recently fixed ^{13}C can be detected in the soil soluble C pool and root-derived CO_2 efflux already within one hour after fixation and the exudation commonly peaks between 3-48 hours after fixation (Warembourg & Estelrich 2000; Domanski et al. 2001; Leake et al. 2006; **Kastovska & Santruckova 2007; Kastovska et al. 2017**). The exudation of recent assimilates can reach from 1 % up to 15 % of net C fixation by the plant (Farrar et al. 2003; **Kastovska & Santruckova 2007; Kastovska et al. 2017**).

Root exudates released to the soil solution have very short turnover time in the order of minutes or hours because these are rapidly utilized by soil microbes (Jones & Kielland 2002; Boddy et al. 2007; **Kastovska & Santruckova 2007**; Rousk & Jones 2010). Microbial C pool is always highly enriched in new C and commonly contains about 40-80 % of the labeled C found in the soil. It indicates that soil microbial community plays a crucial role in exudate transformation (Kuzyakov et al. 2001; Butler et al. 2003). Microbes use exudates primarily in energy metabolism and rapidly respire about 60-80 % of them (Hutsch et al. 2002; Kuzyakov & Domanski 2002; Leake et al. 2006). This emphasizes the proposed role of recent assimilates as the driver of soil microbial activity (Warembourg & Estelrich 2000). Microbes actively growing on exudates have turnover time of 3-5 days (Butler et al. 2003; Boddy et al. 2007; **Kastovska & Santruckova 2007**; Malik et al. 2015). Such short biomass turnover is comparable to values measured using growth kinetics after glucose amendment under optimum laboratory conditions (Anderson & Domsch 1986; Blagodatskaya et al. 2014). This indicates that the rhizosphere contains a sufficient amount of substrate to supply microbial growth differently from the bulk soil, which is commonly considered C limited (Alden et al. 2001; De Nobili et al. 2001).

The allocation of labeled assimilates from shoots below ground continues in smaller amounts for about one to two weeks after their fixation (Leake et al. 2006) before the aboveground–belowground partitioning of recent assimilates is completed (Warembourg & Estelrich 2000). During that time, the ^{13}C amount in root biomass may still increase but the soil ^{13}C commonly decreases due to its microbial respiration. Finally, only about 1-5 % of plant ^{13}C fixation remains in the soil - in the soil microbial biomass and stable SOM (Kuzyakov & Domanski 2000; **Kastovska et al. 2017**; Pausch & Kuzyakov 2018). The "microbial stabilization" seems to be the main mechanism of exudate sequestration in soil.

OTHER COMPONENTS OF RHIZODEPOSITION DERIVED FROM "OLD C"

The components of rhizodeposition derived from old root C cannot be tracked using $^{13}CO_2$ pulse labeling approach. Continuous labeling which traces all the compounds released from roots gives an information about the net root C input to the soil but does not discriminate between particular components of rhizodeposition and does not include the respired portion of rhizodeposition. Therefore, the information about the contribution of recent assimilates and other C sources in the rhizodeposition flux is very sparse and requires specific approaches.

Using continuous $^{13}CO_2$ labeling of plants grown in liquid nutrient media, Thornton et al. (2004) estimated that recent assimilates form ca 50-60 % of the rhizodeposition flux. Jones et al. (2009) estimated the root turnover-derived C flux from common standing root

biomass and its turnover in grasslands. It reached 2-10 g C kg^{-1} soil $month^{-1}$, which is comparable to his calculated range of exudation flux of 0.1-5 g C kg^{-1} soil $month^{-1}$.

In **Kastovska et al. (2017)**, we proposed an approach, which enabled the estimation of exudation and root death (a source of root lysates), and the total rhizodeposition flux as the sum of the two components, under conditions when plant grow in soil. We combined ^{13}C pulse labeling to assess the exudation flux and gross root growth rate (calculated from the final ^{13}C allocation to roots in the system with completed ^{13}C fluxes) with direct measurements of net root growth rate. In this way, we could roughly estimate root death. In our systems, the exudation flux reached 5-20 µg C g^{-1} root-C day^{-1}. The C flux derived from dead roots was 4-20 µg C g^{-1} root-C day^{-1}, being in the same order of magnitude as the exudation flux. Root exudates accounted for 20-70 % of the rhizodeposition flux, with much lower contribution under conditions of increasing soil N availability. This wide range of values is similar to the 10-60 % of rhizodeposition found in other studies (Paterson 2003; Thornton et al. 2004; Jones et al. 2009).

THE IMPORTANCE OF RHIZODEPOSITION IN PLANT C BUDGET

Using isotopic tracer methods, it was shown that plants allocate a large portion of their gross primary production below ground. Annual plants, mainly represented by crops breed for aboveground biomass production, allocate about 20-30 % of total assimilated C below ground. Perennial plants, mainly grasses, which rely on C reserves in their roots for regrowth in spring and after cutting or grazing, allocate 30-50 % or even more (up to 70 %) of assimilates below ground (Meharg 1994; Kuzyakov & Domanski 2000; Kuzyakov 2001; Kuzyakov et al. 2001; **Kastovska et al. 2015**). In absolute numbers, the total belowground C flux can reach 1500-2200 g C m^{-2} during the vegetation season (Kuzyakov & Domanski 2000; Saggar & Hedley 2001). Similar results were observed for trees, which allocate from 20 % to 60 % of the fixed C below ground, which represents ca 400-2400 g C m^{-2} yr^{-1} (Litton et al. 2007; Litton & Giardina 2008).

The carbon allocated below ground is then partitioned among several pools and processes depending on the inherent strategy of the plant species and current environmental conditions. On average, half of the belowground allocated assimilates is invested into the root growth (including growth of mycorrhiza and endosymbionts), through which the plant explores the surrounding soil to gain nutrients and water. The other half of belowground C is lost through rhizodeposition and root respiration (Kuzyakov & Domanski 2000; Kuzyakov et al. 2001). Both “C losses” are unavoidable for the plant. Root respiration relates to root energy metabolism and maintenance, while rhizodeposition promotes the activity of the rhizosphere microbial community, which is connected with the release of limiting nutrients from SOM.

In summary, rhizodeposition is a non-negligible part of the plant C economy, which may account for 5-30 % of C fixation (Farrar et al. 2003; Jones et al. 2004). Although the rhizodeposition C flux is commonly less than C investments to root growth: e.g. reaching 30-70 % of investments into root production in case of our studied plant species *Carex acuta* and *Glyceria maxima* (**Kastovska et al. 2017**), there are ecosystems like montane and alpine pasture systems, where rhizodeposition C loss may even exceed the investments into root

production (Pausch & Kuzyakov 2018). Therefore, rhizodeposition represents a highly significant soil C input, which is quantitatively comparable to that from senescing root mass (root litter).

FACTORS INFLUENCING PLANT C ALLOCATION PATTERN AND RHIZODEPOSITION

Plant C allocation pattern and, consecutively, also rhizodeposition are primarily determined by inherent plant factors: plant species and its developmental stage (Kuzyakov & Domanski 2002; Cheng et al. 2003). Moreover, the exudation of simple compounds as an important part of the rhizodeposition flux largely depends on plant photosynthetic activity (Kuzyakov & Cheng 2001), and thus on controlling factors such as light intensity and temperature. Consequently, exudation has a strong diurnal and seasonal pattern in the flux as well as in compound composition (Aulakh et al. 2001; Kuzyakov et al. 2001; Kuzyakov et al. 2002; Staley et al. 2017; **Edwards et al. 2018**).

Additionally, many other environmental factors importantly impact plant growth and subsequent C distribution: atmospheric CO_2 concentration, soil physical and chemical conditions such as soil pH, availability of limiting nutrients or toxic compounds, soil aeration and soil moisture (Cheng et al. 2003; Nguyen 2003). Similarly important are biotic environmental factors such as microbial biomass, activity and composition of the soil microbial populations, symbionts and phytopathogens living in plant tissues or in the root surroundings (Lambers et al. 2009; Friesen et al. 2011). In summary, changes in plant C allocation enable a plant to adapt to the given environmental conditions as well as to their changes. At the same time, the changes in rhizodeposition and namely exudation flux and its composition are the most rapid and dynamic plant reactions to changing environmental conditions.

PART II. PLANT–MICROBIAL–SOIL INTERACTIONS IN THE CONTEXT OF ECOSYSTEM C AND N CYCLING

The following part of the thesis consists of our research focused on processes involved in C and N cycling in two types of ecosystems, peatlands and grasslands. In peatlands, we aimed to understand the links between the spatial heterogeneity of the plant and soil characteristics and associated soil processes. In grasslands, we studied the functioning of habitats differing in soil organic matter content and, consequently, in plant dominants under medium-to-long term fertilization. We always combined a field work with detailed studies on the quantification and characterization of plant C allocation patterns and rhizodeposition fluxes of targeted species. It enabled us to identify key changes in plant–microbial–soil interactions and to understand the mechanisms driving C and nutrient cycling at the ecosystem level under changing environmental conditions.

PLANT–SOIL INTERACTIONS AS A MAIN CONTRIBUTOR TO SPATIAL FUNCTIONAL DIVERSITY IN PEATLANDS

Peatlands are in the center of researchers´ interest due to their vegetation diversity, hydrologic function and accumulation of large amounts of C thanks to specific conditions

such as the prevailing soil anoxia and low mean temperatures associated with the high altitudes or latitudes where peatlands are predominantly found (Limpens et al. 2008). Understanding peatland functioning and its changes after drying and rewetting is a traditional research area of our team. Our previous work has been mainly focused on the characterization of greenhouse gas emissions (Urbanova et al. 2013a; Urbanova et al. 2013b), soil microbial communities (Urbanova & Barta 2014; Urbanova & Barta 2016) and characterization of dissolved organic matter in pristine and disturbed peatlands (Mastny et al. 2016; Mastný et al. 2018; Urbanova et al. 2018).

Recently, a few studies emerged which stressed the importance of plant species for gas exchange and decomposition processes in peatlands. The papers mostly investigated the cascade effects of different plant species (or plant functional types) on the composition of soil microbial communities in the context of ongoing global climate changes (Jassey et al. 2013; Bragazza et al. 2015; Robroek et al. 2015) or on the chemistry and biodegradability of dissolved organic matter, with an implication for its losses via microbial respiration and export from peatlands (Pinsonneault et al. 2016; Robroek et al. 2016). We contributed to this research field by studies linking common peatland plant species with characteristics of the peat formed in their presence and ongoing soil processes. Our experimental sites were spruce swamp forests, which are some of the most common but rarely studied types of peatlands in Europe. The spruce swamp forests occurring in Central Europe and parts of Western Europe belong to nutrient-poorer types of peatlands due to their location in mountain areas formed by old, nutrient-poor, high-grade metamorphic rocks (the so-called Moldanubicum zone). Their understory vegetation is commonly dominated by *Sphagnum* mosses on the wettest places, followed by cotton-grass (*Eriophorum vaginatum*) lawn occurring with high coverages on less wet open areas and by ericoids, namely blueberries (*Vaccinium myrtillus*) co-dominating on drier hummocks. The patchy distribution of the vegetation formed by these three different functional types - mosses, graminoids and ericoids, which reflects water level variations in the system, provides a perfect experimental design for studying plant–microbial–soil interactions in field conditions.

We have shown that the patchy distribution of vegetation visible above ground is significantly reflected below ground in peat characteristics, microbial communities as well as dominant soil processes **(Edwards et al. 2018;** Chroňáková et al. in prep.**; Kastovska et al. 2018**). The peat formed in habitats dominated by different species have its specific fingerprints of organic compound composition, reflecting differences in the biomass composition of the three plant dominants (**Kastovska et al. 2018**). Significant differences were also found in the peat C:N:P stoichiometry, nutrient availabilities and microbial activities in particular habitats. Specifically, the habitats occupied solely by *Spagnum* were richest in ammonia and had the highest pH within the whole peatland but their microbial biomass and activities were low. Differently, the drier, more aerated habitats co-dominated by blueberries had the highest soil microbial biomass, with important contribution by saprotrophic and mycorrhizal fungi (Chroňáková et al., in preparation) with enhanced activities of extracellular enzymes, increased P availability and promoted peat decomposition (**Kastovska et al. 2018**). These results agreed with observations of other authors documenting a stimulative effect of an ericoid spread on peat decomposition and C loss from peatlands (Bragazza et al. 2013; Jassey et al. 2013; Bragazza et al. 2015). A similar

stimulative effect is generally anticipated in the presence of graminoids, which are known for even higher photosynthetic capacities (Leppala et al. 2008), exudation rates (Jassey et al. 2013) but lower contents of phenolics and lignin in their litter than ericoids (Hobbie 1996). However, contrary to these expectations, we did not observe any sign of an enhanced peat decomposition in the presence of cotton-grass (**Kastovska et al. 2018**). The likely reasons can be the prevailing anoxia due to the high water level in cotton-grass dominated habitats, which may limit microbial activity similarly as in the *Sphagnum*-dominated habitats but also some inherent attributes of the plant alone. Primarilly, cotton-grass has the most efficient nutrient resorption from the studied species (**Kastovska et al. 2018**) and is able to immobilize nutrients very effectively due to its large biomass productivity (Tuittila et al. 1999), significant allocation of nutrients in the slowly decomposing storage organs (Cholewa & Griffith 2004) and a long life span of individual tussocks (Shaver et al. 1986). We further found that the soil microbial biomass in cotton-grass dominated habitats bounds relatively large proportions of total soil N and P, while the residual peat was strongly nutrient depleted. In fact, the peat formed in the presence of cotton-grass was poorest in nutrients within the peatland. This is a sign of tightly coupled plant–microbial–soil relations causing an efficient nutrient mining from the peat and its subsequent immobilization in the plant–microbial system (**Kastovska et al. 2018**). Additionally, the root litter of cotton-grass decomposed even slower than *Sphagnum* tissue, potentially contributing to the C accumulation in these habitats. In summary, we showed that plant–microbial–soil interactions are the main contributors to the spatial functional diversity inside a peatland ecosystem. The potential effect of the presence and spreading of vascular plants on ecosystem functioning is species-specific and, contrary to general expectations, need not necessarily result in increased organic matter decomposition.

We continued to uncover the exceptional ability of cotton-grass and associated microbes to acquire nutrients from the nutrient-limited environment and to clarify differences in nutrient cycling inside the spruce swamp forest. We compared the seasonal quality and quantity of exudates released by the three plant dominants and how this could possibly affect their ability for nutrient acquisition from the peat (**Edwards et al. 2018**). Keeping the conditions close to natural, we collected the exudates from large plant clumps, which were brought from the field shortly before exudate sampling. We found that plant species significantly affected exudate quantity and quality as well as timing. Cotton-grass as the most efficient photosynthesizer from the studied species (Leppala et al. 2008) released the highest amounts of organic C on a root-mass basis during the whole vegetation season, followed by blueberry and *Sphagnum* (exudation expressed per tissue-mass basis in this case). Both vascular species further released significantly greater amounts of N (up to several mg N g^{-1} root h^{-1}) and thus their exudates were of significantly lower C:N ratios and greater degradability compared to *Sphagnum* (**Edwards et al. 2018**).

Our data about N exudation of the three species is an important contribution to the limited dataset and incomplete information about this phenomenon (Hinsinger et al. 2009). Generally, the rhizosphere is thought to be a C-excess environment and rhizodeposits are expected to be composed of C-rich but N-poor compounds (Paterson 2003; Badri & Vivanco 2009), with modelled C:N ratios ranging from 8 to 100 (Drake et al. 2013). However, the majority of our stoichiometric results varied from 8 to 25, with the exception of the fall

Sphagnum exudation with a C:N of 80 (**Edwards et al. 2018**). This documents significant "N losses" in a nutrient-poor habitat like peatland, in which plants would be expected to retain nutrients and limit their costs (Aerts & Chapin 2000). Our data support the idea that the exudation of compounds containing a certain amount of N is a plant strategy to improve its own nutrition in a nutrient poor environment because it enables synthesis of N-demanding microbial exoenzymes needed to decompose stable SOM and mine the limiting nutrients from it (Drake et al. 2013). The total N flux from the plants to the rhizosphere represented more than 10 % of the dissolved N present in the soil of the spruce swamp forest in the summer period (**Edwards et al. 2018**). The efficient use of the delivered N by microbes for growth and nutrient mining, with a positive feedback for plant nutrition, is supported by the relatively large proportion of total ecosystem N and P immobilized in plant and microbial biomass in habitats dominated by vascular plants, especially cotton-grass (**Kastovska et al. 2018**).

Although there were significant temporal and species differences in exudate composition, organic acids (in particular ascorbic+oxalic, acetic+glycolic, lactic and formic acids) comprised the majority of the identified C, while sugars and amino acids each comprised < 20% of the exuded C. Especially cotton-grass exudates were rich in citric acid, most notably in summer at the time of maximum plant biomass, and amino acids compared to those of blueberry or *Sphagnum* (**Edwards et al. 2018**). The usually large proportion of organic acids in exudates is typically found when plants are growing in nutrient-limited habitats (Grayston & Campbell 1996; Dakora & Phillips 2002) and a greater release of citric acid and other chelating compounds help plants to relieve P deficiency (Marschner et al. 2011). The large exudation flux with a greater proportion of amino acids and chelating compounds from cotton-grass roots could help explain its much larger N and P uptake efficiencies compared to the other species and therefore the consequent significant depletion of these nutrients from the peat in cotton-grass dominated habitats (**Kastovska et al. 2018**). The large exudation flux rich in organic acids also fits to observations linking cotton-grass presence in peatlands with enhanced net ecosystem CO_2 exchange (Riutta et al. 2007; Laine et al. 2012) and methane efflux (Strom et al. 2003; Robroek et al. 2015). However, our exudate composition data significantly contrast to Saarnio et al. (2004), who studied exudation of cotton-grass grown in sand supplied with a weak nutrient solution, and noted a majority proportion of sugars (neutral compounds) in the identified exudates. Therefore, we stress a critical impact of sampling conditions on the quantity and quality of root exudates, as pointed out in other works (Grayston & Campbell 1996; Aulakh et al. 2001), and highlight the necessity of field work and studies conducted in conditions close to natural to get reliable data.

PLANT–SOIL INTERACTIONS CONTROLLING C AND N CYCLING AND SEQUESTRATION IN GRASSLANDS

Besides the unique peatland systems, my research effort is focused on understanding the functioning of temperate grasslands and pastures, which are an integral part of the Bohemian cultural landscape. While historically their production function was prioritized, nowadays they are rather considered as ecosystems supporting unique communities of plants, soil organisms, insects and birds, and together with woods and wetlands form the

frame for the ecological stability of the landscape. Moreover, grasslands represent significant carbon stocks in the global C cycle, of which more than 80 % is stored belowground as SOM (Conant et al. 2001; Pete 2014). Except for very limited areas of natural grasslands, the sustainability of these human-influenced biotopes and the protection of their numerous functions in the landscape including the C store function fully depend on suitable management.

European grasslands were traditionally maintained through regular extensive cutting or grazing, keeping their plant species richness (Galvanek & Leps 2008; **Kotas et al. 2017**; Smith et al. 2018) and preserving their inherently high SOM contents (**Uhlirova et al. 2005; Kotas et al. 2017**). The positive impacts of these managements occur through defoliation effects on the below ground food web, and thus SOM transformation and nutrient cycling. Regular defoliation leads to a short-term redistribution of assimilated C into shoot regrowth, but also into exudation, which enhances microbial activity and its substrate use efficiency (**Uhlirova et al. 2005**). This increased exudation feeds back to the plant through enhanced nutrient supply from the rhizosphere necessary for regrowth (Hamilton & Frank 2001; Kuzyakov et al. 2002; Hamilton et al. 2008). Therefore, in the longer-term, regular defoliation enhances the concentration of nutrients in the plant biomass and litter (Hamilton & Frank 2001) and structures the plant community (Grime & Mackey 2002; Mládková et al. 2015). However, changes in agricultural practices and land use pressures mean that grasslands are disappearing at an alarming rate due to abandonment or afforestation or they are impacted by intensification of their management or management of the surrounding arable and built-up areas. Consequently, semi-natural grasslands are among Europe's most threatened ecosystems. Grassland research is focused on studying the long-term impacts of management changes or alternative practices, which should be economically more convenient than mowing, on grassland performance.

In our research, we have focused mainly on the effects of mineral fertilization on grassland functioning. Targeted grassland fertilization aims to improve forage production and its nutritional quality and compensates for the slow depletion of soil nutrients (mainly N and K) connected with long-term mowing and biomass removal (Olde Venterink et al. 2009). In addition, mainly wet grasslands often face enhanced nutrient inputs from surrounding arable land, causing their eutrophication. The consequences of enhanced nutrient inputs are similar in both cases. Longer-term fertilization commonly causes changes in vegetation composition and loss of plant species richness (Hector et al. 1999; Hautier et al. 2014), while its impact on the soil microbial community and processes is less predictable and sometimes even contradictory. The results of our field observations mainly showed negative fertilization impacts on grassland performance and are summarized in several publications (**Picek et al. 2008**; Kaplova et al. 2011; **Kastovska et al. 2012**; Edwards 2015; Edwards et al. 2015; **Kotas et al. 2017**). In all the studied grasslands, enhanced nutrient input lasting more than five years accelerated soil N cycling and enhanced the potential for N losses as indicated by faster net nitrification and denitrification rates, and nitrate concentrations in the soil. The changes in soil N cycling were closely linked with C transformation processes and negatively impacted SOM sequestration. An early manifestation of the detrimental changes in soil functions was a declining soil microbial biomass, which is in accord with the results of other studies (Treseder 2008; Sillen &

Dieleman 2012). The negative fertilization effects rose from a combination of shifted plant resource allocation, soil acidification and lowered soil cation exchange capacity. Consequently, the potential for microbial C immobilization and stabilization of organic compounds decreased and resulted in decreased SOM levels in the grasslands over the long-term (**Kastovska et al. 2012; Kotas et al. 2017**).

The above described fertilization effects occurred in all studied grasslands, however, the extent and significance of the changes visible above- and below ground varied among studied sites (**Picek et al. 2008; Kastovska et al. 2012**). To understand the different vulnerabilities of the systems, we did few complementary laboratory experiments. In the first one, we focused on differences in the microbial utilization of glucose as a typical representative of root exudates under conditions of enhanced soil N availability (**Kastovska et al. 2010**). We have shown that enhanced N input shifted soil microbial metabolism towards a preferential utilization of soluble substrates over pre-existing stable SOM but, at the same time, decreased the microbial growth rate and the efficiency of glucose utilization, which resulted in a lower potential for stabilization of new C in the soil. This negative effect of N fertilization was more pronounced in the soil richer in organic matter with larger soil microbial biomass, which agreed with our field observations.

We further studied C budgets and economics of two main plant dominants of our experimental sites, *Carex acuta* typical for the oligotrophic sites richer in organic matter (Ellenberg indicator value N 4) and *Glyceria maxima* preferring mesotrophic and more mineral sites (Ellenberg indicator value N 9). The variety of their functional traits known from the literature (Grime & Mackey 2002) and our field observations (Kaplova et al. 2011; **Kastovska et al. 2012**; Edwards 2015) showed that *Carex acuta* and *Glyceria maxima* are representatives of conservative and competitive wetland species, respectively. They show most of the following criteria separating the two plant life strategies. Generally, a conservative life strategy, based on a high level of resource conservation is characteristic for plant species that persist in stressful conditions (e.g. low availability of nutrients, light, water, oxygen, extremes in soil pH). Conservative species preferentially allocate biomass below ground, where they form nutrient poor long-lived root systems with a large proportion of structural and reserve compounds and often involve mycorrhiza. Differently, competitive (or exploitative) species dominating in more plentiful conditions grow faster and preferentially allocate resources above ground to build photosynthetically active tissues. The faster growth trades off with a shorter lifespan, lower dry matter and C content in the tissues in comparison to slow-growing plants (Aerts et al. 1992; Hobbie 1992; Aerts & Chapin 2000). The plant life strategy (predominance of conservative versus competitive traits) thus determines the relative inputs of above- and belowground litter, and strongly controls overall litter quality and litter decomposition rates (Freschet et al. 2013). Consequently, dominance by exploitative species links with faster soil C and N cycling provided by the bacteria-dominated soil microbial community, while the opposite is true for slower growing conservative plants often associated with fungal-dominated soil microbial communities (Hobbie 1992; Craine et al. 2002; Orwin et al. 2010; de Vries & Bardgett 2012; Grigulis et al. 2013b).

Different attributes of plant growth rate, plant biomass chemistry and soil N cycling linked to plant growth strategy strongly indicate differences in rhizodeposition. Competitive

plants are thought to lose more C from roots than conservative ones because of (i) their larger photosynthetic capacity (Personeni & Loiseau 2005; Personeni et al. 2005; De Deyn et al. 2008) and thus larger quantities of assimilates, which can be allocated to roots and (ii) root structures with low levels of structural C allowing passive diffusion of exudates through the root tissue (Vale et al. 2005), with shorter life span and faster turnover, and possibly losing more root lysates. We were the first, who directly measured specific exudation fluxes of competitive and conservative species and linked them to microbial N transformation associated with such plants in the field (**Kastovska et al. 2015**). In agreement with expectations, we showed that the competitive *Glyceria*, as compared to the conservative *Carex*, invested significantly more C into root exudation under N limiting as well as N excess conditions. Therefore, *Glyceria* was associated with larger and temporally highly dynamic microbial biomass, providing faster N cycling. At the ecosystem level, this was manifested by higher N availability and pronounced seasonal N redistribution between plants and microbes in the *Glyceria*-dominated than the *Carex*-dominated grassland. *Glyceria*, through its high exudation flux, was more strongly coupled with soil microbes thereby covering plant N requirements. The conservative *Carex*, irrespective of its larger root system, invested less C to exudation. In this case, the plant–microbe relationships appeared to be less-coupled in time and space with slower plant N supply, relying mainly on the relatively slow microbial mineralization of organic matter than on the rhizosphere priming effect. In addition, a ^{15}N labeling study confirmed the greater competitiveness of *Glyceria* with soil microbes for all available N forms compared to *Carex* (**Kastovska & Santruckova 2011**). Our results also indicated that both plants can take up organic N forms but these cover only negligible portion of their N demand under conditions of strong microbial competition for the organic N occurring in soil. Therefore, a majority of N demand of both species was covered by the uptake of mineral N forms, mainly nitrate-N.

Under conditions of enhanced soil nutrient availability, both species shifted partitioning of belowground resources towards higher mass-specific root production but invested less into root exudation (**Kastovska et al. 2017**). In other words, they invested more in their own ability to acquire nutrients from soil than in supporting the plant–microbial cooperative system as the source of their nutrition. This is one of the likely reasons why other researchers point to a weakening of plant–microbial–soil interactions in nutrient luxury conditions (Bai et al. 2013; Wei et al. 2013). Since the faster growing roots of fertilized plants turned over faster, the total rhizodeposition flux of both species was enriched in predominantly more complex root lysates over soluble root exudates (**Kastovska et al. 2017**). This N fertilization-induced change in the quality of the daily root-derived C input to the soil implies differences in microbial utilization of the substrate and its potentially distinct role in soil organic matter dynamics (Jaeger et al. 1999). The changes found in soils either in the field (**Kastovska et al. 2012**) or in a microcosm laboratory experiment (**Kastovska et al. 2017**) indicated stimulation of exoenzymatic activity and thus faster decomposition, which resulted in increased concentrations of soil soluble C and consequent higher C losses from the soils. When comparing the response of the two species to N loading, *Carex* showed a higher plasticity in its allocation pattern and root physiological traits than *Glyceria*, such as a more pronounced increase in the shoot/root ratio, but also in the gross root growth rate and consequently the mass-specific rhizodeposition flux. This

higher plasticity in observed traits could be how conservative plants, being superior inhabitants of oligotrophic systems, can cope with faster-growing species in competition for light when growing in mesotrophic conditions (Wedin & Tilman 1990). However, at the same time, the excessive expenses for maintaining the root system are disadvantageous in productive N-rich environments. Therefore, *Carex* is finally replaced by stronger competitors for N such as *Glyceria* at a certain level of soil fertility, which was visible in our fertilized grasslands.

In summary, we propose a combination of studies at the ecosystem level with detailed experiments at the level of individual plants as a tool for learning the mechanisms which drive C and N cycling in the studied ecosystems. We showed that the total C input via rhizodeposition is closely related to the total root mass but its composition is greatly affected by important species-specific differences. This mainly impacts the exudation flux, which provides the coupling of plant–microbial–soil interactions over time and space and governs the differences in the velocity of soil N cycling associated with plants. We further suggest that fertilization mediated changes in soil C sequestration are modulated by root responses to N availability. Therefore, fertilization effects on the soil C levels will be always detrimental, if the total root mass will decrease as a consequence of changed allocation pattern of fertilized plants. Finally, belowground C partitioning and mainly the C costs connected with the maintenance of root functioning at a particular level of soil fertility, are important for potential plant species survival or replacement within the existing community.

CONCLUSIONS AND FUTURE PERSPECTIVES

Plant–microbial–soil interactions control C and N cycling in ecosystems. This interaction is mediated through rhizodeposition, the "hidden part" of the plant C budget. Our research presented in this thesis contributed to knowledge about rhizodeposition, which was repeatedly found to be an important part of the plant C budget and a significant C input into the soil, supporting primarily microbial energy metabolism and nutrient mining from SOM. We advantageously combined isotope labeling experiments held in laboratory conditions with the field work to understand in detail the mechanisms involved in the functioning of the studied systems – mainly grasslands and peatlands. In this way, the found species-specific differences in plant resource allocation and investments into nutrient acquisition through plant–microbial–soil relations can be linked with differences in C and nutrient cycling at the habitat or ecosystem level.

We continue to study the role of rhizodeposition flux and quality in soil processes in the frame of the running GA CZ project "C:N stoichiometry in plant-soil interactions: effects on plant metabolism and processes in the rhizosphere". There we focused mainly on the links between plant C:N stoichiometry and the C:N ratio and composition of root exudation and its consequent impact on soil C and N cycling. Besides using isotopic labeling for the quantification of exudation and the rhizodeposition flux, we newly focus on characterizing the root metabolic pool and released exudates by metabolomics and on the assimilation and channeling of the labeled C-compounds by various functional (and notional) groups of soil microorganisms using the PLFA-stable isotope probing method. Our first results show

that plant species can partly control the composition of exudates and modifies it sensitively in response to N availability. Although these changes do not impact on microbial community composition, they influence microbial processes related with SOM decomposition and nutrient mining.

Within the running project and also in our future research, we want to go further in studying the role of rhizodeposits as a substrate utilized by microbes in energy gaining processes and stimulating decomposition of pre-existing SOM but also as a source of new microbially stabilized SOM.

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PAPER 1

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REGULAR ARTICLE

Fate and dynamics of recently fixed C in pasture plant–soil system under field conditions

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Abstract The flow of photosynthetically fixed C from plants to selected soil C pools was studied after $^{13}CO_2$ pulse labeling of pasture plants under field conditions, dynamics of root-derived C in soil was assessed and turnover times of the soil C pools were estimated. The transport of the fixed C from shoots to the roots and into the soil was very fast. During 27 h, net C belowground allocation reached more than 10% of the fixed C and most of the C was already found in soil. Soil microbial biomass (C_{MIC}) was the major sink of the fixed C within soil C pools (ca 40–70% of soil ^{13}C depending on sampling time). Significant amounts of ^{13}C were also found in other labile soil C pools connected with microbial activity, in soluble organic C and C associated with microbial biomass (hot-water extract from the soil residue after chloroform fumigation-extraction) and the ^{13}C dynamics of all these pools followed that of the shoots. When the labelling (2 h) finished, the fixed ^{13}C was exponentially lost from the plant–soil system. The loss had two phases; the first rapid phase corresponded to the immediate respiration of ^{13}C during the first 24 h and the second slower loss was attributable to the turnover of ^{13}C assimilated in C_{MIC}. The corresponding turnover times for C_{MIC} were 1.1 days and 3.4 days respectively. Such short turnover times are comparable to those measured by growth kinetics after the substrate amendment in other studies, which indicates that microbial growth in the rhizosphere is probably not limited by substrate availability. Our results further confirmed the main role of the soil microbial community in the transformation of recently fixed C, short turnover time of the easily degradable C in the rhizosphere, and its negligible contribution to more stable soil C storage.

Keywords Carbon cycle · Carbon allocation · Microbial biomass · Pulse labelling

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Introduction

Rhizodeposition is the key process of C input into the soil. Experiments on perennial grasses showed that 10–20% of photosynthetically fixed C is released into the soil during the vegetation season (Hütsch et al. 2002; Kuzyakov and Domanski 2002; Nguyen 2003), which could represent ca 1500 kg C ha^{-2} in the case of *Lolium perenne* at the end of development (Kuzyakov and Domanski 2000; Kuzyakov et al. 2001). The majority of rhizodeposits are represented by root exudates, which are low molecular weight

water-soluble substances (Hütsch et al. 2002; Nguyen 2003). Their transformation dominates the rhizosphere C flow (Warembourg and Estelrich 2000). Root exudates, mainly composed of carbohydrates, amino-acids and organic acids (Hütsch et al. 2002), originate from newly assimilated C, which is rapidly transported from shoots to belowground (Ostle et al. 2003; Rangel-Castro et al. 2004), where they passively diffuse from intact root cells into the soil solution. Already in 1 h after photosynthetic fixation, new C could be detected in labile soil organic C (Leake et al. 2006), where it greatly influences the size and activity of the rhizosphere microbial community and microbially mediated C turnover.

Exudates in soil solution are known to have very short turnover times (in the order of minutes or hours) (Nguyen 2003; Jones et al. 2004; Boddy et al. 2007) and 64–86% of them are rapidly lost by rhizomicrobial respiration (Kuzyakov et al. 2001, 2003; Hütsch et al. 2002; Kuzyakov and Domanski 2002; Dilkes et al. 2004; Leake et al. 2006). Only about 2–5% of net plant C assimilation is kept in the soil (Hütsch et al. 2002). Its allocation and use in the belowground system reaches a maximum after 20–48 h after photosynthetic assimilation (Warembourg and Estelrich 2000; Leake et al. 2006), and most of the efflux is completed within a week (Warembourg and Estelrich 2000; Kuzyakov et al. 2001). Microbial biomass plays a crucial role in exudate C transformation and is always highly labelled by the new C (Kuzyakov et al. 2001; Ostle et al. 2003; Butler et al. 2004). This "biological C immobilization" seems to be the main way of exudate C fixation in soil. The new C turnover rate in the C_{MIC} and RNA pools was estimated from the label dynamic to be only 3–5 days (Ostle et al. 2003; Butler et al. 2004; Rangel-Castro et al. 2005). There is also a lack of information on the new C input to other soil C pools and its potential contribution to more stable soil C storage.

The majority of these data were obtained under controlled laboratory conditions from pot or mesocosm studies while there have been only a few studies conducted under field conditions. We studied the flow of the new assimilates, labelled by the $^{13}CO_2$ pulse, from pasture plants to the soil under field conditions. Our goal was to quantify the net transport of the fixed C from plants to the soil and the C flow through selected soil C pools. Soluble organic C, microbial biomass C and labile C associated with microbial biomass were chosen as representatives of labile organic C pools, and the C non-hydrolyzed in hot acid as a stable C pool. We calculated amounts of new C in these pools, estimated turnover times of the new carbon from the ^{13}C dynamics in these pools, and assessed a possible contribution of the exudates to C sequestration in soil.

Materials and methods

Study site

The study site is located in South Bohemia (near České Budějovice), Czech Republic, at about 400 m above sea level. The climate is moderate (mean annual temperature, 8°C) and rather dry (annual rainfall, 623 mm m^{-2}). The site is a regularly cut cultural grassland dominated by *Lolium perenne* and *Trifolium repens*. The soil is a sandy loam with bulk density of 1.16 g cm^{-3}, pH of 5.6, 1.87±0.17% C_{ORG}, and 0.15±0.02% N_{TOT}. Preliminary analysis with 0.1 M HCl showed that the soil was free of carbonate.

^{13}C pulse labelling

Labelling was done on September 17, 2006, between 11:00 and13:00 (sunny weather, maximum temperature 21°C). At that time (a month after cutting), the mean height of the vegetation was 12 cm. Four gas-tight chambers (50×50×50 cm) constructed of plexiglass (4 mm thickness), with the holes fitted with rubber-septa, were placed on selected sites of grassland with a similar vegetation cover. After the ambient CO_2 concentration in the chambers decreased below 200 ppm (checked by GC), $^{13}CO_2$ tracer (99.9 atom % ^{13}C, Cambridge Isotope Laboratories, GB) was repeatedly pulsed into the chambers by a syringe to keep the internal CO_2 concentration at 400–500 ppm for a period of 2 h. Total amount of 400 ml of $^{13}CO2$ was added to each chamber during the labelling. Air circulation during labelling was ensured by two, 6 cm^2 12 volt fans. The chambers were removed after labelling.

Harvesting procedure and soil sampling

Samples of soil and plant biomass were taken 2, 9, 26, 50, 74, and 148 h after the beginning of pulse-labelling

to include the first ^{13}C signal in soil and the quick flow through available C pools including microbial biomass. The first three days of sampling were sunny (daily maximum temperatures of 20°C), followed by partly cloudy days with a few showers (maximum temperatures of 15–17°C). Control samples for the ^{13}C natural abundance determination in the studied C pools were collected next to the chambers at the beginning of the labelling procedure. At each sampling time, above-ground biomass (shoots) was clipped from the area of 25 cm^2 (in duplicates) and two soil cores (5 cm diameter, 10 cm depth) per chamber were taken. The soil cores were pooled and homogenized, and roots were separated by hand. Plant biomass was washed to remove soil particles, dried at 60°C, and weighed. A part of the soil samples (5 g) were oven-dried at 105°C to determine water content and the remaining soil was processed immediately as follows.

Separation and determination of selected soil C pools

Fresh soil samples were treated immediately after sampling. Chloroform fumigation-extraction was used to determine microbial biomass carbon (C_{MIC}) (Vance et al. 1987; modified for ^{13}C analysis by Bruulsema and Duxbury 1996). Briefly, 10 g of soil was extracted with 20 ml of 50 mM K_2SO_4 for 30 min, centrifuged for 10 min at 4,000 g, and supernatant was filtered through a glass-fibre filter (GF/F). C_{MIC} was calculated as the difference between C content in fumigated (24 h) and non-fumigated extracts using K_{EC} of 0.45 to convert chloroform flush C to C_{MIC} (Vance et al. 1987). Other two labile C pools related to the microbial metabolism were separated: soluble organic c pool (C_{SOL}), represented by the C content in the 50 mM K_2SO_4 extract from the non-fumigated soil, and material derived from the microbial biomass ($C_{PAST\text{-}MIC}$), obtained by extraction of the soil residue after fumigation-extraction with 20 ml of hot distilled water (120°C for 1 h in autoclave) according to Lemaitre et al. (1995a, b). These two labile C pools differ mainly in chemical composition and origin of compounds they included. C_{SOL} is an important labile and mobile SOM fraction and the immediate source of energy for microorganisms (Zsolnay 2003). Major source of C_{SOL} is leaching of the old humified C, whereas new degradable C components make only minor contribution (Gregorich et al. 2000; Hagedorn et al. 2004). $C_{PAST\text{-}MIC}$ includes material mainly derived from C_{MIC}, which is not extractable by 50 mM K_2SO_4 after chloroform fumigation. It contains mainly amino sugars, amino acids and neutral sugars, which differ from the microbial components in C_{MIC} by their higher percentage of non-hydrolysable C (acid hydrolysis with 3 M HCl and 1.25 M H_2SO_4) and higher degree of transformation (Lemaitre et al. 1995b). Acid hydrolysis of the soil according to Leavitt et al. (1996) was used (6 M HCl at 110°C for 18 h; 1:10, soil to acid, w/v) to obtain non-hydrolysable C pool (residual C, $C_{NON\text{-}HYDR}$), which is mainly composed of stable humus (Stevenson and Cole 1999). After the acid hydrolysis, the non-hydrolysable soil fraction was kept on the glass-fibre filter GF/F (0.45μm), and chlorides were washed out with 50 ml of hot distilled water. The samples were dried at 105°C.

Dried shoot, root and soil samples, and freeze-dried soil extracts were finely ground in a mill and weighed in tin cups. Analyses of C and ^{13}C were conducted on an NC Elemental analyzer (ThermoQuest, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany). The ^{13}C atomic percent of the sample was determined and ^{13}C enrichments were also expressed as $\delta^{13}C$ in parts per thousand (‰) in relation to Pee Dee Belemnite as the reference standard material.

Calculation and statistical analysis

The following equation was used to calculate the $\delta^{13}C$ value of microbial biomass (Šantrůčková et al. 2000):

$$\delta^{13}C_{MIC} = \left(\delta^{13}C_{FLUSH} * C_{FLUSH} - \delta^{13}C_{SOL} * C_{SOL}\right)/\left(C_{FLUSH} - C_{SOL}\right) \quad (1)$$

where C_{FLUSH} and C_{SOL} refer to the amounts of C extracted from fumigated and non-fumigated soil, respectively, and $\delta^{13}C_{FLUSH}$ and $\delta^{13}C$ C_{SOL} to their $\delta^{13}C$ values.

A binary mixing model with ^{13}C atomic percent was used to calculate the amount of the pulse-derived ^{13}C in various studied C pools:

$$^{13}C\left(\mu g\, g^{-1}\right) = \left[\left(\text{at.}\%_{\text{sample}} - \text{at.}\%_{\text{control}}\right)/\left(99.9 - \text{at.}\%_{\text{control}}\right)\right] * \text{C pool size}\left(\mu g\, C\, g^{-1}\right) \quad (2)$$

where $_{control}$ is natural abundance in control samples and 99.9 is the pulse ^{13}C atomic percent.

Data on temporal changes in ^{13}C amounts in the studied pools were fitted using a one-phase exponential decay model in SigmaPlot 9.0 (Fig. 1). Turnover (mean life-time) was then calculated as the reciprocal value of the fitted decay constant k. The ^{13}C turnover in the second slower phase of decay (24–48 h) was calculated according to Ostle et al. (2003):

$$^{13}\text{C turnover (days)} = (^{13}\text{C \% ae at T1})/[(^{13}\text{C \% ae at T1} - ^{13}\text{C \% ae at T2})/(\text{T2} - \text{T1})] \quad (3)$$

where ^{13}C % ae is ^{13}C atomic percent excess of sample at times T1 (26 h) and T2 (50 hod), and (T2−T1) is time difference given in days.

The T-test (STATISTICA 7.0 Software) was used to determine whether significant differences existed between labelled and natural abundance control samples.

Results

Amount and temporal variability of the studied C pools

Shoot biomass reached 637±45 g m^{-2} (dry mass), with C content of 40.37±0.09%, and root biomass was 191±24 g m^{-2} (dry mass), with mean C content of 44.50±0.15%. Average amounts of C in the studied soil pools were 46.0±12.1 mg C kg^{-1} in C_{SOL}, representing 0.3% of soil C_{ORG}; 830.9±177.6 mg C kg^{-1} in C_{MIC}, representing 4.4% of soil C_{ORG}; and 803.0±77.6 mg C kg^{-1} in $C_{PAST\text{-}MIC}$ (4.3% of soil C_{ORG}). These values did not change significantly during the sampling period of 6 days. Soil C_{ORG} contained 56.0±4.6% of $C_{NON\text{-}HYDR}$.

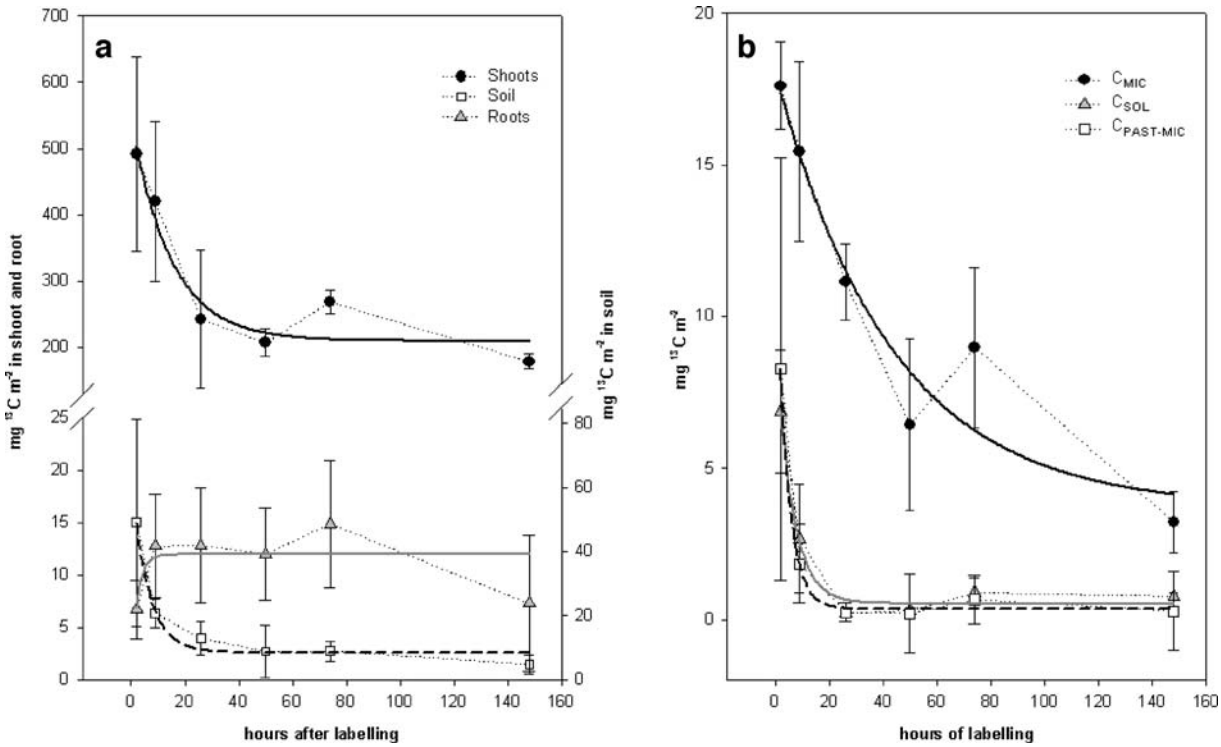

Fig. 1 Temporal changes in ^{13}C amount (mg ^{13}C m^{-2}) within the plant-soil system during the 6-day experiment, given for **a** shoots, roots, and soil total C, and **b** soil microbial biomass (C_{MIC}), soluble organic carbon (C_{SOL}), and microbial metabolites ($C_{PAST\text{-}MIC}$) extracted by hot water from the soil residue after chloroform fumigation. Mean values (n=4) and standard deviations are given. Data are fitted using a one-phase exponential decay model

^{13}C plant fixation

The labelled $^{13}CO_2$ was added to the chamber in the average amount of 1.3 mg ^{13}C g^{-1} shoot dry mass. During the 2-h labelling period, plants fixed 807.3±207.3 μg ^{13}C g^{-1} shoot dry mass or 547.4±85.3 mg ^{13}C m^{-2} (62% of ^{13}C added), which was assumed to be 100% of net plant ^{13}C fixation. At that time, almost 0.2% of shoot biomass was labelled by new ^{13}C. Shoot respiratory losses of $^{13}CO_2$ occurring concurrently with partitioning were not estimated.

^{13}C allocation within the plant–soil system during the experiment

Allocation of the fixed ^{13}C occurred shortly after ^{13}C fixation, as documented in samples collected at the end of 2-h labelling. Increases in $\delta^{13}C$ were found in all studied C pools within the pasture plant–soil system. The $\delta^{13}C$ changes were highly significant in shoots (233‰), roots (9‰) and dynamic soil C pools (C_{MIC} and C_{SOL}, changes of 104‰ and 70‰, respectively) but statistically non-significant in larger soil pools (C_{ORG}, $C_{PAST\text{-}MIC}$, $C_{NON\text{-}HYDR}$, $\delta^{13}C$ changes of 2‰, 5‰, and less than 1‰, respectively) due to high between-chamber variability.

The amount of the fixed ^{13}C remaining in the system and its distribution among studied C pools changed over time (Table 1). The highest proportion of ^{13}C in the system always occurred in shoots (Table 1). The amount of ^{13}C in shoots decreased exponentially over time (Fig. 1a). During the 6-d experiment, shoots lost more than 67% of the fixed ^{13}C either by shoot respiration or belowground transport. From that amount, 10.2% (more than 50 mg ^{13}C m^{-2}) already represented net belowground transport during the first 2 h of the experiment and was found in roots and soil (Table 1). The ^{13}C allocation to the roots continued because the ^{13}C content of roots increased in next samplings, reached its maximum between 9 and 26 h and then only slowly decreased (Table 1, Fig. 1a). Total system ^{13}C loss reached 356.6±21.4 mg ^{13}C m^{-2} (64.3% of the fixed ^{13}C) by the end of the experiment (Table 1). The highest amount of ^{13}C remaining in the system was in shoots (almost 33% of the fixed ^{13}C). C_{MIC} kept the highest amount of ^{13}C within all soil pools (0.6% of the fixed ^{13}C), corresponding to 68% of the soil ^{13}C at that time. There was one exception from the temporal ^{13}C decrease in all studied pools found during day three (74 h after the labelling ended, Fig. 1). At that time, the ^{13}C amount significantly increased in shoots, which was followed by non-significant increases in all other compartments (Fig. 1). As the same trend was found in all four chambers (detailed data were not shown), we excluded possible errors in preparation or analyses of samples. We suggest that it could be a response of the pasture plant–soil system to a quite strong night shower before sampling. Other explanations are also possible.

Within the studied soil C pools, C_{MIC} was the most ^{13}C enriched pool during the whole experiment. In the first samples, C_{MIC} contained ca 35.9% of soil ^{13}C (3.2% of the fixed ^{13}C). The proportion of the root-derived ^{13}C in C_{MIC} was 0.16% at that time.

Table 1 Temporal changes in the amount of fixed ^{13}C in the whole plant–soil system and its distribution (in %) among the studied C pools

	2 h	9 h	26 h	50 h	74 h	148 h
^{13}C in plant soil system (mg ^{13}C m^{-2})						
Total C	547.4±162.5	453.9±130.0	268.6±114.5	228.3±23.2	292.6±19.0	190.8±12.0
^{13}C in plant–soil system (%)						
Shoot	89.8±26.7	76.8±21.9	44.7±18.9	37.9±3.9	49.1±3.2	32.7±2.1
Root	1.2±0.5	2.3±0.9	2.3±1.0	2.2±0.8	2.7±1.1	1.3±1.2
Soil C_{ORG}	9.0±5.9	3.8±0.8	2.4±0.9	1.6±1.5	1.6±0.6	0.9±0.5
^{13}C within soil C pools (%)						
C_{SOL}	1.3±0.3	0.5±0.2	0.1±0.0	0.1±0.0	0.2±0.1	0.1±0.0
C_{MIC}	3.2±0.2	2.8±0.4	2.0±0.1	1.2±0.4	1.5±0.3	0.6±0.1
$C_{PAST\text{-}MIC}$	1.5±0.8	0.4±0.2	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.1

The ^{13}C amount fixed during 2 h of labelling represents 100%. Mean values for all chambers (n=4) and standard deviations are given.

Significant amounts of soil ^{13}C were found also in the other dynamic soil C pools, C_{SOL} and $C_{PAST\text{-}MIC}$ (13.9% and 16.9% of soil ^{13}C, respectively). The corresponding contribution of the new ^{13}C was 0.15% in C_{SOL} and 0.01% in $C_{PAST\text{-}MIC}$. Between 10 to 20% of soil ^{13}C occurred in more stable soil C pools, with a detectable labelling even in the $C_{NON\text{-}HYDR}$ fraction, being ca 5% of soil ^{13}C (less than 1% of fixed ^{13}C). After 6 days, soil lost more than 90% of the ^{13}C found in soil C_{ORG} at the beginning of the experiment (Table 1). The ^{13}C loss from microbial biomass was slower than from C_{SOL} and $C_{PAST\text{-}MIC}$ and the proportion of the soil ^{13}C amount in these pools diminished in favour of C_{MIC} during this time (Table 1). The $C_{NON\text{-}HYDR}$ pool lost only about 33% of its initial ^{13}C (data not shown). However, the ^{13}C amount in this pool was quite low and variable and the calculated results were not highly reliable. Temporal changes of ^{13}C amounts in all soil compartments were similar to that of shoots (Fig. 1), which documented microbial assimilation of root derived C, its supply to the $C_{PAST\text{-}MIC}$ pool and quick C transfer between these pools.

Discussion

Variability in labelling of the studied C pools

Regardless of the efforts to select similar labelling plots with homogeneous plant cover, the plant labelling in four chambers was quite variable, especially in early samplings made within 24 h after the labeling period (see Fig. 1). This variability in plant ^{13}C assimilation was reflected also in the amount of ^{13}C transported to soil and thus in soil C labelling. In spite of this, ^{13}C flow through the plant–soil system, as expressed by changes in $\delta^{13}C$ of the studied pools, was similar in all four chambers, which allowed us to generalize about the observed trends. Feeding the plants 2 h with ^{13}C–CO_2 of 99.9 at%, however, was sufficient only for the significant labelling of plant biomass (shoot and root) and dynamic C pools (C_{SOL} and C_{MIC}). At the first sampling, the proportion of the new ^{13}C reached 0.15% in both C pools. The label was detected also in soil C_{ORG}, $C_{PAST\text{-}MIC}$ and $C_{NON\text{-}HYDR}$ but the high between-chamber data variability made the corresponding differences in $\delta^{13}C$ from the natural abundance control (in units of ‰) non-significant. Studying dynamics of larger, less active soil C pools thus requires an extension of the labelling period in exchange for missing the first ^{13}C allocated belowground or running the experiment in the high vegetation season, when exudation should be higher than in September (Kuzyakov and Domanski 2000).

^{13}C fixation and allocation in plant

The amount of ^{13}C fixed by plants was estimated to be 547.4±85.3 mg ^{13}C m^{-2} by summation of ^{13}C found in shoots, roots and soil (per area of m^2) immediately after the short pulse-labelling. The summation of the label in plant and soil pools likely provides more precise estimation of fixed ^{13}C than only the amount of label in shoots as proposed by Leake et al. (2006). During the whole experiment, shoots were 40–50 times more enriched in ^{13}C than roots as in Ostle et al. (2003), which corresponded to the ^{13}C content in hundreds of mg m^{-2} in shoots and only to 15 mg m^{-2} in roots (see Fig. 1a). A total of 67% of the fixed ^{13}C was exported from the shoots during the 6 days of the experiment, which is close to the 76% found by Hill et al. (2007) and within the wide range ca 30–90% of total fixation estimated for grasses (Baxter and Farrar 1999; Dilkes et al. 2004). The main proportion of assimilate transport from shoots (>50% of fixation) occurred during the first day, which is in accordance with Butler et al. (2004) and Leake et al. (2006), who found losses of 31–70% of fixation from shoots within 24 h. It is known that the newly fixed C is preferentially respired by plants, as CO_2 evolved is much more enriched in ^{13}C in comparison to plant tissue (Staddon et al. 2003), but we had no possibility to measure shoot respiration and its $\delta^{13}C$ signature. Therefore, we could only roughly estimate the partitioning of ^{13}C exported from shoots between shoot respiration and gross belowground transport. As shoot respiration is estimated to be around 30–36% of fixation (Domanski et al. 2001; Kuzyakov et al. 2001) or even higher (31–70% of fixation; Leake et al. 2006), we can assume that at least half of the ^{13}C transported from shoots (30–35% of the fixed ^{13}C) was lost by shoot respiration and the rest (ca 30–35% of fixation) was transported belowground. This estimation of the gross belowground transport lies within the wide range of 8–67% of fixation given by Meharg and Killham (1990) for

Lolium perenne in different growth stages, but is a bit lower than the 40–80% of fixed C reported for perennial plants (Kuzyakov and Domanski 2000).

The allocation of ^{13}C from shoots to roots occurred already during the first hours after its fixation. However, the label dynamics differed between shoots and root biomass. The ^{13}C content in shoots exponentially decreased after the high enrichment at the beginning, while it slowly increased in roots and reached the maximum between 9–26 h after the first ^{13}C–CO_2 pulse. Similar results were found by Domanski et al. (2001), Johnson et al. (2002) and Rangel-Castro et al. (2004), who found that the maximum incorporation of the assimilated C to roots occurred between 24–48 h. According to Leake et al. (2006), the allocation of the fixed carbon from shoots to roots continues for about one week, which indicates that exudation of the "new C" can occur during this time. At the end of the experiment, 1.3% of the fixed C still remained in the roots, which is in accordance with results of Hill et al. (2007) from a similar 6-day experiment. This indicates that roots provided a rapid transport of new assimilates to soil and only slowly but gradually incorporated a proportion of them to the root biomass.

^{13}C rhizodeposition and flow in soil C pools

We found very rapid and quite large exudation of the root-derived ^{13}C, which is in accordance with Cheng (1996), Domanski et al. (2001), Johnson et al. (2002), Leake et al. (2006), and others. Nine percent of the fixed ^{13}C occurred in soil within 2 h and this amount was probably even higher because the C allocation to roots continued for at least 24 h after labelling (see above). The rhizodeposition was similar to the 10% for *Lolium perenne* (Butler et al. 2004), the 9% for *Bromus erectus* (Warembourg and Estelrich 2000), and the 7–12% for *Zea mays* (Haller and Stolp 1985; Jones and Darrah 1993).

Higher amounts of ^{13}C were allocated into the labile C pools, C_{MIC} and C_{SOL} (and $C_{PAST\text{-}MIC}$), than into the rest of the soil C_{ORG}. C_{MIC} was the pool with the highest ^{13}C enrichment during the whole experiment, as also found by Pelz et al. (2005), Yevdokimov et al. (2006), and Leake et al. (2006). It contained almost 4% of the fixed C at the beginning of the experiment and 0.6% of the fixed C at the end (comparable to Rattray et al. 1995; Domanski et al. 2001; Kuzyakov et al. 1999, 2001; Lu et al. 2004; Hill et al. 2007), which represented 40–70% of soil ^{13}C (Butler et al. 2004). The highest enrichment of C_{MIC} within soil C pools and the slower loss of ^{13}C from C_{MIC} than from other labile C pools (see Fig. 1b) indicate the importance of root-derived C as microbial C substrate and its partial immobilization in the soil by assimilation into microbial biomass. A rapid microbial consumption of exuded ^{13}C is also documented by very similar dynamics of ^{13}C temporal changes in C_{MIC} and shoots. ^{13}C temporal changes in C_{MIC} were closely related also to $C_{PAST\text{-}MIC}$. The $C_{PAST\text{-}MIC}$ pool was described as a labile pool, which is intermediate between microbial biomass and humic substances, containing metabolites derived from microbial biomass (Lemaitre et al. 1995b). Fast changes in ^{13}C content in $C_{PAST\text{-}MIC}$ (see Fig. 1b) in response to C_{MIC} indicate an active communication between these pools. It is possible that the excess C from exudates, which have higher C/N ratio than microbial biomass, is immobilized by transformation to storage carbohydrates (Knapp et al. 1983; Nickels et al. 1979).

Root-derived C was found also in more stable soil C. Except for the first sampling immediately after labelling, the amount of ^{13}C in this soil C was almost constant, about 0.2–0.3% of the fixed C. Less than one half of this amount was found in the non-hydrolysable soil C – $C_{NON\text{-}HYDR}$. According to Kuzyakov and Demin (1998), this could be caused by adsorption of exuded C compounds to soil particles, in their case being 0.6–0.7% of the amount of glucose and glycine added to the soil. A longer labelling period should be used to estimate the root-derived C input to the stable soil C storage, so that significant amount of the ^{13}C in those soil pools may be reached. However, we suggest that the contribution of root exudates to the stable soil C is negligible.

^{13}C turnover in studied C pools

The ^{13}C dynamics in the studied pools (except roots) were estimated by fitting a one-phase exponential decay curve. The calculated turnover was 18.1 h for shoots, 4.4 h for C_{SOL}, 26.3 h for C_{MIC}, and 6.1 h for $C_{PAST\text{-}MIC}$. These very short turnover times are produced mainly by the quick loss of label during the first 24 h. However, it can be seen from the Fig. 1 that there was a second slower phase of ^{13}C loss occurring after 24 h and the exponential ^{13}C decrease

is rather two-phase. Boddy et al. (2007) suggested that the first rapid ^{13}C loss corresponds to the immediate use of ^{13}C substrate in catabolic processes whereas the slower second phase is attributable to the subsequent microbial biomass turnover. Because we did not have sufficient amount of data for fitting a double exponential decay curve, we calculated the turnover in the second phase according to the equation 3 (Ostle et al. 2003) from the difference between the ^{13}C atomic percent excess of the samples from 26 and 50 h after labelling (before the increase in ^{13}C content). The calculated turnover was than 3.1 days for shoots, 2.3 days for C_{SOL}, 3.4 days for C_{MIC}, and 3.1 h for $C_{PAST\text{-}MIC}$. The C_{MIC} turnover in the second phase was similar to results of Ostle et al. (2003) and Butler et al. (2004), who used ^{13}C labelling for the calculation. The found C_{MIC} turnover was also comparable to e.g. Anderson and Domsch (1986), who calculated turnover 3–11 days using classical microbiological methods (growth kinetics after the substrate amendment). This could indicate that rhizosphere contains sufficient amount of substrate to supply active microbial growth. We further used the known concentration of root exudates in soil solution, which is in order of tens to hundreds μmol l^{-1} (Kuzyakov and Domanski 2000; Hütsch et al. 2002; Nguyen 2003) for a rough estimation of the amount of biodegradable C in the rhizosphere. Calculated for glucose as the typical representative of root exudates (molar weight of 180 g mol^{-1}, 40% C content), the amount of biodegradable C corresponds to tenths to units mg C l^{-1}, which highly exceeds the range of 15–100 μg available C l^{-1} characteristic for the oligotrophic environment (Morita 1993). Based on these results, we consider that microbial growth in the rhizosphere is not limited by substrate availability, as already suggested by Helal and Sauerbeck (1986), Van Veen et al. (1989) or Cheng et al. (1996).

Conclusions

The use of ^{13}C as a label for recently fixed photosynthate allowed us to estimate its approximate allocation among different plant and soil C pools connected with microbial metabolism under unmodified field conditions. We found similar dynamic of the root-derived C in labile soil C pools – C_{MIC}, C_{SOL} and $C_{PAST\text{-}MIC}$, both later serving as sources and sinks of microbial substrates and metabolites, indicating rapid communication between them. We confirmed the main role of the soil microbial community in the immobilization of recently fixed C in its biomass and based on short C_{MIC} turnover time we proposed that microbial growth in the rhizosphere is not limited by substrate availability. The recently fixed C contributes only insignificantly to stable C storage (likely stabilization by physico-chemical sorption).

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PAPER 2

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A larger investment into exudation by competitive versus conservative plants is connected to more coupled plant–microbe N cycling

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Abstract Plant–microbe interactions actively control nitrogen (N) cycling in the ecosystem. We hypothesize that the investment into exudation and the coupling of plant–microbe N cycling will be larger in competitive plants compared to the more conservative species. Root exudation of competitive (*Glyceria maxima*) and conservative (*Carex acuta*) plants was estimated by ^{13}C-CO_2 labeling. Seasonal changes in plant, microbial, and soil soluble N pools as well as potential net microbial N transformations were determined to interconnect the C and N cycling within grassland ecosystems dominated by these species. We showed that competitive *Glyceria*, as compared to conservative *Carex*, appears to affect soil N cycling through a more direct temporal and spatial influence on soil microbes due to a larger investment into root exudation. This makes the system highly dynamic, with faster soil N cycling and pronounced seasonal N redistribution between plants and microbes. The conservative *Carex*, irrespective of its larger root system, invested less C to exudation. In this case, the plant–microbe relationships appear to be less-coupled in time and space with the plant N supply likely relying mainly on the relatively slow microbial mineralization of organic matter than on rhizosphere priming effect. We showed that differences in soil N cycling associated with competitive versus conservative plants are closely connected with their different investments into root exudation, which govern the coupling of plant–microbe interactions in time and space.

Keywords Plant growth strategy · Exudation · Grassland · Labile N · Microbial biomass · The plant–microbe interaction

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Introduction

Over the last 50 years, empirical studies have documented the importance of plant species in affecting N cycling and soil N availability through changes in plant growth rate, nutrient use efficiency and litter quality, and biodegradability (Wedin and Tilman 1990; Hobbie 1992; Binkley and Giardina 1998; Craine et al. 2002; Personeni et al. 2005; Fornara et al.

2011). Other works have linked soil N cycling and N availability with the composition of soil microbial communities (Wardle et al. 2004; Hogberg et al. 2007; Strickland and Rousk 2010). A growing body of evidence shows that the functioning of plants and soil microbes are closely linked (Hartmann et al. 2009; Friesen et al. 2011) and their interactions play a major role in controlling ecosystem N cycling and retention (Knops et al. 2002; Bardgett et al. 2005; Chapman et al. 2006; Orwin et al. 2010; de Vries and Bardgett 2012; Grigulis et al. 2013).

Fast-growing plant species with higher photosynthetic capacity and rapid rates of N acquisition are called competitive (exploitative, N-rich) species in contrast to slower-growing conservative (stress tolerant, N-poor) species with lower biomass N concentrations but a longer lifespan (Grime 1977; Tilman 1990; Hobbie 1992; Aerts and Chapin 2000). To cover the larger N requirements, competitive plants support bacterial-dominated soil microbial communities, providing fast soil N cycling (Grigulis et al. 2013). This is manifested by higher rates of N mineralization, higher concentrations of mineral N forms in soil (Orwin et al. 2010), and higher net microbial N immobilization (Personeni et al. 2005). The opposite is true for conservative plants which have a strategy of high resource conservation, large below-ground allocation, long-lived tissues and poor litter quality, often associated with fungal-dominated soil microbial communities (Hobbie 1992; Grigulis et al. 2013).

In addition to the well-documented effect of plant biomass chemistry (Scott and Binkley 1997; Binkley and Giardina 1998; Fornara et al. 2011), different attributes of soil N cycling linked to plant growth strategy strongly indicate differences in plant rhizodeposition, a key factor of plant–microbe interactions in the rhizosphere (Jones et al. 2004; Hartmann et al. 2009). Competitive plants are thought to invest more to root exudation than conservative ones due to their larger photosynthetic capacity (Personeni and Loiseau 2005; De Deyn et al. 2008). However, plant exudation is poorly understood relative to other processes in the terrestrial C cycle (Jones et al. 2004). To the best of our knowledge, there are currently no available data, which would directly compare exudation of competitive and conservative plant species in connection to the microbial N transformation associated with such plants.

Plant–microbe interactions, driven by root exudation, continuously provide roots with available N through the microbial loop and protect the ecosystem from N losses (Kuzyakov and Xu 2013). The closeness of their cooperation in labile N cycling can be demonstrated in the seasonal partitioning of N between plants and soil microbes. While microbial immobilization of N from plant litter decomposition is dominant in the autumn–spring period (no plant N requirements), a redistribution of the majority of labile N to plant biomass is observed in the vegetation period (Jaeger et al. 1999; Lipson et al. 1999; Bardgett et al. 2002, 2005; Lipson and Schmidt 2004). If competitive plants invest more into exudation to fuel the microbial loop and are able to compete for the released N effectively, we may also expect a larger direct effect of the plants on microbial biomass and activity in the rhizosphere during the vegetation season connected with pronounced seasonal N redistribution between plants and microbes.

Our objective was to compare the effect of plant nutritional strategy on the investment into exudation, the "tightness" (coupling in time and space) of the plant–microbe interactions covering plant N requirement, and the consequences for C and N cycling associated with conservative versus competitive plants. Our study sites were two temperate wet grasslands with different nutrient status, vegetation composition, and nutritional strategy of the dominant vegetation. These site features were demonstrated by differences in the concentration and composition of the soil soluble N pool, in the net rates of microbial N transformation processes, and in the plant biomass characteristics. *Carex acuta* and *Glyceria maxima*, which dominated the oligotrophic and mesotrophic grasslands, respectively, are typical representatives of conservative and competitive wetland plant species. They were used in a labeling study to assess their investments into exudation. We hypothesized that *Glyceria,* as a competitive species, will invest more fixed C into exudation than the conservative *Carex*. Higher root exudation by *Glyceria* will govern intensive, near-time and near-space microbial activity and will result in closer, well coupled plant–microbial cooperation on the covering of plant N requirements. This will be demonstrated by a larger proportion of microbial N in the ecosystem labile N pool and its larger seasonality leading to a significant seasonal N redistribution between microbes and plants.

Materials and methods

Study sites

The two wet grassland sites are located in the Trebon Basin Biosphere Reserve, South Bohemia, Czech Republic. The first site is a nutrient poorer sedge meadow on organic soil (Histosol, FAO-WRB classification), dominated by *C. acuta* and *C. vesicaria* (referred to as *Carex*-dominated grassland). The basic chemical characteristics of the soil are: SOM content 42.4 ± 8.9 %, Ctot 22.3 ± 2.3 % (46.8 ± 4.8 mg C cm^{-3}), Ntot 1.2 ± 0.1 % (2.5 ± 0.2 mg N cm^{-3}), pH(H_2O) 4.93 ± 0.23, bulk density 0.21 ± 0.02 g cm^{-3}. The second site is a nutrient richer wet meadow in the floodplain of a small river, dominated by *G. maxima*, with *C. acuta* comprising up to 30 % of the aboveground biomass (referred to as *Glyceria*-dominated grassland). It lies on a silt-loam alluvial soil, classified as Gleysol (FAO-WRB classification). The basic chemical characteristics of the soil are: SOM content 20.0 ± 2.0 %, Ctot 10.3 ± 2.0 % (53.6 ± 10.4 mg C cm^{-3}), Ntot 0.7 ± 0.1 % (3.6 ± 0.5 mg N cm^{-3}), pH(H_2O) 4.6 ± 0.2, bulk density 0.52 ± 0.04 g cm^{-3}. Soil profiles on both sites are relatively deep (>60 cm), diminishing an effect of the parent material on soil properties. The significantly higher SOM content in the *Carex*- versus *Glyceria*-dominated grassland could be ascribed mainly to significantly slower decomposition rate of plant litter, resulting from lower quality of plant litter with a higher C/N ratio (unpublished results), different composition of soil microbial community displaying a lower activity (Kastovska et al. 2012) and commonly higher water level at this site.

Sampling of plants for labeling experiment

To study the effect of plant nutritional strategy on the C allocation and investment into exudation, *G. maxima* and *C. acuta* plants (hereafter referred to as *Glyceria* and *Carex*, respectively) were collected in early May 2008. Plants were sampled in the mesotrophic site, where both species co-exist together and prosper well. *Carex* was not sampled from the *Carex*-dominated site because it could bring a confound effect of a different plant origin to the comparison of C economics between both plant species. Individual plants were grown in a mixture of the field soil and sand (3/1; soil/sand weight ratio, mixing necessary to facilitate a complete separation of roots and soil for the ^{13}C measurement) in pots (in 300 g of fresh substrate) in open air for 2 months.

Pulse labeling of plants by $^{13}CO_2$

Pulse labeling of plants with $^{13}CO_2$ was done after 50 days of plant growth in pots, on 8 July 2008 (10:00–14:00, mostly sunny weather, maximum temperature 23 °C). Pots with plants were placed in two gas-tight plexiglass chambers (50 × 50 × 50 cm) with holes fitted with rubber-septa. After the ambient CO_2 concentration in the chambers decreased below 200 ppm (verified by GC), $^{13}CO_2$ tracer [99.9 atomic percent (at.%) ^{13}C, Cambridge Isotope Laboratories, GB] was repeatedly pulsed into the chambers by a syringe to keep the internal CO_2 concentration at 400–500 ppm for a period of 4 h. A total amount of 400 ml of $^{13}CO_2$ was added to each chamber during the labeling. Air circulation during labeling was ensured by two 6 cm^2 12 V fans. The chambers were removed after labeling and pots with plants were left in fresh air. Each chamber contained 10 randomly selected pots of each plant species. The remaining pots were not labeled, but used as control samples for the ^{13}C natural abundance determination.

Harvests and analyses of labeled plant–soil systems

The first harvest was done immediately after the end of the 4-h labeling period and then 1, 3, 8, and 15 days after the beginning of pulse-labeling. At each sampling time, four pots with plants of each species were harvested. The above-ground part (shoot) of each plant was cut off. The corresponding below-ground parts (roots and rhizomes) were carefully cleaned by hand to remove soil and then quickly washed under tap water. Plant material was dried (60 °C), weighed and ground. All soil from a pot was thoroughly mixed. A part of each soil sample (5 g) was oven-dried at 105 °C to determine water content. Another subsample was dried at 60 °C and ground. Dried and ground shoot, root, and soil samples were weighed in tin cups. Analyses of C, N and ^{13}C (in ^{13}C at.%) contents were conducted on an NC Elemental analyzer (ThermoQuest, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany).

Calculations

A binary mixing model with ^{13}C at.% was used to estimate the amount of the pulse-derived ^{13}C in the various C pools investigated:

$$^{13}C\ (\mu g\ g^{-1}) = \left[\left(\text{at.}\%_{\text{sample}} - \text{at.}\%_{\text{control}}\right)/(99.9 - 1.10)\right] \times \text{C pool size}\ (\mu g\ C\ g^{-1}),$$

where $\text{at.}\%_{\text{control}}$ is the natural abundance in the control samples, 99.9 is the pulse ^{13}C at.% and 1.10 is the at.% of the atmosphere (corresponding to average value of −8.5 ‰).

Net C fixation (mg ^{13}C $plant^{-1}$) was calculated as the sum of ^{13}C amount in shoots, roots, and soil after the 4-h labeling period; respiratory losses occurring during the labeling were not estimated. Differences between the net C fixation and the amounts of ^{13}C in the system during the subsequent samplings were considered as respiration losses. The maximum soil ^{13}C content, occurring between 4 and 48 h after labeling represented net exudation (without microbial respiration of exudates). The ^{13}C distribution in the plant–soil system was expressed on relative bases (% net C fixation).

Soil and plant sampling in the field

Soils for soil analyses were sampled three times a year in spring (early May, beginning of plant growth), summer (mid-July, high season) and autumn (October, plant senescence) in 2008. Four independent replicates were taken from four treatment plots in both grasslands, each resulting from the pooling of 10 samples taken by a soil core (diameter of 3 cm) from 0 to 20 cm depth. The samples were sieved through a 5-mm mesh and kept at 4 °C until analyzed. At the same time, aboveground plant biomass was harvested from eight randomly-selected quadrants (0.25 × 0.25 m). Below-ground plant biomass was collected using soil corers. Roots were kept at 4 °C until they were washed to remove soil.

Plant N pools and their turnover rates

All sampled plant material was dried at 60 °C for 48 h, weighed, and analyzed for C and N contents using an NC Elemental analyzer (Elementar, Germany). Mean net annual aboveground plant production (NAPP; g m^{-2} $year^{-1}$) was calculated as the difference between the maximum and initial plant biomass in each site; calculation of mean net annual belowground plant production (NBPP; g m^{-2} $year^{-1}$) was analogous. The aboveground and belowground plant biomass and their N concentrations were used to calculate the aboveground and belowground plant N pools on a square meter basis (g N m^{-2}) for each sampling event. Turnover rates of aboveground and belowground plant N ($year^{-1}$) were calculated as the ratio of seasonal increase in aboveground plant N to the maximum aboveground plant N and seasonal increase in belowground plant N to the maximum belowground plant N, respectively (analogous to Gill and Jackson 2000).

Soil analyses

Soil was sieved immediately after sampling. A part of the soil was dried at 60 °C for 48 h, weighed and analyzed for total N content using an NC Elemental analyzer (Elementar, Germany). The other part was kept wet in a cold room until the following day and analyzed. Soil soluble N was extracted in 0.5 M K_2SO_4 (soil:extractant 1:4) for 30 min on a shaker and measured in filtered extracts on a LiquiTOCII (Elementar, Germany). Concentrations of ammonium and nitrate (NH_4-N and NO_3-N, respectively) were measured using flow injection analysis (FIA Lachat QC8500, Lachat Instruments, USA). The amount of soluble organic N (SON) was calculated as the difference between total soil soluble N and the sum of inorganic N in sulfate extracts. Soil microbial biomass N (Nmic) was measured by chloroform-fumigation and extraction by 0.5 M K_2SO_4, and calculated as the difference between soil soluble N in the extracts from fumigated and non-fumigated soils, using $K_{EN} = 0.54$ (Brookes et al. 1985). The amounts of all soil N fractions were recalculated on a square meter basis (g N m^{-2}) to a depth of 20 cm, respecting the sampling depth of soils and the depth of the dense rooting zone.

Rates of potential net ammonification, nitrification, and N immobilization into microbial biomass (expressed in mg N m^{-2} day^{-1}) were calculated from temporal changes in concentrations of particular N pools (NH_4-N, NO_3-N and Nmic, respectively) after aerobic incubation of soil samples in the laboratory (2 weeks, without N addition, 20 °C). Net N

mineralization was expressed as the sum of ammonification and nitrification. Relative N immobilization was estimated as the ratio of potential net microbial N immobilization to the sum of the processed N (net microbial N immobilization and net N mineralization). This shows the ability of the microbial community to use available N; a higher value indicates a larger potential for immobilization and lower potential for losses of available N. If relative N immobilization is lower than 0.5, release of mineral N prevails over N immobilization.

Ecosystem labile N pool and its seasonal changes

The ecosystem labile N pool (g N m^{-2}) was composed of the plant, microbial, and soil soluble N fractions expressed per square meter to a 20 cm depth. The amounts and proportions of these fractions within the ecosystem labile N pool were calculated for each sampling time.

Statistical analyses

Repeated measures ANOVA with sampling time as a covariate was used to assess the effect of plant species on biomass characteristics of plants used in the labeling experiment (shoot and root biomass and their C and N contents) and the effect of grassland type (*Glyceria*- versus *Carex*-dominated grassland) on characteristics of the ecosystem N cycling (sizes of labile N pools, plant biomass characteristics measured in the field and microbial N transformation rates). The *T* test was used to determine whether significant differences existed in ^{13}C fixation and C allocation patterns between the two plant species and in the NAPP, NBPP and plant N turnover between the *Glyceria*- and *Carex*-dominated grasslands.

Results

Characteristics of plants in labeling experiment

Carex plants had lower shoot, but comparable root biomass to *Glyceria*, which resulted in a significantly lower shoot/root ratio for *Carex* (Table 1). Further, *Carex* shoot and root biomass had higher C but lower N concentrations than *Glyceria* biomass, thus higher biomass C/N ratios (Table 1). Temporal changes in plant biomass characteristics during the 15-day experiment were non-significant (Table 1).

Plant ^{13}C fixation and its distribution in plant–soil system

Net ^{13}C fixation tended to be lower for conservative *Carex* versus competitive *Glyceria* plants, in correspondence to its lower shoot biomass ($P = 0.051$, Fig. 1a). Identically for both plants, 70 % of net fixation was found in shoots and 30 % was already transported below ground after 4 h of labeling (Fig. 1b). The investment into exudation, however, differed significantly between the plants. While *Carex* kept the vast majority of ^{13}C in its roots and exuded only 4 % of net fixation, the exudation of *Glyceria* reached 11 % of net fixation (Table 2).

Shoots of both plants lost fixed ^{13}C quickly during the first days as its transport below ground continued, reaching a maximum of 58 % of net fixation on the second day for *Glyceria* and 72 % of net fixation on day 3 for *Carex* (Fig. 1b; Table 2). Later, there were only minor changes in shoot ^{13}C, likely indicating only maintenance respiration of the fixed ^{13}C from shoots, but large losses of ^{13}C from below-ground caused by root-derived respiration in this period (Fig. 1b).

The final distribution of net fixation after 15 days showed similar losses from both systems but different distribution of the remaining ^{13}C. While *Carex* kept a larger percentage of the remaining ^{13}C in roots against shoots, the proportions in roots and shoots were comparable for *Glyceria.* The *Glyceria* systems' processes resulted in a larger proportion of ^{13}C net fixation in the soil (Table 2).

Field plant N production and biomass characteristics

The *Glyceria*-dominated grassland had higher NAPP but significantly lower NBPP than the *Carex*-dominated one (Table 3). Above- and below-ground plant biomass had higher N concentrations, lower biomass C/N ratios, and faster N turnover in the *Glyceria*- versus *Carex*-dominated grassland (Table 3), which coincided with the expected difference in the nutritional plant strategy between both grasslands.

Table 1 Shoot and root biomass of labeled plants and their C and N concentrations (%); mean ± standard deviation ($n = 24$); and results of Repeated measures ANOVA with plant species as categorical predictor

	Carex acuta	*Glyceria maxima*	Plant	Time	P × T
Shoot biomass (g)	0.27 ± 0.14	0.48 ± 0.20	*	ns	ns
Root biomass (g)	0.57 ± 0.27	0.60 ± 0.31	ns	ns	ns
Shoot/root ratio	0.50 ± 0.16	0.83 ± 0.25	**	ns	ns
Shoots					
C (%)	44.1 ± 1.1	42.0 ± 0.6	***	ns	ns
N (%)	1.41 ± 0.37	1.73 ± 0.28	***	ns	ns
C/N	33.5 ± 12.1	24.1 ± 8.1	**	ns	ns
Roots					
C (%)	42.3 ± 1.1	41.0 ± 1.1	***	ns	ns
N (%)	0.63 ± 0.12	0.78 ± 0.15	*	ns	ns
C/N	67.9 ± 11.9	54.5 ± 11.1	**	ns	ns

ns Non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

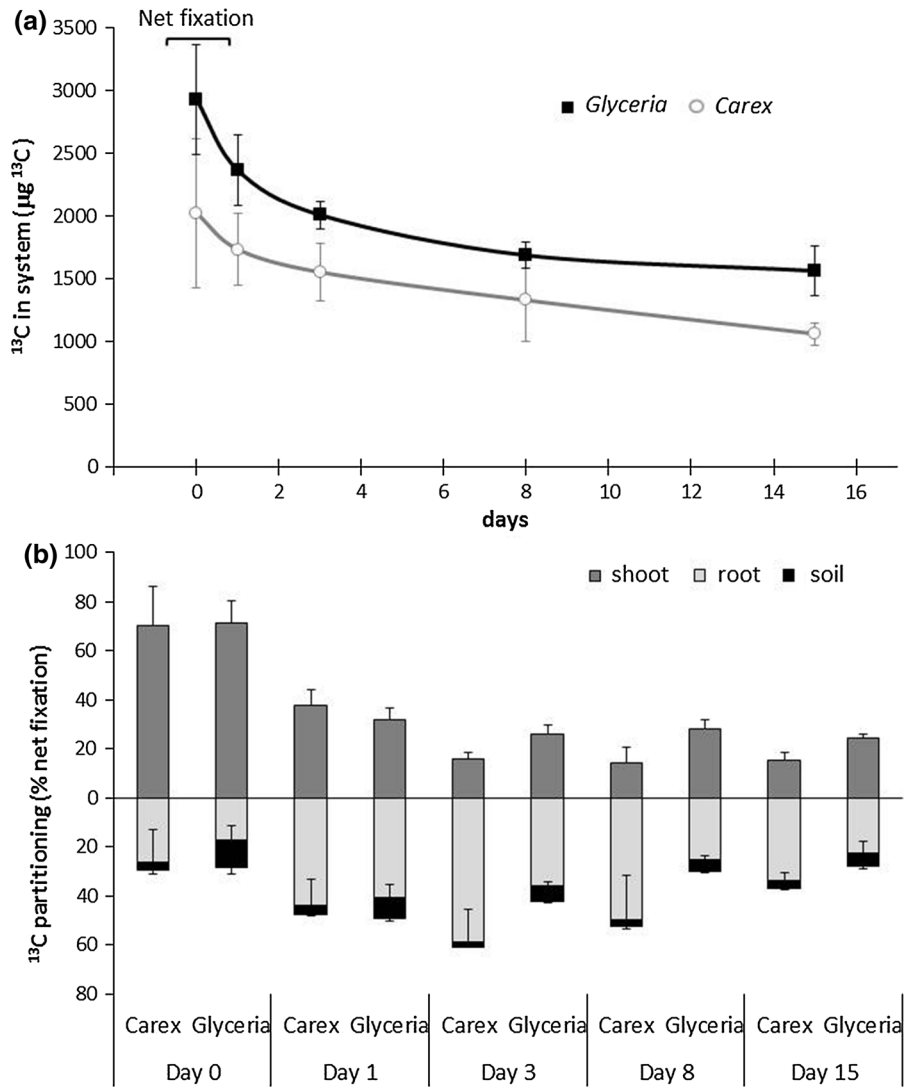

Fig. 1 Temporal changes in: **a** the amount of the fixed ^{13}C in plant–soil systems with *Carex* and *Glyceria* during 15 days of incubation, where the amount of the fixed ^{13}C in plant–soil systems at day 0 represents 100 % of net fixation for the systems, and **b** the percentage of net fixation allocated in shoots, roots and soil in the systems at each sampling time. Means and standard deviations ($n = 4$) are given for each sampling time

Table 2 Total ^{13}C distribution among particular pools and fluxes in the systems with *Carex* and *Glyceria* plants 15 days after fixation expressed as % of net ^{13}C fixation; mean ± standard deviation ($n = 4$); and results of *T*-test

	Carex acuta	*Glyceria maxima*	Plant
C distribution in pools and fluxes after 15 days (% net fixation)			
Total losses	47.5 ± 1.7	46.5 ± 6.8	ns
Shoot C	15.5 ± 3.0	24.2 ± 1.6	**
Root C	34.0 ± 3.7	22.4 ± 4.6	**
Soil C	3.1 ± 0.5	5.5 ± 1.0	**
Total C transport below ground	72.7 ± 10.1	58.1 ± 6.0	*
Net exudation	4.0 ± 0.4	11.4 ± 2.4	***

Net exudation corresponds to the maximum ^{13}C allocation into soil, occurring 4–48 h after labelling

ns Non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Composition and seasonal changes in ecosystem labile N pool

A size of the ecosystem labile N pool (sum of plant, microbial and soil soluble N) was comparable between both grasslands (Fig. 2a). The pool showed significant seasonal variability ($P < 0.001$); an increase towards summer and a decrease in autumn was observed together with differences in its composition between both grasslands (Fig. 2a). In the *Carex*-dominated grassland, the mean contributions of N fractions to the ecosystem labile N pool were: below-ground plant N ≫ microbial N ≥ aboveground plant N > soluble soil N. However, this differed in the *Glyceria*-dominated grassland, with microbial N ≥ total plant N (below-ground plant N = aboveground plant N) > soluble soil N (Fig. 2b). In both grasslands, a seasonal redistribution of labile N between plants and microbes appeared—plant N pool prevailed in summer, while microbial N pool in early spring and autumn, with more pronounced seasonal dynamics in the *Glyceria*-versus *Carex*-dominated grassland (Fig. 2b).

Soil soluble N represented the smallest and the only seasonally invariable fraction of the ecosystem labile N in both grasslands (Fig. 2; Table 3). It was higher in the *Glyceria*- versus *Carex*-dominated grassland (Fig. 2a, $P < 0.001$), which corresponded with its larger proportional contribution to the ecosystem labile N pool in the *Glyceria*-dominated grassland (Fig. 2b, $P < 0.001$). In both grasslands, soil soluble N was dominated by organic forms (SON), with the rest found in mineral forms, with ammonium N exceeding nitrate N concentrations (Fig. 2c). The

Table 3 Net annual above- and belowground plant production (NAPP and NBPP, respectively) and characteristics of above- (AG) and below-ground (BG) plant biomass in the *Carex*- and *Glyceria*-dominated grassland in 2008

Grassland	*Carex*-dominated	*Glyceria*-dominated	Site	Time	S × T
NAPP (g m^{-2} $year^{-1}$)	424.9 ± 47.3	514.1 ± 59.8	*	–	–
NBPP (g m^{-2} $year^{-1}$)	984.8 ± 169.0	435.5 ± 17.1	**	–	–
AG plant biomass					
N concentration (%)	1.90 ± 0.15	2.65 ± 0.51	***	*	***
C/N ratio	22.6 ± 10.5	16.4 ± 3.4	***	*	***
N turnover ($year^{-1}$)	0.61 ± 0.10	0.72 ± 0.04	***	–	–
BG plant biomass					
N concentration (%)	0.93 ± 0.05	1.14 ± 0.07	***	ns	ns
C/N ratio	49.3 ± 5.8	37.3 ± 4.9	***	*	ns
N turnover ($year^{-1}$)	0.32 ± 0.10	0.54 ± 0.11	*	–	–

Mean ± standard deviations ($n = 12$) followed by results of *T*-test (for NAPP, NBPP and N turnover) or Repeated measures ANOVA (N concentration and C/N) are given. Plant N turnover was calculated as the ratio of annual N production (the difference between plant N contents in the maximum standing crop and in the biomass in the beginning of growing season) to maximum biomass N

ns Non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

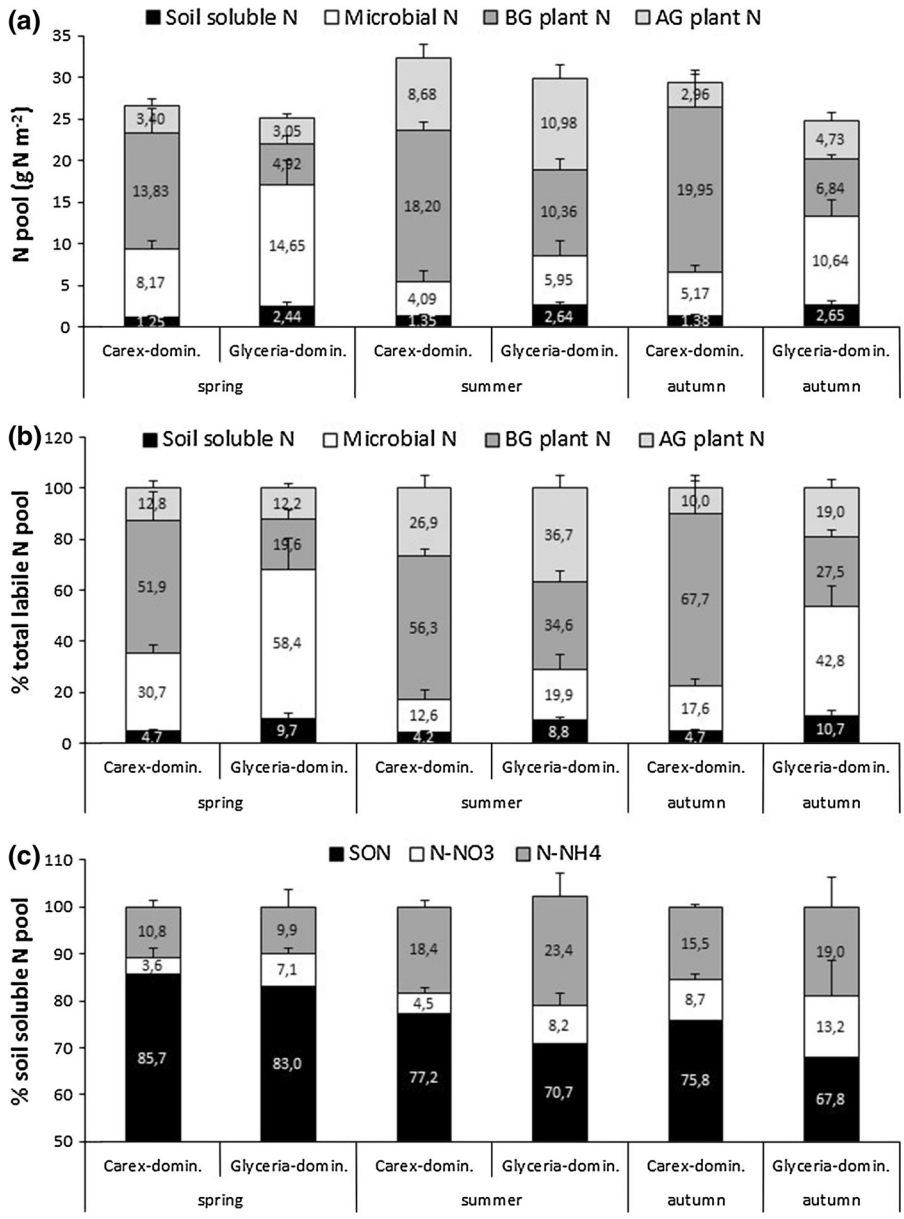

Fig. 2 Seasonal changes in the composition of ecosystem total labile N pool in the *Glyceria*- and *Carex*-dominated grasslands, composed of the N in aboveground (AG) and belowground (BG) plant, microbial and soil soluble N fractions expressed in **a** absolute numbers (g N m^{-2}) and **b** relative values (percentage of total labile N pool), and **c** seasonal changes in proportions of soil organic N (SON), nitrate N ($N\text{-}NO_3$) and ammonium N ($N\text{-}NH_4$) within soil soluble N in the respective grasslands. Means and positive standard deviations are given for each N fraction ($n = 4$)

concentrations of both mineral N forms, as well as their proportions in soil soluble N were significantly higher in the *Glyceria*- versus *Carex*-dominated grassland ($P < 0.001$ for both N forms). The proportions of N forms in soil soluble N were variable in time ($P < 0.01$). In both soils, the proportion of SON was highest in spring versus summer and autumn, when the proportions of mineral N forms increased. The ammonium N pool was highest in summer, while nitrate N increased in autumn (Fig. 2c).

Potential net microbial N transformations (laboratory measurements)

Potential microbial processes were faster and relative N immobilization (the ratio of immobilized N to totally processed N) tended to be higher in the soil of *Glyceria*- versus *Carex*-dominated grassland (Table 4). There was significant seasonal variability in all the measured parameters. Net microbial N immobilization was negative in spring and all N released from microbial

Table 4 Potential net microbial N immobilization, N mineralization and nitrification rates, and relative N immobilization (ratio of immobilized-to-processed N) in soils of *Carex*- and *Glyceria*-dominated grasslands in spring, summer and autumn sampling 2008

Grassland	*Carex*-dominated	*Glyceria*-dominated	Site	Time	T × S
Net N immobilization (mg N m^{-2} day^{-1})					
Spring	−27.9 ± 3.0	−125.8 ± 96.0	*	***	**
Summer	62.7 ± 26.3	179.7 ± 76.3			
Autumn	81.6 ± 26.1	211.8 ± 76.1			
Net N mineralization (mg N m^{-2} day^{-1})					
Spring	57.2 ± 9.5	125.5 ± 14.9	***	***	ns
Summer	53.8 ± 14.2	105.4 ± 19.5			
Autumn	39.8 ± 8.1	72.6 ± 27.3			
Net nitrification (% net N mineralization)					
Spring	97.6 ± 16.3	100.1 ± 11.8	***	***	ns
Summer	97.2 ± 25.7	98.2 ± 18.6			
Autumn	98.5 ± 20.0	103.0 ± 37.6			
Relative N immobilization					
Spring	0	0	ns	**	ns
Summer	0.54 ± 0.11	0.63 ± 0.13			
Autumn	0.67 ± 0.13	0.74 ± 0.15			

Mean ± standard deviations ($n = 4$) followed by results of Repeated measures ANOVA are given

ns Non-significant

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

biomass was nitrified. On the contrary, there were significant net microbial N immobilization and high net N mineralization rates in summer and autumn (Table 4), which indicated much higher gross N mineralization. Relative N immobilization was zero in spring and tended to be higher in autumn versus summer (Table 4).

Discussion

Plant and soil characteristics of grasslands

Characteristics of plants exhibiting different degree of dominance at the two study sites demonstrate differences in C uptake and nutritional strategies consistent with soil characteristics. *Glyceria*-dominated grassland exhibited a higher concentration of soil soluble N, with a larger percentage of mineral N forms than the *Carex*-dominated grassland, indicating higher N availability. The higher soil trophy was connected with significantly higher N concentrations, lower C/N ratios, and faster N turnover in plant tissues and preferential plant biomass allocation aboveground, which indicated a shift from the competitive plant strategy in the *Glyceria*-dominated mesotrophic grassland towards a conservative strategy in the *Carex*-dominated oligotrophic grassland. Similar differences were also found in the biomass stoichiometry and allocation patterns of plant dominants from both grasslands, grown in pots for the purpose of the labelling experiment. In addition, a larger competitive ability of *Glyceria* compared to *Carex* was shown by Kastovska and Santruckova (2011) studying plant N nutrition preferences.

The ecosystem labile N pool and microbial N transformations

While the average turnover time of soil organic N is in the order of decades (Jenkinson 1990), the internal N cycling driven by plant–microbe interactions, commonly including ca. 5 % of the total N storage (referred to as the ecosystem labile N pool), is faster. Turnover times of components of the ecosystem labile N pool range from hours to days for the soluble N pool (Boddy et al. 2007) and active microbial biomass N (Schmidt et al. 2007; Kastovska and Santruckova 2011) to weeks/years for plant N (here; Fisk et al. 1998; Gill and Jackson 2000) and N in an inactive microbial biomass (Paul and Juma 1981). Therefore, a partitioning of the ecosystem labile N among these components can be a good indicator of the rate of N cycling in the system. In the *Glyceria*-dominated grassland, large proportion of microbial N and a significant contribution of soil soluble N to the ecosystem labile N pool pointed to significantly faster N cycling compared to the *Carex*-dominated grassland. There, plant N, especially below-ground plant N

with slow turnover, dominated the ecosystem labile N pool and exceeded microbial N. In contrast, soil soluble N pool formed very small proportion of the ecosystem labile N pool. The faster N cycling in the *Glyceria*- versus *Carex*-dominated grassland was further documented by significantly higher rates of net N mineralization, nitrification, and microbial N immobilization/release. The findings about the association of faster N cycling with competitive versus conservative plants are in accord with Hobbie (1992), Orwin et al. (2010), de Vries and Bardgett (2012) and Grigulis et al. (2013).

Seasonal plant–microbe redistribution of labile N

The faster N cycling in the *Glyceria*- versus *Carex*-dominated grassland was further associated with more pronounced seasonal redistribution of the labile N between microbes and plants (Fig. 2b). The seasonal N redistribution within the ecosystem labile N is consistent with the release of the majority of microbially immobilized N and its immobilization in growing plant biomass from spring until summer, followed by its immobilization back into the microbial biomass after plant senescence and litter decomposition in autumn–winter period. The seasonal N redistribution has an important role in the retention of labile N in the ecosystem (Bardgett et al. 2005) and was described also for N limited ecosystems in dry tropical forests and savannas (Singh et al. 1989), tallgrass prairie (Garcia and Rice 1994), northern hardwood forests (Zak et al. 1990), and alpine meadows (Jaeger et al. 1999; Lipson et al. 1999; Bardgett 2002; Schmidt and Lipson 2004; Bardgett et al. 2005).

The more pronounced seasonal N redistribution between microbes and plants in the *Glyceria*- versus *Carex*-dominated grassland was likely induced by a combination of the following factors: A larger plant N requirement connected with faster plant growth, plant N turnover and larger competitive ability of vegetation, and larger, more responsive soil microbial biomass with higher specific rates of N transformation per unit of microbial biomass. Higher specific microbial activity would enable closer plant–microbe cooperation, and an effective, fast coverage of plant N demands. At the same time, it points to a more direct plant effect on soil N cycling in the *Glyceria*- versus *Carex*-dominated grassland, in which plant exudation appears to play an important role.

Plant investment into exudation in connection to its growth strategy

A possible difference in root exudation between competitive and conservative plants was suggested in the literature due to differences in plant photosynthetic capacity and relative growth rate (Personeni and Loiseau 2004; De Deyn et al. 2008) as well as in soil N cycling associated with them (Orwin et al. 2010). Results from our $^{13}CO_2$ pulse labeling study lend support to this assumption. The competitive *Glyceria*, in spite of its smaller below-ground C allocation, invested significantly more of the net C fixation to exudation than the conservative *Carex*. On the other hand, *Carex* contributed to soil C pool mainly through the input of low-quality, nutrient poor root tissue with apparently slower turnover. Based on the final distribution of ^{13}C in plant–soil systems, measured investment into exudation (Table 2) and field measurements of the above- and below-ground plant production in both grasslands (Table 3), we roughly estimated the exudation 50 g C m^{-2} per season for the *Carex*-dominated grassland and 95 g C m^{-2} for the *Glyceria*-dominated grassland (Fig. 3).

A response of rhizomicroflora to root exudation and the amount of available N depends not only on the amount of exudates released to the soil but also on their C/N stoichiometry (Dalenberg and Jager 1989; Drake et al. 2013). In N limited conditions of the rhizosphere, the plant released N is used by microbes for exoenzyme synthesis driving organic matter mineralization (Drake et al. 2013). According to this logic, a lower C/N ratio of plant-released compounds would induce a relatively larger rhizosphere priming effect, which in turn could prompt a release of N available for plant uptake. In accordance with this, Cheng et al. (2003) and Fornara et al. (2011) showed that the rhizosphere priming effect is plant species specific and higher for plants with lower biomass C/N ratios. In the current study, it fits to the finding that *Glyceria* with lower plant C/N ratio promotes faster microbial N transformations in comparison with *Carex*.

Consequences for C and N cycling

The quantity and quality of plant input into the soil, which can be characterized by plant litterfall and rhizodeposition, serve as one link connecting ecosystem C and N cycles. The differential connectedness of

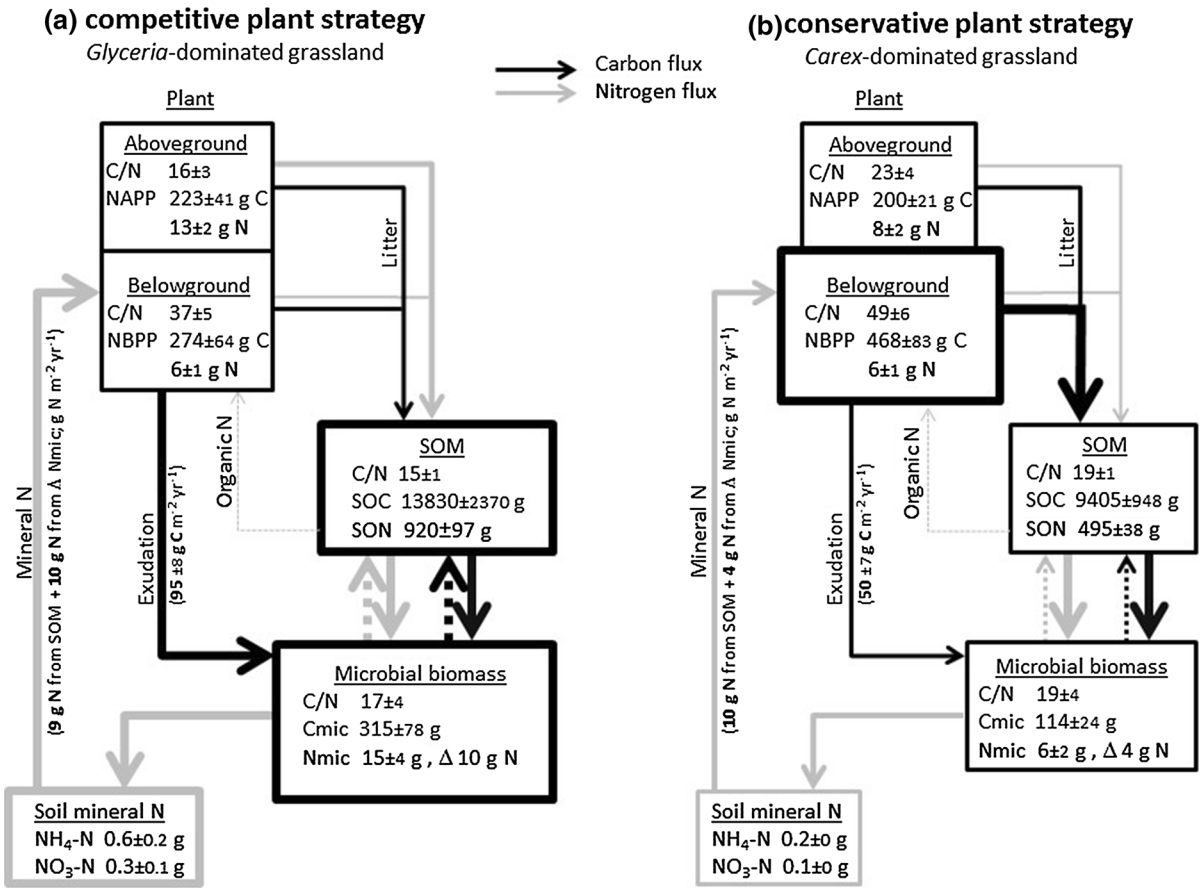

Fig. 3 C and N cycling in connection with **a** competitive plant strategy represented by the *Glyceria*-dominated grassland and **b** conservative strategy represented by the *Carex*-dominated grassland. *Box sizes* indicate quantitative differences in C and N pool between the grasslands; N contents and C/N ratios of the pools come from data presented here, C contents are from Kastovska et al. (2012). Means and standard deviations ($n = 12$) are given. *Arrow widths* indicate qualitative differences in C and N fluxes between the grasslands. Root exudation was estimated from the ^{13}C allocation pattern and its relative investment into exudation (Table 2) combined with the net above- and below-ground plant production in both grasslands (Table 3). Plant N uptake was put equal to the increase of plant N pool from spring to summer. The vast majority of the N was taken in mineral forms because the uptake of organic N by both plants is negligible (Kastovska and Santruckova 2011). The N taken up by plants could originate partly from the relatively labile N pool (a portion equal to a decrease of the microbial N pool from spring to summer; Fig. 2) and partly from the decomposition of soil organic matter. Other fluxes were not calculated. The *arrows* with *dashed lines* show an expected contribution of microbially transformed material to SOM, assuming to be larger in *Glyceria*- versus *Carex*-dominated grassland due to larger root exudation, higher litter decomposition rates (unpublished results) and enzymatic activity (Kastovska et al. 2012)

the plant–microbial interaction through different plant exudation in the *Glyceria*- versus *Carex*-dominated grassland has important consequences for the sizes of their respective C and N pools and fluxes. The competitive *Glyceria* has a large direct effect on soil C and N cycling through the large daily input of root exudates to the soil (Fig. 3). Through intensive exudation, it closely cooperates with the large and responsive microbial community, thereby facilitating plant N requirements about equally through a relatively large seasonal N redistribution from microbes to plants and N release after SOM decomposition (Fig. 3) induced mainly by the intensive rhizosphere priming effect (Personeni and Loiseau 2005; Personeni et al. 2005). At the ecosystem level, fast N turnover within the microbial loop is manifested by faster net N mineralization and larger total soil soluble N with a high proportion of mineral N forms. In autumn, a

higher capacity for microbial N immobilization reduces potential losses of labile N from the ecosystem (Schmidt et al. 2007; Kuzyakov and Xu 2013).

In contrast, the conservative *Carex* invested more C into the building of a large, nutrient poor root system, while decreasing C losses through exudation (Fig. 3). Production of low quality plant organic matter, with slow N turnover (Table 3), likely feeds back into maintaining the present vegetation–soil system (Wedin and Tilman 1990; Craine et al. 2002). The N supply of *Carex* likely relies mainly on slow SOM decomposition with less primed by root exudation, while the contribution of the seasonal decrease in microbial N was less important (Fig. 3). At the ecosystem level, the lower plant N requirements and less coupled plant–microbe interactions are manifested as lower net N mineralization, lower microbial N immobilization potential and a less pronounced seasonal N redistribution between plants and microbes in the system. The conservative plant has a smaller direct effect on the soil N cycle through exudation, which makes the plant–soil relation less dynamic, less responsive to environmental changes, but also less susceptible to N leakage after disturbance, compared to systems with competitive plants with large investments into exudation.

Conclusions

In this work, we demonstrate a variation in the degree of interconnection in time and space among features of ecosystem soil N cycling, soil N availability, plant biomass C/N ratios and plant exudation, as driven by plant–microbe interactions. We showed that competitive plants appear to affect soil N cycling through more direct temporal and spatial influence on soil microbes due to the larger investment into root exudation than more conservative ones. Differently, relationships between conservative plants and soil microbes appear to be less-coupled in time and space and N supply of the conservative plants likely relies mainly on the relatively slow microbial mineralization of organic matter, less dependent on rhizosphere priming effect. Therefore, soil microbial biomass associated with conservative plants is smaller, temporally less variable, with lower specific activity than microbes connected with competitive plants.

Our work suggests that soil N cycle associated with conservative plants is likely slower, with lower seasonal variability and fewer losses of bioavailable N compared to those dominated by more competitive, N-rich plants, and that these differences are driven by the degree to which competitive plants govern soil microbial activity via root exudation. We further showed that temporal variation in the composition of ecosystem labile N, especially the range of seasonal N redistribution between plants and microbes, can indicate well the coupling of the plant–microbial N relationship in time and space, dynamics of ecosystem N cycling and a propensity of the ecosystem to N leaching.

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PAPER 3

Kastovska E, Edwards K, Santruckova H (2017): Rhizodeposition flux of competitive versus conservative graminoid: contribution of exudates and root lysates as affected by N loading. *Plant and Soil* 412: 331-344

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REGULAR ARTICLE

Rhizodeposition flux of competitive versus conservative graminoid: contribution of exudates and root lysates as affected by N loading

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Abstract
Background and aims Carbon distribution between root production and rhizodeposition represents the plant strategy for growth and nutrient capture. It can shift in response to changed availability of limiting nutrients, with important consequences for ecosystem functioning. We studied the influence of nitrogen (N) availability on the belowground C fluxes of two wetland graminoids, the competitive *Glyceria maxima* and the conservative *Carex acuta*.
Methods Plants grown in pots under two levels of N availability were pulse-labeled with $^{13}CO_2$ and the ^{13}C distribution in the plant–soil systems was followed for 15 days. Together with ^{13}C allocation measurements, root production and death were estimated to constrain the belowground C fluxes, including rhizodeposition.
Results Higher N supply enhanced root biomass and, subsequently, the total rhizodeposition. Both species shifted partitioning of belowground C towards higher mass-specific root production and turnover, with lower investments into root exudation. Therefore, the rhizodeposition was enriched in root-derived lysates over soluble exudates. Increased total rhizodeposition and its changed quality enhanced the concentration of soluble organic C. The N fertilization induced changes in belowground C fluxes were species-specific. Contrary to *Glyceria*, *Carex* enhanced mass-specific root growth rate, which implied a markedly larger root-derived C flux to soil.
Conclusions In general, soil N loading enhanced total C rhizodeposition and, simultaneously, the proportion of predominantly more complex root lysates over soluble root exudates, with consequences for soil organic matter dynamics. Our results also underline the importance of species-specific responses to N loading in predicting total rhizodeposition flux and changes in its quality.

Keywords Fertilization · Allocation pattern · Root exudation · Root lysates · Rhizodeposition · Belowground C fluxes

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Introduction

Carbon allocation represents the ecological strategy of plant species for growth, reproduction and resource capture and is an important determinant of ecosystem functioning (Knops et al. 2002; De Deyn et al. 2008;

Orwin et al. 2010; Metcalfe et al. 2011; Grigulis et al. 2013). Plants exhibit relatively high plasticity in their allocation pattern in response to changes in the availability of nutrients limiting their production, namely nitrogen (N). Increased N loading enhances primary productivity and relative biomass allocation to aboveground parts in response to relieved competition for N below ground but increasing competition for light above ground (Robinson and Rorison 1988; Tilman and Wedin 1991; Liu and Greaver 2010; Pan et al. 2011). The N enrichment also changes belowground C dynamics (Liu and Greaver 2010; Bardgett et al. 2014; Legay et al. 2014). Roots of fertilized plants commonly have higher specific root length, area and N concentration, and lower root tissue mass density (Ryser 1996; Leuschner et al. 2013). It is connected with their enhanced mass-specific respiration (Paterson et al. 2006; Reich et al. 2008) and faster turnover (Ryser 1996), which implies an increased C input from the root tissue into N fertilized soils.

Beside the continuous turnover of root tissue (mainly of root cell caps and root hairs), root exudates significantly contribute to root C efflux in the soil. Root exudates, mostly simple soluble compounds originating from recent assimilates released from roots, can account for 10–60 % of rhizodeposition (Paterson 2003; Thornton et al. 2004; Jones et al. 2009). Plant C allocation into root exudation is also sensitive to N loading but the observed plant responses range from a decrease (Hodge et al. 1996; Cotrufo and Gorissen 1997; Paterson and Sim 1999; Kuzyakov et al. 2002), across no change (Warembourg and Estelrich 2000) to an increased C allocation to exudation after N fertilization (Warembourg and Estelrich 2001; Paterson et al. 2006). Consequently, the resulting effect of N fertilization on the total rhizodeposition flux, representing an important daily C input in the soil with large effect on soil organic matter turnover (Kuzyakov 2010) is virtually unknown. Further, potential fertilization-induced shift in contribution of soluble exudates and predominantly more complex root lysates to rhizodeposition can imply different microbial activity (Jaeger et al. 1999). Therefore, any shift in the belowground C fluxes and partitioning between root production, exudation and other rhizodeposits could affect the turnover and sequestration of soil organic matter (Dijkstra et al. 2005; Adair et al. 2009; Eisenhauer et al. 2012; Carrillo et al. 2014).

The belowground C partitioning between root production and different components of rhizodeposition could also vary with plant nutrient acquisition strategy (Personeni and Loiseau 2005). Generally, the total rhizodeposition is closely related to total root mass (Van der Krift et al. 2001; Baptist et al. 2015). However, competitive species are likely to have high exudation (Van der Krift et al. 2001; De Deyn et al. 2008), as their faster growth is linked to large quantities of assimilates, which are allocated to roots with lower tissue density, enabling their diffusion to soil (Vale et al. 2005). Differently, conservative plants have lower relative growth rates and allocate assimilates to roots of higher tissue density and slow turnover, thus their exudation may be lower. The differences in photosynthetic activity and root biomass characteristics between competitive and conservative species imply potentially various contributions of root lysates and exudates to rhizodeposition flux. Baptist et al. (2015) and Van der Krift et al. (2001) further documented changes in the total rhizodeposition caused by N fertilization were species specific, modulated by root responses to N availability. Also changes in mass-specific rhizodeposition, decomposition of rhizodeposits and related soil microbial activity were species specific, indicating potential fertility-induced shift in rhizodeposition quality. However, as both teams used long-term continuous ^{13}C (^{14}C) labeling, they could not get any additional information about the quality of soil C efflux to the soil.

In this study, we aimed to elucidate the effects of two levels of N availability on plant allocation patterns with emphasis on belowground C dynamics. We wanted to quantify how the belowground C is distributed between root production and rhizodeposition as well as between different components of rhizodeposition (exudation of recent assimilate and losses related to root death). We used two plant species differing in C allocation pattern and competitive ability for N, the fast-growing competitive *Glyceria* versus the slower growing, more conservative *Carex* (Kastovska and Santruckova 2011). Both species commonly dominate oligo-mesotrophic wet grasslands, often subjected to fertilization and/or eutrophication by flooding water, but no information exists about the influence of N availability on their C economics. In previous study we showed that under N limitation, *Carex*, irrespective of its larger root system, invested less C to exudation than *Glyceria*. It implied for less-coupled relation with soil microbes in time and space, lower seasonal N redistribution between plants ad microbes and slower soil N cycling (Kastovska et al. 2014). In this study, we wanted to extend the knowledge on the whole belowground C dynamics and its potential

fertility-induced changes. We used ^{13}C-CO_2 pulse labeling of plants in a pot experiment to study utilization of recent assimilates and their partitioning to root growth and exudation. We combined the ^{13}C allocation study with an estimation of net root production, which enabled us to assess root death and its contribution to rhizodeposition flux, and complete the information on belowground C fluxes. We hypothesize that fertilized plants will allocate a lower proportion of net fixed C below ground and shift the partitioning of the belowground C towards faster root production and turnover at the expense of exudation compared to control plants. This pattern would be more pronounced in the conservative *Carex*. The expected shift in the distribution of belowground C will increase the proportions of root lysates over exudates in the rhizodeposition flux, with an unknown effect on the total amount of organic C released from roots.

Material and methods

Experimental material

Experimental plants were taken from a non-fertilized wet grassland, located in the Třeboň Basin Biosphere Reserve, Czech Republic. Fifty individual plants of *Glyceria* and *Carex* were sampled in early May 2008. Their roots were washed with tap water to remove soil particles and plants were grown in a mixture of the field soil and sand (3/1; soil/sand weight ratio, mixing was necessary to facilitate a complete separation of roots and soil for the ^{13}C measurement) in pots (in 300 g of fresh substrate) in open air for 2 months. Half of the pots with each species were fertilized with a solution containing a mineral NPK fertilizer in a dose of 300 kg NPK ha^{-1} yr^{-1} (N:P:K ratio 2.3:1:2, corresponding N dose of 45 kg ha^{-1} yr^{-1}) divided into two half-doses. The first dose of fertilizer was applied a week after planting and second one after 30 days.

Pulse-labeling experiment

Pulse labeling of plants with $^{13}CO_2$ was used to trace dynamics of recent assimilate and its final distribution among compartments of plant-soil system, with an emphasis to track inputs derived from recent assimilate in growing roots and in the soil. The pulse labeling was done after 50 days of plant growth in pots, on July 8, 2008 (10:00–14:00, mostly sunny weather, max. temperature 23 °C). Pots with plants were placed in four gas-tight plexiglass chambers (50 × 50 × 50 cm) with holes fitted with rubber septa. After the ambient CO_2 concentration in the chambers decreased below 200 ppm (verified by gas chromatograph), $^{13}CO_2$ tracer (99.9 atom % ^{13}C, Cambridge Isotope Laboratories, GB) was repeatedly pulsed into the chambers by a syringe to keep the internal CO_2 concentration at 400–500 ppm for a period of 4 h. The CO_2 concentration in chambers was checked regularly using gas chromatograph. A total amount of 200 ml (8.9 mmol) of $^{13}CO_2$ was added to each chamber during the labeling, which corresponded to 116 mg ^{13}C-CO_2 per chamber and ca 4.6 mg ^{13}C-CO_2 per plant. Air circulation during labeling was ensured by two 1 watt fans. The chambers were removed after labeling and pots with plants were left in fresh air. The remaining pots were not labeled, but used as a control for determining the ^{13}C natural abundance.

Harvests and analyses

The first harvest was done immediately after the end of the 4-h labeling period and then 1, 3, 8, and 15 days after the beginning of pulse-labeling. At each sampling time, four fertilized and four non-fertilized pots with plants of each species were harvested (always one fertilized and one non-fertilized pot from each chamber). The aboveground part (shoot) of each plant was cut off. The corresponding belowground parts (roots and rhizomes) were carefully cleaned by hand to remove soil and then quickly washed under tap water. Plant material was dried (60 °C), weighed and ground. All soil from a pot was thoroughly homogenized. A subsample of each soil sample (5 g) was oven-dried at 105 °C to determine water content. Another subsample was dried at 60 °C and ground. The remaining fresh soil was sieved through a 5-mm sieve. Subsamples (10 g) were fumigated by chloroform (24 h) and then extracted with 50 mM K_2SO_4 (20 ml) for 30 min to obtain the chloroform-labile C pool, which contains soil soluble C and microbial biomass C, the most labile soil C pools. The extracts were filtered and soluble organic C was analyzed on a LiquicTOC II (Elementar, Germany). The remaining extract was freeze-dried. Dried and ground shoot, root and soil samples, and freeze-dried soil extracts (representing the chloroform-labile C pool) were weighed in tin cups.

Analyses of C, N and ^{13}C (in ^{13}C atomic percent, at.%) contents were conducted on an NC Elemental analyzer (ThermoQuest, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany).

Calculations

A binary mixing model with ^{13}C at.% was used to estimate the amount of the pulse-derived ^{13}C in the various C pools investigated:

$$^{13}C\ (\mu g\ g^{-1}) = \left[\left(at.\%_{sample} - at.\%_{control}\right)/(99.9 - 1.10)\right] * C\ pool\ size\ (\mu g\ C\ g^{-1})$$

where $at.\%_{control}$ is the natural abundance in the control samples, 99.9 is the pulse ^{13}C at.% and 1.10 is the at.% of the ambient atmosphere.

Net C fixation (μg ^{13}C plant^{-1}) was calculated as the sum of ^{13}C amount in shoots, roots, and soil after the 4-h labeling period. Differences between the net C fixation and amounts of ^{13}C in the system on the following sampling dates were considered as respiration losses. They were divided between the shoot and root-derived respiration as follows. The shoot respiration in the first period of rapid ^{13}C loss from the systems (0–3 days) was calculated as the portion of the daily respiration loss corresponding to the aboveground ^{13}C/total system ^{13}C ratio. Later, when negligible changes occurred in the shoot ^{13}C, a maintenance respiration was ascribed to shoots being 0.1 % shoot ^{13}C d^{-1} (Kuzyakov et al. 2001). The difference between the total and shoot respiration was ascribed to the root-derived respiration. The total amount of ^{13}C allocated below ground was calculated as the sum of the final root and soil ^{13}C, and the root-derived respiration. The ^{13}C distribution in the plant–soil system was expressed on relative basis (% net C fixation). Kinetics of distribution and turnover rate of newly fixed ^{13}C in particular compartments of the system was estimated from its temporal changes. For this purpose, the daily net C fixation (μg ^{13}C plant$^{-1}d^{-1}$) was taken as the starting point for each plant soil system. The daily net C fixation was estimated from the net ^{13}C fixation divided by 4 h of labeling-period, assuming 16 h of day light.

The maximum soil ^{13}C content, which occurred between 4 and 48 h after labeling in all treatments, was ascribed to root exudation of simple compounds (net exudation). Later, turnover of simple root structures such as root cap cells and root hairs, derived from recent assimilate, contributed to the input of ^{13}C to soil (Warembourg and Estelrich 2000; Kuzyakov 2002). We are aware of the conservative estimation of the exudation due to the rapid turnover of the exuded compounds and microbial biomass in soil (Boddy et al. 2007; Kastovska and Santruckova 2007). However, the mistake in estimation of root exudation will be relatively small shortly after labelling.

Physiological root traits were calculated to show any shift in costs of root functioning after fertilization and to complete the estimation of belowground C fluxes. Net root growth rate (μg C plant^{-1} day^{-1}) for control and fertilized plants was assessed from the slope of the regression of temporal changes in root C biomass of plants sampled during the 15 days chase period after labeling. Gross root growth rate (μg ^{13}C plant^{-1} day^{-1}) was put equal to the part of daily net ^{13}C plant fixation (μg ^{13}C plant^{-1} d^{-1}) allocated to roots. The ^{13}C allocation to roots was taken from final ^{13}C partitioning 15 days after labeling (given in Figs. 1, 2 and 3), when belowground C fluxes should be in equilibrium (Warembourg and Estelrich 2000; Leake et al. 2006). The difference between gross and net root growth rates represented the root death rate (μg C plant^{-1} day^{-1}) and, at the same time, represented a rough estimate of the lysate flux originating from dead, unlabeled roots. Root turnover rate was estimated as the proportion of daily dying roots per to total root biomass (% C day^{-1}). Exudation rate (μg C plant^{-1} day^{-1}) corresponded to the part of the daily net ^{13}C plant fixation released by roots during 24 h after labeling (net exudation, Fig. 3). The sum of daily exudation flux and daily C loss via root turnover equaled to the rhizodeposition rate (μg C plant^{-1} day^{-1}). All the fluxes were then expressed per mass unit of root C (μg C g^{-1} root C day^{-1}), which enabled better comparison of C economics of both species in dependence on different N availability.

Statistics

Means and standard deviations ($n = 24$ for characteristics of plant biomass, $n = 4$ for data from the labeling experiment and field measurements) were calculated for

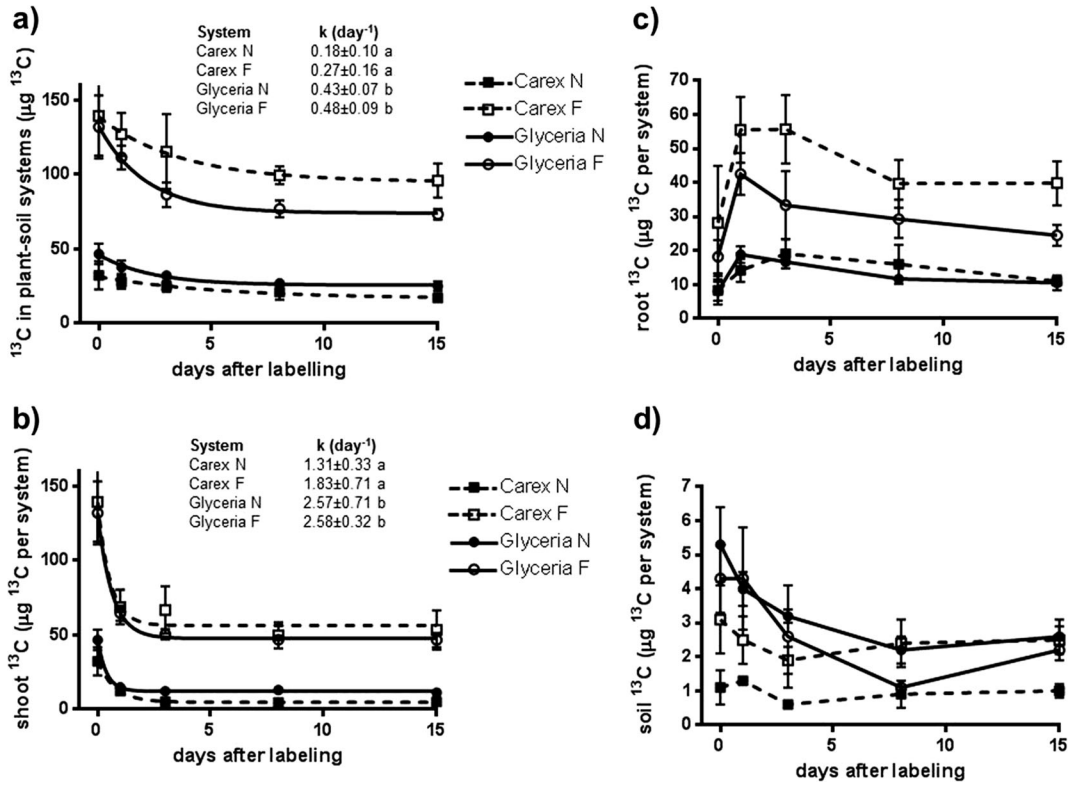

Fig. 1 Temporal dynamics of **a** ^{13}C in the plant-soil systems, **b** the shoot ^{13}C, **c** the root ^{13}C and **d** the soil ^{13}C pool in the non-fertilized (N) and fertilized (F) plant-soil systems with *Carex acuta* and *Glyceria maxima* during 15 days after labelling. The daily net ^{13}C fixation in particular plant-soil systems are shown (day 0), followed by changes in absolute amounts of ^{13}C in the given compartments. The ^{13}C kinetics in the plant-soil systems and shoots were fitted by the equation of one-dose exponential decay. The decay constants k (day^{-1}) are given for each fit, together with results of One-way ANOVA and Tukey test marked by lower case letters

all data. Repeated measures ANOVA was used to assess effects of plant species and fertilization on plant biomass characteristics in the pot experiment. Data on temporal changes in ^{13}C amounts in the studied pools were fitted using a one-phase exponential decay model in GraphPad Prism 6.0 (Fig. 1) to get the ^{13}C decay rate k in the plant-soil system and shoots. Factorial Two Way ANOVA was used to assess effects of plant species and fertilization of the net ^{13}C fixation and its distribution between plant and soil compartments and physiological root traits.

Results

Plant characteristics and soil microbial biomass

At the time of the labeling experiment, the growth and biomass characteristics of *Carex* and *Glyceria* control plants differed in accordance with their different exploitation type. The conservative *Carex* had lower shoot/root ratio, and significantly higher C/N tissue ratios compared to competitive *Glyceria* (Table 1). This was mirrored in the *Carex* soil containing larger microbial C pool and higher C/N ratio of microbial biomass than in the *Glyceria* system (Table 1). Fertilization increased plant biomass and also the shoot/root ratios of both plants, with a more pronounced effect for *Carex* (Table 1). Fertilized plants had higher N concentrations and thus significantly lower C/N ratios in the tissues, again with a larger shift in *Carex* than *Glyceria*. Temporal changes in shoot biomass and plant tissue C and N concentrations were negligible, while roots grew significantly during the 15 day after labeling (Table 1; significant effect of time on root biomass). Fertilization also enhanced concentration of soluble organic C in soils.

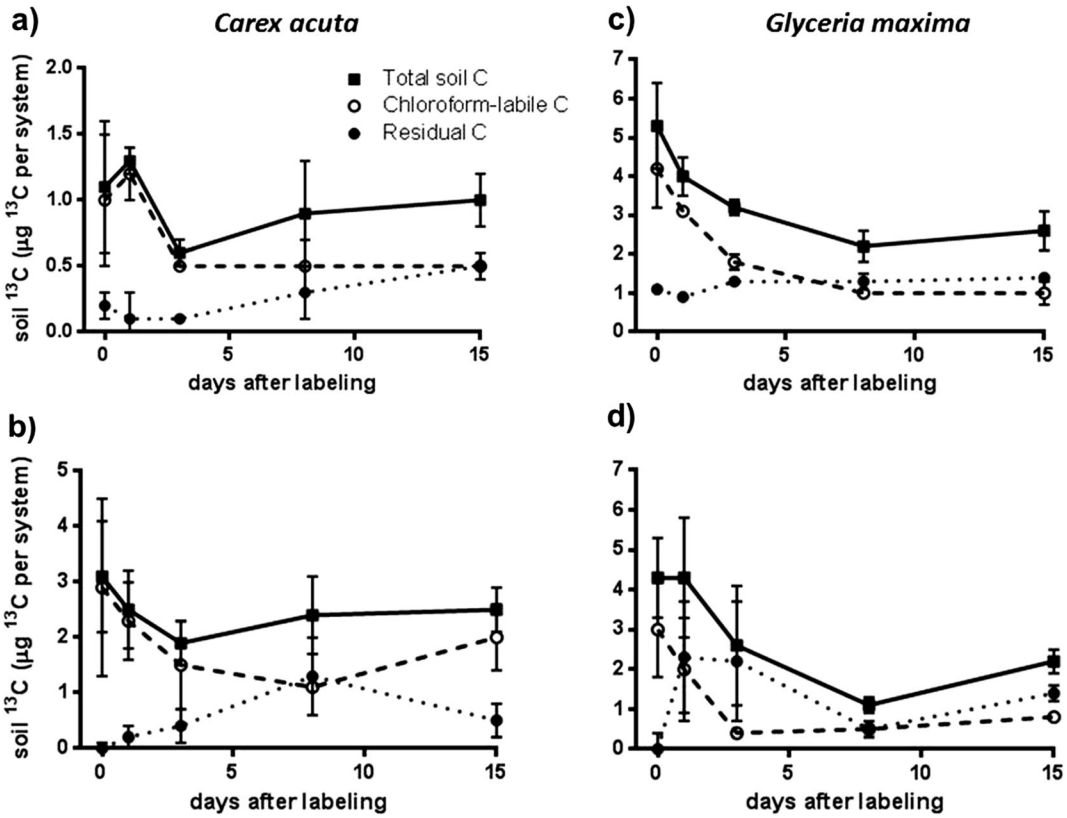

Fig. 2 Temporal changes of the total soil ^{13}C and its distribution between the chloroform-labile and residual C pool in **a** non-fertilized and **b** fertilized soil with *Carex acuta*, and **c** non-fertilized and **d** fertilized soil with *Glyceria maxima* during 15 days after labeling. Mean and standard deviations ($n = 4$) are given for each sampling time

Temporal changes in distribution of the fixed ^{13}C in plant-soil systems

The net ^{13}C fixation rate did not differ between species but was significantly enhanced after fertilization (Fig. 1a, Table 2), through the positive effect of higher N availability on their plant biomass production (Table 1). Therefore, fertilized plants contained significantly more ^{13}C in shoots than non-fertilized ones (Fig. 1b). Consequently, more ^{13}C was transported to roots and released to soils after N loading (Fig. 1c, d). However, the differences between fertilized and non-fertilized systems in ^{13}C amounts diminished from shoots, across roots, to soils. In case of *Glyceria*, the difference in the soil ^{13}C between N rich and poor soil was not significant (Fig. 1d). Otherwise, fertilization effect on the ^{13}C dynamics in plant-soil systems was weak. Generally, fertilized systems showed a tendency for the acceleration of ^{13}C turnover in the plant-soil system in the case of both species (Fig. 1a).

There was a significant species effect on ^{13}C dynamics within the plant-soil systems. In the *Carex*-system, lower turnover rate constant (k) indicated slower utilization and turnover of the fixed ^{13}C, and resulted in lower ^{13}C losses in comparison to the *Glyceria*-system (Fig. 1a). Although the amount of ^{13}C transported by *Carex* to roots was comparable with *Glyceria* in non-fertilized systems or even significantly higher in fertilized systems (Fig. 1c), *Carex* released a lower amount of ^{13}C from roots to soil (Fig. 1d). Independently of fertilization, the amount soil ^{13}C declined faster in system with *Glyceria* than with *Carex* (Fig. 1d). For both plants independent of fertilization, chloroform-labile compounds formed the majority of the soil ^{13}C shortly after labeling. The proportion of residual (chloroform non-labile) fraction within the soil ^{13}C increased in all systems later over time, indicating a contribution of non-soluble forms of rhizodeposits and microbial transformation of released compounds (Fig. 2).

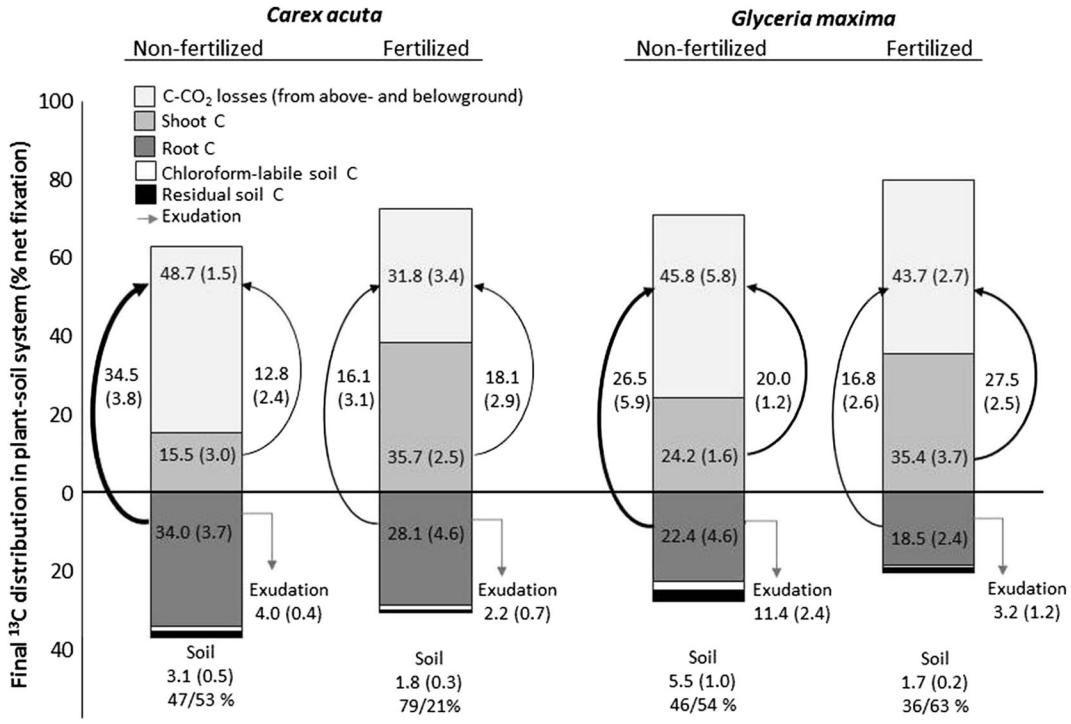

Fig. 3 The final ^{13}C distribution (% net ^{13}C fixation) in the non-fertilized and fertilized systems with *Carex acuta* and *Glyceria maxima* 15 days after labeling. Contributions of root-derived and shoot respiration to total ^{13}C-CO_2 losses and ^{13}C partitioning to net exudation are also shown using arrows. Means and standard deviations ($n = 4$) in brackets are shown. Results of factorial ANOVA with plant species (P) and fertilization (F) as categorical predictors are given in Table 1 in supplement

The final relative ^{13}C partitioning in plant-soil systems

Fertilization significantly shifted relative ^{13}C partitioning in plant–soil systems. Both plant species markedly increased their ^{13}C partitioning aboveground, while suppressing the relative belowground ^{13}C allocation (Fig. 3). Consequently, a contribution of root-derived respiration to total C-CO_2 loss declined, while that of shoot respiration increased. The final shift in ^{13}C partitioning towards aboveground was more pronounced in *Carex*, consistently with the larger shift in its biomass shoot/root ratio observed after fertilization (Table 1). The reduced belowground ^{13}C partitioning was mirrored by a decrease in relative net exudation by both fertilized plant species and especially by *Glyceria*.

Independent of fertilization, *Carex* largely exceeded *Glyceria* in the proportion of ^{13}C transported below ground and kept in roots (Fig. 3) but lagged behind in the proportion of ^{13}C released in the form of exudates during 24 h after labeling (exudation, Fig. 3).

Fertilization effect on belowground C fluxes

The belowground C dynamics was significantly affected after N fertilization. Fertilization enhanced gross and net root growth, with more pronounced effect in the case of *Carex* (Table 2). The larger difference between the gross and net root growth rate resulted in a faster root death rate and concomitant release of root-derived lysates to the soil in fertilized than control systems (Table 2). The C flux derived from dead roots reached 4–7 μg C g^{-1} root-C day^{-1} in unfertilized soils and 14–36 μg C g^{-1} root-C day^{-1} in fertilized soils, being comparable or even larger than the root exudation fluxes. The fertilization effect of belowground C dynamics was more pronounced in *Carex*, where it markedly increased rhizodeposition flux per unit of root mass due to stronger effect on the root turnover rate, while keeping the exudation flux almost unchanged (Table 2). In the case of *Glyceria*, the significant increase in root C losses due to enhanced mass-specific root death rate was compensated by a decrease in the exudation flux. Therefore, the resulting rhizodeposition flux per unit of root mass was

Table 1 Shoot and root biomass of the plants in the pot experiment, their tissue C and N concentrations and shoot and root growth rates, soil microbial biomass C and its molar C/N ratio, and concentration of soluble organic C in the soil; means and standard deviations ($n = 24$)

	Carex acuta				*Glyceria maxima*				Plant	Fert	Time	P×F	P×T	F×T	P×F×T
	Non-fertilized		Fertilized		Non-fertilized		Fertilized		(P)	(F)	(T)				
Shoots (g)	0.27^{a}	(0.14)	1.18^{b}	(0.64)	0.48^{a}	(0.20)	1.36^{b}	(0.48)	*	***	ns	ns	ns	ns	ns
Roots (g)	0.57^{a}	(0.27)	0.87^{ab}	(0.55)	0.60^{a}	(0.31)	1.08^{b}	(0.52)	ns	***	*	ns	ns	ns	ns
Shoot/root ratio	0.50^{a}	(0.16)	1.59^{c}	(0.51)	0.83^{b}	(0.25)	1.38^{c}	(0.34)	(*)	***	ns	***	ns	ns	ns
Shoots:															
C (%)	44.1^{a}	(1.1)	44.1^{a}	(0.6)	42.0^{b}	(0.6)	42.6^{b}	(0.6)	***	ns	ns	ns	ns	ns	ns
N (%)	1.41^{a}	(0.37)	1.95^{bc}	(0.44)	1.73^{b}	(0.28)	1.83^{c}	(0.46)	ns	*	ns	**	ns	ns	ns
C/N	33.5^{a}	(12.1)	23.8^{b}	(5.5)	24.1^{b}	(8.1)	23.6^{b}	(9.9)	*	**	ns	*	ns	ns	(*)
Roots:															
C (%)	42.3^{a}	(1.1)	41.5^{b}	(1.3)	41.0^{b}	(1.1)	41.3^{b}	(0.6)	**	ns	ns	ns	ns	ns	ns
N (%)	0.63^{a}	(0.12)	0.92^{b}	(0.32)	0.78^{a}	(0.15)	0.95^{b}	(0.16)	(*)	**	ns	ns	ns	ns	ns
C/N	67.9^{a}	(11.9)	50.0^{bc}	(15.7)	54.5^{b}	(11.1)	44.7^{c}	(6.9)	**	***	ns	ns	ns	ns	ns
Soil:															
Microbial C (μg C g^{-1})	647^{b}	(23)	816^{c}	(79)	488^{a}	(88)	496^{a}	(13)	***	*	ns	*	ns	ns	ns
Microbial C/N ratio	15.3^{b}	(1.2)	13.7^{b}	(2.0)	7.1^{a}	(0.8)	6.7^{a}	(0.2)	***	ns	ns	ns	ns	ns	ns
Soluble org. C (μg C g^{-1})	45.5^{a}	(5.6)	60.8^{b}	(7.8)	36.5^{a}	(2.8)	72.1^{b}	(11.1)	ns	**	ns	ns	ns	ns	ns

Results of factorial ANOVA with plant species (P), fertilization (F), and sampling time (T) as categorical predictors and post-hoc test (indicated by lower case letters, $P < 0.05$) are also shown

ns non-significant

(*) $P < 0.1$

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

not affected by fertilization (Table 2). The C loss via rhizodeposition reached 7–14 % of daily net C fixation by plants and corresponded to 23–72 % of daily C investments into the net root growth (Table 2).

Discussion

Increased N loading has been recognized as an important factor influencing ecosystem C sequestration through increases in plant biomass and changed plant-derived C input to soil (Liu and Greaver 2010; Sillen and Dieleman 2012). While such effects of N addition are well-known on the aboveground part of the system, N effects on belowground processes remain substantially less clear (Liu and Greaver 2010). Therefore, we aimed to determine the fertility-induced changes in belowground C dynamics and quality of root-derived C input to the soil. Specifically, we wanted to know how belowground C is distributed between net root production and the two most important components of rhizodeposition, root lysates supplied by root turnover and exudation. Root tissue senescence and rhizodeposition represent soil C inputs differing in the timing when they enter the soil, quality, turnover and functioning in the soil system (Nguyen 2003; Jones et al. 2009). Their quantification can help to fill the gaps in the understanding of C cycling belowground.

Belowground C dynamics and their components

Continuous labeling is commonly used to get information on total root C input to the soil, because it assures homogeneous labeling of the plant tissue and tracing all compounds released from roots (Meharg 1994). However, continuous labeling does not discriminate between root exudation and root turnover, formed by distinct materials with potentially different effects on rhizosphere microbial activity (Jaeger et al. 1999). We thus

Table 2 The daily net ^{13}C fixation per plant and mass-specific root physiological traits expressed on a root C basis of *Carex* and *Glyceria* plants; means and standard deviations ($n = 4$) are given

		Carex acuta				*Glyceria maxima*				Plant	Fert	P×F
		Non-fertilized		Fertilized		Non-fertilized		Fertilized		(P)	(F)	
Daily net ^{13}C fixation	(μg ^{13}C plant^{-1} d^{-1})	32.32^{a}	(9.60)	139.36^{b}	(26.56)	46.72^{a}	(6.88)	132.25^{b}	(21.28)	ns	***	ns
Mass-specific net root growth rate[1]	(μg C g^{-1} root C d^{-1})	41.58	(17.19)	72.64	(23.91)	35.33	(17.17)	40.93	(17.96)	ns	(*)	(*)
Mass-specific gross root growth rate[2]	(μg C g^{-1} root C d^{-1})	45.58^{a}	(4.96)	108.46^{b}	(17.76)	42.54^{a}	(8.74)	54.75^{a}	(7.10)	ns	***	**
Mass-specific root death rate[3]	(μg C g^{-1} root C d^{-1})	4.00^{a}	(1.43)	35.82^{c}	(5.86)	7.21^{a}	(3.07)	13.82^{b}	(3.79)	*	***	**
Root turnover rate[4]	(% C day^{-1})	0.40^{a}	(0.04)	3.58^{c}	(0.59)	0.69^{a}	(0.14)	1.38^{b}	(0.18)	*	***	**
Root turnover time	(days)	250^{c}	(27)	28^{a}	(5)	145^{c}	(39)	72^{b}	(9)	ns	***	**
Mass-specific exudation rate[5]	(μg C g^{-1} root C d^{-1})	5.36^{a}	(0.54)	8.49^{a}	(2.70)	19.65^{b}	(4.56)	9.47^{a}	(3.55)	***	*	***
Mass-specific rhizodeposition rate[6]	(μg C g^{-1} root C d^{-1})	9.36^{a}	(0.94)	44.31^{c}	(14.10)	26.86^{b}	(5.65)	23.29^{b}	(8.73)	ns	*	**
	(% net C fixation)	7.0^{a}	(0.7)	11.5^{a}	(3.7)	14.1^{b}	(3.0)	7.9^{a}	(3.0)	*	*	*
	(% net root growth)	22.5		61.0		72.0		56.9				

Results of a Two-Way ANOVA with plant species (P) and fertilization (F) as categorical predictors and post-hoc test (indicated by lower case letters, $P < 0.05$) are also shown

[1] Mass-specific net root growth rate was assessed from temporal changes in root C amount using regression

[2] Mass-specific gross root growth rate was equal to the proportion of daily net ^{13}C fixation allocated to root growth (in Fig. 3)

[3] Mass-specific root death rate is the difference between gross and net root growth rates

[4] Root turnover rate was calculated from a ratio of dying root C to total root C amount and expressed in %C per day, root turnover time is a reciprocal value of turnover rate

[5] Mass-specific exudation rate was equal to the proportional flux to exudation (in Fig. 3) from daily net ^{13}C fixation

[6] Mass-specific rhizodeposition rate is the sum of daily C fluxes from dead roots and exudation

ns non-significant

(*) $P < 0.1$

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

used a different approach, which enabled both the estimation of exudation and root death, and total rhizodeposition flux as the sum of the two components. We combined ^{13}C pulse labeling to assess the root exudation flux and gross root growth rate with measurements of net root growth rate. In this way, we could roughly estimate root death, which represents a source of root lysates entering the soil.

When quantifying the exudation flux of the recently fixed assimilates to the soil, we took advantage of the massive labeling of assimilates by the ^{13}C pulse labeling and directly measured the amount of ^{13}C released from root to the soil. We used a conservative estimate of exudation based on the peak of the fixed ^{13}C occurring within 24 h after pulse labeling of plants in the soil until microbial uptake and mineralization decreased the amount of soil ^{13}C (Kuzyakov 2002; Hill et al. 2007; Clayton et al. 2010). In our systems, the exudation flux reached 5–20 μg C g^{-1} root-C day^{-1}, being similar to the values observed for other grasses, which typically range from 1 to 10 μg C g^{-1} root-C day^{-1} (Hodge et al. 1997; Paterson and Sim 1999; Paterson 2003). The exudation flux represented 2–11 % of the net assimilated C, which is in accord with the 1–10 % of net C fixation estimated by Jones et al. (2004) and Farrar et al. (2003).

The root C efflux supplied by "older" C from continuous root tissue turnover (mainly of root cell caps and root hairs) cannot be tracked using pulse labeling. We put it equal to the root death rate, estimated as the difference between gross and net root growth rates (see methods for detailed calculations). While the net root growth was measured directly, the gross root growth rate was calculated as a part of the net daily ^{13}C fixation incorporated to roots. To obtain a reasonable estimation, we took the ^{13}C allocation to roots from the final ^{13}C partitioning 15 days after labeling, when belowground C fluxes should be in equilibrium (Warembourg and Estelrich 2000; Leake et al. 2006) and all the root ^{13}C should be incorporated into the root biomass. We further stress the importance of complete root sampling for the estimation of gross root growth rate, because especially young fine roots have a high concentration of recently assimilated C (Thornton et al. 2004). The C flux derived from dead roots reached 4–5 μg C g^{-1} root-C day^{-1} in unfertilized soils and increased to 14–20 μg C g^{-1} root-C day^{-1} in N fertilized soils, being in the same order of magnitude as the exudation flux. The total rhizodeposition flux (9–44 μg C g^{-1} root-C day^{-1}) is within the range of values measured for four grasses under different N availabilities by Baptist et al. (2015), who used a very precise method of ^{13}C continuous labeling. The good agreement with the direct measurement of rhizodeposition shows that the combined approach proposed here could give a good estimation of the amount of C released from roots together with an insight into its sorting between exudation and root lysate flux.

Root exudates accounted for 60–70 % of the rhizodeposition flux in non-fertilized soils but their contribution decreased to 20–40 % after N loading. This wide range of values is similar to the 10–60 % of rhizodeposition found in other studies (Meharg and Killham 1988; Paterson 2003; Thornton et al. 2004; Jones et al. 2009). We are aware that the estimated C partitioning between the two rhizodeposition components based on pulse labeling is specific for the given plant growth stage, which was the period of active plant growth in our case. Still, we can generalize that rhizodeposition C loss is an important and non-negligible portion of the plant C economy. Although being less than the plant investment to root growth, rhizodeposition still corresponded to 22–72 % of the net belowground plant production. Therefore, along with senescing root mass, it represents a highly significant soil C input.

Fertilization-induced shifts in plant C allocation and its belowground distribution

N fertilization induced changes in C allocation in our plant-soil systems, which are potentially important for soil microbial activity and soil organic matter dynamics. As in other studies (Warembourg and Estelrich 2000; Henry et al. 2005; Adair et al. 2009; Eisenhauer et al. 2012), N fertilization stimulated plant growth. Thus, despite reduced relative C allocation below ground by ca 20–25 % of net fixation (similar to Cotrufo and Gorissen 1997; Kuzyakov and Domanski 2000; Nguyen 2003; Henry et al. 2005), fertilized plants had larger root biomass than non-fertilized ones. Given the importance of belowground litter input into the soil for soil C storage (Rasse et al. 2005; Freschet et al. 2013), the increased root production may have a positive effect on soil organic matter content in the fertilized soil.

The larger root biomass implied a larger total rhizodeposition flux to the soil (Fig. 4), similarly as shown by Baptist et al. (2015) and Van der Krift et al. (2001). Additionally, we showed that the N richer roots of the fertilized plants contained more C originating from recent assimilates, similarly as found by

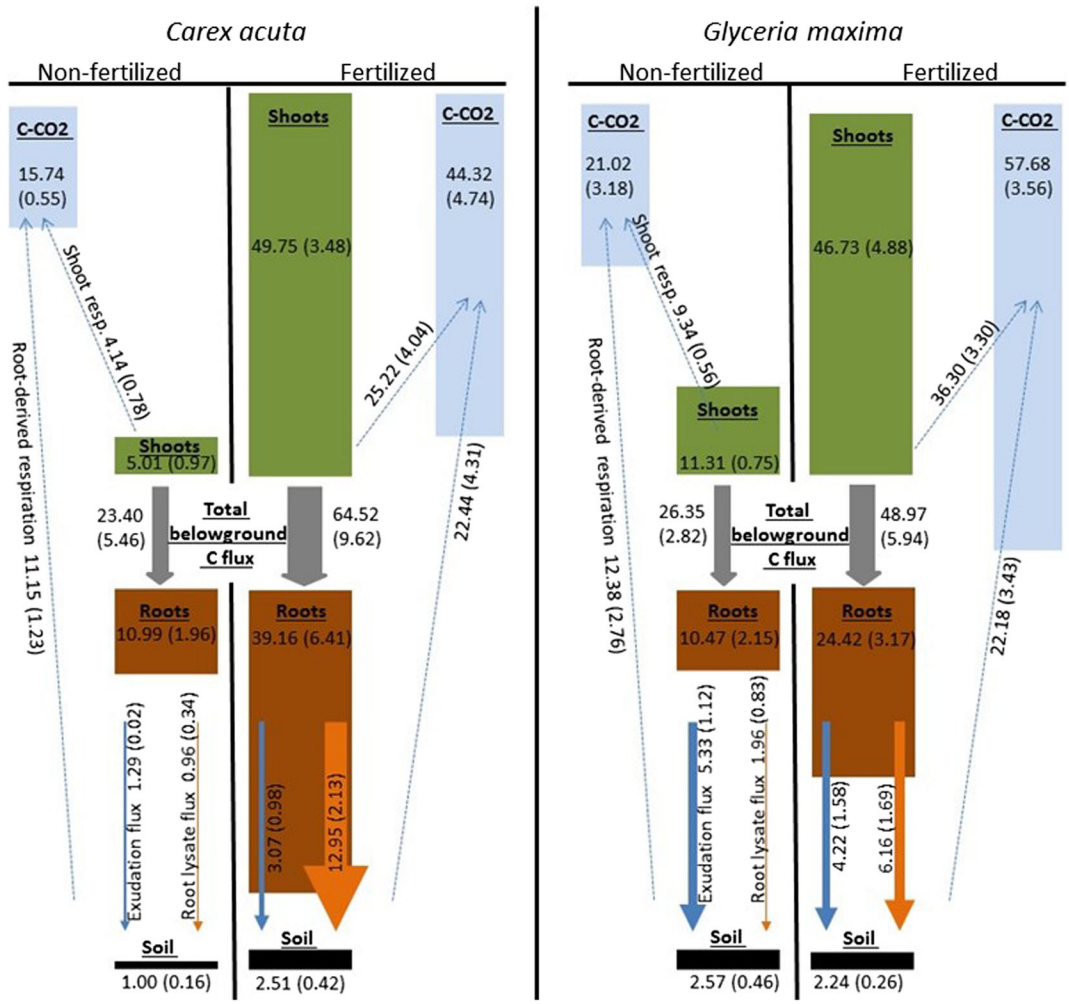

Fig. 4 Daily C fluxes of recent assimilates (μg C d^{-1}) in the non-fertilized and fertilized systems with *Carex acuta* and *Glyceria maxima*. The fluxes were estimated from the daily net ^{13}C fixation (assuming 16 h of day-light) and final ^{13}C distribution among particular compartments of plant-soil systems (given in Fig. 3). The rhizodeposition flux supplied by root-lysates is also shown. The box sizes (for shoots, roots, soil and CO_2 losses) are proportional to representative values, similarly as the arrows representing fluxes of root exudates and root lysates to soil. The most significant effect of N fertilization on ecosystem functioning and decomposition processes in soil is its stimulating effect on primary productivity. It implies larger input of the above- and belowground litter to the soil but also larger total rhizodeposition flux. Fertility-induced root growth results in larger contribution of root lysates to total rhizodeposition flux, while the portion of root exudates diminishes. A response to N fertilization was more pronounced for *Carex* investing significantly more recent assimilates to root growth in accord with its conservative nutritional strategy, which was related to large C losses via rhizodeposition

Henry et al. (2005), and turned over faster than roots of plants grown in N-poorer conditions. The results are similar to those of Ryser (1996), who proposed a shorter lifespan for more active roots, connected with their higher N concentration and lower root tissue mass density (Ryser 1996; Leuschner et al. 2013). Together with larger investments to root growth, fertilized plants reduced the partitioning of recent assimilates to exudation in comparison to non-fertilized ones, which resulted in an enhanced proportion of root lysates in the rhizodeposition flux by ca 30–40 %. Therefore, the majority of the root C efflux of fertilized plants was supplied by root turnover (Fig. 4). This result likely means that there is a predominance of more complex compounds released from cell autolysis, including structural compounds from sloughed root cap cells and root hairs (Leigh et al. 2002; Meier et al. 2009), requiring enzymatic

degradation before the C becomes generally available to soil microbes.

This N fertilization-induced change in the quality of the daily root-derived C input to the soil implies differences in microbial utilization of the substrate and its potentially distinct role in soil organic matter dynamics (Jaeger et al. 1999). Concomitantly, we found a 50–70 % increase in concentrations of soil soluble C in the case of both plant species in fertilized versus N-poorer soils. This could be related to increased total rhizodeposition flux but also to accelerated decomposition of soil organic matter due to enhanced exoenzymatic activity, stimulated by the presence of suitable complex substrates. Unfortunately, we did not measure soil enzymatic activities to test this hypothesis. Still, the fertilization-induced increase in microbial biomass C could indicate enhanced enzymatic activity after N loading at least in the *Carex* system. We suggest that the N fertilization-induced changes in rhizodeposition quality towards more complex compounds from root turnover could stimulate the activity of extracellular enzymes, which could have a potential impact also on soil organic matter decomposition.

Plant species effect on ^{13}C dynamics and belowground C fluxes

We found some species specific differences in C dynamics and distribution in the plant-soil system. The system associated with *Carex* utilized the fixed C more slowly than that of the faster-growing *Glyceria*, with the differences in ^{13}C dynamics occurring both above and below ground. The slower turnover of recent assimilates in *Carex* shoots was accompanied with their prolonged transport below ground, slower release from roots and low exudation flux. It may be related to the conservative strategy of the plant, which is characterized by higher tissue density and a longer lifespan (Ryser 1996; De Deyn et al. 2008; de Vries and Bardgett 2012). The exudation flux can be further reduced due to the presence of "dark septate endophytic fungi", which were detected microscopically in the roots of *Carex* plants sampled in the field. These fungi may have large C demands analogous to classical mycorrhiza (Johnson et al. 2002), thus diminishing root exudation.

Independent of N fertilization, the compounds released from *Carex* roots were of a different quality in comparison to *Glyceria*. They contained ca 10–20 % less exudates but a larger proportion of lysates from root turnover (Fig. 4). This could have an impact on soil microbial biomass, which was larger but of significantly higher C/N ratio in the system with *Carex* in comparison to the *Glyceria*-system.

Finally, *Carex* showed a higher plasticity in allocation pattern and root physiological traits in response to N loading than *Glyceria*, such as a more pronounced increase in the shoot/root ratio, but also in gross root growth rate, root turnover and consequently total as well as mass-specific rhizodeposition flux (for species comparison see Fig. 4). This higher plasticity in the biomass allocation pattern could be the reason how conservative plants, being superior "inhabitants" of oligotrophic systems, can cope with faster-growing species in competition for light when growing in mesotrophic conditions (Wedin and Tilman 1990). However, excessive expenses for maintaining the functioning of the root system are disadvantageous in productive N-rich environments. Therefore, *Carex* is finally replaced by stronger competitors for N such as *Glyceria* at a certain level of soil fertility. We thus suggest that belowground C partitioning and mainly the C costs (losses) connected with the maintenance of root functioning at a particular level of soil fertility are important for potential plant species survival or replacement within the existing community.

Conclusions

We propose a combination of pulse-labeling to track recent assimilates with estimation of the root death rate calculated from the difference between gross and net root growth rates for assessing the rhizodeposition flux to soil, and the contribution of root exudates and lysates from root turnover. The rhizodeposition flux quantified in this way reached 9–44 μg C g^{-1} root-C day^{-1}, which was fully comparable with values obtained using continual labeling in other studies. Root exudates accounted for 20–70 % of the flux in the given plant growth stage (fast plant growth), with most data variability related to N availability.

N loading enhanced root biomass and, consequently, total rhizodeposition flux to the soil. Due to the lower partitioning of recent assimilates to exudation and faster root turnover of fertilized plants, their rhizodeposits predominantly contained root lysates. The fertility-induced changes in the amount and quality of root-derived C input to the soil implied different microbial activity which could impact soil organic matter dynamics, as demonstrated by the increased concentration of

dissolved organic C in N-fertilized soils. The effect of N loading on belowground C distribution was species specific. Mass-specific root growth and rhizodeposition did not change in the faster-growing competitive *Glyceria*, while those investments significantly increased in the conservative *Carex*. This puts *Carex* at a disadvantage in eutrophic conditions and leads to its replacement by more competitive species. These results provide an interesting perspective for further studies, which should include more detailed analyses of rhizodeposition quality and related microbial activity under different soil N availability.

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Table 1. Results of factorial ANOVA with plant species (P), fertilization (F), and sampling time (T) as categorical predictors are given for the final ^{13}C distribution (% net fixation) in the non-fertilized and fertilized systems with *Carex* and *Glyceria* 15 days after labeling, including net exudation.

	Plant (P)	**Fert (F)**	**P×F**
Total C losses	ns	**	*
By root-derived respiration	ns	***	(*)
By shoot respiration	***	*	ns
Shoot C	*	***	*
Root C	***	*	ns
Soil C	**	***	**
Net C exudation[2]	***	***	***

[1] Total C allocation below ground is the sum of root C, soil C, and root-derived respiration, expressed as % of net fixation
[2] Net C exudation corresponds to the maximum soil ^{13}C, occurring within 24 hours after labelling, expressed as % of net fixation

ns	non-significant
(*)	P<0.1
*	P<0.05
**	P<0.01
***	P<0.001

PAPER 4

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ECOSYSTEMS

Cotton-Grass and Blueberry have Opposite Effect on Peat Characteristics and Nutrient Transformation in Peatland

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Abstract

Peatlands are large repositories of carbon (C). *Sphagnum* mosses play a key role in C sequestration, whereas the presence of vascular plants is generally thought to stimulate peat decomposition. Recent studies stress the importance of plant species for peat quality and soil microbial activity. Thus, learning about specific plant–microbe–soil relations and their potential feedbacks for C and nutrient cycling are important for a correct understanding of C sequestration in peatlands and its potential shift associated with vegetation change. We studied how the long-term presence of blueberry and cotton-grass, the main vascular dominants of spruce swamp forests, is reflected in the peat characteristics, soil microbial biomass and activities, and the possible implications of their spread for nutrient cycling and C storage in these systems. We showed that the potential effect of vascular plants on ecosystem functioning is species specific and need not necessarily result in increased organic matter decomposition. Although the presence of blueberry enhanced phosphorus availability, soil microbial biomass and the activities of C-acquiring enzymes, cotton-grass strongly depleted phosphorus and nitrogen from the peat. The harsh conditions and prevailing anoxia retarded the decomposition of cotton-grass litter and caused no significant enhancement in microbial biomass and exoenzymatic activity. Therefore, the spread of blueberry in peatlands may stimulate organic matter decomposition and negatively affect the C sequestration process, whereas the potential spread of cotton-grass would not likely change the functioning of peatlands as C sinks.

Key words: peatlands; C/N/P stoichiometry; vascular plants; Sphagnum; nutrient; availability; decomposition; enzymatic activity.

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Authors' Contributions: EK, HŠ and TP conceived and designed the study as well as the field methods, KE sampled plant material and measured decomposition rates in the field and performed language corrections, PS analyzed plant material using IR, JB analyzed enzymatic activities, ZU and JM performed the field and laboratory portion of the research, and EK wrote the paper with editorial help of other co-authors.

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Introduction

Peatlands are nutrient-deficient systems that can accumulate large amounts of carbon (C) due to slow peat decomposition. This slow rate of decomposition is regulated by the prevailing anaerobic soil conditions and low mean temperatures associated with the high altitudes or latitudes where peatlands are predominantly found (Limpens and others 2008). *Sphagnum* mosses play a key role in this C sequestration due to the production of decay-resistant litter (Hájek and others 2011). Peat accumulation changes the local hydrology and pore water biogeochemistry, which generally positively feeds back to support *Sphagnum* (van Breemen 1995). Therefore, *Sphagnum*-dominated vegetation is considered to be fundamental to many ecosystem functions, including C storage (Limpens and others 2008; Bragazza and others 2013; Kuiper and others 2014), providing the stability and resilience of peatland ecosystems (Turetsky and others 2012).

Besides *Sphagnum* mosses, vascular plants are typical inhabitants of peatlands, with water table, nutrient status and temperature being the dominant factors controlling species composition and biomass (Bragazza 2006; Breeuwer and others 2009; Laine and others 2012; Dieleman and others 2015). Peatland vascular plants are more efficient photosynthetizers under all light levels than *Sphagnum* mosses (Leppala and others 2008), enhancing net ecosystem primary production and net CO_2 exchange (Tuittila and others 1999; Riutta and others 2007; Laine and others 2012). Through the labile C released in root exudates, vascular plants shape soil microbial community structure (Bragazza and others 2015) and their presence can be associated with greater microbial biomass and decomposition activity (Bragazza and others 2013; Jassey and others 2013; Bragazza and others 2015). Moreover, peatland vascular plants contain more N and P in their living tissues in comparison with peat mosses (Wang and Moore 2014), with a positive effect on the decomposability of their tissues (Hobbie 1996; Dorrepaal 2007) and nutrient cycling in peatlands (Jassey and others 2013; Bragazza and others 2015).

Most peatland vascular plants belong to two plant functional types, graminoids (sedges) and ericoid shrubs. Deeply rooting graminoids with aerenchyma generally occur in wetter habitats with a mean water table ranging from −10 to −20 cm and are strong competitors in such habitats, whereas ericoid shrubs are abundant further above the water table, avoiding soil anaerobiosis (Bragazza 2006; Laine and others 2012). The differing life strategies of the two functional types are connected with differences in their tissue and litter chemistry (Moore and others 2007; Wang and Moore 2014). Graminoids have higher photosynthetic capacities and respiration rates (Bubier and others 2003; Riutta and others 2007; Leppala and others 2008) and lower contents of phenolics and lignin in the litter than ericoids (Hobbie 1996; Dorrepaal 2007), suggesting their higher tissue decomposability. Moreover, the plant functional types are associated with soil microbial communities of different composition (Haichar and others 2008; Bragazza and others 2015; Robroek and others 2015b). Therefore, the presence of graminoids or ericoids differentially impacts net ecosystem CO_2 exchange, its drought response (Riutta and others 2007; Laine and others 2012; Kuiper and others 2014) and CH_4 dynamics (Robroek and others 2015b; Strom and others 2015), and very likely also peat composition, soil microbial biomass and nutrient cycling.

It is expected that changing climate conditions will shift the functional composition of peatland vegetation toward a graminoid-dominated system under elevated temperatures or increased precipitation (Wahren and others 2005; Dieleman and others 2015) and toward a shrub-dominated community under a combination of drier and warmer seasons with decreased water levels (Bragazza and others 2013; Heijmans and others 2013). Therefore, learning about specific plant–microbe–soil relations and their potential feedbacks for peat quality and nutrient cycling are important for correctly understanding peatland ecosystem functioning as a C sink and its potential shift associated with vegetation change (Heijmans and others 2013; Bragazza and others 2015). We found only a few papers about peatlands that investigated the cascade effects of different plant species or plant functional types on the characteristics of soil organic matter and microbial biomass. Bragazza and others (2013) linked increasing ericoid shrub abundance in peatlands to the structure, C/N stoichiometry and activity of the soil microbial community and dissolved organic matter chemistry, whereas Robroek and others (2015b) demonstrated a direct effect of the removal of different plant functional types on microbial community composition. Jassey and others (2013) related changes in the peatland vascular plant community resulting from warming effects to the structure of microbial food webs. Recently, Robroek and others (2015a) and Pinsonneault and others (2016) stressed the impor-

tance of plant species (or more generally plant functional types) in dissolved organic matter chemistry and its biodegradability, with a possible influence on microbial respiration and dissolved organic matter export from peatlands.

We selected spruce swamp forests (SSF) as a representative type of peatland for the purpose of linking the specific effect of plant species presence on the characteristics of peat and the soil microbial community. SSF are widespread peatland systems, characterized by a patchy distribution of understory vegetation of different plant functional types reflecting site microtopography. In Central Europe and parts of Western Europe, SSF are considered nutrient-poorer types of peatlands because they are located in mountain areas formed by very old, nutrient-poor, high-grade metamorphic rocks (the so-called Moldanubicum zone). This is reflected in their vegetation composition, characterized by a high presence of cotton-grass (*Eriophorum vaginatum*), a species with a highly developed tolerance to low resources and a large capacity for nutrient immobilization (Cholewa and Griffith 2004; Silvan and others 2004). The wettest places of these SSF are covered only by *Sphagnum* mosses, followed by cotton-grass codominating less wet areas, whereas ericoids—namely blueberry (*Vaccinium myrtillus*)—are codominant on drier hummocks. We wanted to determine how the presence of particular vascular plant species is reflected in peat and soil microbial characteristics and thus to determine the possible implications of their spread for nutrient cycling and C storage in the SSF system. Our particular hypotheses were as follows: (1) The non-mycorrhizal cotton-grass and ericoid blueberry will differ in their tissue chemistry from *Sphagnum* mosses, causing faster decomposition of their litter. Their presence will influence peat chemistry, enhance nutrient availability and stimulate microbial activity in comparison with *Sphagnum* peat. (2) There will also be differences between the vascular plant species. Cotton-grass biomass will contain more nutrients and less polyphenolic and lignin compounds than that of blueberry, which will be reflected in its lower litter C/N/P stoichiometry and higher decomposition rate. Therefore, the stimulating effect on the nutrient cycling rate, microbial biomass and its activity will be more pronounced in the presence of cotton-grass than of blueberry. To attain this, we measured and compared C, N and P contents and their stoichiometric ratios of live and senescent aboveground and belowground tissues of the studied plant species—*Sphagnum*, cotton-grass and blueberry, and in the peat, dissolved organic matter and microbial biomass taken in the patches covered only by peat moss or dominated by one of the particular plant species. The plant and peat samples were also characterized by their organic compound composition using infrared spectroscopy. The activities of extracellular enzymes gaining C, N and P, and microbial respiration measured in peat samples were used to assess the possible changes in peat transformation under different vegetation.

Materials and Methods

Study Sites

The study sites are located in the Šumava Mountains, southwest Czech Republic (48°59′N, 13°28′E). Three spruce swamp forest (SSF) sites are located in the catchments of three different small brooks, situated on an upland plateau at an altitude of approximately 1100 m a.s.l, with a cold and humid climate. The mean annual temperature is 4.0°C with mean annual precipitation of 1100 mm (years 1961–1990, statistics by the Czech Hydro-Meteorological Institute). The SSF are covered by a continuous layer of *Sphagnum* mosses (dominated by *S. fallax* with the rare presence of *S. flexuosum* and *S. girgensohnii*) with wet open patches occupied by *Eriophorum vaginatum* L. (with a coverage of 25–50%) and drier microhabitats with shrubs of *Vaccinium myrtillus* L (with a coverage of 45–75%). Other plant species like *Vaccinium vitis-idaea* L., *Vaccinium oxycoccos L.*, sedges and grasses are also rarely present. The patchy distribution of the three dominants in the understory reflects variations in terrain microtopography and water level in the SSF. The tree canopy cover (*Picea abies*) varies from 0% to 80% with tree height ranging from 8 to 15 m. Total N deposition is 0.5–1 g N m^{-2} y^{-1} (2011, statistics by the Czech Hydro-Meteorological Institute).

Aboveground and Belowground Plant Tissue Sampling and Analyses

Plant samples for the purpose of this study were collected from only one of the studied sites, Tetrevska. This site was chosen due to its easy accessibility and the fact that temperature and water level dataloggers were already located there. Preliminary vegetation biomass sampling (both above- and belowground) found no significant differences between the sites (Edwards, unpublished data). Because the sites are protected areas, the aim was to minimize disturbance to the sites as much as possible. The *Sphagnum* capitula, and the fully expanded sun-exposed mature leaves from the top canopy of blueberry and cotton-grass were

randomly sampled (n = 10) in May (beginning of the growing season), July (top season) and September (end of vegetation season) in 2013 and 2014. The senescent leaves were obtained as follows. In the case of cotton-grass, senescent leaves still attached to plants were sampled in September 2013 and in May, July and September 2014. For blueberry, recently senesced, but still attached, reddish brown leaves were sampled in September 2013 and 2014. For *Sphagnum*, the part of the stem 2–3 cm below the capitulum was considered to represent recently senescent tissue.

Belowground biomass of blueberry and cotton-grass was sampled just below the sampled plant using a soil corer (6.5 × 5.5 cm inner dimension; n = 4) at the same sampling times as the aboveground plant biomass in the patches where particular plants dominated. The roots were carefully separated from the peat by hand, washed and assigned to the studied plant species. Roots from other species were discarded. The belowground samples were further separated to living and senescent tissues according to their color, structure and strength. The above- and belowground plant materials were dried at 60°C for 72 h.

To address the likely differences in the quality of living and senescent biomass between *Sphagnum*, cotton-grass and blueberry, their total C and N concentrations were determined by dry combustion on an elemental analyzer (ThermoQuest, Italy). Total P was measured colorimetrically using the ammonium molybdate–ascorbic acid method on a flow injection analyzer (FIA, Lachat QC8500, Lachat Instruments, USA) after perchloric acid digestion (Kopáček and Hejzlar 1995). Differences in N and P concentrations between live and senescent tissues of the particular plant species were used to estimate nutrient resorption efficiencies; these were calculated separately for the above- and belowground plant tissues.

The chemical composition of the plant material sampled in 2013 was assessed using infrared spectroscopy. Infrared spectra were obtained with a Bruker VERTEX 70 series FTIR (Fourier Transform InfraRed) spectrometer (Bruker Optics, Germany) equipped with a horizontal attenuated total reflectance (ATR) sampling accessory. Dried and powdered samples were inserted directly on the ATR crystal, and a MIRacle high-pressure digital clamp was used to achieve even distribution and contact of the sample and crystal. Each spectrum consisted of 65 averaged absorbance measurements between 4000 and 650 cm^{-1}, with a 4 cm^{-1} resolution. Offsets in baseline and slope between the different runs (samples) were removed by standard normal variate transformation and the second derivative using the Unscrambler software (CAMO, Norway). The individual bands were assigned according to Artz and others (2008). Summed absorbance values of the following bands were used as representative of the different organic compounds in the ordination diagrams of Figure 1: 2920 and 2850 cm^{-1} (fats, wax, lipids); 1515, 1454 and 1265 cm^{-1} (phenolics, lignin); 1550 and 1650 cm^{-1} (polypeptides); 1153, 1030 and 900 cm^{-1} (polysaccharides).

Field Decomposition Study

A decomposition study was conducted at the Tetrevska study site to determine whether the observed changes in the litter quality were reflected in the rate of mass loss under field conditions. Senesced leaves of cotton-grass and blueberry were collected at the end of the 2014 growing season (October) as well as roots of the two species and *Sphagnum* thalli. Subsamples of the litter material were dried at 60°C for 48 h and weighed to determine initial litter dry weight, whereas fresh litter material (1.5 g) was placed into separate litter bags (8 × 8 cm; mesh size = 1 mm). These bags were placed in the field in November 2014 with litter bags containing the leaf litter or *Sphagnum* lain on the ground within clumps of the respective species. Bags containing roots were installed in the top 15 cm of the peat within clumps of the respective species using a shovel to produce a slit into which the litter bags were carefully slid so to ensure contact with the peat. Four replicate bags per each species and litter type were collected after 175 and 345 days of exposure (May and October 2015, respectively). The remaining litter was carefully removed from the mesh bags, gently washed, dried at 60°C for 48 h and weighed.

Peat and Soil Solution Sampling and Analyses

Peat was sampled in all three SSF sites in May, July and September of 2013 and 2014 using a soil corer (6.5 × 5.5 cm inner dimension; n = 4) to a depth of 30 cm in randomly selected places covered only by *Sphagnum* and in patches of cotton-grass or blueberry. The samples were homogenized by hand, and roots and other woody material were removed. A portion of the soil was dried at 60°C to constant weight, milled and analyzed for total C, N and P contents and their organic compounds composition by infrared spectroscopy as described above for plant material.

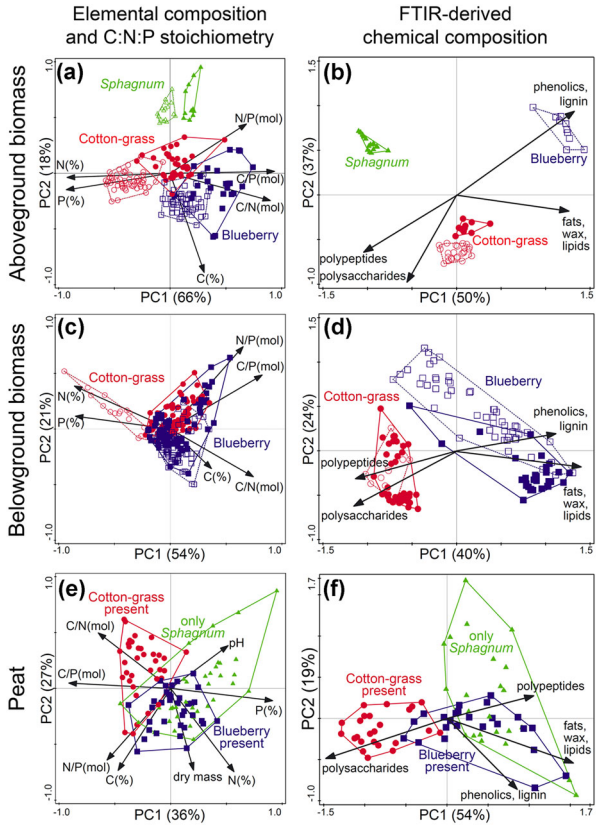

Figure 1. Plant tissue and peat chemistry: ordination diagrams from principal component analysis (PCA) showing the relations between tissue elemental concentrations and the stoichiometry of *Sphagnum*, cotton-grass and blueberry, and FTIR-derived chemical composition for their (**A**, **B**) aboveground and (**C**, **D**) belowground biomass, divided into living (*open symbols*) and senescent (*full symbols*) tissue, and for (**E**, **F**) peat formed in their presence. Graphs **A**–**D** are based on plant data from the Tetrevska site only; graphs **E**–**F** show peat data from all three studied sites.

The soil solution was extracted by centrifugal drainage at 4000 g for 1 h at 4°C (Giesler and Lundstrom 1993) from undisturbed peat cores, sampled from a depth of 5–15 cm from the above-defined patches with different vegetation. The extraction was done within 24 h after peat sampling. After filtration through a low-protein-binding Express PLUS polyethersulfone membrane (MILLGPWP) with a 0.22-µm pore size (Merck Millipore Ltd., Ireland), the soil solution was analyzed for organic C (DOC) and N (DN) concentrations on a LiquiTOC II (Elementar, Germany), whereas soluble reactive P (SRP), ammonium and nitrate N were analyzed colorimetrically on a flow injection analyzer (FIA Lachat QC8500, Lachat Instruments, USA). Total soluble P was measured colorimetrically as orthophosphates on a flow injection analyzer after perchloric acid digestion (Kopáček and Hejzlar 1995). The same solution was used to measure pH by a glass pH electrode.

Microbial Biomass and Activity

In fresh peat samples, microbial biomass carbon (MB-C), nitrogen (MB-N) and phosphorus (MB-P) were determined using the chloroform fumigation extraction method (Brookes and others 1982, 1985; Vance and others 1987) within 48 h after sampling. Samples were extracted by 0.5 M potassium sulfate (1:4 w/v) in the case of MB-C and N and by 0.5 M sodium bicarbonate with a pH of 8.5 (1:15 w/v) in the case of MB-P before and after chloroform fumigation for 24 h. The dissolved organic C and dissolved N concentrations in the soil extracts were measured with a TOC/TN analyzer (LiquiTOC II, Elementar, Germany). The P content was measured spectrophotometrically at 886 nm wavelength by the ammonium molybdate–ascorbic acid method. MB-C, MB-N and MB-P were calculated by subtracting the C, N and P concentrations in extracts from the fumigated and nonfumigated samples using correction factors of 0.3, 0.54 and 0.4, respectively.

Microbial respiration was measured as the increase in CO_2 concentration over 48 h during incubation of fresh peat at 10°C in bottles sealed with rubber covers. Anaerobic microbial respiration was assessed similarly, but the headspace of the bottles was flushed with nitrogen. CO_2 concentrations were measured on a gas chromatograph (Agilent 6850 Series, Agilent, USA).

Potential extracellular enzyme activities were determined by microplate fluorometric assays under standardized laboratory conditions. For determination of hydrolytic enzyme activities, 0.5 g of soil was suspended in 50 ml distilled water and sonicated for 4 min to disrupt the soil particles. 200 µl of the soil suspension was added to 50 µl methylumbelliferyl substrate solution for *β*-glucosidase (BG), phosphatase (AP) or *N*-acetylglucosaminidase (NAG) determination or to 50 µl 7-aminomethyl-4-coumarin substrate solution for leucine aminopeptidase (LAP) determination (Marx and others 2001). Plates were incubated at 20°C for 2 h. Fluorescence was quantified at an excitation wavelength of 365 nm and an emission wavelength of 450 nm using the INFINITE F200 microplate reader (TECAN, Germany). All the enzymatic activities were summed.

The activity of BG represented investments into C acquisition, the sum of LAP and NAG showed N acquisition and AP was a measure of P acquisition (Sinsabaugh and others 2009).

Statistics

The molar C/N, C/P and N/P ratios were calculated for the living and dead tissues from each of the three plant dominants, and the peat, soil microbial biomass and soil solution occurring under these dominants (Sterner and Elser 2002). For the peat and microbial characteristics, mean values from all three sites are presented in ''Results,'' whereas data from individual sites are in Supplement.

Variations in plant biomass, peat, MB and soil solution characteristics among patches covered only by *Sphagnum* or with the presence of cotton-grass or blueberry were explored by principal component analysis (PCA, Canoco 5). The measured C, N and P concentrations and their stoichiometric ratios in the samples, or infrared absorbance data, were used as response variables, while variables describing plant species and the live/dead status of the plant tissue (only for analyses of plant tissues) were passive explanatory variables. A constrained analysis (RDA) was then used to determine the proportion of data variability connected with the explanatory variables, plant species, live/dead status, site, time of sampling within the growing season (May, July, September) and sampling year (2013, 2014), from which the best predictors were then selected by interactive forward selection, with a false discovery rate used to adjust the significance of the multiple tests.

The effects of plant species on plant biomass, peat and microbial biomass characteristics were further assessed using a general linear model (with site, vegetation season and sampling year used as covariates), followed by post hoc testing with the Tukey HSD test when the effect was significant (Statistica 10, USA). When necessary, the data were log-transformed to meet the requirements for normality and variance homogeneity.

Between-species differences in decomposition rate were determined by running repeated measures ANOVAs on the relative remaining dry mass data (%) separately for each litter type. The data had normal distributions and homogeneous variances; thus, no data transformations were necessary. The rate of relative mass loss for each species and litter type was determined as the slope of a linear regression (Statistica 10, USA).

Results

Elemental and Stoichiometric Characteristics of the Aboveground and Belowground Plant Tissues

Carbon content of the above- and belowground plant tissues increased in the order *Sphagnum* < cotton-grass < blueberry. The cotton-grass shoots had the highest N and P concentrations and thus the lowest C/N and C/P ratios among the studied plants (Table 1). The two other species, the living *Sphagnum* and blueberry leaves, had lower, but similar, nutrient concentrations. However, the significantly lower C concentration in *Sphagnum* resulted in tissue C/N and C/P ratios markedly lower in comparison with those of blueberry leaves. The N/P tissue ratio ranged from 19 to 23 with no significant difference among the species.

The living belowground parts, although being generally nutrient poorer than the aboveground tissues, mirrored the stoichiometric differences between both vascular plants. Cotton-grass roots were nutrient richer, with lower C/N, C/P and N/P ratios, than the belowground parts of blueberry (Table 1).

The PCA clearly separated all three plants according to the nutrient and stoichiometric characteristics of their living aboveground (pseudo-F = 90.5, $p = 0.002$, Figure 1A) and belowground tissues (pseudo-F = 17.9, $p = 0.002$, Figure 1C). In the first case, the differences among plant species explained 48% of the data variability ($p = 0.005$), whereas this factor explained only 12% of the belowground data variability ($p = 0.005$, results of interactive forward selection). The nutrient and stoichiometric characteristics of the plant tissues for all species changed during the season. In both years, the higher N and P concentrations in May decreased toward autumn leading to higher tissue C/N and C/P ratios in September. These temporal changes explained an additional 3–6% of the data variability ($p < 0.01$ for both above- and belowground biomass).

The ordination diagrams further showed significant nutrient, especially P, depletion of the above- and belowground tissues after senescence, whereas their C concentration was not changed (Figure 1A, C). Therefore, senescent plant parts had markedly higher C/N and C/P, and also N/P ratios than their living tissues (Table 1). The live/dead status of the tissue explained 23% of the aboveground data variability ($p = 0.004$) and 7.3% of the belowground data variability ($p = 0.005$). Because the sampled senescent plant material was still attached

Table 1. Plant Tissue Chemistry

Plant biomass		*Sphagnum*	Cotton-grass	Blueberry
Aboveground biomass				
Live	C (%)	$41.2^{aA} \pm 0.1$	$46.5^{bA} \pm 0.1$	$48.7^{cA} \pm 0.1$
	N (%)	$1.00^{bA} \pm 0.03$	$2.15^{cA} \pm 0.05$	$0.77^{aA} \pm 0.03$
	P (%)	$0.09^{aA} \pm 0.00$	$0.26^{bA} \pm 0.01$	$0.08^{aA} \pm 0.00$
	C/N	$48.7^{bA} \pm 1.3$	$26.3^{aA} \pm 0.8$	$78.6^{cA} \pm 2.9$
	C/P	$1157^{bA} \pm 36$	$501^{aA} \pm 20$	$1641^{cA} \pm 65$
	N/P	$23.4^{bA} \pm 0.8$	$19.0^{aA} \pm 0.4$	$21.1^{aA} \pm 0.6$
Senescent	C (%)	$42.0^{aA} \pm 0.3$	$46.7^{bA} \pm 0.1$	$49.2^{cA} \pm 0.1$
	N (%)	$0.76^{aB} \pm 0.03$	$1.09^{bB} \pm 0.04$	$0.64^{aB} \pm 0.04$
	P (%)	$0.06^{bB} \pm 0.00$	$0.09^{cB} \pm 0.01$	$0.04^{aB} \pm 0.00$
	C/N	$64.2^{bB} \pm 2.1$	$52.6^{aB} \pm 2.0$	$96.2^{cB} \pm 5.2$
	C/P	$1925^{bB} \pm 75$	$1440^{aB} \pm 86$	$3225^{cB} \pm 202$
	N/P	$32.5^{bB} \pm 1.2$	$26.9^{aB} \pm 0.7$	$34.3^{bB} \pm 1.8$
Belowground biomass				
Live	C (%)		$45.7^{aA} \pm 0.7$	$49.6^{bA} \pm 0.2$
	N (%)		$1.46^{bA} \pm 0.17$	$0.56^{aA} \pm 0.02$
	P (%)		$0.27^{bA} \pm 0.03$	$0.05^{aA} \pm 0.00$
	C/N		$51.2^{aA} \pm 10.1$	$114.2^{bA} \pm 4.2$
	C/P		$580^{aA} \pm 81$	$3324^{bA} \pm 209$
	N/P		$13.7^{aA} \pm 1.0$	$30.2^{bA} \pm 1.6$
Senescent	C (%)		$47.4^{aB} \pm 0.1$	$49.4^{bA} \pm 0.1$
	N (%)		$0.67^{aB} \pm 0.02$	$0.71^{aB} \pm 0.03$
	P (%)		$0.03^{aB} \pm 0.00$	$0.05^{aA} \pm 0.00$
	C/N		$88.2^{aB} \pm 2.8$	$93.5^{aB} \pm 4.0$
	C/P		$4784^{bB} \pm 271$	$4227^{aA} \pm 380$
	N/P		$52.8^{aB} \pm 2.2$	$43.0^{aB} \pm 2.6$

Average concentrations of C, N and P (%), and their molar stoichiometric ratios in the aboveground and belowground plant tissues of Sphagnum, cotton-grass and blueberry, either living or senescent (still attached to plant). Plant material was sampled in May, June and September 2013 and 2014 in the site Tetrevska (mean, standard error of the mean SEM, n = 60). Capital letters refer to significant differences in particular characteristics between live and senescent tissues, whereas lowercase letters show differences among plant species (results of one-way ANOVAs and post hoc comparisons, $p < 0.05$).

to plants, its nutrient depletion was mainly ascribed to nutrient resorption into living tissues. Generally, cotton-grass displayed the most efficient nutrient resorption with P resorption being greater than that of N. The aboveground P resorption was 65, 52 and 41%, whereas N resorption was 50, 17 and 25% for cotton-grass, blueberry and *Sphagnum*, respectively. Below ground, cotton-grass resorbed even more, 89% of P and 55% of N, whereas no significant nutrient resorption was found from senescent roots of blueberry. In summary, the characteristics of the aboveground senescent tissues still reflected the stoichiometric differences found among the living tissues, with cotton-grass being nutrient-richer litter than blueberry. Below ground, the initially differing nutrient and stoichiometric characteristics converged in the senescent material of both vascular plants (Figure 1C), making the senescent *Sphagnum* tissue relatively the nutrient richest and senescent cotton-grass roots the most P-depleted sources for microbial decomposition (Table 1).

Infrared Spectra of the Aboveground and Belowground Plant Tissues

All three plant dominants differed in both their aboveground (pseudo-F = 109, $p = 0.002$, Figure 1B) and belowground (pseudo-F = 33.8, $p = 0.002$, Figure 1D) tissue chemical composition. The differences among plant species explained 74% of the aboveground FTIR data variability ($p = 0.002$), whereas it explained only 37% of the variability for the belowground FTIR data ($p = 0.002$). *Sphagnum* biomass had a high relative content of polysaccharides (Figure 2A, bands at 3340, 1153, 1030 and 900 cm^{-1}) and the lowest content of aliphatic and aromatic compounds: fats, waxes, lipids (bands at 2920 and 2850 cm^{-1}), phenolic and lignin-like compounds (bands in the region 1735–1265 cm^{-1}). Cotton-grass had a similarly high content of polysaccharides as *Sphagnum*, but differed (leaves more than roots) by having a somewhat higher content of aliphatic and aromatic

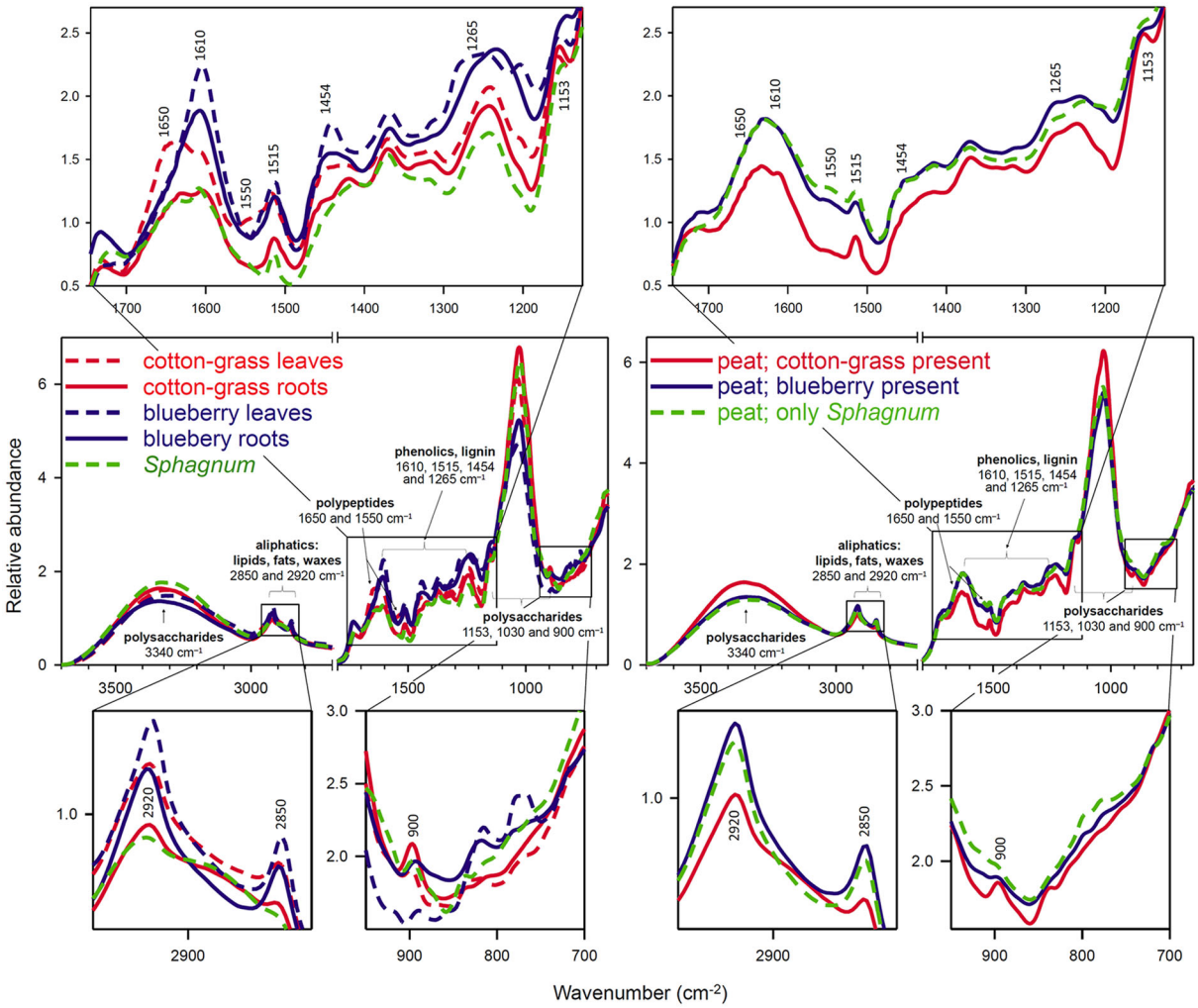

Figure 2. Infrared spectra of (**A**) leaves and roots of cotton-grass and blueberry, and *Sphagnum* capitula and (**B**) peat formed in the prevalence of particular plants, with detailed views of the parts of the spectra showing significant differences among plant tissues. Band assignments as according to Artz and others (2008).

compounds, and polypeptides (bands at 1650 and 1550 cm^{-1}). Blueberry biomass had the highest content of aliphatic and aromatic compounds (again, leaves more than roots) and the lowest content of polysaccharides and polypeptides.

As the plant material used for IR analysis did not contain living aboveground biomass of *Sphagnum* and blueberry, we cannot document any shifts in the contents of the chemical compounds caused by senescence of these species. In the case of cotton-grass, living shoots contained more polypeptides (Figure 2A, bands at 1650 and 1550 cm^{-1}) and a larger proportion of lignin to polysaccharides than dead ones (Figure 1B).

The FTIR-derived characteristics of plant tissues of all species also changed during the season. However, these temporal changes explained only 1–2% of data variability. The contents of phenolic and lignin-like structures tended to increase from May to September. Polysaccharides showed the highest abundance in July and the lowest in September. Polypeptides had an opposite seasonal pattern than that of polysaccharides.

Litter Decomposition of Different Plant Species

The field data on relative mass loss were fitted by linear regression, which is relevant for the early stage of litter decomposition. Different slopes for the fitted lines pointed to significantly different litter decomposition rates of the studied species. The mass loss of aboveground litter was fastest for cotton-grass leaves, which lost 68.9 ± 14.3% of

Table 2. Peat Chemistry

	Sphagnum	Cotton-grass	Blueberry	Plant	Site	Time
Ctot	$43.8^{a} \pm 0.9$	$45.1^{a} \pm 0.2$	$46.8^{a} \pm 0.3$	ns	***	ns
Ntot	$1.63^{b} \pm 0.05$	$1.12^{a} \pm 0.05$	$1.59^{b} \pm 0.03$	***	**	ns
Ptot	$0.09^{b} \pm 0.00$	$0.05^{a} \pm 0.00$	$0.08^{b} \pm 0.00$	***	ns	ns
C/N	$25.0^{a} \pm 0.6$	$44.3^{b} \pm 1.8$	$29.2^{a} \pm 0.8$	***	ns	ns
C/P	$1457^{a} \pm 132$	$2226^{b} \pm 71$	$1609^{a} \pm 69$	***	**	ns
N/P	$44.0^{a} \pm 2.6$	$46.0^{a} \pm 1.5$	$46.1^{a} \pm 1.5$	ns	***	ns
pH	$4.31^{a} \pm 0.04$	$4.01^{a} \pm 0.03$	$3.94^{b} \pm 0.02$	***	***	**

Average concentrations of C, N and P (%), their molar stoichiometric ratios and pH of the peat formed in patches covered only by Sphagnum or affected by the presence of cotton-grass or blueberry. Peat cores were sampled in May, June and September 2013 and 2014 in three spruce swamp forest sites (mean, standard error of the mean SEM, n = 72). Results of GLM on the effect of plant dominants are shown, with site and sampling time as covariates. Lowercase letters show differences among peat characteristics formed in the presence of different plant dominants ($p < 0.05$).
*ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

their weight during the first year of incubation in the field, whereas the decomposition rates for blueberry leaves ($37.9 \pm 3.7\%\ y^{-1}$) and *Sphagnum* ($24.2 \pm 8.7\%\ y^{-1}$) were significantly lower ($p < 0.001$). Between-species differences for the roots showed the opposite result, with blueberry roots decomposing at a significantly faster rate ($42.2 \pm 7.6\%\ y^{-1}$) than those of cotton-grass ($18.1 \pm 13.4\%\ y^{-1}$, $p < 0.001$). As a result, cotton-grass roots decomposed much more slowly than its leaves, and, in fact, as slowly as the decomposition-resistant *Sphagnum* litter. The decomposition rates of blueberry leaves and roots were only slightly different.

Peat and Soil Solution Characteristics and C/N/P Stoichiometry

The presence of cotton-grass significantly lowered the N and P contents in peat (Table 2), enhancing its C/N and C/P ratios (Figure 1E). The nutrient-depriving effect of cotton-grass was consistent at all three sites (Table S1). The cotton-grass further had a potentially acidifying effect on peat (Table 2), which occurred on two of the three sites (Table S1). The blueberry, located on drier patches in the SSF sites (relation with soil dry mass in Figure 1E), did not affect peat nutrient content but significantly acidified it (Table 1) at all three sites (Table S1). Generally, the total N and P contents were correlated ($r = 0.418$, $p < 0.05$) across all peat types. Accordingly, the peat N/P ratio remained relatively constant (Table 2). Using RDA, we were able to explain 41% of the variability in the peat characteristics, of which 28% can be ascribed to the presence of vascular plants (namely cotton-grass, 21%), 9% to the site and 4% to seasonal variations, mainly related to changes in soil water content (results of interactive forward selection).

The PCA ordination diagram for the FTIR-derived peat chemical composition separated the peats formed under particular plant species relatively well (Figure 1F). The chemical composition of the peat formed in the presence of the two vascular plant dominants differed in a similar manner as found for their biomass. Compared to *Sphagnum* peat, the peat formed under the polysaccharide-rich cotton-grass was enriched in polysaccharides, whereas the peat with the contribution of blueberry was enriched in aliphatic and aromatic compounds (fats, waxes, lipids, lignin-like and phenolic compounds) (Figure 2B). Using RDA, we were able to explain 43% of FTIR data variability, of which 38% can be ascribed to the presence of vascular plants, namely cotton-grass, and 5% to the site. Generally, our results on peat elemental and organic compound composition show that differences in peat characteristics attributed to plant dominants are significant and larger than those caused by site differences.

In comparison with bulk peat, the soil solution contained more P, but less N, relative to C, as shown by the higher C/N but lower C/P and N/P ratios (Table 3). Similarly to the situation in the peat, each plant dominant had a specific effect also on the soil solution chemistry. The presence of blueberry in the understory enhanced the concentration of DOC (Table 3) at two of the three sites (Table S2), which indicated higher mobility and lability of soil C. Because the soluble N concentration did not change under blueberry, this soil solution had a higher C/N ratio in comparison with other plants. Overall, the content of mineral N forms in the soil solution was intermediate for blueberry peat, but the increased content of nitrates as compared to *Sphagnum* and cotton-grass peat indicated more oxic conditions (Table 3).

Table 3. Soil Solution Chemistry

	Sphagnum	Cotton-grass	Blueberry	Plant	Site	Time
DOC	$66.5^{a} \pm 5.5$	$72.9^{a} \pm 5.2$	$92.3^{b} \pm 6.8$	**	**	***
SN	$1.92^{a} \pm 0.15$	$1.59^{a} \pm 0.10$	$1.78^{a} \pm 0.14$	ns	***	ns
SP	$0.28^{a} \pm 0.04$	$0.23^{a} \pm 0.02$	$0.51^{b} \pm 0.12$	*	***	ns
C/N	$51.3^{a} \pm 4.6$	$67.4^{ab} \pm 6.2$	$77.8^{b} \pm 7.8$	**	ns	***
C/P	$889^{a} \pm 72$	$1124^{a} \pm 106$	$927^{a} \pm 76$	*	***	***
N/P	$21.5^{b} \pm 1.5$	$20.6^{b} \pm 1.7$	$14.9^{a} \pm 1.1$	**	*	***
NH_4	$0.59^{c} \pm 0.06$	$0.15^{a} \pm 0.01$	$0.26^{b} \pm 0.03$	***	***	***
NO_3	$0.09^{a} \pm 0.01$	$0.10^{a} \pm 0.01$	$0.17^{b} \pm 0.03$	**	ns	*
SRP	$0.17^{a} \pm 0.03$	$0.11^{a} \pm 0.01$	$0.27^{a} \pm 0.09$	ns	***	ns

Average concentrations of dissolved organic C (DOC; mg l^{-1}), soluble N (SN; mg l^{-1}) and P (SP; μg l^{-1}), their molar stoichiometric ratios, concentrations of mineral N forms (N-NH4, N-NO3; mg l^{-1}) and soluble reactive P (SRP, μg l^{-1}) in the soil solution extracted from peat cores from patches covered by Sphagnum and affected by the presence of cotton-grass or blueberry. Peat cores were sampled in May, June and September 2013 and 2014 in the three spruce swamp forest sites (mean, standard error of the mean SEM, n = 72). Results of GLM on the effect of plant dominants are shown, with site and sampling time as covariates. Lowercase letters show differences among solution characteristics in the presence of different plant dominants ($p < 0.05$).
*ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

Blueberry presence further had the potential to mobilize P, evidenced by an enhanced concentration of soluble P found at two of the three sites, and also by a higher concentration of soluble reactive P at the P richest Tetrevska site (Table S2). This ability of blueberry resulted in the systematically lowest soil solution N/P ratio from all plant dominants (Table 3). The effect of cotton-grass on the soil solution was not significant, but was coincident with its nutrient-depleting effect on the bulk peat. Overall, the soil solution in the cotton-grass peat had the lowest concentration of mineral N at all three sites (Table 3 and S2). The concentrations of soluble P and soluble reactive P were lowered by cotton-grass presence only at the Tetrevska site, characterized by the highest concentrations of soluble P from the three sites (Table S2). The soil solution in patches covered only by *Sphagnum* always had the highest ammonium-N concentration indicating the highest N availability but lack of oxic conditions. In total, however, only 7% of the variability in the soil solution data can be ascribed to the dominant vegetation (RDA analysis and interactive forward selection, $p = 0.003$), with site explaining 9%, and changes within vegetation season, mainly the increase in DOC concentration in summer ($p = 0.004$), and inter-annual differences in the nitrate concentration in soil solution ($p = 0.004$) (detailed data not shown) explaining 12% of data variability.

MB, Its Elemental Stoichiometry and Activity

Microbial biomass C, N and P were higher in the patches with blueberry in comparison with the other two dominants (Table 4). This blueberry effect was consistent in all three sites, although the increase in microbial C and N was not always statistically significant (Table S3). In two of the three sites, microbial P, but no other element bound in the microbial biomass, was also enhanced under cotton-grass (Table S3). The changes in microbial biomass did not significantly affect its elemental stoichiometry, although the microbial biomass under blueberry appeared to have the lowest C/P ratio, whereas that under cotton-grass the highest C/N ratio (Table 4). In comparison with *Sphagnum* peat, the portion of peat N and P bound in microbial biomass was higher, exceeding 2% for N and 20% for P in the presence of both vascular plants, making microbial biomass an important nutrient pool in those types of peat. The interactive forward selection of explanatory variables showed that only 5% of the variability in the microbial biomass and its elemental stoichiometry could be ascribed to the effect of vegetation of the sampled patch, with a similar portion of variability explained by site (5%) and temporal changes in MB. A decrease in the microbial C/P and N/P ratios from May to the end of the growing season explained 5% of data variability and the year-to-year decrease in MB-N content from 2013 to 2014 explained an additional 3% ($p < 0.05$ in all cases, detailed data not shown).

Aerobic and anaerobic microbial respiration and total enzymatic activity were comparable among the patches with different vegetation (Table 4), with the exception of the Kvilda site, where microbial respiration was higher under cotton-grass than in the other types of vegetation (Table S4). A majority of enzymatic activity (≥75%) was directed

Table 4. Microbial Biomass

	Sphagnum	Cotton-grass	Blueberry	Plant	Site	Time
MB-C	$3217^{a} \pm 321$	$3613^{ab} \pm 240$	$4354^{b} \pm 336$	*	***	ns
MB-N	$276.2^{a} \pm 25.1$	$301.3^{a} \pm 31.3$	$459.0^{b} \pm 39.4$	***	**	ns
MB-P	$123.3^{a} \pm 9.7$	$150.1^{a} \pm 12.4$	$201.6^{b} \pm 14.4$	***	ns	**
C/N	$17.35^{a} \pm 1.77$	$24.24^{b} \pm 4.67$	$15.34^{a} \pm 1.58$	*	*	**
C/P	$90.15^{a} \pm 15.34$	$97.82^{a} \pm 19.07$	$68.4^{a} \pm 6.39$	ns	**	**
N/P	$9.23^{a} \pm 2.36$	$6.41^{a} \pm 0.95$	$6.23^{a} \pm 0.63$	ns	**	**
MB-C/Ctot	$0.75^{a} \pm 0.08$	$0.82^{a} \pm 0.05$	$0.94^{a} \pm 0.07$	ns	***	*
MB-N/Ntot	$1.42^{a} \pm 0.15$	$2.12^{b} \pm 0.22$	$2.26^{b} \pm 0.19$	***	***	ns
MB-P/Ptot	$15.07^{a} \pm 1.63$	$27.62^{b} \pm 2.37$	$26.51^{b} \pm 2.05$	***	ns	***
Microbial respiration aerobic	$7.78^{b} \pm 0.48$	$8.31^{b} \pm 0.44$	$6.02^{a} \pm 0.32$	**	***	*
Microbial resp. anaerobic	$1.32^{a} \pm 0.13$	$1.35^{a} \pm 0.13$	$1.17^{a} \pm 0.09$	ns	ns	***
Sum of enzymatic activity	933.1 ± 83.6	754.1 ± 49.9	866.5 ± 72.0	ns	**	*
% C-gaining enzymes	$14.5^{a} \pm 1.0$	$12.8^{a} \pm 0.7$	$18.1^{b} \pm 1.4$	**	ns	ns
% N-gaining enzymes	$2.5^{a} \pm 0.3$	$3.6^{a} \pm 0.3$	$3.5^{a} \pm 0.4$	*	***	ns
% P-gaining enzymes	$83.0^{b} \pm 1.2$	$83.6^{b} \pm 0.9$	$78.5^{a} \pm 1.5$	**	*	ns

C, N and P (MB-C, MB-N, MB-P; $\mu g\ g^{-1}$) and their molar stoichiometric ratios, proportions of peat C, N and P bound in the microbial biomass (%), microbial respiration in aerobic and anaerobic conditions ($\mu l\ CO_2\ g^{-1}\ h^{-1}$) and the sum of hydrolytic enzymatic activity and proportions of C-, N- and P-gaining enzymatic activities in the peat formed in patches covered only by Sphagnum or affected by the presence of cotton-grass or blueberry. Peat cores were sampled in May, June and September 2013 and 2014 in the three spruce swamp forest sites (mean, standard error of the mean SEM, n = 72). Results of GLM on the effect of plant dominants are shown, with site and sampling time as covariates. Lowercase letters show differences among peat characteristics formed in the presence of different plant dominants.
*ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

to P acquisition under all types of vegetation. However, in two of the sites, the portion of P-gaining enzymes was lower, whereas the portion of enzymes mining C was higher, in the peat formed in the presence of blueberry in comparison with both other peat types (Table S4).

Discussion

Shifts in the peatland plant community toward vascular plant dominance, driven by climate change, are predicted to have negative consequences for peatland C sink functioning (Bragazza and others 2013; Jassey and others 2013; Buttler and others 2015; Dieleman and others 2015). However, we demonstrated here that the implications on ecosystem behavior are species specific and markedly differ between blueberry and cotton-grass, the most common vascular plants codominating spruce swamp forests together with *Sphagnum* spp.

Sphagnum Mosses Formed the N Richest Environment within the Generally Nutrient-poor Spruce Swamp Forest System

Against the general assumption that *Sphagnum* is a low-quality substrate (Hájek and others 2011; Turetsky and others 2012), we found that *Sphagnum* biomass is rather rich in polysaccharides and nutrients. *Sphagnum* peat, formed in the absence of any vascular plants, was also relatively nutrient rich and its soil solution contained the highest concentration of mineral (ammonium) N from the three peat types studied (Figure 3). We suggest that the relatively high N availability in the *Sphagnum* peat could be related to several specific characteristics of *Sphagnum* mosses: biological atmospheric N_2 fixation by cyanobacteria and methanotrophs associated with *Sphagnum* (Larmola and others 2014), high cation exchange capacity (Verhoeven and Liefveld 1997) and low N resorption efficiency from the senescing *Sphagnum* (only approximately 25% of N was resorbed, calculated on a mass basis). Despite the highest N availability in the *Sphagnum* peat within the studied peatland, the *Sphagnum* litter decomposed very slowly in comparison with the other types of litters, and microbial biomass and activity were also low. Therefore, these microsites undoubtedly acted as C sinks. Besides the possible suppression of microbial activity by polyphenolics (e.g., Verhoeven and Liefveld) and the prevailing anaerobic conditions, another reason is that the system is also P limited. This limitation is indicated by the high peat C/P and N/P ratios in comparison with common soils (Cleveland and Liptzin 2007) and also by the large investments in P acquisition, which exceeded 80% of the measured hydrolytic enzyme activity. These findings agree with the

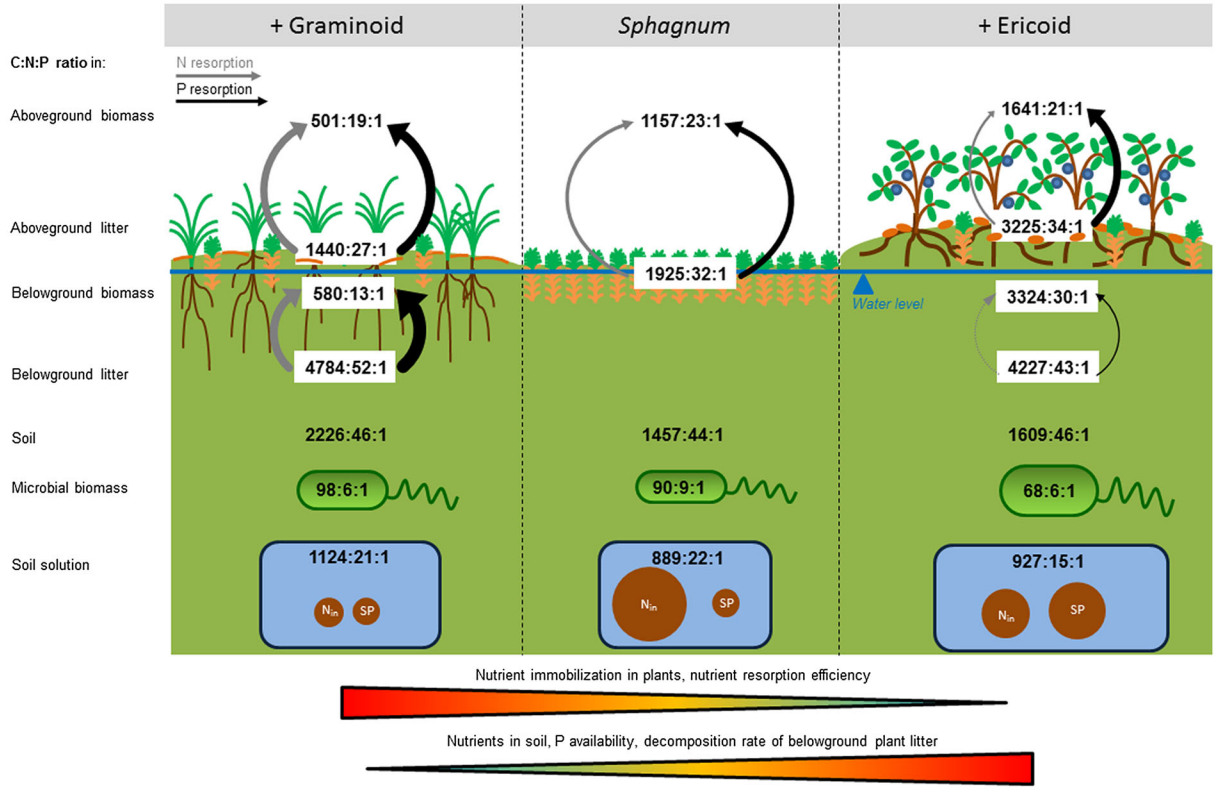

Figure 3. Scheme of the functioning of the spruce swamp forest system in the presence of the studied plant dominants; with *Sphagnum* only (*in the center*), in the presence of cotton-grass (*on the left*) and blueberry (*on the right*). The C/N/P stoichiometry of the living and senescent biomass of particular plant dominant is shown; their nutrient resorption efficiencies are indicated by the width of the *arrows*. Belowground, the C/N/P stoichiometry of the peat, microbial biomass and soil solution formed in the presence of particular plant dominant is shown, based on average data from the three study sites. Sizes of *ovals* and *circles* show the relative differences in the microbial biomass, soil organic C pool and concentration of mineral N and soluble phosphorus among the areas affected by different plant dominants. The figure demonstrates the decreasing concentration of nutrients in the living plant tissues but also decreasing nutrient efficiencies from cotton-grass over *Sphagnum* to blueberry. At the same time, P availability increased in the soil, which was connected with enhanced microbial biomass and the decomposition rate of belowground plant litter in the same direction.

suggestion that primary production and microbial growth are P limited in peatlands (Hill and others 2014). Therefore, environmental changes affecting P cycling and relieving P limitation of the microbial activity in the *Sphagnum*-dominated peatland should be expected to impact its ability to act as a C sink.

Cotton-Grass Depleted Nutrients from the Peat and Its Presence did not Stimulate Peat Decomposition in Spruce Swamp Forests

Both above- and belowground living cotton-grass biomass was rich in polysaccharides and formed significant nutrient pools within the spruce swamp forest system. Such biomass characteristics suggest that cotton-grass presence should be connected with fast litter decomposition, the enhancement of nutrient concentrations in soil solution and acceleration of peat decomposition (Chapin and others 2003). However, our results did not support any of these expectations and showed that the system with cotton-grass functioned differently.

Cotton-grass appeared to be the center of a closed nutrient pool, with only small losses into the soil solution and bulk peat. The high nutrient resorption of cotton-grass, reaching 50% for N and 60–80% of P, secured effective internal N and P recycling within the plant (Figure 3). Its large nutrient immobilization capacity can be ascribed to effective biomass production (Tuittila and others 1999), significant allocation of nutrients in the slowly decomposing storage organs (Silvan and others

2004), unusual vascular system of cotton-grass enabling efficient internal nutrient recycling (Cholewa and Griffith 2004) and the long life span of individual tussocks (Shaver and others 1986). The deeply rooting system of cotton-grass facilitated nutrient uptake by the plant. The mechanisms of efficient nutrient uptake and immobilization within the plant resulted in N and P depletion from the soil solution and the formation of the nutrient poorest peat within the spruce swamp forest system (Figure 3).

Cotton-grass is further thought to have large root exudation, which is deduced from increased CO_2 and CH_4 effluxes in its presence (Saarnio and others 2004; Laine and others 2012; Kuiper and others 2014; Robroek and others 2015b). The exudation of low molecular weight compounds commonly enhances microbial activity in the vicinity of roots and accelerates soil organic matter decomposition by the rhizosphere priming effect (Kuzyakov 2002). We found that cotton-grass presence enhanced the proportions of total peat N and P bound in the soil microbial biomass in comparison with the *Sphagnum* peat, which documented the high efficiency of plant–microbe relations in mining of nutrients from the peat and subsequent immobilization. However, we did not find any significant increase in either soil microbial biomass or exoenzymatic activity, which could be a sign of increased peat decomposition in the presence of cotton-grass. Still, the fact that cotton-grass enriched the peat with easily decomposable compounds can be deduced from enhanced microbial respiration under aerobic conditions, as found in one of the three studied sites. In anaerobic conditions, however, this cotton-grass effect disappeared. Lack of the expected stimulation effect of cotton-grass might be explained by strong nutrient limitation of microbial activity, because of the nutrient depletion by cotton-grass, as well as the prevailing anoxic conditions, which disable the functioning of oxidative enzymes and lower the energetic gain of microbial metabolism.

The decomposition rate of the polysaccharide-rich but nutrient-poor cotton-grass root litter was very low under such conditions, similar to that of the decay-resistant *Sphagnum* litter (Hájek and others 2011). This seems to be a general phenomenon because decomposition of cotton-grass root litter was markedly slower in comparison with other root litter types (*Carex* sp., *Betula nana*, fine roots of *Pinus sylvestris*) as found in a field study in boreal peatlands (Straková and others 2012). Well recognizable residues of its dead organs can be found even in peat layers formed thousands of years ago (Kalnina and others 2015). As a result, a combination of high primary productivity and slow tissue decomposition makes cotton-grass a typical peat-forming species with C sink function (Tuittila and others 1999; Silvan and others 2004; Kivimaki and others 2008).

Based on these results, we suggest that cotton-grass presence does not relieve the microbial community from nutrient limitation and supports the peatland C sink function. Supportive data about its efficient C and nutrient economics suggest that cotton-grass will have a similar effect on peat chemistry over a wide range of peatlands in which it can typically be found, including nutrient-poor open bogs and pine bogs, boreal peatlands, cutaway and also restored peatlands.

Blueberry Promoted Soil Organic Matter Decomposition by Significantly Enhancing P Availability

As hypothesized, blueberry biomass and attributes varied from that of the two other plant species. Noteworthy, blueberry was the nutrient poorest plant dominant but still it was not efficient in the internal recycling of nutrients within its biomass (Figure 3). It resorbed only 17% of N and approximately 52% of P from senescing leaves and almost no nutrients from its dying belowground biomass. The lower need for closed nutrient cycling within blueberry biomass could be due to nutrient income via ericoid mycorrhizal symbionts, which are able to mobilize N and P complexed in recalcitrant organic matter and facilitate plant uptake (Cairney and Meharg 2003; Read and others 2004).

The more open nutrient cycling within blueberry biomass increased P availability, resulting in the highest concentrations of P in the soil solution among all three plant species (Figure 3). In relation to this, microorganisms decreased their costs expenditure for gaining P but invested more into enzymes connected to C acquisition. Ericoid mycorrhizae associated with blueberry roots can play an important role in extracellular enzyme production and increased P availability (Read 1996; Read and others 2004). Enhanced concentrations of nitrate–N in the soil solution and its decreasing pH indicated more oxic conditions in the peat under blueberry. The blueberry litter, although rather nutrient poor and rich in aliphatic and aromatic compounds, thus decomposed relatively faster. The increasing amount of dissolved organic C in the soil solution supported the assumption about faster decomposition.

The higher nutrient availability and presence of ericoid mycorrhizae were mirrored in the microbial biomass, which was larger than in the other peat types and had lower C/N/P stoichiometry (Figure 3). Therefore, the microbial biomass under blueberry represented an important sink/source of nutrients with relatively fast turnover in the range of days to weeks (Schmidt and others 2007). This made nutrient cycling under blueberry more dynamic with the nutrients released during microbial turnover more accessible to plant uptake.

In summary, the blueberry tissues represented a less concentrated but, thanks to the large biomass, still significant nutrient stock within the spruce swamp forest, similar to the cotton-grass biomass. However, contrary to cotton-grass, the blueberry nutrient pool seemed to be more open, with larger losses of N and P to the peat, which could reduce nutrient limitation of the microbial decomposers and potentially stimulate peat decomposition. Our results thus support the projection of others (Bragazza and others 2013, 2015) that spreading of ericoid shrubs in peatlands would enhance organic matter decomposition and increase nutrient cycling, with negative implications for C sequestration.

Conclusions

Vascular plant cover in peatlands will likely increase with climate change (Elmendorf and others 2012). Previous studies demonstrated that this spreading can feed back to change microbial activity in the peat (Bragazza and others 2015; Robroek and others 2015b). Our data showed that the potential effect of vascular plants on peat properties, and soil microbial biomass and activity is species specific and need not necessarily result in increased organic matter decomposition.

The presence of blueberry enhanced the availability of the limiting nutrient P, connected with an increase in soil microbial biomass and the activities of C-acquiring enzymes. Its spread in peatlands, which could occur with lower water levels and the formation of an oxic upper peat layer, thus may result in increased organic matter decomposition, which would negatively affect the capacity of peatlands to act as C sinks, as proposed by others (Bragazza and others 2013, 2015).

Differently, the potential spread of cotton-grass can occur in nutrient-poorer peatlands with increasing temperatures but stable water levels (Wahren and others 2005; Salmon and others 2016). Cotton-grass is thought to provide large amounts of easily degradable compounds to the soil microbial community. However, at the same time, it has a high capacity to immobilize nutrients and strongly depletes P and N from the peat, which is further reflected in the C/N/P stoichiometry of the microbial biomass. The harsh conditions and prevailing anoxia in the upper peat layer retard decomposition of cotton-grass litter and result in no significant enhancement in microbial biomass and exoenzymatic activity. Therefore, the spread of cotton-grass would not change or may even enhance the functioning of peatlands as C sinks.

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Table S1. Peat Chemistry Average concentrations of C, N and P (%), their molar stoichiometric ratios and pH of the peat formed in patches covered only by *Sphagnum* or affected by the presence of cotton-grass or blueberry in three spruce swamp forest sites. Peat cores were sampled in May, June and September 2013 and 2014 (Mean, standard error of the mean SEM, n=72). Results of GLM on the effect of plant dominants are shown. Lower case letters show differences among peat characteristics formed in the presence of different plant dominants ($p<0.05$).

	Kvilda									**GLM**	**Filipova**									**GLM**	**Tetrevska**									**GLM**
	Sphagnum			Cotton-grass			Blueberry				Sphagnum			Cotton-grass			Blueberry				Sphagnum			Cotton-grass			Blueberry			
Ctot	45.6^{a}	±	0.5	44.0^{a}	±	0.2	46.2^{a}	±	0.29	**ns**	38.6^{a}	±	1.7	44.6^{b}	±	0.2	46.6^{b}	±	0.5	**	47.2^{a}	±	1.0	46.6^{a}	±	0.4	47.6^{a}	±	0.5	**ns**
Ntot	1.61^{b}	±	0.04	1.05^{a}	±	0.06	1.62^{b}	±	0.05	***	1.43^{b}	±	0.08	1.01^{a}	±	0.08	1.61^{b}	±	0.06	***	1.85^{b}	±	0.11	1.31^{a}	±	0.09	1.56^{ab}	±	0.05	***
Ptot	0.08^{b}	±	0.003	0.06^{a}	±	0.004	0.08^{b}	±	0.004	**	0.11^{b}	±	0.005	0.05^{a}	±	0.002	0.08^{b}	±	0.007	***	0.08^{a}	±	0.01	0.05^{a}	±	0.00	0.07^{a}	±	0.01	*
C:N	28.2^{a}	±	0.9	46.5^{b}	±	3.6	27.8^{a}	±	1.04	***	24.9^{a}	±	0.44	47.3^{b}	±	2.6	29.0^{a}	±	1.3	***	22.0^{a}	±	0.5	38.9^{c}	±	2.4	30.9^{b}	±	1.6	***
C:P	1545^{ab}	±	87	1948^{a}	±	118	1470^{b}	±	84	**	976^{a}	±	71	2400^{b}	±	123	1532^{ab}	±	106	***	1885^{a}	±	360	2330^{a}	±	91	1827^{a}	±	142	**ns**
N:P	46.6^{a}	±	2.8	38.5^{b}	±	1.5	43.7^{ab}	±	2.0	***	40.3^{a}	±	1.5	44.8^{a}	±	1.5	44.1^{a}	±	2.1	**ns**	56.1^{a}	±	5.2	54.7^{a}	±	2.3	50.3^{a}	±	3.3	**ns**
pH	4.08^{a}	±	0.03	4.08^{a}	±	0.03	3.92^{b}	±	0.04	**	4.49^{a}	±	0.09	4.10^{ab}	±	0.04	3.93^{b}	±	0.18	***	4.3^{c}	±	0.3	3.8^{a}	±	0.2	4.0^{b}	±	0.3	***

ns nonsignificant
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Table S2. Soil Solution Chemistry Average concentrations of dissolved organic C (DOC; mg l^{-1}), soluble N (SN; mg l^{-1}) and P (SP; µg l^{-1}), their molar stoichiometric ratios, concentrations of mineral N forms (N-NH4, N-NO3; mg l^{-1}) and soluble reactive P (SRP, µg l^{-1}) in the soil solution extracted from peat cores from patches covered by *Sphagnum* and affected by the presence of cotton-grass or blueberry in three spruce swamp forest sites. Peat cores were sampled in May, June and September 2013 and 2014 in the three spruce swamp forest sites. (Mean, standard error of the mean SEM, n=72). Results of GLM on the effect of plant dominants are shown. Lower case letters show differences among solution characteristics in the presence of different plant dominants ($p<0.05$).

	Kvilda									**GLM**	**Filipova**									**GLM**	**Tetrevska**									**GLM**
	Sphagnum			Cotton-grass			Blueberry				Sphagnum			Cotton-grass			Blueberry				Sphagnum			Cotton-grass			Blueberry			
DOC	76.0^{ab}	±	12.7	58.2^{a}	±	4.0	90.3^{b}	±	9.8	*	49.4^{a}	±	4.2	73.4^{b}	±	8.0	75.0^{b}	±	7.6	*	74.2^{a}	±	9.1	87.0^{a}	±	12.4	111.7^{a}	±	15.7	ns
SN	2.10^{b}	±	0.26	1.50^{a}	±	0.12	1.37^{a}	±	0.10	ns	1.34^{a}	±	0.11	1.35^{a}	±	0.19	1.61^{a}	±	0.16	ns	2.32^{a}	±	0.34	1.89^{a}	±	0.16	2.35^{a}	±	0.36	ns
SP	0.27^{a}	±	0.03	0.25^{a}	±	0.04	0.24^{a}	±	0.02	ns	0.13^{a}	±	0.01	0.17^{ab}	±	0.03	0.23^{b}	±	0.04	*	0.46^{ab}	±	0.09	0.28^{a}	±	0.03	1.04^{b}	±	0.3	*
C:N	55.5^{a}	±	8.0	50.3^{a}	±	3.8	79.2^{b}	±	6.9	*	55.2^{a}	±	10.3	94.8^{a}	±	15.5	70.9^{a}	±	13.8	ns	43.2^{a}	±	4.4	58.2^{ab}	±	7.7	83.2^{b}	±	17.6	*
C:P	791^{a}	±	68	858^{a}	±	92	1057^{a}	±	103	ns	1225^{a}	±	173	1561^{a}	±	265	1148^{a}	±	172	ns	650^{ab}	±	72	952^{b}	±	114	584^{a}	±	82	*
N:P	19.2^{a}	±	2.3	20.8^{a}	±	3.3	14.8^{a}	±	1.6	ns	25.7^{a}	±	1.9	20.7^{a}	±	2.9	19.3^{a}	±	1.7	ns	19.5^{a}	±	3.2	20.3^{a}	±	2.8	10.8^{a}	±	1.8	ns
NH4	0.52^{b}	±	0.09	0.15^{a}	±	0.02	0.21^{a}	±	0.03	***	0.40^{b}	±	0.07	0.13^{a}	±	0.01	0.17^{a}	±	0.02	***	0.86^{c}	±	0.12	0.18^{a}	±	0.03	0.40^{b}	±	0.06	***
NO3	0.10^{a}	±	0.02	0.08^{a}	±	0.01	0.14^{a}	±	0.03	ns	0.07^{a}	±	0.00	0.08^{a}	±	0.01	0.20^{a}	±	0.08	ns	0.11^{a}	±	0.02	0.13^{a}	±	0.02	0.17^{a}	±	0.03	ns
SRP	0.14^{a}	±	0.02	0.11^{a}	±	0.03	0.09^{a}	±	0.01	ns	0.03^{a}	±	0.00	0.08^{a}	±	0.02	0.08^{a}	±	0.02	ns	0.32^{ab}	±	0.09	0.14^{a}	±	0.03	0.64^{b}	±	0.25	*

ns nonsignificant
* p<0.05
** p<0.01
*** p<0.001

Table S3. Microbial Biomass C, N and P (MB-C, MB-N, MB-P; μg g^{-1}) and their molar stoichiometric ratios, proportions of peat C, N, and P bound in the microbial biomass (%), microbial respiration in aerobic and anaerobic conditions (μl CO_2 g^{-1} h^{-1}) and the sum of hydrolytic enzymatic activity and proportions of C, N and P-gaining enzymatic activities in the peat formed in patches covered only by *Sphagnum* or affected by the presence of cotton-grass or blueberry in the three spruce swamp forest sites. Peat cores were sampled in May, June and September 2013 and 2014 in the three spruce swamp forest sites. (Mean, standard error of the mean SEM, n=72). Results of GLM on the effect of plant dominants are shown. Lower case letters show differences among peat characteristics formed in the presence of different plant dominants.

	Kvilda			GLM	Filipova			GLM	Tetrevska			GLM
	Sphagnum	Cotton-grass	Blueberry		Sphagnum	Cotton-grass	Blueberry		Sphagnum	Cotton-grass	Blueberry	
MB-C	3272^{a} ± 405	4602^{ab} ± 422	5876^{b} ± 719	**	4080^{a} ± 734	3634^{a} ± 391	4087^{a} ± 368	ns	2215^{a} ± 400	2559^{a} ± 331	3163^{a} ± 494	ns
MB-N	206.3^{a} ± 24.8	279.2^{a} ± 48.7	420.9^{b} ± 45.8	**	289.5^{a} ± 51.0	245.7^{a} ± 43.8	370.8^{a} ± 53.9	ns	325.1^{a} ± 48.2	380.9^{a} ± 66.5	583.8^{a} ± 89.7	ns
MB-P	117.1^{a} ± 13.3	193.6^{b} ± 26.3	187.2^{b} ± 21.1	**	137.8^{a} ± 17.1	150.4a$_{b}$ ± 17.4	217.7^{b} ± 29.0	*	112.2^{a} ± 21.3	102.2^{a} ± 14.3	191.5^{b} ± 24.5	**
C:N	18.92^{a} ± 1.24	31.12^{b} ± 4.80	17.69^{a} ± 1.68	**	23.03^{a} ± 4.46	25.89^{a} ± 5.62	18.44^{a} ± 2.55	ns	9.50^{a} ± 1.24	13.52^{a} ± 3.24	9.98^{a} ± 3.38	ns
C:P	118.16^{a} ± 37.36	146.90^{a} ± 33.20	93.88^{a} ± 15.00	ns	83.00^{a} ± 17.20	73.73^{a} ± 10.50	62.96^{a} ± 7.80	ns	54.41^{a} ± 14.50	68.80^{a} ± 9.74	49.51^{a} ± 7.55	ns
N:P	5.75^{a} ± 1.52	6.79^{a} ± 2.49	5.90^{a} ± 1.02	ns	5.32^{a} ± 1.08	4.05^{a} ± 0.64	4.34^{a} ± 0.59	ns	19.77^{a} ± 8.55	8.48^{a} ± 1.24	8.45^{a} ± 1.41	ns
MB-C/Ctot	0.72^{a} ± 0.08	1.04^{ab} ± 0.09	1.27^{b} ± 0.15	**	1.04^{a} ± 0.18	0.81^{a} ± 0.08	0.88^{a} ± 0.08	ns	0.47^{a} ± 0.08	0.55^{a} ± 0.07	0.66^{a} ± 0.10	ns
MB-N/Ntot	1.28^{a} ± 0.15	2.54^{b} ± 0.38	2.65^{b} ± 0.30	**	2.04^{a} ± 0.36	2.50^{a} ± 0.46	2.44^{a} ± 0.40	ns	1.76^{a} ± 0.26	2.91^{a} ± 0.51	3.75^{b} ± 0.58	*
MB-P/Ptot	15.12^{a} ± 1.64	32.11^{b} ± 4.64	23.19^{ab} ± 2.38	**	13.44^{a} ± 1.73	31.67^{b} ± 3.95	29.39^{b} ± 4.80	**	13.6^{a} ± 2.60	19.4^{a} ± 2.71	26.6^{b} ± 3.41	ns
Microb. respiration aerobic	9.05^{b} ± 0.68	11.56^{c} ± 0.79	5.60^{a} ± 0.35	***	7.27^{a} ± 1.02	6.96^{a} ± 0.45	6.81^{a} ± 0.64	ns	7.03^{a} ± 0.72	6.41^{a} ± 0.51	5.65^{a} ± 0.59	ns
Microb. respiration anaerobic	1.48^{ab} ± 0.19	1.59^{b} ± 0.30	0.91^{a} ± 0.11	*	1.41^{a} ± 0.30	1.25^{a} ± 0.15	1.27^{a} ± 0.16	ns	1.06^{a} ± 0.13	0.81^{a} ± 0.14	1.31^{a} ± 0.18	ns
Sum of enzymatic activity	969.5^{a} ± 174.7	839.0^{a} ± 52.8	1009.0^{a} ± 134.5	ns	1184.7^{b} ± 122.8	911.3^{ab} ± 106.3	744.6^{a} ± 116.1	*	645.0^{a} ± 87.5	512.0^{a} ± 37.1	846.0^{a} ± 120.9	ns
% C-enzymes	14.9^{a} ± 1.7	10.9^{a} ± 0.7	15.2^{a} ± 2.3	ns	15.2^{a} ± 2.4	14.2^{a} ± 1.6	21.5^{a} ± 3.1	ns	13.5^{a} ± 1.2	13.2^{a} ± 1.1	17.6^{b} ± 2.7	**
% N-enzymes	1.7^{a} ± 0.4	2.5^{a} ± 0.4	1.9^{a} ± 0.2	ns	3.1^{a} ± 0.5	4.2^{a} ± 0.4	3.0^{a} ± 0.5	ns	2.6^{a} ± 0.7	4.0^{ab} ± 0.8	5.5^{b} ± 0.8	*
% P-enzymes	83.4^{a} ± 1.8	86.6^{a} ± 1.0	82.9^{a} ± 2.4	ns	81.7^{b} ± 2.7	81.5^{b} ± 1.8	75.5^{a} ± 3.3	*	83.9^{b} ± 1.5	82.8^{b} ± 1.8	77.0^{a} ± 1.4	**

ns	nonsignificant
*	p<0.05
**	p<0.01
***	p<0.001

PAPER 5

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REGULAR ARTICLE

Species effects and seasonal trends on plant efflux quantity and quality in a spruce swamp forest

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Abstract
Aims We aimed to compare seasonal exudate quality and quantity between *Sphagnum* moss, *Eriophorum vaginatum* (graminoid) and *Vaccinium myrtillus* (ericoid shrub).
Methods Exudates were collected in May, July and September 2014 using a culture-based method and characterized by total organic carbon (TOC) and nitrogen (TN) contents with exudation fluxes expressed on a root-mass basis. Organic acids, sugars and amino acids in the exudates were identified by ion exchange chromatography. C and N exudate fluxes, in situ exudation fluxes and exudate contribution to soil dissolved organic matter (DOM) were estimated. Differences in exudate biodegradability were assessed by ^{13}C pulse labeling.
Results E. vaginatum had the largest exudation fluxes, *Sphagnum* the lowest, and *V. myrtillus* intermediate, being the greatest in July. All species mostly exuded organic acids except *Sphagnum* in September when sugars (allose, xylose) and amino acids (cystine) dominated. *Sphagnum* exudates were more C-rich and less degradable than the vascular species exudates, which released both organic and inorganic N forms. *E. vaginatum* exudates were richer in amino acids and citrate especially in July. Exudates contributed up to 20% to soil DOM.
Conclusions Plant species composition greatly affects exudate quantity, quality and timing. Plant exudates represent considerable contributions to soil DOM.

Keywords Biodegradability · Dissolved organic matter · Peatlands · Root exudates · *Sphagnum* · Vascular plants

Abbreviations

C	Carbon
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DN	Dissolved nitrogen
LMW	Low molecular weight
N	Nitrogen
TOC	Total organic carbon
TN	Total nitrogen

Introduction

Peatlands represent large repositories of carbon (C) (Yu 2012), with *Sphagnum* mosses playing a dominant role in peat formation due to their unusual physiological and biochemical features. *Sphagnum* can maintain waterlogging in surface peat (Hayward and Clymo 1982) and its tissue also contains phenolic compounds

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and polysaccharides that suppress decomposition of dead plant matter (Verhoeven and Liefveld 1997; Hájek et al. 2011).

In addition to *Sphagnum*, vascular plants, mainly sedges and dwarf shrubs, commonly inhabit peatlands. The presence of vascular plants generally increases net ecosystem CO_2 exchange (Laine et al. 2012) and methane efflux (Robroek et al. 2015b), as well as soil microbial biomass, the decomposition rate of organic matter and nutrient availability (Bragazza et al. 2015). Vascular plants also influence the quality of dissolved organic matter (Robroek et al. 2015a) and its export from peatlands (Freeman et al. 2004). Vegetation composition is recognized as an important factor affecting the magnitude of these changes (Breeuwer et al. 2009; Laine et al. 2012; Kuiper et al. 2014; Robroek et al. 2015a; Robroek et al. 2015b). The above described effects on peatland functioning connected with the presence of vascular plants and the differences in these effects among plant species or plant functional groups are commonly ascribed to the different quality and quantity of root exudates.

The release of low molecular weight (LMW = compounds <1000 Da; Warren 2016) root exudates represents an important and continuous carbon source to the soil environment during the growing season (Grayston et al. 1996; Jones et al. 2004). As with the release of other rhizodeposits, such as root mucilage and root lysates, exudates can greatly impact the carbon and nutrient cycles in an ecosystem by stimulating rhizosphere microbial activity and inducing the priming effect (van der Krift et al. 2001; Jones et al. 2009). Interestingly, despite the recognized role of root exudates in shaping microbial community composition and consequently biogeochemical processes in peatlands, information about their fluxes, differences in the composition among characteristic peatland species and their potential contribution to dissolved organic matter (DOM) is very limited.

Most studies of the compounds released by *Sphagnum* mainly focused on their inhibitory and allelopathic potential, especially the roles of phenolic and uronic acids (Verhoeven and Liefveld 1997). Fenner et al. (2004), using ^{13}C pulse labeling, showed that fresh *Sphagnum* exudates contribute up to 4% of the total dissolved organic carbon (DOC) content, however these authors did not provide deeper information about the composition or fate of the exudates. The exudates of peatland vascular plants were shown to significantly contribute to CO_2 efflux from peatlands (Crow and Wieder 2005), indicating their higher decomposability in comparison to the DOM in peatlands. Recent root exudates of some vascular plants, namely sedges, were found to be a primary source of methane emissions from peatlands (King et al. 2002; Ström et al. 2003). Only the exudates of *Eriophorum vaginatum* were studied in more detail in this regard, with carbohydrates and organic acids being the major compounds released (Saarnio et al. 2004). However, these plants were grown in waterlogged quartz sand and supplied with a nutrient solution, creating artificial conditions quite different from natural ones.

For this study, we chose representatives of the three main plant functional groups characteristic of peatlands: *Sphagnum* moss, graminoids and ericaceous shrubs, represented in our case by *Sphagnum fallax*, *Eriophorum vaginatum* and *Vaccinium myrtillus*, respectively (the three species are hereafter referred to respectively as *Sphagnum*, *Eriophorum* and *Vaccinium*). *Sphagnum fallax* is widespread in nutrient-poor to intermediate peatlands. *Eriophorum* (cotton grass) is a tussock forming sedge common in nutrient-poor temperate to boreal peatlands in Europe, Asia and North America (Mitsch and Gosselink 2000). It was the first non-mycorrhizal species found to be able to take up organic forms of N, usually as amino acids (Chapin et al. 1993; Leadley et al. 1997), as well as having a high nutrient resorption capacity (Shaver et al. 1986; Kaštovská et al. 2017). *Vaccinium* (blueberry) commonly inhabits drier parts of peatlands, facilitating its nutrient uptake by symbiosis with ericoid mycorrhiza. The onset of drier conditions resulting from human and/or climate changes would likely result in the spread of *Vaccinium* in the affected peatlands (Bragazza 2006; Dieleman et al. 2015). A previous study (Kaštovská et al. 2017) found species-specific effects on peat properties and soil microbial biomass and activity, with these differences likely related to differences in the quantity and quality of root exudates.

The aim of our study was to compare the fluxes and composition of exudates released by living *Sphagnum* with that released from the roots of the two vascular species, *Eriophorum* and *Vaccinium*, in conditions close to natural ones. We estimated the contribution of organic acids, sugars and amino acids to these fluxes and determined the significance of species and seasonal effects on their amounts and composition. Additionally, we estimated the contribution of exudates to DOM during the

vegetation season and compared the biodegradability of the exudates released at the peak of the vegetation season. We tested the following hypotheses: 1) the two vascular plant species, *Eriophorum* and *Vaccinium*, will have larger exudate fluxes per mg root mass, with the exudates being more biodegradable, compared to *Sphagnum*. 2) There will be significant species as well as seasonal differences in the composition of the identified exudate compounds. Still, organic acids will be the main compounds in the exudates of all three species, as their release increases P availability in P-limited systems (Höffland et al. 1992; Gerke 1994; Neumann and Romheld 1999; Abrahao et al. 2014), which is common for many peatlands (Hill et al. 2014). Lastly, 3) the contribution of the exudates to peatland DOC will approach several percents, with the largest contribution occurring at the peak of the vegetation season in summer.

Methods

Study site

The Tetřevská spruce swamp forest (SSF) is located in the Šumava Mountains, south-west Czech Republic (48°59'N, 13°28′E). The SSF is situated on an upland plateau at an altitude of 1100 m.a.s.l, with a cold and humid climate (mean annual temperature = 4.0 °C and mean annual precipitation = 1200 mm; average data from 1961 to 1990, Czech Hydro-Meteorological Institute). The SSF is covered by a continuous layer of *Sphagnum* mosses (dominated by *S. fallax* and rarely by *S. flexuosum* and *S. girgensohnii*) in a sparse matrix of *Picea abies* trees. Within this matrix, wet open patches are occupied by *Eriophorum* while *Vaccinium* dominates drier microhabitats. Other plant species, such as *V. vitis-ideae*, *Oxycoccus palustris,* and other sedges and grasses, are also rarely present. The patchy distribution of the three dominants in the understory of the SSF reflects variations in terrain, water level and the location of springs.

Plant sampling and exudate collecting

In the field, whole clumps (shoots and roots) of *Eriophorum,* shoots with attached rhizomes and roots of *Vaccinium,* and blocks of *Sphagnum* (20 cm W * 30 cm L * 15 cm D) were collected in May, July and September 2014 and brought back to the laboratory. All plant and *Sphagnum* samples were kept moist and shaded during the ca 1 h transport. Root exudates were collected using a modified version of the method developed by Phillips et al. (2008). For these analyses, three-to-four shoots of *Eriophorum*, with attached roots, were separated from each clump the day following field collection. The same was done for individual roots of *Vaccinium* except we cleaned the end 20 cm of three-to- four intact roots which were still connected to the plant. The roots were carefully cleaned of any adhering material using distilled water. The clean roots were then wrapped in moist *Sphagnum* collected from the field site, which still contained pore water from the study site, placed within an aluminum foil envelope and kept overnight for equilibration. Wrapping the roots in *Sphagnum* with pore water from the site helped to maintain the plant samples in conditions closer to those in the field site compared to experiments conducted entirely in the laboratory or using hydroponic solutions. The next day, the samples (entire roots of one shoot for *Eriophorum* and the individual root sections for *Vaccinium*) were uncovered, quickly cleaned of any adhering material and placed in 20 ml syringes filled with distilled water. In addition, selected *Sphagnum* shoots were cleaned and placed into 50 ml beakers filled with distilled water. The samples were placed in an area with slight tree shading, similar to field conditions, and exposed to daylight conditions for four hours, from 9:00–13:00. After the exposition period, the selected roots were sampled, scanned and then dried at 65 °C for 24 h and weighed. The root scans were analyzed for root surface area and volume using WinRhizo (Regent Instruments, Canada). Root surface area and root dry weight were highly correlated ($r^2 = 0.98$). The solution with exudates was immediately filtered through 0.2 μm Express Plus PES membrane filters (Merck-Millipore, Ireland), collected in sterilized 5 ml vials. All collected exudate samples were kept cooled (+4 °C) until they were analyzed, which was usually within 24 h after collection.

We are aware that cleaning the roots with distilled water of adhering soil particles and using a short exposition time of four hours reduced but did not eliminate the impact of root-associated microbes. Therefore, the exudates sampled in the described manner represent a mixture of compounds of plant origin, influenced in their amount and composition by the activity of present rhizoplane microbes (Warren 2016). Since the aim of our study was to determine the species and seasonal

effects of plant effluxes to the soil, and the potential contribution of these inputs to soil DOM, the presence of even reduced microbial numbers would result in closer-to-natural experimental conditions, which would provide a more realistic picture than if completely sterile conditions were applied.

In addition, given the analytical method employed, distilled water was considered as the best medium for producing clear results even though we are aware that distilled water could influence the exudate flux from the plants (Aulakh et al. 2001). However, the short duration of root exposure and maintaining the roots in site DOM prior to exposure should have minimized such an effect.

Exudate analyses

The collected exudate samples were analyzed for total organic carbon (TOC) and total nitrogen (TN) contents on a LiquiTOC (Elementar, Germany). Ion exchange chromatography (IC; Thermo ICS-5000, USA) was used to separate organic acids, sugars and amino acids. Organic acids and other anion forms (including nitrite and nitrate-N) were separated by injecting 3 μl of each sample into a Dionex AS-11 HC capillary column (0.4 × 250 mm) using a non-linear gradient elution of distilled water and KOH (0.5–110 mM) with a flow rate of 10 μl min^{-1} and detected on a conductivity detector. Sugars and amino acids (50 μl of each sample) were separated on a Pac PA10 column using a non-linear elution of 10–250 mM NaOH, which included 1 M NaOAc and a 100 mM acetic acid wash. The flow rate was 0.25 mL min^{-1} and compounds were detected amperometrically. Particular compounds were determined by comparison to the retention times of known standards and their concentration quantified after peak integration and comparison to peak areas of the respective standards. In most cases, there were distinct peaks for individual compounds. However, the peaks for acetic and glycolic acids, as well as for ascorbic and oxalic acids, had very similar retention times and could not be distinguished. Therefore, a common peak area was used to calculate the concentrations of these combined compounds, which are hereafter noted as acetic+glycolic and ascorbic+oxalic, respectively. A common peak area was also used for benzoic+succinic+malic acids. The measured TOC and TN exudate concentrations were relativized to a root mass/living biomass basis and to sampling time (mg g^{-1} h^{-1}). *Eriophorum* had the smallest roots (4.5–6.6 mg), followed by *Vaccinium* roots (20.0–77.1 mg), while the *Sphagnum* shoots were the heaviest (135.5–213.8 mg). We further summed the C concentrations for the identified organic acids, amino acids and sugars, and calculated their contribution to the total TOC exudation flux for the respective species and samplings. Similarly, we calculated the contributions of inorganic (nitrite and nitrate-N) and organic N (contained in amino acids and amino sugars) to the total TN exudation fluxes.

Estimation of in situ exudation fluxes

We estimated the C and N exudation fluxes (mg m^{-2}) which would be released by the three species in the field conditions of the SSF, by multiplying the relativized TOC and TN released by particular species with their mean live root biomass (or mean living biomass for *Sphagnum*). The live root biomass data were obtained from ten belowground samples (5.5 cm W * 6.5 cm L * 30 cm D), collected from habitats dominated either by *Vaccinium* or *Eriophorum* at the same time as the plants sampled for the exudate analyses. The belowground materials in the cores were carefully cleaned and then separated to live and dead roots and rhizomes. *Sphagnum* living biomass was obtained from the blocks sampled for the exudate analyses. All samples were dried at 65 °C for 48 h, weighed and mean dry weights per area (g m^{-2}) calculated for each species and season (Supplemental Table 1).

We further estimated the potential contributions of the C and N exudation fluxes to peatland DOM, using DOC and DN contents for the respective habitats. To obtain the soil solution, we sampled four intact cylindrical peat cores (72 cm^3 volume) to a depth of 10 cm (the main rooting zone) under each plant dominant in May, July and September 2014. The sampled peat cores were immediately inserted into extraction tubes, closed with aluminum foil and put into a cooling box with ice. The soil solution was extracted by the centrifugal-drainage method at 4000 g for 1 h at 4 °C (Giesler and Lundstrom 1993) within 24 h. The solution was filtered through a low-protein-binding Express PLUS Polyethersulfone membrane with a 0.22 μm pore size (Merck Millipore Ltd., Ireland) and analysed for TOC and TN on a LiquiTOC II (Elementar, Germany). TOC and TN were calculated per volume of soil per m^2 to a 10 cm depth using peat bulk density. The proportion of soil DOC and DN attributed to the estimated field C and N exudate fluxes were calculated for each season.

Exudate biodegradability in natural DOM

The exudate biodegradability in natural DOM was measured after ^{13}C pulse labeling of plants in July 2014. Plants were collected in the field and prepared similarly as for the exudate sampling and then put into boxes. The roots and rhizomes of the herbaceous species and the whole *Sphagnum* shoots except the upper ca 1 cm were submerged into a known volume of DOM sampled in the field at the same time as the plants. Four replicate boxes were prepared for each species. The boxes were placed in a gas-tight plexiglass chamber with a hole fitted with a rubber-septa and two 1watt fans. The $^{13}CO_2$ tracer (99.9 atom % 13C, Cambridge Isotope Laboratories, GB) was repeatedly added into the chamber by a syringe to keep the internal CO_2 concentration at 400–500 ppm (verified by GC) for a period of 4 h, from 9:00–13:00. Immediately after the labeling, and after 8, 24, and 48 h, all the DOM in the boxes was sampled and replaced by fresh solution. A portion of each DOM sample was immediately freeze-dried and the same was done to the rest of the sample after incubation in the dark at 20 °C for 3 days. All the freeze-dried samples were analyzed for total C and ^{13}C contents (in ^{13}C atomic percent, at.%) on an NC Elemental analyzer (ThermoQuest, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany). The difference in ^{13}C content between the immediately frozen and incubated samples was used to calculate the biodegradability of the released ^{13}C compounds (the percentage of mineralized to original ^{13}C in a particular sample).

Statistical evaluation of the data

The data on the relativized TOC and TN exudations, contributions of organic acids, amino acids and sugars, as well as inorganic N to the TOC and TN exudation, estimated field C and N exudation fluxes, and exudate biodegradability were natural log transformed (ln + 1) when necessary in order to meet the conditions of normality and variance homogeneity. Repeated measures ANOVAs were run to evaluate species and seasonal (temporal) effects. Due to the small number of samples, Scheffé's comparison of means test was used when there were significant species effects. A Bonferroni correction was used for comparing the differences between means in the case of significant seasonal or species*season (time) interaction terms. Analyses were conducted in SYSTAT v. 11.

The possible connections between individual identified LMW compounds in the exudates and particular species and/or season were examined by constrained multivariate analysis (RDA with species*season interaction as the explanatory variable) using the data on proportional contribution of individual compounds to total C in these LMW compounds. The analysis was run in CANOCO v.5.

Results

TOC / TN amounts released per root mass

The TOC and TN contents of the root exudates significantly differed between the three species ($p < 0.001$) as well as seasonally (TOC: $p < 0.001$; TN: $p = 0.011$). In general, *Eriophorum* with the smallest roots released the largest TOC and TN amounts per root mass and its C and N fluxes were the most seasonally variable of the studied species (Fig. 1a, b) ranging from 1.47 to 50.48 mg C g^{-1} h^{-1} and 0.08 to 4.26 mg N g^{-1} h^{-1} respectively (Fig. 1a, b). On the contrary, *Vaccinium* and *Sphagnum* C fluxes were significantly less (0.34–3.35 and 0.16–0.79 mg C g^{-1} h^{-1}, respectively) as were the respective N fluxes (0.02–0.34 and 0.01–0.06 mg N g^{-1} h^{-1}). *Eriophorum* and *Sphagnum* released the most TOC in July, but *Vaccinium* in September. The two vascular plants generally released more TN per root mass than *Sphagnum* in May and especially in September, when the TN release of *Sphagnum* was significantly reduced (Fig. 1b). In July, the *Eriophorum* TN release highly exceeded those from the other two species, which were similar. As a result, the C:N ratios of the released compounds were relatively low from May to July, ranging on average between 17 and 25, with *Sphagnum* always leaching compounds with a higher C:N ratio than the vascular plants. The lowest C:N of 8.6 occurred for *Vaccinium* exudates in May (Fig. 1c; Table 1). The C:N ratio then increased in September for all three species, but most notably for *Sphagnum* exudates, which had an average ratio > 80.

Identified N forms in the released TN

The identified N forms, containing both organic (amino acids, amino sugars) and inorganic (nitrite and nitrate

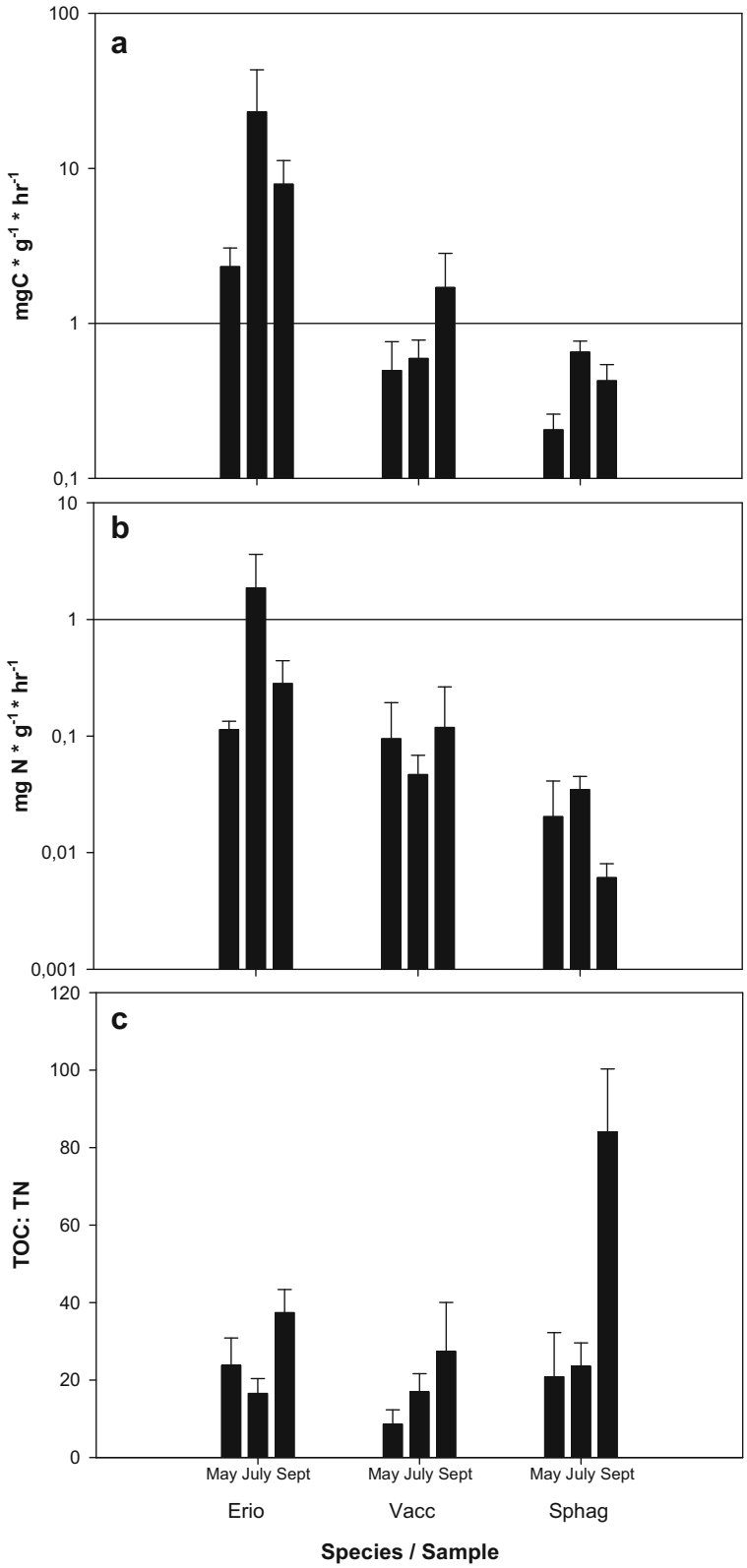

Fig. 1 The amounts of **a**) TOC and **b**) TN released per g root mass per hour of vascular plants or living mass of *Sphagnum* in May, July and September 2014, and C) their molar C:N ratios. Mean and standard deviations ($n = 4$) are shown. Note the logarithmic scale on the Y axes for both the TOC and TN graphs. Species: Erio = *Eriophorum vaginatum*; Vacc = *Vaccinium myrtillus*; Sphag = *Sphagnum* (mostly *S. fallax*).

Table 1 The C:N ratios for the identified (Ident) LMW and the remaining, unidentified (Other) compounds in the collected exudates from three dominant understory plant species from a spruce swamp forest

	Ident			Other			Total		
	M	J	S	M	J	S	M	J	S
Erio	9.33 ± 5.48bc	3.34 ± 1.09c	26.09 ± 4.26a	36.05 ± 13.54b	24.38 ± 7.54bc	43.62 ± 7.72b	23.83 ± 5.72bc	16.56 ± 3.80bc	37.42 ± 5.92ab
Vacc	21.16 ± 2.02ab	5.42 ± 3.94c	15.65 ± 4.04ab	8.16 ± 3.73c	23.32 ± 9.45bc	63.22 ± 49.08b	8.61 ± 3.68c	16.97 ± 4.65bc	36.66 ± 11.52ab
Sphag	47.09 ± 18.61a	53.38 ± 37.52a	19.81 ± 6.40ab	20.48 ± 11.37bc	21.92 ± 5.23bc	271.06 ± 172.26a	20.86 ± 11.38bc	23.58 ± 5.99bc	84.08 ± 16.19a

Total = total TOC:TN. Species: Erio = *Eriophorum vaginatum*; Vacc = *Vaccinium myrtillus*; Sphag = *Sphagnum* (mostly *S. fallax*). Seasons: M = May; J = July; S = September. Different letters in each C:N ratio type indicate significant differences at the $p < 0.05$ level

but not ammonium, which was not measured) N accounted for 3.2–64.7% of the TN flux, differing by species and season (Fig. 2a). The proportion of inorganic N in the *Sphagnum* TN flux was very low in all samplings. In fact, all identified N forms released by *Sphagnum* accounted only for <7% of the TN flux in May and July but then significantly increased to almost 65% of the TN in September (Fig. 2a), mainly due to a large (15% of all identified compounds in that sampling) contribution of cystine. The collected exudates of both vascular plants contained more inorganic N than *Sphagnum*. In July, the inorganic N flux from *Eriophorum* and *Vaccinium* even exceeded the release of organic N, forming 25–27% of the TN flux. By September, the identified organic and inorganic N forms contributed equally to the TN flux, both forms accounting for 10 to 18% of the flux (Fig. 2a).

Identified LMW compounds in the released TOC

The identified LMW compounds formed 5.8–22.7% of the TOC flux, with no clear species or seasonal effect (Fig. 2b). Organic acids represented the majority of the identified compounds in the TOC fluxes of all species, with *Sphagnum* exudates containing a significantly higher proportion of organic acids than either *Eriophorum* or *Vaccinium,* except in the September sampling when there was a large increase in the release of sugars. Sugars and amino acids each contributed 4–35% to the identified TOC compounds (Fig. 2b). While their contributions were relatively stable across the season for *Vaccinium*, being 13% for amino acids and 7–22% for sugars, they varied over time for the other two species. The identified TOC of *Sphagnum* contained small portions of amino acids and sugars in May and July (each ca 5% of identified TOC) but both increased towards September. On the contrary, the identified *Eriophorum* exudates contained larger portions of sugars and namely amino acids in May but both decreased towards autumn, when its exudates were largely dominated by organic acids (> 90% of the identified TOC; Fig. 2c). The C:N ratios of the identified LMW compounds therefore differed significantly between the species as well as seasonally (Table 1).

Composition of identified LMW exudates

We identified 39 compounds in total, 15 organic acids (individual and also composite peaks), 15 sugars and 9 amino acids (Supplementary Figure 1). Of the organic

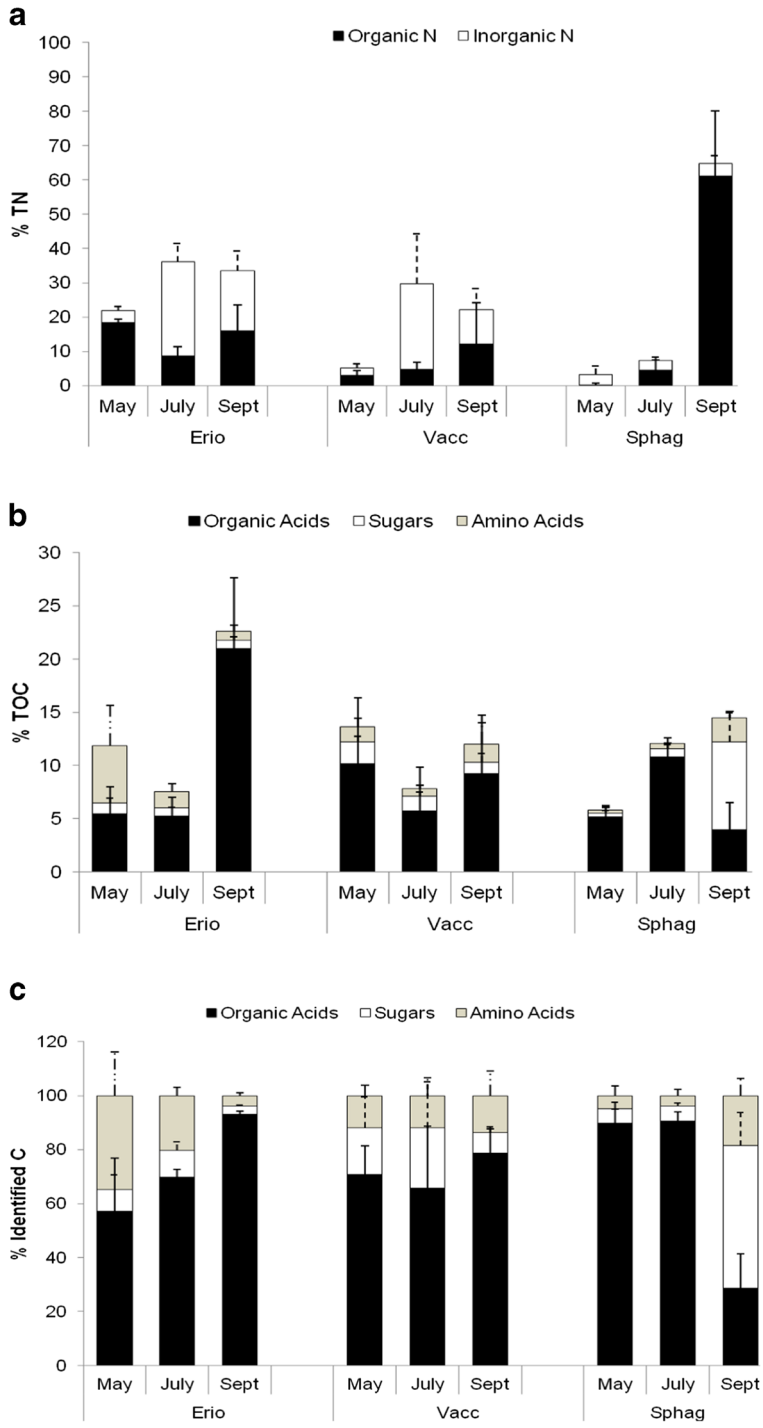

Fig. 2 **a** Contributions of organic (amino acids + amino sugars) and inorganic (nitrite + nitrate) N to the exudate TN fluxes from three common understory species in a spruce swamp forest peatland. **b** Contribution of identified organic compounds, organic acids, amino acids and sugars, to the exudate TOC fluxes from the studied plant species during the year. **c** Proportion of organic acids, sugars and amino acids comprising the identified exudate compounds. Species: Erio = *Eriophorum vaginatum*; Vacc = *Vaccinium myrtillus*; Sphag = *Sphagnum* (mostly *S. fallax*). Sept = September.

acids, lactic, formic and the combined peaks of acetic+ glycolic and ascorbic+oxalic acids dominated in all three species during the whole season. Other acids, such as quinic, iso-butyric, adipic and benzyl+succinic+malic, were also produced by all the species but in lower amounts. The production of other organic acids, such as citric and iso-citric acids, were related either to a specific sampling time or particular species. The commonly released sugars were mainly alcohol sugars such as glycerol and mannitol, supplemented by glucosamine

and lactose in both vascular species and also glucose namely in *Vaccinium*. The exudation of other sugars was again either species or season specific. All species preferentially released cystine, being the dominant amino acid released, supplemented by methionine and cysteine in the exudates of both vascular plants. The production of other amino acids was related to particular seasons, such as serine in summer and leucine and proline in autumn.

The composition of exudates differed among the seasons (pseudo-F = 7.4, $p = 0.02$) as well as among the species (pseudo-F = 2.9, p = 0.02). The separation of the samples is visualized in Fig. 3a (RDA analysis with species*seasonal interaction as the explanatory variable, pseudo-F = 5.2, p = 0.02, 52.6% of variability explained). The September sampling was clearly separated from those in May and July along axis 1 (Fig. 3a) mainly due to the enhanced proportion of sugars in especially the *Sphagnum* exudates, which were the richest in sugars in particular allose and to a lesser extent xylose, ribose and glucose (Fig. 3b). This large contribution of specific sugars, as well as the enhanced proportion of amino acids in the September *Sphagnum* exudates, clearly distinguished it from those of the two vascular plants. The May and July samplings were well separated along the second axis. Both seasons were characterized by larger contributions of ascorbic+oxalic acids and glycerol in the exudates of all three plants than in September. The exudates sampled in May exclusively contained raffinose and also large peaks of benzoic+succinic+malic acids, while fructose was completely absent. Also, the amount of formic acid in the May exudates was much less than for the exudates from the other seasons (Fig. 3b). *Sphagnum* exudates were again well distinguished from those of the two vascular species as they contained only 11–19 identified compounds in comparison to the richer exudates of the vascular plants, and dominated by acetic+glycolic acid (38% C in the identified compounds). On the contrary, the July exudates of all species exclusively contained alanine and serine, and were also richer in citric and iso-citric acids than exudates released in the other seasons (Fig. 3b). The two acids, which are potentially important in enhancing P availability in the root surroundings, were produced in larger amounts by *Eriophorum*, separating it from the other two species ($p < 0.001$) (Fig. 3a). In fact, the largest species differences in exudate composition occurred in July. Variation partitioning analysis showed that seasonal variation explained 26% and species-differences 7.9% of total data variability.

Estimated exudation fluxes and their contribution to DOM in the field

We estimated the size of the C and N exudate fluxes in the patches dominated by the studied species in the

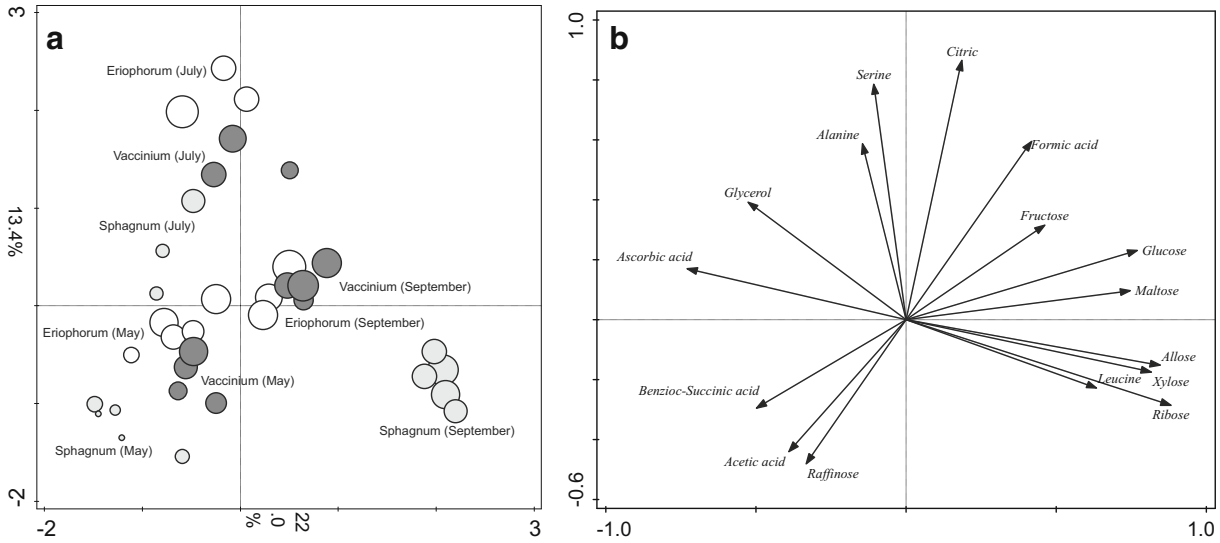

Fig. 3 **a** Separation of the exudates released by *Sphagnum*, *Vaccinium* and *Eriophorum* in May, July and September 2014 according to the composition of identified LMW compounds (RDA analysis with species*time interaction as the explanatory variable). Size of the circles correspond to the proportion of exuded compounds. **b** The 16 compounds mostly related to the explained variability in the data

peatland. The exudate C fluxes per m^2 were mainly controlled by the biomass of the particular species in the field, with *Sphagnum* having much greater biomass than either vascular species. The spring exudation fluxes were similar for all three species, being about 150 mg C m^{-2} (Fig. 4a). However, due to plant growth and increased exudation in the vegetation season, the C flux from *Sphagnum* significantly increased to about 800 mg C m^{-2} in July ($p < 0.001$) with a small decrease in September. However, the fluxes from *Eriophorum* and *Vaccinium* roots remained similar during the season, ranging from 100 to 200 mg C m^{-2} (Fig. 4a). Accordingly, the potential contribution of exudate flux to soil DOC in the field was estimated to be <5% for both vascular plants, but this would greatly increase up to 18% in July in *Sphagnum*-dominated patches while still accounting for 12% of the DOC in September (Table 2a).

Sphagnum also had a significantly larger overall TN flux ($p < 0.001$) compared to *Eriophorum* and *Vaccinium* (Fig. 4b), exceeding 40 mg N m^{-2}. Both *Eriophorum* and *Sphagnum* had similar TN flux patterns, with low fluxes in May and September with the maxima in July. The pattern differed for *Vaccinium* in that the maximum TN flux occurred in May and then decreased to similar levels in both July and September. The TN flux pattern was mostly mirrored in the potential contribution of this flux to soil DN (Table 2b). Maximum contributions from both *Eriophorum* and *Sphagnum* occurred in July (7–10% of field DN), while it was

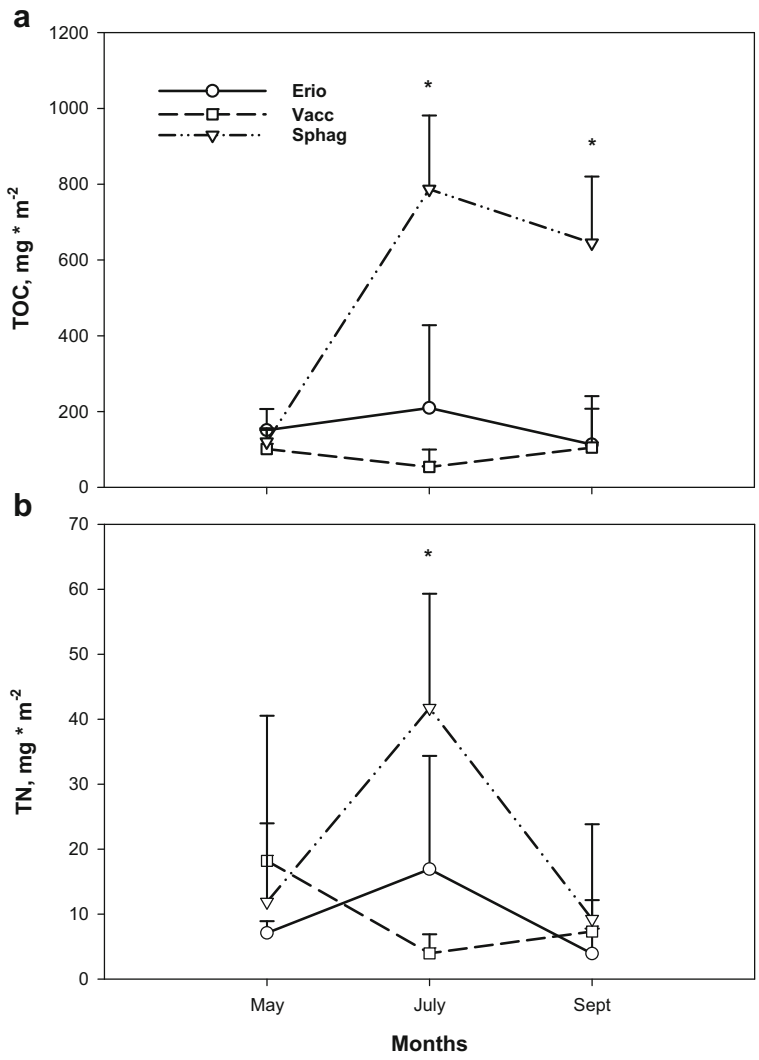

Fig. 4 Estimated field **a**) **C** and **b**) N exudate fluxes from roots of vascular plants and living *Sphagnum* biomass per m^2 (mg C or N m^{-2}, mean ± standard deviation, $n = 4$) in habitats dominated by those species during the year. Asterisks represent species samples significantly different from those of other species in the same time (* = $p < 0.05$; ** = $p < 0.01$). Species: Erio = *Eriophorum vaginatum*; Vacc = *Vaccinium myrtillus*; Sphag = *Sphagnum* (mostly *S. fallax*).

Table 2 Estimated percent contribution of carbon and nitrogen exudate fluxes (TOC, TN respectively) to the (A) dissolved organic carbon (DOC) and (B) dissolved nitrogen (DN) in the peatland in patches dominated by particular plant species during the year

Season	Spring			Summer			Autumn		
	TOC	DOC	%	TOC	DOC	%	TOC	DOC	%
A.									
Eriophorum	150.90	3321.69	4.54	209.79	5266.22	3.98	113.27	5901.58	1.92
Vaccinium	101.42	3873.04	2.62	54.05	5414.97	1.00	105.12	5691.25	1.85
Sphagnum	120.26	3871.43	3.68	787.13	4285.87	18.37	645.13	5284.15	12.21
Season	Spring			Summer			Autumn		
	TN	DN	%	TN	DN	%	TN	DN	%
B.									
Eriophorum	7.09	151.56	4.68	16.91	249.30	6.78	3.91	133.06	2.94
Vaccinium	18.19	138.03	13.18	3.95	313.36	1.26	7.32	205.50	3.56
Sphagnum	11.86	144.80	8.19	41.71	408.32	10.22	9.24	273.71	3.38

Values were calculated by combining TOC or TN fluxes per root mass measured in this study with data about the average living root biomass of vascular species or living *Sphagnum* biomass per m^2 area and DOC or DN amounts in peat cores measured in spring, summer and autumn of 2014 in the field. Flux units are mg * m^{-2}

May for *Vaccinium* (up to 13% of field DN). Potential TN flux contributions to soil DN in *Sphagnum*-dominated patches were also relatively high in May.

Exudate biodegradability in natural DOM

We further measured biodegradability of the exudates released by the three species in July. The degradability of the ^{13}C exudates significantly differed between the species and over the two days of exudate collection (Fig. 5). The average biodegradability of the *Sphagnum* exudates, for the full two days of the experiment, was significantly lower ($F = 68.223$; $p < 0.001$) than the *Eriophorum* and *Vaccinium* exudates, which were similar in biodegradability. Over time, the exudates of the two vascular species released only four hours after ^{13}C fixation were highly biodegradable, losing more than 50% of their ^{13}C in three days, while *Sphagnum* exudates were slowly degradable (losing ca 10% of ^{13}C in three days). For all species, the biodegradability of the compounds released between 4 and 8, 8–24 and 24–48 h after ^{13}C fixation decreased over time ($F_{3,\ 27} = 4.003$; $p = 0.018$). The *Eriophorum* exudates had the most pronounced change, with those released after four hours being similar

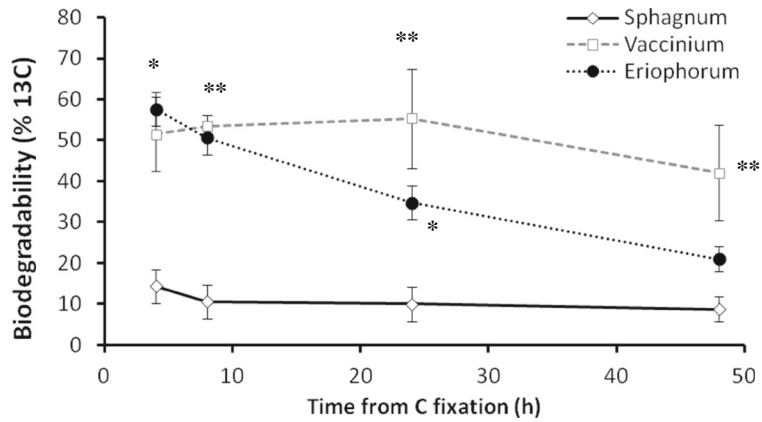

Fig. 5 Changes in the biodegradability (% of initial C content) of the ^{13}C exudates released from the studied species between 0 and 4, 4–8, 8–24, and 24–48 h after plant ^{13}C fixation. Asterisks represent species samples significantly different from those of other species in the same time (* = $p < 0.05$; ** = $p < 0.01$). Species: Eriophorum = *Eriophorum vaginatum*; Vaccinium = *Vaccinium myrtillus*; Sphagnum = *Sphagnum* (mostly *S. fallax*).

to *Vaccinium* exudates while the degradability of those produced after 48 h were closer to those released by *Sphagnum.*

Discussion

Plant species composition, and the associated quality and quantity of root exudates, can greatly affect the functioning of peatlands (Breeuwer et al. 2009; Laine et al. 2012; Robroek et al. 2015a). In this study, we compared the fluxes and composition of exudates released from representative species of three main plant functional types occurring in peatlands: *Sphagnum* moss, the graminoid *Eriophorum vaginatum* and the ericoid shrub *Vaccinium myrtillus*. We always sampled the plants in large clumps in the peatland shortly before exudate sampling and watered them with fresh field DOM to keep the conditions very close to natural. This action differentiates our study from previous ones, when the studied plants were grown in artificial conditions (e.g. Saarnio et al. 2004, working with *Eriophorum* plants grown in a sand culture). Further, we studied not only the differences between species, but also seasonal changes in exudate fluxes and composition, and further estimated the potential contribution of the exudate flux to peatland DOM. Use of completely sterile conditions (e.g., Kuijken et al. 2015) would not have provided a realistic estimate of these fluxes. Therefore, our results are rather unique and enlarge the information about peatland DOM sources and the potential role of exudates in peatland functioning.

Sphagnum exudates differ from those of the two vascular species

As hypothesized, the C and N exudate fluxes of the two vascular plant species were significantly greater, with different chemistry and degree of biodegradability, than those from *Sphagnum*. The *Sphagnum* exudates were more C-rich, with corresponding significantly larger C:N ratios, and were also significantly less degradable. Part of this difference is due to the large proportion of organic acids which comprised the LMW fraction of these exudates, especially in May and July. The release of organic acids in order to acidify their environment is a well-known trait of *Sphagnum* mosses (Clymo and Hayward 1982). In addition, it is likely that more recalcitrant compounds, which are known to be produced by *Sphagnum* and include various phenolics, uronic acids and polysaccharides (Verhoeven and Liefveld 1997; Kaštovská et al. 2017), may have also been substantial components of these exudates, since the identified LMW fraction comprised at most 14% of the TOC in the *Sphagnum* exudates. Although these secondary metabolites were not measured in our study, these highly soluble compounds are important components of cell walls and thus can form a large part of the excretions from *Sphagnum* shoots (Verhoeven and Liefveld 1997). Therefore, their presence in our *Sphagnum* exudate samples would not be surprising. In addition, these compounds are highly resistant to degradation due to their chemical composition. The presence of these compounds would thus likely explain the low degradability (10% on average) of the *Sphagnum* exudates.

The root exudates of the two vascular plant species were significantly more degradable than those from *Sphagnum*, with biodegradation of the vascular exudates reaching 57% after three days. The exudates in all three species became less degradable over time due to the likely larger contribution of more recalcitrant compounds in the later exudates (Gransee and Wittenmayer 2000; Dessureault-Rompre et al. 2007; Chaparro et al. 2013). Unfortunately, these compounds were not identified in our study, thus we cannot determine the contribution of particular compounds. This decrease was most notable for *Eriophorum*, in which the biodegradability of the exudates sampled after 48 h was only 21% compared to the 57% degradation of the exudates sampled after 4 h, a decrease of 64%. In comparison, degradability decreased only by 27% and 38% for the *Vaccinium* and *Sphagnum* exudates, respectively.

Compared to *Sphagnum*, the two vascular species released significantly greater amounts of N (up to several mg N g^{-1} root h^{-1}), resulting in their significantly lower C:N ratios and likely contributing to their greater degradability. Unlike *Sphagnum*, both *Eriophorum* and *Vaccinium* released inorganic (nitrates) as well as organic (amino acids, sugar amines) N compounds. Both vascular species released significant amounts of nitrates, which reached a maximum in July when nitrate-N formed ca 25% of the total root N flux of these species. Simple amino acids accounted for a maximum of only 20% of TN, a percentage similar to that found in other studies (e.g., Jones 1998; Lesuffleur and Cliquet 2010), but less than what was found for wheat (Warren 2015). Thus, the largest portion of exuded N must be composed of other

unidentified N compounds, such as ammonium compounds, polyamines, nucleobases and polypeptides, several of which were identified in the exudates from wheat and other species and therefore could be common components of plant N exudates (Dakora and Phillips 2002; Warren 2015). Although it was not measured, it is likely that ammonium-N was exuded as appreciable amounts were found to be a common component of root exudates in other cases (see Beauchemin et al. 2012; Lesuffleur et al. 2013). Peptides and enzymes, especially phosphatases, may also have been important components of the May and July excretions of *Vaccinium* and *Eriophorum*, which is implied by the low C:N ratio of the unidentified compounds in those samples (Table 1) as well as the presence of polypeptides in the *Eriophorum* plants from our study site (Kaštovská et al. 2017). In addition, the synthesis and release of phosphatases are known to increase with greater P limitation (Neumann and Romheld 2012).

The May and July stoichiometric results, especially those of the two vascular plant species, are contrary to expectations that plants release N-poor exudates (Deubel et al. 2000; Paterson 2003; Badri and Vivanco 2009). In fact, there is little information about the C:N ratio of exuded or rhizodeposited compounds (Hinsinger et al. 2009), with modelled values ranging from 8 to 100 (Drake et al. 2013). The majority of our results fall in the lower range of these expected values.

Such relatively large N fluxes to the soil, especially from the two vascular species, raises questions about the fate of this released N. Rhizosphere soil is typically a C-excess environment (Hinsinger et al. 2009), a situation also found in our study site (Kaštovská et al. 2017). According to Jones et al. (2009), the plants may recapture the N, however, Warren (2016) found that uptake of N compounds by wheat is an active process. Since the release of amino acids is likely also an active process under direct plant control (Lesuffleur et al. 2007; Lesuffleur and Cliquet 2010), re-uptake of exuded N compounds would represent a double cost to the plants, a situation which would seem to be counterproductive for plants growing in nutrient-poor habitats, in which the plants would be expected to retain nutrients and limit their costs (Aerts and Chapin 2000). It thus seems more likely that the N is captured by soil microbes, which is a common occurrence in nutrient-poor conditions (Raynaud et al. 2006; Kuzyakov and Xu 2013).

Generally, all three species exuded almost the same identified LMW compounds, with organic acids, in particular ascorbic+oxalic, acetic+glycolic, lactic and formic acids, comprising the majority of the identified C, while sugars and amino acids each comprised <20% of the exudates. The usually large proportion of organic acids is similar to the findings of other exudate studies in nutrient-limited habitats (Grayston et al. 1996; Dakora and Phillips 2002; Yong Wu et al. 2012). In contrast, organic acids comprised <1% of exudates from wheat plants grown in non-limiting conditions, with the majority of those exudates being composed of amino acids and other N-containing metabolites (Warren 2016). Our results also differ from those of Saarnio et al. (2004), who noted a majority proportion of sugars (neutral compounds) in the identified exudates of *E. vaginatum*, a species included in our study. Saarnio et al. (2004) grew their plants in sand supplied with a weak nutrient solution, conditions far from natural. Environmental characteristics, both biotic and abiotic, as well as the collection medium used, have been shown to influence the quantity and quality of root exudates (Grayston et al. 1996; Aulakh et al. 2001; Carvalhais et al. 2011). There is a chance that, by using distilled water as the collection medium, instead of a weak salt solution, there could have been an increased release of particular compounds, especially sugars, due to changed osmotic potentials (Aulakh et al. 2001). However, as noted above, the composition and proportion of our identified exudates were very similar to those of other studies conducted in nutrient-limited conditions (Grayston et al. 1996; Neumann and Romheld 1999; Dakora and Phillips 2002). In addition, the fact that our plants were kept in DOM from the field site to just before exudate collection and the short time of exposure to the distilled water solution (4 h) seemed to minimize any such physiological effect on our plants. Therefore, our results, which were derived from plants kept in more natural conditions, likely represent what is actually occurring in the field.

The composition of the *Sphagnum* exudates changed over the growing season in a manner different from those of the two vascular species, with sugars, especially allose and xylose, supplemented by ribose, lactose, glucose and fructose, and the amino acid cystine, forming a majority of the identified *Sphagnum* exudates in September. Xylose is commonly found in the exudates of many plant species (Grayston et al. 1996; DeLarue et al. 2011), but can also be produced microbially (Macko et al. 1991; Kazda et al. 1992). Allose, a C-3 epimer of glucose, is a rare sugar not usually found in nature (Fukumoto et al. 2013), although it has been identified

in other peatlands (Comont et al. 2006). It may have a role in promoting plant defenses against particular soil pathogens and nematodes (Kano et al. 2010, 2013; Sakoguchi et al. 2016), increasing plant stress resistance (Hu et al. 2015) as well as affecting plant growth (Fukumoto et al. 2011). Likewise, cystine and its close congener cysteine also increase plant stress resistance, especially to the onset of hypoxia brought about by prolonged flooding (White et al. 2017), as well as being important in the development of anti-microbial mechanisms (Tam et al. 2015). The large release of both allose and cystine in September could indicate the need by *Sphagnum* to increase its plant defenses against pathogenic organisms. A similar need could explain the large release of amino acids, especially cystine and cysteine, by *Eriophorum* in May and July, although this may also be connected to plant growth in the spring and summer. Future studies are needed to connect the exudation of specific compounds to particular environmental stimuli.

Differences in graminoid and ericoid shrub exudates

While the exudates of the two vascular species significantly differed from those of *Sphagnum* in many ways, there were also important differences between the two vascular species. *Eriophorum* had markedly larger mass-specific C and N fluxes than either *Vaccinium* or *Sphagnum*, which were sometimes similar. The larger exudation of carbon compounds from *Eriophorum* could be related to the higher photosynthetic capacity of graminoids in comparison to ericoid shrubs and peat mosses (Riutta et al. 2007; Leppala et al. 2008), as well as its smaller root mass. In addition, *Eriophorum* exudates were richer in citric acid, most notably in July at the time of maximum plant biomass, and amino acids than those of *Vaccinium* or *Sphagnum*. A greater release of citric acid and other chelating compounds is a common response of plants to P deficiency (Höffland et al. 1992; Jones 1998; Marschner et al. 2011). In fact, soil P content was the lowest in the *Eriophorum*-dominated patches in our study site (Kaštovská et al. 2017). Also, the greater exudation flux of chelating compounds from *Eriophorum* could help explain its much larger N and P uptake efficiencies, as noted by its significantly lower shoot and root C:N ratios (Cholewa and Griffith 2004; Kaštovská et al. 2017). However, these phytochelators may only indirectly affect P availability by supplying C substrates to enhance soil microbial activity as well as acidifying the rhizosphere soil (Jones and Darrah 1994; Jones 1998; Neumann and Romheld 1999; but see Höffland et al. 1992). Phosphatases and other P-acquiring enzymes accounted for at least 80% of hydrolytic enzyme activity in our study site (Kaštovská et al. 2017), but further studies would be required to determine whether these compounds have a greater direct impact on P availability.

The reduced quantity of chelating compounds and amino acids in the *Vaccinium* exudates is likely connected to the mycorrhizal status of this species. In contrast to *Eriophorum*, *Vaccinium* roots are infected by ericoid mycorrhizae, which allow the host plants to access a larger substrate area and thereby improve N and P uptake (Cairney and Meharg 2003; Finlay 2005). By increasing the area from which available nutrients can be obtained, mycorrhizal species do not have to rely as much on nutrient resorption in comparison to non-mycorrhizal graminoid species, such as *Eriophorum*, which require high nutrient resorption efficiencies to survive in nutrient-limited conditions (Gavazov et al. 2016; Kaštovská et al. 2017). However, this increased nutrient supply comes at a cost to the host plant (Aerts and Chapin 2000; Lynch and Ho 2005). While we did not measure the cost in new photoassimilates to *Vaccinium* in maintaining the ericoid mycorrhizal connections, the C requirements of ectomycorrhizal fungi have been estimated to range between 15% and 28% of net plant C fixation (reviewed by Finlay 2008). Increased nutrient availability and the costs associated with maintaining the mycorrhizal symbiont would then likely have a negative impact on the production and release of citric acid and other chelating compounds, as shown by the greatly reduced exudation of organic acids in mycorrhizal-infected species compared to non-mycorrhizal species in P-deficient soils (Ryan et al. 2012). There are similar costs in the production and release of amino acids. If amino acid release is an active process under direct plant control (Lesuffleur et al. 2007; Lesuffleur and Cliquet 2010), then this would be an additional cost to the plant. *Eriophorum* is not burdened with maintaining a mycorrhizal association and thus can expend more of its available energy to exporting amino acids and chelating compounds, while such production and release would represent a double cost for *Vaccinium*. This may also help to explain the more stable C and N exudation flux patterns exhibited by *Vaccinium*.

Contribution of root exudates to peatland DOM

In agreement with our third hypothesis, the root exudates of the three studied species represented an important

potential input of C substrates to soil DOC. Peatlands are a major source of DOC contributing an estimated 20% of all terrestrially-derived DOC to the oceans (Fenner et al. 2007), however only a few studies have explicitly investigated the contribution of exuded compounds to peatland DOC (e.g., King et al. 2002; Fenner et al. 2004) or DN (Bragazza and Limpens 2004; Fustec et al. 2010). In our study, the *Eriophorum* and *Vaccinium* exudates could potentially contribute between 1 and 5% to the DOC measured within their respective patches, but, because of their higher biodegradability, microbes will likely metabolize them rapidly. However, the possible contribution was greater for *Sphagnum*, reaching 18% in July, a value which is much larger than the 4% calculated by Fenner et al. (2004). The release of these compounds by living *Sphagnum* may be an important source of DOC in the peatland system, due to their low degradability, which is comparable to the degradability of the original DOC (10–25% C in 40 days, unpublished results).

The TN flux from the plants to the rhizosphere represents a considerable input of N to an otherwise N-limited environment, representing 10–13% of the DN in May and July. The addition of N-enriched substrates can have a priming effect on soil microbes, stimulating microbial metabolic activity and the production of exoenzymes resulting in increased SOM decomposition (Hinsinger et al. 2009; Kuzyakov 2010; Drake et al. 2013), which can facilitate plant nutrient uptake.

Conclusions

The quantity and quality of the *Sphagnum* exudates differed greatly from those of the two vascular species especially in regards to the C and N fluxes, role of inorganic N sources, exudate stoichiometry and biodegradability. The exudate flux from *Sphagnum* represents a quantitatively important carbon and nitrogen input to peatland DOM (King et al. 2002; Fenner et al. 2004), which can remain as part of site DOM for a relatively long time due to its low degradability. The exudates of the vascular plants contribute a smaller percentage to DOM of the peat, but since these have a higher degradability, they would fuel microbial activity in the vicinity of their roots, thus producing hotspots of microbial activity. This greater difference between the moss and two vascular plant species is apparent, however there were also important differences between *Eriophorum* and *Vaccinium*. *Eriophorum* released a greater amount of organic acids, especially chelators, which resulted in increased P availability. Coupled with the greater nutrient uptake efficiency of *Eriophorum*, this resulted in greater nutrient capture, and thus poorer soil conditions with slower turnover rates, in patches dominated by *Eriophorum* (Kaštovská et al. 2017). On the contrary, the greater biodegradability of *Vaccinium* exudates would likely lead to faster nutrient turnover rates in patches dominated by the ericoid shrub. In addition, the more stable input of exudates from *Vaccinium* would be expected to lead to changed nutrient dynamics and a decreased ability of peatlands dominated by ericoid shrubs to capture and store C (Fenner et al. 2007; Dunn et al. 2016), an event predicted to occur under the expected future warmer and drier conditions resulting from climate change (Bragazza et al. 2013; Heijmans et al. 2013).

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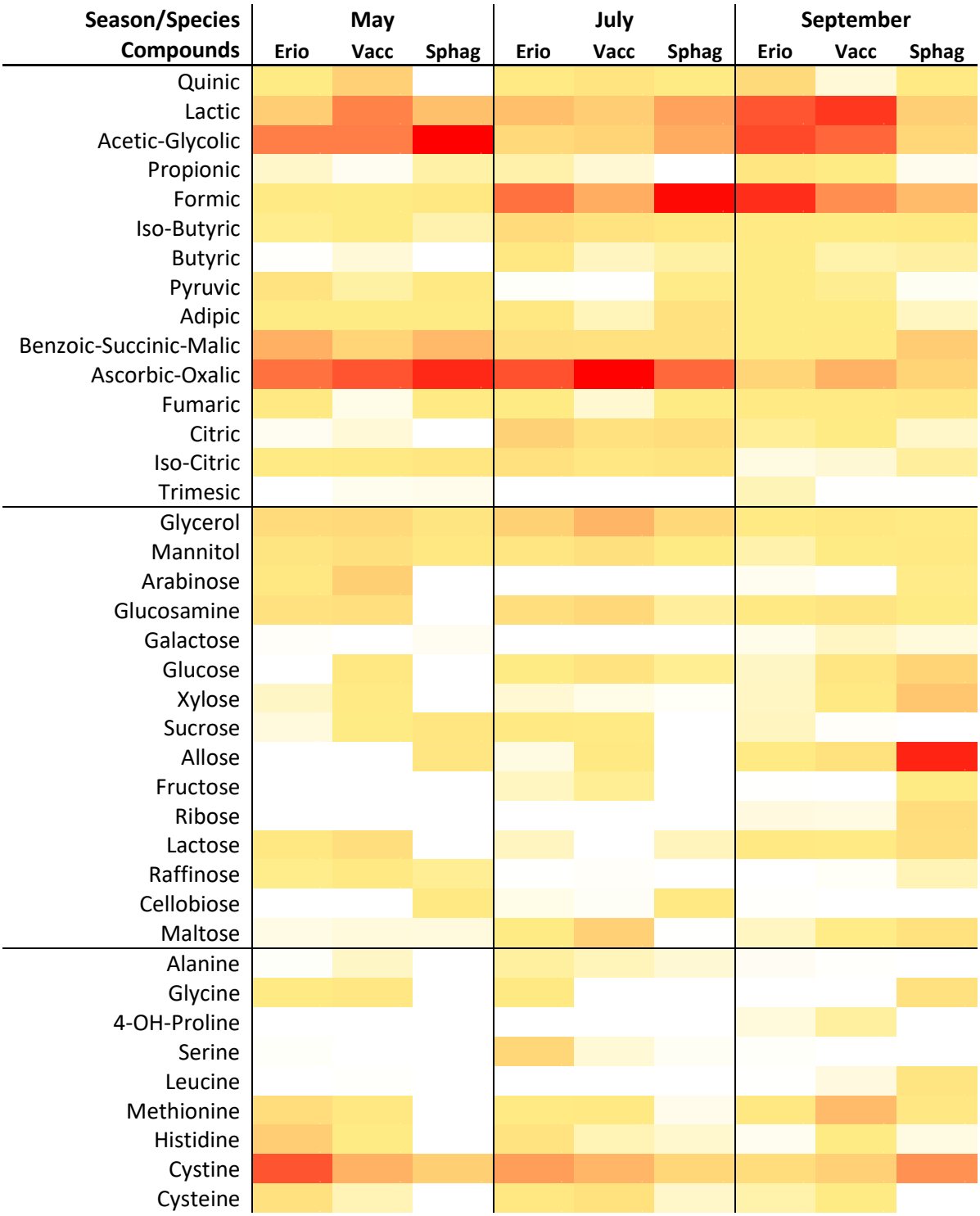
Supplement Table 1. Mean live root weight (g dry weight * m^{-2}) for each plant species in the studied spruce swamp forest per sampling time in the 2014 growing season. Eriophorum = *Eriophorum vaginatum*; Vaccinium = *Vaccinium myrtillus*.

Species / Season	May	July	September
Eriophorum	14.71	2.26	3.68
Vaccinium	538.03	241.33	153.93
Sphagnum	145.74	300.333	377.75

Supplement Figure 1. Contribution of individual identified compounds to total flux of identified LMW compounds for *Eriophorum vaginatum* (= Erio), *Vaccinium myrtillus* (= Vacc) and *Sphagnum* (mostly *S. fallax* = Sphag) in May, July and September 2014. The color scale white-yellow-red corresponds to increasing relative presence within the exudate sample of the respective species at a particular time (white = not present, deep red= 0.38).



PAPER 6

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Microbial transformation of organic matter in soils of montane grasslands under different management

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Abstract

The study compared the effects of mowing, mulching, and no-treatment, applied to a mountain grassland over five years, on microbial transformation of soil organic matter (SOM). Microbial biomass, microbial respiration, cellulose decomposition and mineralization were measured in the laboratory eight times during the three-year experiment. In addition, soil phosphatase activity and factors limiting microbial growth were assessed once to complete the results. Mowing increased soil microbial biomass and carbon use efficiency, which supported carbon sequestration in soil. In contrast, mulching led to a decrease in microbial biomass and microbial metabolic efficiency due to the limitation of easily decomposable carbon. This was a consequence of changes in temperature and light conditions under the mulch layer, which suppressed plant growth and rhizodeposition. Processes causing organic matter transformation in the mulched grassland were similar to those of the untreated grassland. Annual mowing appears to be most suitable for maintenance of SOM content and sustainability of montane grasslands.

Keywords: Grassland; Mowing; Mulching; Defoliation; Soil organic matter; Decomposition; Mineralization; Microbial biomass

1. Introduction

Montane and submontane secondary grasslands are an integral part of the cultural landscape of the Czech Republic. Historically, they were used for hay production and sheep or cattle grazing but the demand for hay has declined and traditional cycles of mowing and grazing have lost their economic importance. In spite of this, the importance of permanent grasslands increases, irrespectively of direct biomass production. They represent valuable ecosystems, supporting unique plant and faunal communities and, together with woods and wetlands, form the basis for the ecological stability of the cultural landscape.

Grasslands have a high inherent soil organic matter (SOM) content that supplies plant nutrients, increases

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soil aggregation, limits soil erosion, and also increases cation exchange and water holding capacities (Miller and Donahue, 1990). Thus, maintenance of SOM and the quality of soils are the key factors in the sustainability of grassland ecosystems (Conant et al., 2001) and productivity of plant communities (Bending et al., 2000). Most of the organic carbon found in the soil is primarily plant derived. Pasture plants translocate about 30–50% of assimilates below ground (Swinnen et al., 1995; Kuzyakov and Domanski, 2000a,b). One-half of the translocated C is found in the roots, while one-third is evolved as CO_2 from the soil by microbial and root respiration. The remaining C is incorporated into soil microorganisms and SOM (Kuzyakov et al., 1999; Domanski et al., 2001).

Total C input to grassland soil has been calculated as being from 2450 to 4430 kg C ha^{-1} during the vegetation season (Saggar et al., 1997; Domanski et al., 2001; Hütsch et al., 2002). Two main sources of C input to the soil are root/shoot remains and root exudates plus other root-borne substances. Actual rhizodeposition under field conditions depends on many different factors including climatic conditions, such as temperature, photo-period, and light intensity, as well as soil characteristics like moisture content, nutrient status, pH, or oxygen availability (Hütsch et al., 2002).

There is an increasing amount of data showing that grassland management may substantially influence the below ground food-web, and thus SOM transformation and nutrient cycling (Mikola et al., 2001). Bardgett et al. (1998) considered two broad ways by which defoliation caused by grazing or mowing may affect soil biotic communities. The first one occurs through defoliation effects on the pattern of root exudation and carbon allocation (short-term effects) and on root biomass and morphology (long-term effects). Defoliation leads to a complex redistribution of assimilated C (Schnyder and de Visser, 1999; Kuzyakov et al., 2002). An important part of C is allocated into growing shoots, while another part remains in the roots and is used for root growth, respiration, and exudation (Schaufele and Schnyder, 2001). Although no marked changes were usually found in underground phytomass (McNaughton et al., 1998; Guitian and Bardgett, 2000; Hejduk and Hrabě, 2003), many authors reported an increased exudation after plant defoliation (Holland, 1996; Hamilton and Frank, 2001; Kuzyakov et al., 2002). The increased rhizodeposition positively influences the size and activity of the soil biotic community (Rice et al., 1996) and enhances the supply of nutrients in the rhizosphere for plant uptake and regrowth (McNaughton et al., 1997; Hamilton and Frank, 2001). The second way is the influence of defoliation on soil biota through altering the quality of the plant litter input. This involves enhancing the concentration of nutrients in the root and leaf litter (McNaughton et al., 1997; Hamilton and Frank, 2001) and a shift in plant community structure caused by selective grazing (Mikola et al., 2001) or different plant tolerance to defoliation (Kvítek et al., 1998; Mikola et al., 2001; Moog et al., 2002).

Mulching has been suggested as a new alternative practice, which should be economically more convenient than mowing. However, the chopped plant litter, which is left on site, alters the physical and chemical environment. The litter layer intercepts light and reduces the thermal amplitude in the soil. By reducing maximal soil temperatures and creating a barrier to water-vapor diffusion, the presence of litter reduces evaporation from the soil. In contrary, it may also diminish water availability when it retains a large portion of rainfall. The decomposition of litter may release both nutrients and phytotoxic substances (Facelli and Picket, 1991). Kvítek et al. (1998) and Moog et al. (2002) found differences in plant community composition between mulched and mowed grasslands. Mulching has been used namely in tilled soils due to its restorative effects on SOM and reduction of soil erosion (Saroa and Lal, 2003). However, a decline in SOM and effective cation exchange capacity were reported after mulching, too (Tian and Brussaard, 1997). The effects of mulching on the soil microbial community and SOM transformation in grassland ecosystems has received only limited attention.

Total SOM content in a grassland is often unaffected even after long-term management (Hassink and Neeteson, 1991; Hassink, 1994; Bending et al., 2000). As microorganisms are largely responsible for the transformation of SOM and soil nutrient cycling, the effect of grassland management is mediated by microbial biomass (Rice et al., 1996). Therefore, microbial biomass (Powlson et al., 1987; Rice et al.,

1996) and activity (Tracy and Frank, 1998; Bending et al., 2000) can provide an early indication of the changes in total SOM due to management regime. Many authors reported that plant defoliation (grazing/mowing) had a positive effect on microbial biomass, improved nutrient cycling (Bardgett et al., 1998) and on increased C-use efficiency (Guitian and Bardgett, 2000). For the sake of completeness, there are also studies that found no effect of management regime on soil microbial biomass (Wardle and Barker, 1997).

This study reports on a three-year investigation that compared the effects of different management techniques on changes in microbial organic matter transformation in the soil of a mountain meadow. The objectives of our study were to: (1) determine the effect of different management practices (mowing, mulching, and no-treatment) on soil microbial biomass and organic matter transformation (to confirm the positive effect of defoliation on soil microbial biomass and C-use efficiency), (2) compare microbial transformation of SOM in mowed and mulched grasslands, (3) choose the management regime that would be most suitable for maintenance of SOM content and biological activity of montane grasslands. The study is part of a larger project focused also on vegetation and microclimatic changes in mountain grasslands and shifts in soil physico–chemical properties as affected by management practices (Kvítek et al., 2001; Mašková et al., 2001; Smejkal et al., 2001; Zelený et al., 2001).

2. Material and methods

2.1. Research area

The research area was situated on an enclosed mesic grassland, in the central mountain plateau of Bohemian Forest National Park, Czech Republic, at an altitude of 1150–1180 m, on a SW-facing slope. The whole site was subjected to the same long-term management, which consisted of random mowing and very rare cattle grazing between 1952–1990 and annual mowing since 1990. The oligotrophic plant community of the grassland belongs to the *Cardaminopsio halleri-Agrostietum* ass. The experimental area was tested for spatial heterogeneity in selected soil physico–chemical characteristics (pH, SOM content, total C, N and P, amounts of available nutrients P, K, Ca, and Mg) in 1997. The coefficients of variation were 10–30%. The experimental area (150 m × 100 m) was established on the grassland and divided into three 50 m × 100 m plots. Different management practices have been applied to the plots since 1997. The first plot was regularly mowed (C) once a year in July, and the cut plant biomass removed. The second plot was regularly mulched (M) once a year in July and the cut plant biomass was left in place. Mowing and mulching were done using a tractor. The third plot was left untreated (O). The present study was initiated in 1999, two years after the different practices were first applied.

The soil was a sandy loam (11% clay, 66% sand), acid distric cambisol with pH (H_2O) of 4.67 ± 0.22, total C of 66.86 ± 2.48 mg g^{-1} and total N of 4.87 ± 0.21 mg g^{-1} (average ± standard deviation, n = 15). Detailed information on the physico–chemical soil characteristics is given by Kvítek et al. (2001). Sampling occurred eight times during 1999–2001 (17 May 1999, 7 July 1999, 18 October 1999, 18 May 2000, 17 July 2000, 23 October 2000, 10 May 2001, 29 October 2001). Summer samplings were done after mowing/mulching. Soils from experimental areas C, M, and O were sampled from pits 15 cm × 15 cm in area and from a depth of 5–20 cm. Fifteen soil samples were taken from each plot along five vertical transects. Soils from three sampling points chosen randomly along each vertical transect were always mixed into one composite sample. Thus, five composite soil samples per experimental treatment were collected on each sampling date and analyzed separately. This way of sampling reduced substantially the effect of micro-scale variability (mm to cm), which can be larger than mezo-scale (meters) or macro-scale (within and among plots) variability (Morris, 1999; Morris and Boerner, 1999). The fresh soils were sieved through a 2 mm mesh and stored in polyethylene bags at 4 °C for three weeks prior to analyses.

2.2. Analytical procedures

The amount of C and N bound in soil microbial biomass was determined using the chloroform fumigation-extraction method (Vance et al., 1987). Soils were extracted with 0.5 M K_2SO_4. Organic C in the sulfate extracts was oxidized by acid dichromate digestion. The excess dichromate was determined by

back-titration with $(NH_4)_2Fe(SO_4)_2$. Microbial carbon (C_{MIC}) was calculated as the difference between organic C content in fumigated and non-fumigated soils using k_{EC} 0.38 (Vance et al., 1987). Microbial nitrogen (N_{MIC}) was measured using the method of Cabrera and Beare (1993). Briefly, the sulfate extract was autoclaved together with persulfate, oxidizing all N forms to nitrate. Nitrate in the extract was measured after reduction to nitrite, using a flow injection analyzer (FIA STAR 5023, Sweden). N_{MIC} was calculated as the difference between the N concentration in extracts from the fumigated and non-fumigated soils using k_{EN} 0.45 (Jenkinson, 1988).

Soil respiration and cellulose decomposition and mineralization were measured as follows. Two subsamples containing 200 g of fresh soil wetted to 60% of water-holding capacity were prepared from each composite soil sample. To one of the samples, an ash-less cellulose filter paper of known weight (ca 0.5 g) was buried in a nylon bag (mesh size 1 mm). Soils were incubated in airtight closed 0.75 l glass bottles at 20 °C for 35 days. Soil respiration was measured by the alkali absorption method six times during the incubation. Average basal (r_0, soil only) and cellulose induced (r_P, soil with a filter paper) respiration rates were calculated at the end of the experiment. Cellulose decomposition was calculated from the weight difference of the filter paper before and after incubation. At the end of the incubation, the cellulose filter paper was dried to a constant weight and incinerated at 550 °C for 4 h to correct for ash content. The difference between the cellulose induced and basal respiration rates was considered to be the cellulose mineralization rate; $(r_P - r_0)$. A ratio of mineralized to decomposed cellulose (%) was calculated as the difference between cumulative cellulose induced respiration and cumulative basal respiration divided by the total amount of decomposed cellulose. This shows the proportion of carbon evolved as C-CO_2 from the soil after cellulose addition. All microbial activities were expressed in μg C g^{-1} dry soil h^{-1}.

Phosphatase activity was estimated using a fluorogenic substrate 4-methylumbelliferil-phosphate (MUF-phosphate; Glycosynth, UK), by the method modified for microplates (Marx et al., 2001) as described by Šantrůčková et al. (2004). Briefly, soil was homogenized with distilled water and the suspensions (soil:water, 1:100, w/v, 4 min of sonication) were pipetted into wells of the microplate. MUF-phosphate (final concentration 100, 200, 400 μM) was added and the samples were pre-incubated at 20 °C for 2 h. The relative fluorescence of each well was measured (excitation/emission: 365 nm/445 nm wavelengths, 1 nm/5 nm band passes, respectively) in a spectrofluorometer FluoroMax-3 (Jobin Yvon, Spex-Horiba, USA–France) at 5 min intervals for 2 h. Relative fluorescence rate was determined as the slope of a linear regression.

The nutrient requirements of the microflora were determined by measuring respiratory response (O_2 production; OxiTop system, WTW, Germany) after amendments of C, N, and P (Scheu, 1993) in soil samples from October 2001 only. The nutrients were added in aqueous solutions (5 ml per 10 g dry soil) in four treatments: C (glucose only), CN (glucose and $(NH_4)_2SO_4$), CP (glucose and K_2HPO_4), and CNP (glucose, $(NH_4)_2SO_4$, and K_2HPO_4). A ratio of C:N:P 10:2:1 was always used (1000, 200, and 100 μg g^{-1} dry wt for C, N, and P, respectively), which is close to that in microbial tissue (Anderson and Domsch, 1980). For basal respiration (r_B) measurement (control), only distilled water was added to the soil samples. Samples were incubated at 20 °C for 5 days. Three replicates were set up for each combination of factors. Each sample from the C, CN, CP, and CNP treatments were characterized by active biomass and maximum respiration rate (r_{MAX}). Active biomass was expressed as the respiration rate of the linear phase of the respiration response to the substrate addition during the first 20 h of incubation, and r_{MAX} was calculated from the exponential phase of the respiration curve, which was observed between 20–30 h of incubation in all treatments. For a better comparison of soils, microbial response to nutrient addition was expressed as the relative increase of respiration after nutrient addition; $(r_{MAX} - r_B)/r_B$.

2.3. Statistical analysis

Data were analyzed using STATISTICA 5.5 for Windows (Statsoft, USA). Effects of management regime (mowing, mulching, no-treatment) and sampling time were tested using analysis of variance (Two-way ANOVA). Post hoc comparisons were done using the Tukey's HSD test. A statistical probability of $P < 0.05$ was considered significant.

3. Results

3.1. Microbial biomass

The management practices significantly affected the amount of microbial biomass (C_{MIC} and N_{MIC}) in the grassland soils (Table 1). The C_{MIC} in the soil of the mowed plot was significantly higher in comparison to both the untreated and mulched plots (Tukey's HSD test, $P < 0.0001$), while that in the untreated plot was significantly greater than the C_{MIC} found under mulching treatment (Tukey's HSD test, $P < 0.05$). The N_{MIC} of the mowed plot was also significantly higher than in the mulched and untreated plots (Tukey's HSD test, $P < 0.0001$).

C_{MIC} and N_{MIC} were also significantly affected by sampling time. Sampling time explained a considerable part of the variability for the N_{MIC} data (Table 1). Temporal changes in C_{MIC} were affected by management practice (Fig. 1a). C_{MIC} fluctuated the least in the untreated plot (Fig. 1a) during the 1999–2001 sampling period (coefficient of variation 5%), whereas seasonal changes of C_{MIC} in the mowed and mulched plots were more pronounced (coefficients of variation 9 and 15%, respectively). C_{MIC} in both managed plots followed a very similar trend, but overall C_{MIC} in the mulched plot was significantly lower than that in the mowed plot (Fig. 1a). The temporal fluctuation of N_{MIC} followed a similar pattern in all plots (Fig. 1b).

Table 1
The effect of grassland management (M) and sampling time (T) on soil microbial characteristics — results of statistical analyses (Two-way ANOVA)

Characteristic	Variable	df	*F*	*P*
Microbial biomass carbon	Management	2	47.364	<0.001
	Time	7	9.486	<0.001
	M × T	14	1.743	0.059
Microbial biomass nitrogen	Management	2	47.178	<0.001
	Time	7	46.503	<0.001
	M × T	14	4.276	<0.001
Basal respiration rate	Management	2	1.655	0.196
	Time	7	49.099	<0.001
	M × T	14	0.865	0.599
Cellulose decomposition rate	Management	2	23.788	<0.001
	Time	7	17.211	<0.001
	M × T	14	1.202	0.287
Cellulose mineralization rate	Management	2	34.110	<0.001
	Time	7	25.442	<0.001
	M × T	14	1.944	0.031
Mineralization/decomposition ratio	Management	2	4.499	0.009
	Time	7	6.076	<0.001
	M × T	14	0.404	0.971
Phosphatase activity	Management	2	39.729	<0.001
Active biomass	Management	2	9.204	0.007
	Treatment	3	0.810	0.520
	M × Tr	6	0.795	0.596
Maximum respiration rate	Management	2	12.949	0.002
	Treatment	3	12.832	0.001
	M × Tr	6	5.800	0.010
Relative respiration response to nutrient addition	Management	2	73.105	<0.001
	Treatment	3	14.478	<0.001
	M × Tr	6	7.635	0.004

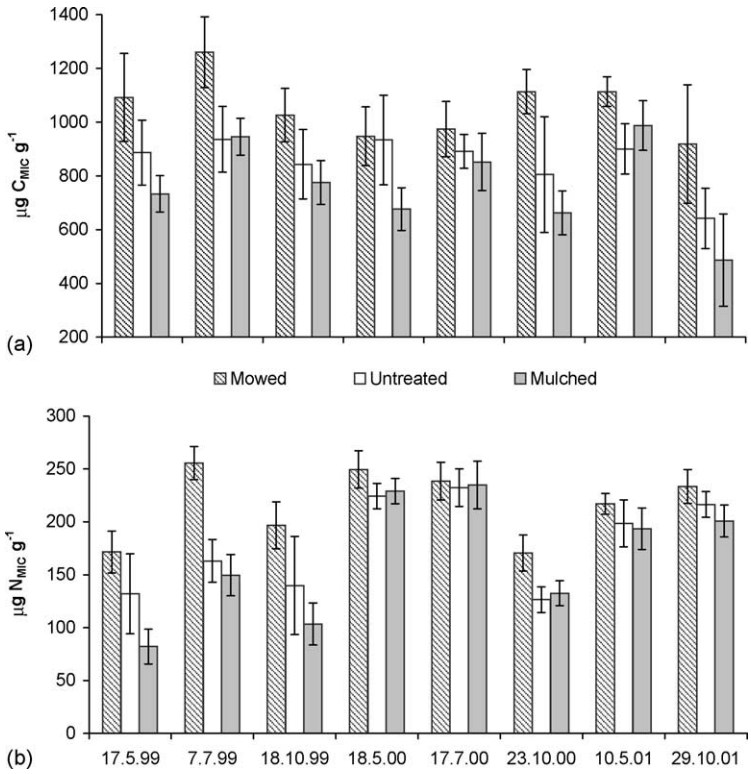

Fig. 1. Microbial biomass carbon (a) and nitrogen (b) in the mowed, untreated, and mulched plots in 1999–2001. Means and standard deviations (n = 5) are given.

3.2. Soil respiration, cellulose decomposition and mineralization

Grassland management practices had no effect on the basal soil respiration rate, while sampling time explained a considerable part of the data variability (Table 1). Temporal fluctuations in the basal respiration rates followed the same pattern for all treatments, with higher respiration rates in May 1999, July 2000, and October 2001 (Fig. 2). Cellulose decomposition and especially mineralization rates showed a high temporal variability; however, the effect of the management type was significant (Table 1). Cellulose decomposition and mineralization rates were significantly higher in the mulched and untreated plots in comparison to the mowed plot (Table 2). This was found in all sampling dates except October 1999, when soil activities were very similar in all three plots. The ratio of mineralized to decomposed cellulose varied in time as well as between particular laboratory replicates (Table 2). Despite the high variability, the values were significantly lower in the mowed plot soil than in the soil of the untreated plot (Table 2), which suggests more effective carbon use at the mowed plot. Values higher than 100% indicate that native carbon in addition to that from the added cellulose was mineralized during the incubation period. This was the case in October 1999 at the untreated and mulched plot, in May 2000 at the untreated plot, and in July 2000 at all plots (Table 2).

3.3. Phosphatase activity

Management practice significantly affected the activity of phosphatase enzymes (biochemical phosphorus mineralization) in the grassland soils (Table 1). The phosphatase activity increased significantly as follows (mean ± standard deviation, n = 5): mowed (3.66 ± 1.38 μmol MUF-P l^{-1} h^{-1}), untreated (9.54 ± 1.12 μmol MUF-P l^{-1} h^{-1}), and mulched (12.77 ±

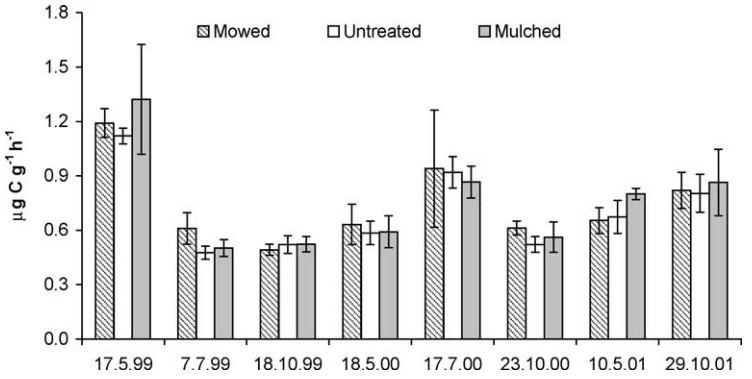

Fig. 2. Basal respiration rates in the mowed, untreated, and mulched plots in 1999–2001. Means and standard deviations ($n = 5$) are given.

1.28 μmol MUF-P l^{-1} h^{-1}) (Tukey's HSD test, $P < 0.01$).

3.4. Microbial nutrient status

Basal respiration rates (r_B) were significantly higher (Tukey's HSD test, $P < 0.05$) at the mowed (0.57 ± 0.02 μl O_2 g^{-1} h^{-1}; mean ± standard deviation, $n = 5$) and mulched (0.54 ± 0.02 μl O_2 g^{-1} h^{-1}) plots than at the untreated plot (0.39 ± 0.05 μl O_2 g^{-1} h^{-1}). We found no differences in active biomass either between soils or between treatments (Table 3) but there were significant differences in r_{MAX} and relative respiration response to nutrient addition among all three plots (Tukey's HSD test, $P < 0.05$). Carbon and nutrient additions led to increased respiration in all of the soils. In general, the response was lowest in the mowed plot soil and highest in the untreated plot (Table 3). In the mowed plot, glucose addition increased the respiration rate 11 times in comparison to the control while nitrogen addition caused another significant increase in respiration. In the mulched plot, glucose addition increased the respiration rate 18 times in comparison to the control but there was no other increase in respiration after nutrient addition. The respiration rate increased 33 times when glucose was added to the untreated soil in comparison to the control. Addition of nutrients in combination (CNP treatment) caused another significant increase in respiration in this plot. Phosphorus addition into the untreated soil significantly lowered the r_{MAX} and relative respiration response (Table 3).

4. Discussion

Soil microbial biomass and microbial transformation of SOM were significantly different between mowed, mulched and untreated grasslands. The differences in soil microbial characteristics appeared over five years, when the treatments were applied to the mountain grassland. Soil microbial biomass was a sensitive early predictor of changes in total SOM caused by management regime as found by Powlson et al. (1987) and Rice et al. (1996). Also some specific microbial activities, such as relative response to substrate addition, carbon use efficiency, and phosphatase activity, indicate changes in SOM transformation under different grassland managements, while basal soil respiration appeared to be an insensitive microbial characteristic.

The highest microbial biomass was found in the soil of the mowed grassland, which agrees with the results of many previous studies (e.g. Tesařová, 1993; Rice et al., 1996; Bardgett et al., 1998). The positive effect of mowing on microbial biomass is often explained as due to increased root exudation after plant defoliation (Holland, 1996; Hamilton and Frank, 2001; Kuzyakov et al., 2002). Increased exudation, resulting in larger supplies of an easily available carbon and energy source, supports the rhizosphere microbial community (Kuzyakov and Domanski, 2000a,b; Hütsch et al., 2002) and enhances microbial biomass and nutrient cycling in the rhizosphere (McNaughton et al., 1997; Bardgett et al., 1998). Although rhizodeposition was not measured directly, the importance of exudates to increasing microbial

Table 2
Cellulose decomposition and mineralization rates and the ratio of mineralized to decomposed cellulose in the soils of mowed (C), untreated (O), and mulched (M) grasslands in sampling period 1999–2001

Parameter	Plot	1999			2000			2001		
		17 May	7 July	18 October	18 May	17 July	23 October	10 May	29 October	Average
Cellulose decomposition (μg C g^{-1} h^{-1})	C	0.40 ± 0.15	0.20 ± 0.09	0.10 ± 0.03	0.23 ± 0.08	0.16 ± 0.12	0.20 ± 0.08	0.33 ± 0.15	0.20 ± 0.11	0.23 ± 0.14^{a}
	O	0.64 ± 0.20	0.36 ± 0.07	0.06 ± 0.04	0.43 ± 0.17	0.36 ± 0.18	0.37 ± 0.12	0.57 ± 0.14	0.39 ± 0.09	0.40 ± 0.21^{b}
	M	0.57 ± 0.08	0.36 ± 0.09	0.04 ± 0.01	0.50 ± 0.04	0.40 ± 0.15	0.49 ± 0.18	0.52 ± 0.08	0.35 ± 0.06	0.40 ± 0.19^{b}
Cellulose mineralization (μg C g^{-1} h^{-1})	C	0.11 ± 0.12	0.03 ± 0.03	0.05 ± 0.05	0.18 ± 0.09	0.17 ± 0.13	0.02 ± 0.04	0.22 ± 0.12	0.11 ± 0.09	0.11 ± 0.11^{a}
	O	0.28 ± 0.17	0.14 ± 0.02	0.05 ± 0.03	0.36 ± 0.06	0.41 ± 0.08	0.24 ± 0.10	0.48 ± 0.10	0.29 ± 0.10	0.28 ± 0.16^{b}
	M	0.15 ± 0.12	0.09 ± 0.03	0.05 ± 0.03	0.41 ± 0.06	0.43 ± 0.07	0.12 ± 0.09	0.46 ± 0.02	0.21 ± 0.07	0.24 ± 0.17^{b}
Mineralization/decomposition (%)	C	24 ± 19	24 ± 7	62 ± 72	80 ± 24	117 ± 67	12 ± 22	60 ± 15	45 ± 24	50 ± 50^{a}
	O	55 ± 43	40 ± 8	134 ± 99	105 ± 65	186 ± 172	66 ± 29	86 ± 19	72 ± 17	93 ± 89^{b}
	M	25 ± 17	26 ± 7	136 ± 95	83 ± 24	124 ± 47	21 ± 10	90 ± 13	60 ± 13	71 ± 58^{ab}

Means and standard deviations ($n = 5$) are given. Different letters show significant differences in average values between management practices ($P < 0.05$).

biomass in the mowed plot was supported indirectly by indices showing a higher amount of available carbon and in the different way of SOM transformation. There was a significantly higher amount of sulfate-extractable carbon in the mowed grassland soil (data not shown), which indicates a higher concentration of an easily oxidizable carbon in this soil. The significantly lower cellulose mineralization in the mowed plot, compared to the mulched and untreated plots suggests a sufficient supply of native, easily degradable carbon and thus a lower necessity of the soil microbiota to use the additional C-substrate (Tempest and Neijssel, 1978). Furthermore, the lower carbon mineralization to decomposition ratio found for the mowed plot (Table 2) could indicate the use of different carbon sources than those used at the mulched and untreated plots (soluble compounds and cellulose, respectively), resulting in higher carbon use efficiency in the mowed plot (Cambardella and Elliot, 1994). The higher carbon use efficiency under defoliation was referred also by Guitian and Bardgett (2000). The higher activity of phosphatase enzymes in the soils of untreated and mulched plots represented greater biochemical phosphorus mineralization in these soils, which was related to increased decomposition of insoluble carbon substrates, than at the mowed plot. Higher soil phosphatase activity is often suggested to indicate phosphorus limitation but we found no phosphorus limitation in the C, M, and O soils (Table 3). Microbial activity was primarily limited by carbon availability in all three soils. Carbon limitation increased in the order mowing $<$ mulching $<<$ no-treatment, based on the growing relative respiration response to glucose addition (Tempest and Neijssel, 1978). Nutrient addition had a significant effect only in the mowed and untreated plots. Soil microbial processes at the mowed plot were apparently limited by nitrogen availability and a combination of nitrogen and phosphorus even promoted soil respiration. Similarly, nitrogen in combination with phosphorus, promoted significantly microbial activity in the soil of the untreated plot. It seems that mulch decomposition can either enhance mineral nutrient turnover (Facelli and Picket, 1991; Kvítek et al., 1998) or diminish competition for nutrients between soil microorganisms and plants by decreasing the ability of plants to grow under a mulch layer.

Table 3
Carbon and nutrient (nitrogen, phosphorus) limitation of microbial growth and activity in the soils of mowed (C), untreated (O), and mulched (M) grasslands, sampled in October 2002

Plot	Treatment	Active biomass ($\mu l\ O_2\ g^{-1}\ h^{-1}$)	r_{MAX} ($\mu l\ O_2\ g^{-1}\ h^{-1}$)	$(r_{MAX} - r_B)/r_B$
C	C	5.74 ± 0.37^{a}	7.03 ± 0.74^{a}	11.34 ± 1.30^{a}
	CN	5.55 ± 0.19^{a}	9.93 ± 0.07^{b}	16.42 ± 0.12^{b}
	CP	5.55 ± 0.56^{a}	7.04 ± 0.74^{a}	11.35 ± 1.29^{a}
	CNP	6.11 ± 0.42^{a}	11.48 ± 0.10^{c}	19.13 ± 0.40^{c}
O	C	5.55 ± 0.19^{ab}	13.33 ± 0.75^{b}	33.40 ± 1.69^{b}
	CN	5.55 ± 0.50^{ab}	11.48 ± 0.74^{b}	28.42 ± 1.89^{b}
	CP	4.60 ± 0.50^{a}	7.40 ± 0.05^{a}	17.97 ± 0.56^{a}
	CNP	6.29 ± 0.56^{ab}	16.29 ± 0.99^{c}	40.77 ± 2.54^{c}
M	C	6.85 ± 0.37^{a}	10.36 ± 0.38^{a}	18.19 ± 0.69^{a}
	CN	6.85 ± 0.50^{a}	10.74 ± 0.30^{a}	18.89 ± 0.41^{a}
	CP	7.03 ± 0.56^{a}	11.11 ± 1.11^{a}	19.58 ± 2.06^{a}
	CNP	6.66 ± 0.19^{a}	11.11 ± 1.12^{a}	19.57 ± 2.07^{a}

Means and standard deviations ($n = 3$) are given. Different letters (a, b, c) indicate significant differences between treatments within a soil ($P < 0.05$).

There was a significant difference in soil microbial biomass and activity between the two defoliated plots. We found no positive effect of plant defoliation on soil microbial biomass in the mulched plot, where the biomass was significantly lower than in the mowed plot. Soil microbial characteristics of the mulched plot were very similar to those of the untreated plot. A significantly higher respiration response to glucose addition, a marked cellulose mineralization and a lower carbon use efficiency indicated lower carbon availability at the mulched plot in comparison to the mowed plot. Accumulated plant litter, the mulch layer, alters some soil physical and chemical characteristics (see Facelli and Picket, 1991), which are important in regulating rhizodeposition (Hütsch et al., 2002). We consider that especially soil temperature, which was 1–2 °C lower in the mulched than the mowed plot during the vegetation seasons of 1999–2001 (Kvítek et al., 2000), and light interception by the mulch layer, which suppressed plant photosynthesis (Facelli and Picket, 1991; Ammerlaan and de Visser, 1993), decreased root exudation in the mulched plot. Other important factors affecting rhizodeposition, like soil moisture, soil pH (Hütsch et al., 2002), root biomass (Tesařová, 1993) or plant community composition (Guitian and Bardgett, 2000; Van der Krift et al., 2001), were unaffected by grassland management in experimental period 1999–2001 (unpublished data). Other unrecognized factors connected to the presence of the mulch layer (oxygen availability, release of phytotoxic substances from mulch) may also play an important role in rhizodeposition control and regulation of soil microbial activity.

In conclusion, mowing appeared to be the most suitable grassland management practice. It increases soil microbial biomass, which represents a labile carbon pool, a rapid nutrient source and sink, and provides SOM transformation. Enhanced carbon use efficiency in microbially mediated SOM transformation processes considerably adds to SOM maintenance and the sustainability of grassland ecosystems.

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PAPER 7

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Short term effects of experimental eutrophication on carbon and nitrogen cycling in two types of wet grassland

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Abstract: Plant biomass production, soil chemical and microbial parameters, microbial processes of C and N cycle and gases emissions were studied in soils at two types of grasslands (wet meadows). Both sites are situated in the Czech Republic: (1) a nutrient poor sedge meadow on organic soil (Z) and (2) a mesotrophic sedge-sweet grass meadow on mineral soil (H). Eutrophication was simulated by the application of NPK fertilizer to selected permanent plots in 2006 and 2007 in amounts of 9 kg N + 4 kg P ha^{-1} $year^{-1}$ (low dose) and 45 kg N + 20 kg P ha^{-1} $year^{-1}$ (high dose). After two years of fertilizer application, we observed an increase in net aboveground plant production (about 9-12 kg ha^{-1} $year^{-1}$) connected with an increase in shoot:root ratio in fertilized plots of both sites, with more pronounced changes in oligotrophic sedge meadow. Total CO_2 efflux from the ecosystem measured in situ was significantly higher at fertilized plots as well as increase in total soil respiration in case of sedge meadow, but we found no significant effect of fertilization on CO_2 efflux from the system at mesotrophic site. Surprisingly, other parameters, like soil microbial biomass C and N content, the rates of respiration, denitrification, nitrification, nitrogen mineralization and nitrogen assimilation were not affected by fertilization. In conclusion, an interesting finding is that despite non significant impact on aboveground component there were significant responses in belowground part which suggest that belowground processes may be suitable early warning signals. Peaty oligotrophic soil seems to be more sensitive to nutrient addition than mineral soil. However, final effect of fertilization on ecosystem C balance stays unknown and longer study is necessary to draw explicit conclusion.

1. Introduction

Increased nutrient inputs as a result of management practices often lead to eutrophication of wetland ecosystems. Eutrophication caused by increased nutrient inputs may change plant species composition and richness (Detenbeck et al. 1999, Bollens et al. 2001, Brinson and Malvarez 2002, Drexler and Bedford 2002), usually as the result of differential effects on plant functional groups (McJannet et al. 1995, Pauli et al. 2002). Greater nutrient input levels favor aboveground plant production over root growth (Detenbeck et al. 1999) and increases plant tissue nutrient contents. The subsequently decreased C:N ratio in plant tissues results in more rapid decomposition rates of the plant litter and a consequent faster cycling of C and N in the wetland as a whole (van Vuuren et al. 1993, van Oorschot 1994, Robarts and Waiser 1998). Greater nutrient availability is negatively related to the occurrence of plant-mycorrhizae associations (Johnson et al. 2003).

While the effects of eutrophication on plant communities are relatively predictable, there is greater uncertainty in regards to the responses of soil organisms and processes to increased nutrient levels. Microbial biomass may increase (Scheu and Schaefer 1998), decrease (Smolander et al. 1994) or have a variable response to increased N loadings (Ettema et al. 1999). Increased N levels may favor fungi at the expense of bacteria, but opposite results are reported too (Smolander et al. 1994, Ettema et al. 1999). Greenhouse gas emissions may also be affected by eutrophying conditions (Borken et al. 2002, de Vries et al. 2003). Methane emissions were significantly higher in eutrophic vs non-eutrophic soils (Giani and Ahrensfeld 2002, Šantrůčková et al. 2001), indicating a positive relationship between methanogenesis rates and fertilization (Čížková et al. 2001, Sorrell et al. 1997).

Nutrient mineralization rates should increase with eutrophication. Mineralization of N and P will become the dominant soil processes when C:N < 20 and C:P < 200 (van Oorschot 1994). N mineralization rates were positively related to N levels in minerotrophic fens (Verhoeven and Arts 1987) and riparian areas with mineral soils (van Oorschot 1994, Ettema et al. 1999), but were negatively affected by increased availability of P (van Oorschot 1994). Organic matter (OM) mineralization rates increased in eutrophic soils, leading to the release of N and P and their greater availability for plant growth. Thus, input of eutrophic waters from external sources may increase or accelerate internal eutrophication of an ecosystem (Koerselman et al. 1993).

Soil type can affect how soil processes are changed by eutrophication, but there is still great uncertainty in how transformation processes are affected by increased nutrient levels in wetlands with different soil types (de Vries et al.

2003). In general, retention of N, P and K is greater in peaty soils (Verhoeven 1986), although mineral soils can sometimes act as sinks for P (Masscheleyn et al. 1992). NH_4^+ and NO_3^- are retained to a much greater extent in peaty than mineral soils (Fenn et al. 1998). The amount of nutrients sequestered in soils is affected by OM (organic matter) decomposition rates. Eutrophication increases OM mineralization, thereby decreasing nutrient retention and increasing nutrient availability (van Oorschot 1994). This effect may be greater in peaty than mineral soils. Increased N levels should stimulate mineralization of soil organic matter (OM) in peaty soils (Verhoeven and Arts 1987, Berendse 1990, van Oorschot 1994, Debusk and Reddy 1998), but lead to greater accumulation in mineral soils (Smolander et al. 1994). However, Aerts and Toet (1997) found decreased OM decomposition in experimental cores of peaty soils enriched with NH_4^+ only.

Understanding ecosystem functioning requires consideration of both above and belowground components, as well as their interactions (Wardle 2002). Soil microbes make nutrients available for plant uptake while plants affect soil properties through litter nutrient content and decomposition rates, among other processes (Hobbie 1992, van den Putten 1997). Although interest in plant-soil interactions has increased, with more and more studies including both above and belowground perspectives (Wardle 2002), there is still much uncertainty concerning how these interactions are affected under differing environmental conditions.

The purpose of the study was to determine the effect of eutrophication on ecosystem functional characteristics in wet meadows with either mineral (siltsand alluvial) or peaty soils.

Our hypothesis was that increased nutrient loading applied within a relatively short time period (two years) will significantly affect the processes responsible for C and N fluxes to and from the wetland, and these effects will be stronger on peaty than mineral soil. The aims of the study were to (i) quantify C and N fluxes in soils receiving different levels of nutrient inputs, (ii) show how soil microbial processes (like respiration, denitrification, nitrification, nitrogen mineralization and nitrogen assimilation) are affected by receiving different levels of nutrient inputs in two different wet meadow soils.

2. Methods

2.1. Study site

Two wet meadows in the Třeboň Basin Biosphere Reserve (TBBR), South Bohemia, Czech Republic, were chosen as the ecosystem in which to study the effect of eutrophication on plant-soil interactions (Figure 1). These two sites are representative of two main types of temperate wet meadows. The first, Záblatské Louky (Z), is on peaty soils while the second is on a silt-sand alluvial substrate and is located near the village of Hamr (H). The altitude is 426 m above sea level for the Z site and 415 m above sea level for the H site. Carbon accumulation is typical of the poorly flushed marginal littoral zones of shallow reservoirs such as fishponds in the TBBR. In floodplains, wetlands on mineral soil tend to predominate on frequently flushed sites. The Záblatské Louky site is a marginal wetland (Prach 2002), located in the inundation area of a large fishpond. As the water level in the fishpond is kept within a narrow range throughout the year, also the water level is usually fairly stable in the adjacent Záblatské Louky wetland (except for long periods of extreme summer drought and several weeks-long periods of fishpond drawdown in autumn). The site is occasionally subject to relatively long-term (for several weeks) shallow flooding when the water level is elevated in the adjacent Záblatský fishpond. The Hamr site is located in the floodplain of a small

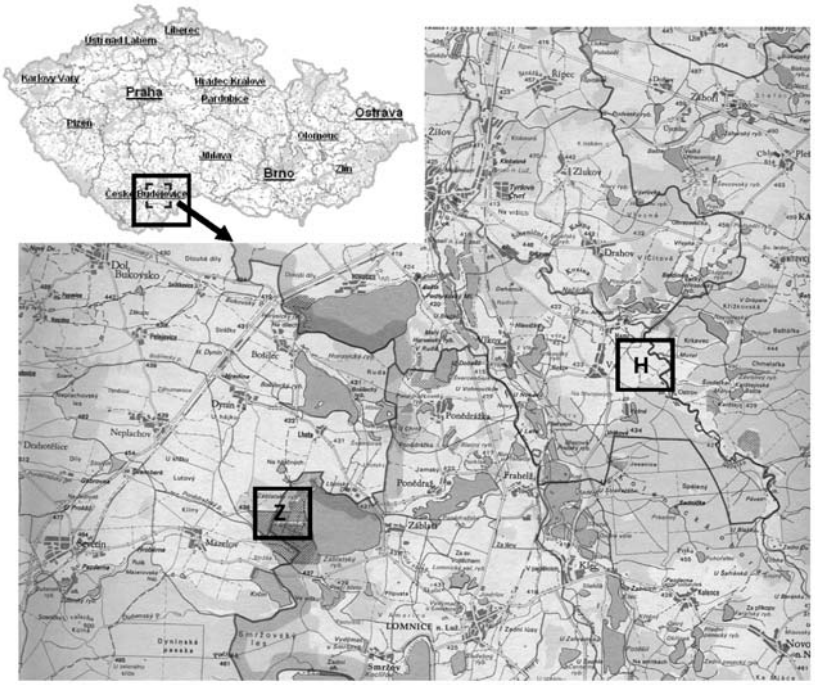

Figure 1. Map of the Třeboň Basin Biosphere Reserve (TBBR) showing the study site locations. Z = Záblatské Louky – peaty soil site; H = Hamr – mineral soil site.

Table 1. Physical and chemical parameters of soils at Hamr (H) and Záblatské Louky (Z) study sites (means ± standard deviations).

Site	H	Z
bulk density [g.cm^{-3}]	0.52 ± 0.04	0.21 ± 0.02
clay [%]	12.5	22.5
sand [%]	15.0	0.0
total C [%]	9.63 ± 1.65	22.33 ± 2.25
total N [%]	0.64 ± 0.1	1.18 ± 0.09
total P [%]	0.18 ± 0.02	0.19± 0.01
C to N ratio	15.0	18.9
pH_{H20}	4.9	5.1
pH_{KCl}	3.8	4.3
NO_3-N (0.5 M K_2SO_4) [g.kg^{-1}]	1.57 ± 0.63	2.28 ± 1.05
NH_4-N (0.5 M K_2SO_4) [g.kg^{-1}]	2.74 ± 0.79	2.72 ± 1.79
PO_4-P (oxalate) [g.kg^{-1}]	0.60 ± 0.12	1.12 ± 0.12
K [g.kg^{-1}]	12.16 ± 0.85	4.36 ± 0.29
Ca [g.kg^{-1}]	0.63 ± 0.10	1.37 ± 0.35
Mg [g.kg^{-1}]	2.23 ± 0.14	1.82 ± 0.09
Fe [g.kg^{-1}]	25.83 ± 1.11	18.80 ± 0.93

river (Nežárka) and the water level is the same as in local drainage ditches connected with the river. Hence, the water level is more variable here than in Záblatské Louky. However, the average water level is lower in the floodplain site (H) than in the marginal fishpond littoral (Z).

Phytosociological relevés conducted in 2007 showed that the Z site is a sedge meadow dominated by *Carex vesicaria* and *C. acuta* (the species having values of 2 and 3, respectively, on the Braun-Blanquet scale). Meanwhile, *Glyceria maxima* and *C. acuta* are dominant in the H site (both species, Braun-Blanquet scale = 3). Both soils in the two study sites are classified as silty-clays; the other physical and chemical parameters are shown in Table 1.

2.2. Experimental design

A complete randomized block design was used in the study. Four blocks were established in each meadow in May 2006, with three treatment plots per block. Each plot was 12.25 m^2 in area (3.5*3.5 m) and subjected to one of three fertilization treatments with a commercial NPK fertilizer (Lovofert 15:15:15 NPK, Lovochemie, a.s.): 1) no fertilizer addition (No), 2) 65 kg NPK * ha^{-1} * yr^{-1} (Low) and 3) 300 kg NPK * ha^{-1} * yr^{-1} (High). The low treatment simulates current management treatments for Czech farms while the high treatment is the dose recommended by agricultural firms for grasslands of this type (European Environmental Agency, 2003). The fertilizer was added in two half doses during the 2007 growing season so to simulate normal agricultural practices; the first application was in mid-May and the second in mid-July. The plots in both sites were mown only in early June, at a similar time as that done by the farmers in the neighboring fields.

2.3. Environmental parameters

Water level was measured using the STELA system, which consists of a field datalogger M4516 with GSM/GPRS modem MG40 and water level sensor (Fiedler, Electronics for Ecology, Czech Republic). Software Most 2.3 was used for data management. The data on air and soil temperatures, rainfall and solar radiation input were recorded at the meteorological station in the Mokré Louky wet grassland 3 km far from Třeboň, which is a similar wetland with similar climate like studied sites.

2.4. Plant biomass and production measurements

Aboveground plant biomass was collected from a randomly-selected quadrat using the harvest method within each plot six times during the growing season (May to September 2007). One quadrat, size 0.4 x 0.4 m, was selected for each treatment plot in the Z site on each sampling date, while, due to greater site heterogeneity, two quadrats (0.25 x 0.25 m each) were harvested in each treatment plot in the Hamr site. The number of quadrats selected in each site on each sampling date was dictated by the size of the treatment plots (12.25 m^2) and the desire to sample a particular quadrat only once over the duration of the three year study. The harvested material was brought back to the University of South Bohemia in České Budějovice, Czech Republic where it was separated into species, dried at 70 ^{0} C for 48 hours and weighed. Standing dead biomass and litter were also collected at the same time as living material but were not separated by species. These were also dried and weighed in a similar manner as the living aboveground plant material. All dry weights were then estimated to a per meter square basis (g DW * m^{-2}). Mean net annual aboveground plant production (NAPP; g DW * m^{-2} * yr^{-1}) was calculated from the dried living, standing dead and litter material for each treatment type in each site. Production of live material was determined as the difference between the maximum DW minus initial mean live stem DW. Since the sites were mown once during the growing season (early June), live production was calculated as the difference between the mean live stem DW in the sampling period just before the site was mown minus the mean initial DW ($LIVE_{Pre\text{-}cut} - LIVE_{Initial}$). To this value was added the difference between the post-mowing sampling period with the maximum mean live DW minus the mean live DW immediately following the mowing. This latter value was assumed to be similar to the initial live DW value. Therefore, total mean live production (LIVEPROD) for each treatment per site was calculated as $LIVEPROD = [(LIVE_{Pre\text{-}cut} - LIVE_{Initial}) + (LIVE_{Post\text{-}cut} - LIVE_{Initial})]$. In addition, production of dead material was determined by adding the standing dead and litter DWs collected in each treatment plot and calculating the mean dead DW for each treatment per site. Differences in mean dead DW per treatment were calculated between subsequent sampling dates. Only differences resulting in positive numbers were included in estimating NAPP. NAPP for each treatment type per site was finally calculated by adding together the total live production (LIVEPROD)

with those positive differences in dead matter (NAPP = LIVEPROD + ” $DEAD_{Positive}$).

Net belowground primary production (NBPP) was measured in both sites in 2007 using the in-growth core bag method (Vogt et al. 1998). Two bags (7 cm diameter x 15 cm depth), and filled with soil from the respective sites, were placed into each treatment plot in both wetlands on 30 April 2007. Between-block differences were not significant, so the samples for each treatment could be combined. This resulted in there being a total of six bags per treatment per site for the analyses. They were left in place until collected on 21 September 2007. Keeping the core bags in the soils over most of the growing season allowed for adequate root growth into the bags without significant root mortality. Once removed, the bags with soil and roots were taken to the laboratory where they were carefully cleaned and the roots washed, dried at 70 ^{o} C for 72 hours and weighed.

2.5. Soil sampling and analysis

Soils from all treatment plots were sampled in May, August and October 2007. One mixed soil sample was prepared per each treatment plot from 10 randomly sampled soil cores. Soil samples were sieved through a 5-mm mesh and stored at 4 °C until analyzed. Soils were characterized for organic C and total N contents (NC Elementar analyzer, ThermoQuest, Italy) and total phosphorus by the perchlorate mineralization method. Soil microbial biomass C and N were measured using the chloroform fumigation-extraction method (Vance et al. 1987) and calculated as the difference between C and N contents in fumigated and non-fumigated samples. Coefficients of 0.38 (Vance et al. 1987) and 0.54 (Brookes et al. 1985) were used to correct the results for soil microbial biomass C and N, respectively. Concentrations of C and N in liquid samples were analyzed on a LiquiTOC II (Elementar, Germany). Soil respiration rate (soil CO_2 efflux) was measured in soil samples pre-incubated for five days at 20 °C and then air-tightly closed and incubated for 24 hours at the same conditions using gas chromatography. Concentrations of available NH_4-N and NO_3-N were measured in 0.5 M potassium sulfate extracts (FIA Lachat QC8500, Lachat Instruments, USA).

2.6. Microbial activities

The rates of net N-mineralization, nitrification and N-assimilation into microbial biomass were calculated from changes in concentrations in particular N pools (NH_4-N, NO_3-N, soil microbial N) after three weeks of aerobic incubation at 20 °C. The rate of denitrifying enzyme activity (DEA) was measured by the acetylene reduction method (Tiedje et al. 1989). All measurements were done in three laboratory replications per each soil sample.

The same procedures were used in laboratory experiments with the soils, to which NPK fertilizer was applied to assess its direct effect on soil microbial activity. NPK fertilizer was always added in a final concentration of 1 mg N g^{-1} dry soil, 0.44 mg P g^{-1} dry soil and 0.83 mg K g^{-1} dry soil, which slightly exceeds the highest dose of NPK applied in the field (300 kg NPK ha^{-1} $year^{-1}$).

2.7. Gas fluxes

Gas emissions were measured at both study sites in situ using the static chamber technique (Livingston and Hutchinson 1995). The chambers were made of plastic and each consisted of two parts. The bottom part was permanently inserted into the soil, while the upper part was like a pot, with a total volume of 30 L and diameter of 334 mm. It was placed on the bottom part of the chamber only in the time when emissions were measured and was sealed by a water-filled ring on the soil surface. Chambers for gas emission measurements were placed in each plot (12 chambers per site). The gases were sampled using a syringe through a septum in a chamber wall after 30 min incubation, then transferred into evacuated vacutainer tube and transported to the laboratory and analyzed using gas chromatographes. Gases were sampled seven times during the 2007 vegetation season: May 7, May 15, June 5, July 18, September 7, October 2, October 22.

CO_2 was determined using an HP 6850 gas chromatograph (Agilent, USA) equipped with a 0.53 mm x 15 m HP-Plot Q column and a 0.53 mm x 15 m HP-Plot Molecular Sieve 5A column, and a thermal conductivity detector, using helium as the carrier gas. CH_4 was determined using an HP 6890 gas chromatograph (Agilent, USA) equipped with a 0.53 mm x 15 m HP-5 column and a flame ionization detector, using nitrogen as the carrier gas.

Nitrous oxide was determined using an HP 6890 gas chromatograph (Agilent, USA) equipped with a 0.53 mm x 15 m HP-Plot Q column and an electron capture detector, using a mixture of 95 % argon and 5% CH_4 as the carrier gas. Data were analyzed by Agilent Chemstation A.08.03 software (Agilent, USA).

Soil respiration was measured *in situ* on May 18 and 21 and August 1 and 3. Two soil collars (10 cm diameter, 7 cm depth) were installed on each treatment plot on the day preceding the measurement. A LiCor 6400 equipped with a soil chamber was used to measure the soil respiration. Mean values of at least 3-5 subsequent measuring cycles were used.

2.8. Data analysis

Complete randomized block ANOVAs were used to test for differences in living aboveground DW among treatment types in each site within each sampling period. Tukey’s multiple comparison of means test was used if significant differences were found. Above to belowground production ratios (A:B) were determined using the NAPP and NBPP data for each treatment type from each site. Differences among treatments in each site were determined by oneway ANOVA. All statistical tests were conducted using SYSTAT, version 11 (SYSTAT, 2002). Soil data were evaluated using ANOVAs, while gas emissions were evaluated using repeated measures ANOVA. Statistical analyses for the soil and gas emission

data were conducted using Statistica 7.1 (StatSoft Inc., USA).

3. Results

3.1. Environmental parameters

Air temperature fluctuated from 1.8 to 27.3 ^{o}C and soil temperature from 6.2 to 23.2 ^{o}C during vegetation season 2007, precipitation was extremely low mainly in spring (April) (Fig. 2a). Water level was bellow soil surface except 2 month period (September and October) at Záblatí study site (Fig. 2b) and most of the vegetation season of 2007 was exceptionally dry, thus favouring aerobic processes in the wetland soils.

3.2. Plant production

Total live aboveground biomass did not differ significantly among the blocks or treatments for any of the sampling periods in Z (Fig. 3a). However, there was a trend of increasing biomass with greater fertilization. This trend was also apparent when comparing NAPP values among the treatments in this site (Table 2).

Usually, there were no significant differences among blocks or treatments within each sampling period in the H site (Fig. 3b). However, there was a slightly significant block effect in the June sampling period ($F_{3, 6} = 6.27$; $p = 0.03$), with there being greater DW values in block 4 compared to the other blocks. There was also a borderline non-significant treatment effect in the September sampling period ($F_{2, 6} = 3.88$; $p = 0.08$), showing the expected trend of increasing biomass with greater fertilization. Overall, this expected trend was less clear for the H site compared to Z. While DW and NAPP levels were highest for the high fertilizer treatment, the Low treatment had the lowest levels, even less than the plots receiving no nutrient additions (Table 2).

Belowground production (NBPP) decreased with increased fertilization in both sites. This was more apparent in Z than in H, where NBPP was greatest in the Low treatment, while it was similar between the No and High treatments (Table 2). Such decreasing allocation of biomass to below-

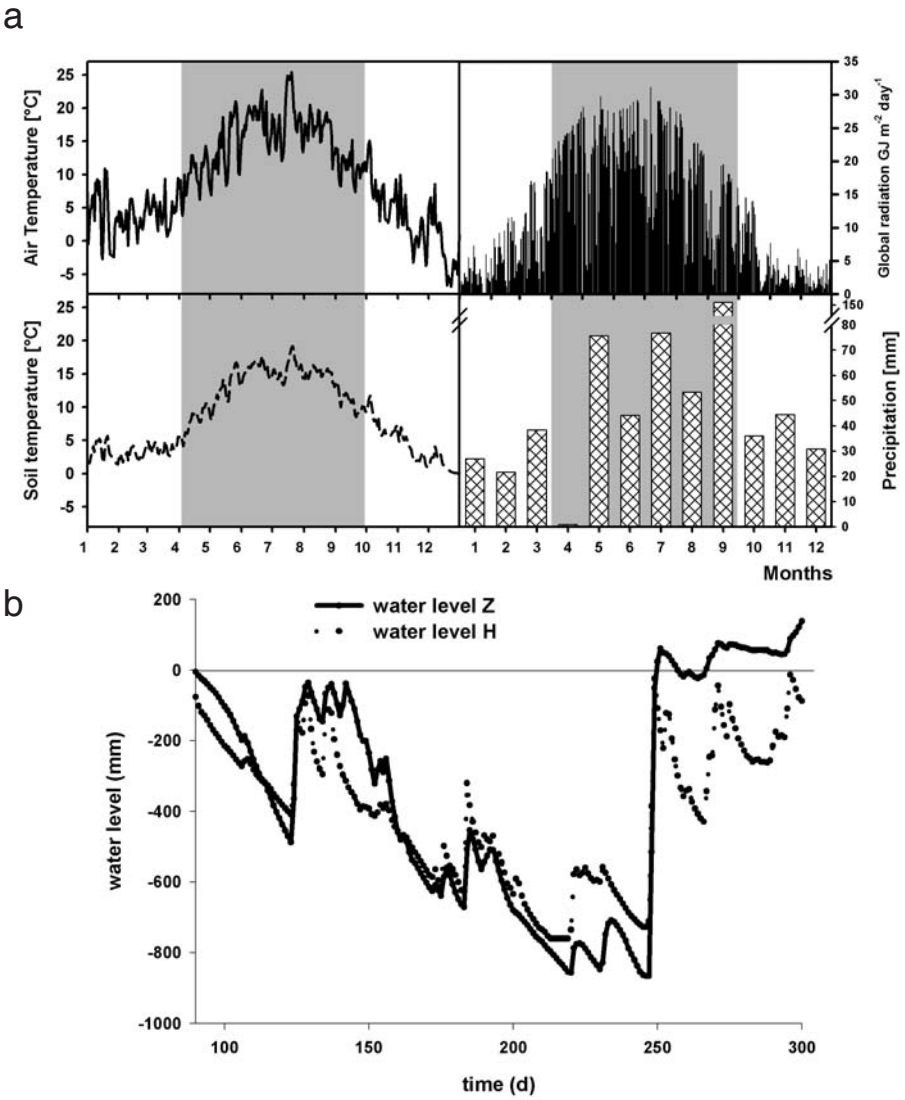

Figure 2. a. Air (2 m height) and soil (50 mm depth) temperatures, energy of global radiation and precipitation during the 2007. Data from wet meadow meteorological station (49°1′28.775”N; 14°46′13254”E), Třeboň, Czech Republic. Growth season marked by gray area. **b.** water levels at Záblatské Louky (Z) and Hamr (H) study sites during the 2007 vegetation season (April 1 to October 31, 0 mm means the soil surface).

Table 2. Above (NAPP; mean ± 1 SD) and belowground (NBPP) plant production (g DW * m^{-2} * yr^{-1}) for Hamr (H) and Záblatské Louky (Z) in 2007. Production allocation measured as the above-to-belowground production ratio (A:B). Fertilizer treatments: No = no nutrient addition; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}.

Site	NAPP			NBPP			A:B		
	No	Low	High	No	Low	High	No	Low	High
Z	735.66 ± 210.69	773.53 ± 103.29	817.41 ± 171.96	417.68 ± 110.31	392.15 ± 139.67	290.00 ± 164.39	1.76	1.97	2.82
H	1033.83 ± 266.70	886.26 ± 117.11	1202.22 ± 201.65	287.83 ± 88.23	327.22 ± 93.37	282.42 ± 147.05	3.59	2.71	4.26

Table 3. Concentration of available organic C and N, nitrate and ammonium nitrogen and total phosphorus content in the soil at Hamr (H) and Záblatské Louky (Z) in 2007. Fertilizer treatments: No = no nutrient addition; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}. (Mean± standard deviation. n = 4).

Site	Sol. organic C [µg g^{-1}]	Sol. organic N [µg g^{-1}]	$N\text{-}NO_3^-$ [µg g^{-1}]	$N\text{-}NH_4^+$ [µg g^{-1}]	P_{TOT} [mg g^{-1}]
Z No	335 ± 36.0	18.7 ± 6.4	19.2 ± 7.6	1.3 ± 1.3	1.89 ± 0.12
Z Low	369 ± 54.2	21.5 ± 9.1	14.8 ± 3.5	1.7 ± 1.3	2.00 ± 0.16
Z High	411 ± 65.5	19.3 ± 11.1	22.2 ± 9.7	1.1 ± 0.4	2.04 ± 0.11
H No	162 ± 21.8	18.7 ± 14.0	18.7 ± 6.7	1.5 ± 1.3	1.51 ± 0.24
H Low	161 ± 39.3	15.9 ± 9.3	14.1 ± 6.8	2.3 ± 2.0	1.70 ± 0.28
H High	179 ± 48.9	21.4 ± 15.8	20.4 ± 10.8	2.0 ± 1.6	1.64 ± 0.19

Table 4. Microbial parameters and rates of soil processes at Hamr (H) and Záblatské Louky (Z) in 2007. Fertilizer treatments: No = no nutrient addition; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}. (Mean± standard deviation. n = 4).

Site	Microb.biomass C [µg g^{-1}]	Microb. biomass N [µg g^{-1}]	Biomass C/N	Soil respiration [µg C g^{-1} h^{-1}]	Nitrification [µg g^{-1} d^{-1}]	N mineralization [µg g^{-1} d^{-1}]	N assimilation [µg g^{-1} d^{-1}]	Denitrification [µg g^{-1} h^{-1}]
Z No	2096 ± 402	171 ± 64.3	13.1 ± 2.33	1.93 ± 0.31	0.75 ± 0.45	-0.04 ± 0.12	-1.62 ± 2.92	722 ± 447
Z Low	2194 ± 486	190 ± 91.6	12.9 ± 3.14	2.02 ± 0.34	0.89 ± 0.68	-0.07 ± 0.12	-3.08 ± 4.90	1051 ± 839
Z High	1993 ± 354	193 ± 70.5	11.1 ± 2.69	1.89 ± 0.32	0.47 ± 1.08	-0.03 ± 0.03	-3.37 ± 2.76	999 ± 640
H No	1795 ± 277	174 ± 56.6	11.2 ± 3.06	1.20 ± 0.24	1.03 ± 0.47	0.00 ± 0.12	-2.55 ± 2.46	695 ± 207
H Low	1520 ± 265	157 ± 48.5	10.2 ± 2.42	1.10 ± 0.30	0.72 ± 0.22	-0.03 ± 0.07	-1.69 ± 3.34	565 ± 215
H High	1692 ± 232	177 ± 73.9	10.0 ± 3.56	1.26 ± 0.29	1.00 ± 0.68	-0.05 ± 0.11	-2.59 ± 3.63	726 ± 185

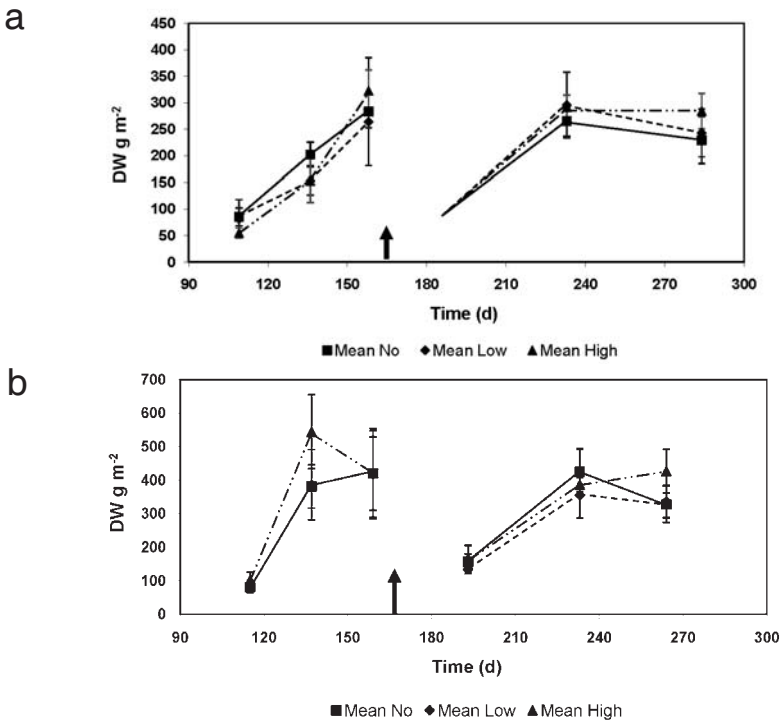

Figure 3. Aboveground plant biomass (mean ± 1 SD; g DW * m^{-2}) during the 2007 growing season for **a**) Záblatské Louky and **b**) Hamr. The arrows mark the time of mowing to simulate management practices of local farmers. Fertilizer treatments: No = no fertilizer added; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}. (Means with standard deviations as error bars, n = 4).

ground structures resulted in increased A:B ratios with greater fertilization, as expected (Table 2).

3.3. Microbial activities

Two years of NPK fertilization caused no significant changes in soil chemical and microbiological characteristics (Tables 3 and 4). In field samples, we found no increase in total C and N content, soluble pools of C and various N forms, soil pH. However, there was a tendency towards an increase in the soluble organic C pool in fertilized soils in Z. This effect of fertilization was repeatedly observed also in laboratory experiments, when NPK fertilizer was applied in higher doses directly to the Z and H soils without plants.

Table 5. Microbial parameters measured in laboratory experiment in Hamr (H) and Záblatské Louky (Z) soils. Fertilizer treatments: No = no nutrient addition; High = 300 kg NPK * g^{-1}. (Mean ± standard deviation. n = 4).

Site	Microb.biomass C [μg g^{-1}]	Microb. biomass N [μg g^{-1}]	Soil respiration [μg C g^{-1} h^{-1}]	Nitrification [μg g^{-1} d^{-1}]	N mineralization [μg g^{-1} d^{-1}]	N assimilation [μg g^{-1} d^{-1}]
Z No	3149 ± 94	213 ± 4.5	0.87 ± 0.02	1.98 ± 0.19	-0.07 ± 0.43	-4.56 ± 1.87
Z High	3208 ± 97	233 ± 23.2	0.68 ± 0.04	3.80 ± 0.17	1.24 ± 0.18	-6.28 ± 2.11
H No	1374 ± 56	112 ± 12.5	0.55 ± 0.10	1.57 ± 0.07	-0.06 ± 0.06	-0.80 ± 0.36
H High	1311 ± 44	116 ± 24.0	0.50 ± 0.08	2.78 ± 0.02	1.59 ± 0.09	-3.41 ± 0.86

Table 6. Soil respiration at Záblatské Louky (Z) and Hamr (H) sites in 2007. Fertilizer treatments: No = no nutrient addition; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}. (Mean ± standard deviation. n = 4).

Site	Soil respiration. May 2007 [mmol C-CO_2 m^{-2} h^{-1}]	Soil respiration. August 2007 [mmol C-CO_2 m^{-2} h^{-1}]
Z No	9.51 ± 1.71	40.06 ± 4.41
Z Low	10.19 ± 1.78	39.23 ± 5.24
Z High	12.26 ± 3.45	41.15 ± 15.52
H No	6.80 ± 1.56	26.58 ± 4.23
H Low	5.89 ± 0.74	23.35 ± 5.96
H High	6.88 ± 1.56	30.87 ± 9.05

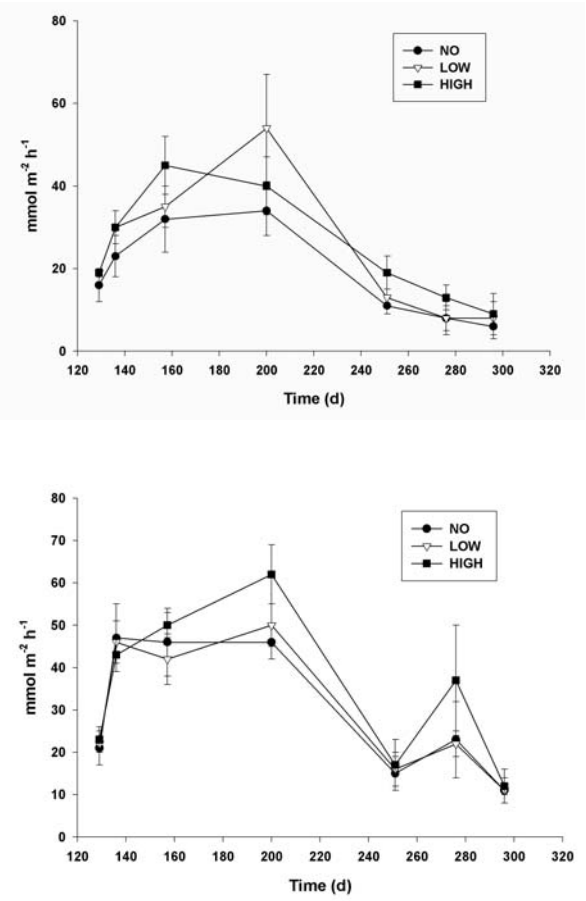

Figure 4. Total CO_2 emissions (means ± 1 SD) for a) Záblatské Louky and b) Hamr. Fertilizer treatments: No = no fertilizer added; Low = 65 kg NPK * ha^{-1} * yr^{-1}; High = 300 kg NPK * ha^{-1} * yr^{-1}. (Means with standard deviations as error bars, n = 4).

Soil microbial characteristics, represented by microbial biomass and its activity measured in field samples, were also unaffected by NPK fertilization. Only the microbial biomass C/N ratio decreased in fertilized treatments; however, this change was also not significant. On the other hand, direct application of NPK fertilizer to the soils in the laboratory experiments caused several significant changes in some of the measured soil microbial characteristics (Table 5). While soil respiration (C mineralization) decreased significantly in fertilized treatments, there were indications of increased microbial N transformations, represented by an increase in N mineralization and nitrification, and a decrease in microbial N assimilation. Soil microbial biomass was not changed.

3.4. Gas emissions

Total CO_2 emissions were significantly higher in the fertilized treatments as compared to the No treatment in Z ($df=2$; $F=7{,}43$; $p<0{,}05$) (Fig. 4a), whereas no significant difference was found at H (Fig. 4b). Also soil respiration (microbial and roots respiration) was significantly higher in the fertilized treatments as compared to the No treatment in the Z site, while no significant differences were found in H (Table 6).

Total CO_2 emissions were higher at H than at Z ($df=1$; $F=32{,}57$; $p<0{,}001$), fluctuating from 6 to 54 mmol m^{-2} h^{-1} at Z (average 19 mmol m^{-2} h^{-1}) and from 11 to 62 mmol m^{-2} h^{-1} at H (average 30 mmol m^{-2} h^{-1}), respectively. The highest CO_2 emissions were measured on 18 July at both sites and the lowest values at the end of the vegetation season (October 22).

Methane and nitrous oxide emissions were under detection limits during the whole vegetation season.

4. Discussion

In our sites, aboveground biomass and production (NAPP) increased with greater levels of nutrient additions, as was expected, while belowground production (NBPP) mostly decreased. These differences in NAPP and NBPP with fertilization resulted in significantly increasing A:B ratios with fertilization, especially in the peaty Z site. This result indicates that fertilization effects can occur within two

years after the start of nutrient additions in peaty soils. The situation appears to be more complicated in the mineral H site.

Standing crop, aboveground production (NAPP) and A:B ratio measured in our study were similar to levels found in other wet meadow systems. Maximum biomass ranged from 600-2600 g DW * m^{-2} for *G. maxima* and 640 g DW * m^{-2} for *Carex* species in other fishpond littoral areas in the TBBR (Pokorný et al. 2002). A:B ratios for these species ranged from 2.3-4.5 from various areas (see Table 2.1, Květ and Westlake 1998).

As expected, two years of nutrient loading was too short time to affect soil parameters like amounts of soil organic C and total soil N. Total soil organic matter content is a robust parameter, which remain unchanged even after long-term management (Hassink and Neeteson 1991, Bending et al. 2000, Emmett 2007). An early indication of changes in soil system due to fertilization can be provided by shifts in small labile soil pools and microbial characteristics changes (Powlson et al. 1987, Bending et al. 2000). However, we found no changes in concentrations of available N-NH_4^+ and N-NO_3^- in the soil after nutrient addition, although nitrates leaching is often referred as a sign of ecosystem eutrophication (Emmett 2007). Neither the processes of microbial N transformation were significantly changed. Due to the non-significant direct effect of fertilization on soil N cycle, we suppose prevailing immobilization of added nutrients in plant biomass (Bardgett et al. 2003, Dunn et al. 2006) and an important role of indirect effects on soil processes mediated by plants in longer-term, when eutrophication of the system will continue. In spite of the absence of clear and significant effect of eutrophication on soil microbial community and its activity, we observed a few changes that could indicate starting changes in the soil system. It was the decreasing tendency in C/N ratio of microbial biomass in eutrophied soils, which can be connected with a shift in composition of microbial community towards bacteria (de Vries et al. 2006, 2007). We further found a slight increase in the amount of soluble carbon, namely in the peaty soil, which was not connected with higher biodegradability of this C pool. This extra C could originate from condensation reaction of added nitrogen and products of OM decomposition, which are unavailable for microbial decomposition (Fog 1988). This phenomenon was found also in laboratory experiments in which N, P and K were added to the soil and direct effect of eutrophication on soil system was studied – the effect was even more pronounced. In such conditions, when plants were absent and more N was available to soil microorganisms, we found also some other significant changes in microbial activity that were not observed in the field. These were i) lowering of mineralization of older soil organic matter, which can be partly explained by negative effect of mineral nutrient addition (mainly N) on fungi with oxidase activity (Waldrop and Zak 2006), and ii) increase in N mineralization rate, nitrification rate and lower microbial N assimilation, showing potential enhancement in the rate of soil N cycle (Dijkstra et al. 2004). If the studied wet-meadow ecosystems is exposed to chronic eutrophication and nutrient loading exceeds plant demands, we suppose the observed changes in soil should likely appear in the field conditions in future.

The rates of total CO_2 emissions measured in situ at our experimental sites (6 to 62 mmol m^{-2} h^{-1}) correspond to the values measured in grassland ecosystems and reported in the literature, e.g., 9.7 mmol CO_2-C m^{-2} h^{-1} in average (Frank et al. 2006), from 20 to 24 mmol CO_2-C m^{-2} h^{-1} (Frank et al. 2002), from 4.3 to 35.4 mmol CO_2-C m^{-2} h^{-1} (Šimek et al. 2004) and from 25 to 44 mmol CO_2 m^{-2} h^{-1} (Kuzyakov et al. 2001).

Total CO_2 emissions increased significantly in fertilized plots as compared to nonfertilized plots at Z site. This effect may be explained by an increase of aboveground plant biomass respiration, root-derived respiration (sum of root respiration and rhizomicrobial respiration) under fertilized conditions and by priming effect (soil organic matter mineralization). It is not possible to measure separately plant-derived respiration and microbial respiration in situ therefore we calculated the rates of soil respiration using the rates measured under laboratory conditions (soil organic matter derived respiration) per square meter of experimental plots. The average soil organic matter derived respiration rates were 12 mmol CO_2-C m^{-2} h^{-1} for H site and 5.2 mmol CO_2-C m^{-2} h^{-1} for Z site, respectively (assuming the active soil layer 200 mm thick).

It means that 25% of total average CO_2 emissions should be soil organic matter derived respiration for H site and 16 % for Z site, respectively. Therefore the estimate of root-derived respiration portion would be 75% of total CO_2 emission for H site and 84% for Z site, respectively. Similar values are reported in literature for grassland soils. For example Xu et al. (2008) estimated that root-derived respiration contributed by 85-92% to bulk soil respiration. The root-derived carbon in the total CO_2 efflux was between 69% and 94% according to Werth and Kuzyakov (2008). Wang and Guo (2006) estimated that the contribution of root respiration to total soil respiration ranged from 24 to 57% in spring and summer, and increased to 73% in autumn. Kuzyakov et al. (2001) calculated that 46% of total CO_2 efflux from the soil originated in root respiration. Kuzyakov and Larionova (2005) concluded for crops and grasses (wheat, ryegrass, barley, buckwheat, maize, meadow fescue, prairie grasses), that root respiration amounts on average to 48% and rhizomicrobial respiration to 52% of root-derived CO_2.

We expected methane and nitrous oxide emissions would be affected by fertilization, too, however emissions of methane and nitrous oxide were under detection limit during the whole vegetation season 2007. The reason was most probably low soil moisture content and oxidized conditions in the soil profile – water level was under the soil surface for most time of vegetation season 2007 at both study sites. The results could be different under different climatic conditions. If the soil is flooded for longer period during vegetation season than it was during the year 2007 then total CO_2 emissions would be probably lower than under low moisture condi-

tions, and methane and nitrous oxide would be other important products produced by microorganisms in soil.

5. Conclusions

Starting eutrophication (2 years period of fertilization) of wet meadow ecosystem was indicated by increase of plants shoot:root ratio, increase of total CO_2 emissions and increase of soil respiration. Many other soil biological characteristics, like microbial biomass, rate of carbon mineralization, rates of nitrogen assimilation and mineralization were not significantly affected by starting eutrophication. Despite non significant impact on aboveground component there were some significant responses in belowground part which suggest that belowground processes may be suitable early warning signals.

Above mentioned plant and soil responses to fertilization indicate on-going changes in C fluxes and its allocation within the system. Peaty oligotrophic soil seems to be more sensitive to nutrient addition than mineral soil. However, final effect of fertilization on ecosystem C balance stays unknown and longer study is necessary to draw explicit conclusion.

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Nutrient addition retards decomposition and C immobilization in two wet grasslands

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Abstract Eutrophication is one of the biggest environmental problems facing wetlands. However, its effect on soil functioning is not yet well understood. We tested the hypothesis that increased nutrient loading into wet grassland ecosystems accelerates soil C and N cycles and decreases microbial immobilization of C and N. Experimental sites were established on two wet grasslands, with either mineral or peaty soils, and fertilized by NPK fertilizer for 3 years. Soils were analyzed for soluble and microbial C and N contents and their transformations, profile of phospholipid fatty acids and number of *nirK* denitrifiers. Fertilization affected C more than N transformations. Opposite to what was predicted, decomposition was retarded, the soil C cycle was based more on labile C compounds, and the soil was more susceptible to C losses in fertilized versus unfertilized treatments in both soils. Fertilization resulted in lower microbial biomass C and microbial C immobilization and also decreased the activity of lignin-degrading enzymes. Shifts in the composition of the microbial communities led to decreased (1) decomposition of complex organic compounds and (2) immobilization of transformed C. Net nitrification and microbial N immobilization tended to increase in fertilized treatments indicating an acceleration of soil N cycling and losses, but only in the more vulnerable organic soil.

Keywords Wet meadows · Eutrophication · Lignin-degrading enzymes · qPCR

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Introduction

Increased nutrient inputs as a result of management practices lead to eutrophication of wetland ecosystems which results in (1) changes in plant species composition and richness (Brinson & Malvarez, 2002), (2) increased aboveground plant production over root growth (Picek et al., 2008), (3) changes in carbon allocation to the belowground parts of plants and changed exudation of soluble organic compounds through plant roots to the soil (Kuzyakov et al., 2002; Paterson et al., 2006), and (4) increased plant tissue nutrient contents which results in changes in stoichiometric ratios of plant litter (e.g., Rejmankova et al., 2008).

Increased nutrient availability further directly or indirectly affects the soil microbial community and the

processes driven by soil microorganisms. High inputs of nutrients, especially nitrogen (N) and phosphorus (P), lead to imbalances in an ecosystem. The effect of eutrophication on soil microbial parameters and processes is less predictable than the effect of eutrophication on vegetation. For example, microbial biomass, as an important soil parameter, may have a variable response or remains unchanged after increased N loading (Bardgett et al., 1999). Measurement of rates of biogeochemical cycles in soil seems to be a better predictor of changes, giving less ambiguous responses to eutrophication than microbial biomass. Eutrophication accelerates the soil N cycle and increases N losses from ecosystems. Net nitrification usually increases (Fortuna et al., 2003; Zhong et al., 2007) and becomes a dominating process (Subbarao et al., 2006). The denitrification rate also increases with nitrogen loads to soil (Niboyet et al., 2010). In contrast, N immobilization decreases with increasing nutrient loadings (Dijkstra et al., 2005; Yevdokimov et al., 2005). However, there are other factors regulating nutrient fluxes in soil, such as water level, N input (Davidsson & Leonardson, 1996), and the chemical form of N, input of easily decomposable organic matter (Garcia-Montiel et al., 2003), N and P stoichiometry in soil organic matter, and nutrient input (van Oorschot, 1994). As a result, they can mask the effect of eutrophication on microbial processes (e.g., Tiemann & Billings, 2008).

Due to the very close connection between C (carbon) and N cycling, C immobilization in the system also usually decreases with increased eutrophication, leading to considerable C mobilization and dissolved organic C (DOC) leaching (Galicia & Garcia-Oliva, 2004—pasture soils; Scheuner & Makeschin, 2005—pine forest soil). However, Evans et al. (2008) reviewed experiments with N-enrichment and found that the effect of N addition on DOC leaching from affected soils was inconsistent. The responses were linked to the form of N added and the resulting changes in soil acidity. Sodium nitrate additions consistently increased DOC leaching, whereas ammonium salts usually caused decreased DOC leaching.

Decomposition is the key process ensuring release of nutrients immobilized in soil organic matter. It is regulated by the activity of extracellular, namely lignin-degrading and cellulolytic enzymes, which are also affected by nutrient levels in soil (Sinsabaugh et al., 2005). The effect of mineral N addition on extracellular enzyme activities was first described by Fog (1988). She observed stimulation of cellulose degradation under high N loads and suppression of ligninolytic activity at the same time. Similarly, Waldrop et al. (2004) found suppressed phenoloxidase and peroxidase activities with high mineral N loads, but only in soil with a higher C:N ratio. In such soils, microbes are N-limited and they receive N from lignin, which is decomposed by ligninolytic enzymes. If mineral N is added to the soil, then the microbial N limitation is removed and the rate of lignin degradation decreases.

Changes in the rate of enzyme activities and subsequent microbial processing of C and N due to eutrophication are connected with shifts in the composition of the microbial community. Again, various responses of soil bacterial and fungal community composition to eutrophication are reported in the literature. Various authors (Högberg et al., 2003; Allison et al., 2007; de Vries et al., 2007) found a shift of microbial community composition towards bacteria. Denef et al. (2009) reported decreased abundance of arbuscular mycorrhizal fungi after N input to grassland soils. In contrast, Bardgett et al. (1999) observed that the structure of the microbial community altered significantly in favor of fungi after N addition to a temperate upland grassland soil. They concluded that root exudation differed among plant species so that microbial biomass and activities were regulated more by root exudates than any direct effect of nitrogen. Similarly, Yevdokimov et al. (2008) found a shift of microbial community composition towards fungi after N addition (in this case as a direct effect of N on the soil microbial community with plant exclusion). Other authors report an increase in the relative abundance of Gram-positive bacterial phospholipid fatty acids (PLFAs) and actinomycetes PLFA (Yevdokimov et al., 2008; Denef et al., 2009) or increase of the abundance of *nirK* denitrifiers as a result of eutrophication (Leonardson, 1996; Henderson et al., 2010; Niboyet et al., 2010).

The effect of eutrophication on C and N cycles may strongly differ in different types of soils. Total organic matter content in a soil especially affects microbial processes. Ammonium, nitrates and phosphorus are retained to a much greater extent in peaty versus mineral soils (Fenn et al., 1998). Therefore, different effects of eutrophication on peaty soils as compared to mineral ones can be expected. However, there is still great uncertainty about the course and extent of

changes in microbial processes in eutrophied wetlands with different soil types (de Vries et al., 2003).

Based on published data, we hypothesized that increased nutrient loading will increase C and N mobilization (mineralization, nitrification, denitrification), while microbial immobilization of C and N in the system will decrease. We further expected a suppression of lignin-degrading enzyme activities as a result of fertilization. In contrast, microbial community composition would likely not change significantly within a short period (2 years of fertilization). All these changes should be stronger on peaty soil rather than on mineral soil.

The aim of our study was to identify the best early indicators of possible eutrophication of two wet meadows with either mineral (silt-sand alluvial) or peaty soils. We focused on (1) net C and N fluxes in soil (C and N mineralization and immobilization, denitrification, nitrification), (2) activities of lignin-degrading enzymes, and (3) changes of microbial community composition in soils receiving different levels of nutrient inputs.

Materials and methods

Study sites

Experimental plots were established on two wet meadows in the Trebon Basin Biosphere Reserve, South Bohemia, Czech Republic. These two sites are representative of two main temperate wet meadow ecosystems. The first is a marginal wetland with organic peaty soil (Histosol, FAO–WRB classification), located in the inundation area of a human-made fishpond. The water level is usually stable, depending on the fishpond management, except during long periods of extreme summer drought. This site is dominated by *Carex vesicaria* and *C. acuta* (values of 2 and 3 on the Braun–Blanquet scale). The second is an alluvial meadow with a mineral substrate (Gleysol, FAO–WRB classification) near a small river. The water level is more variable, depending on the river flow. This site is dominated by *Glyceria maxima* and *Carex acuta* (values of 3 on the Braun–Blanquet scale). Both soils are classified as silt-loams (USDA classification); more physical and chemical parameters are shown in Table 1. More information about the study sites are described by Picek et al. (2008).

Table 1 Physical and chemical parameters of the soils at the Trebon Basin (means ± standard deviation, $n = 4$)

	Mineral soil	Organic soil
Bulk density (g cm^{-3})	0.52 ± 0.04	0.21 ± 0.02
Clay (%)	12.5	22.5
Sand (%)	15.0	0.0
Total C (%)	9.63 ± 1.65	22.33 ± 2.25
Total N (%)	0.64 ± 0.1	1.18 ± 0.09
C:N ratio	15.0	18.9
Total P (%)	0.18 ± 0.02	0.19 ± 0.01
pH_{H_2O}	4.9	5.1
pH_{KCl}	3.8	4.3

Experimental design and soil sampling

Four blocks were established in both meadows in May 2006. This was a manipulative experiment: thus blocks were placed to cover expected heterogeneous conditions in the field sites. Each block was divided into three treatment plots (3.5 × 3.5 m) with different treatments of a commercial NPK fertilizer (containing 15% (w/w) of N, 15% of P_2O_5 and 15% of K_2O; N:P:K ratio 2.3:1:2): (1) no fertilizer addition (control, C), (2) 65 kg NPK ha^{-1} $year^{-1}$ (low, L), and (3) 300 kg NPK ha^{-1} $year^{-1}$ (high, H). The low treatment simulates current management treatments for Czech farms, while the high treatment is the mean dose recommended by agricultural firms for grasslands of these types (European Environmental Agency, 2003). The fertilizer was applied in half-doses two times a year, in mid-July 2006, and mid-May and mid-July in both 2007 and 2008.

Soils from all treatment plots were sampled three times in 2008. The first two samplings were always done 6–7 days after fertilization events (May and July), with the last one at the end of the vegetation season (October). From each treatment plot, 10 randomly sampled soil cores (0–20 cm depth below a litter layer, which was excised) were taken and mixed into one representative soil sample. Soil samples were kept at 4°C, sieved through a 5-mm mesh the next day and immediately prepared for analyses. The soils sampled in May, July, and October were analyzed for soluble N and C, microbial biomass, and microbial N and C transformations, whereas PLFA, quantitative PCR, DNA extraction, and peroxidase and phenoloxidase enzymatic assays were analyzed only in the October samples.

Soluble N and C pools, microbial biomass and microbial N and C transformations

For analyses of soluble organic C (SOC), total soluble N (TSN) and concentrations of ammonium and nitrates (NH_4-N and NO_3-N, respectively), a portion of each soil sample was extracted in 0.5 M K_2SO_4 (soil:extractant ratio 1:4). The extracts were filtered and stored frozen. SOC and TSN were measured on a LiquicTOC II (Elementar, Germany), while NH_4-N and NO_3-N were analyzed using flow injection analysis (FIA Lachat QC8500; Lachat Instruments, USA). The amount of soluble organic N (SON) was calculated as the difference between TSN and the sum of inorganic N forms. Soil microbial biomass C and N were measured using the chloroform fumigation-extraction method (Vance et al., 1987) and calculated as the difference between SOC and TSN contents in fumigated and non-fumigated samples. Coefficients of 0.45 (Vance et al., 1987) and 0.54 (Brookes et al., 1985) were used to correct the results for soil microbial biomass C and N, respectively. The rates of net N mineralization, nitrification, and N and C immobilization into microbial biomass were calculated from temporal changes in concentrations, in particular N and C pools (NH_4-N, NO_3-N, and soil microbial N and C, respectively) after 3 weeks of aerobic incubation of soil samples at 20°C. The rate of C mineralization was measured as an increase of CO_2 concentration in the headspace of air-tight, closed bottles with soil incubated at 20°C for 24 h. The rate of denitrifying enzyme activity (DEA) was measured by the acetylene reduction method (Tiedje et al., 1989). Briefly, DEA was measured under anoxic conditions (helium in the headspace) at 20°C in soil amended with optimal concentrations of glucose and nitrates. Acetylene was added to the headspace to inhibit N_2O reduction to N_2. The increase of N_2O concentration in the headspace of bottles was measured within a 30-min interval. Nitrous oxide was determined using an HP 6890 gas chromatograph (Agilent, USA). All measurements were done in three laboratory replications per soil sample. The results in the tables are arithmetic means of the three sampling times per year, always expressed on an oven-dry soil basis (24 h at 105°C).

Phospholipid fatty acids (PLFA) analysis

PLFA analysis, enzymatic assays (phenoloxidases and peroxidases activities), and qPCR of 16S rDNA and *nirK* genes were analyzed in soil samples from October 2008. PLFA were isolated according to Frostegård et al. (1993). Briefly, 2 g of soil were extracted by a chloroform–methanol–phosphate buffer (1:2:0.8). Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60; Merck) and the samples were subjected to mild alkaline methanolysis. A known amount of methylnonadecanoate (19:0) was added before methanolysis as an internal standard. The free methyl esters of PLFA were analyzed by gas chromatography–mass spectrometry (Varian 3400; ITS-40, Finnigan) (Snajdr et al., 2008). Fungal biomass was quantified based on 18:2ω6 content, while bacterial biomass was quantified as the sum of i14:0, i15:0, a15:0, 16:1ω7, 16:1ω9, 16:1ω5, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0, and cy19:0. Fatty acids found in both bacteria and fungi, for example, 15:0, 16:0, and 18:1ω7, were excluded from the analysis (Snajdr et al., 2008). The B/F (Bacteria/Fungus) ratio was calculated from the content of the above bacterial and fungal biomass-specific PLFA molecules. Branched fatty acids were used to quantify Gram-positive bacteria, monounsaturated and cyclic fatty acids for Gram-negative bacteria, and 10Me fatty acids for actinomycetes. Physiological status was detected using the ratio of cyclopropane PLFA to its monoenoic precursor (cyclo/prec). An increase in the ratio was expected to indicate a shift from exponential to stationary growth, representing substrate limitation (Guckert et al., 1986; Navarrete et al., 2000). The ratio of monoenoic to saturated PLFA (MUFA/STFA) was used as an indicator of substrate availability (Bossio & Scow, 1998).

Extraction of DNA and quantification of 16S rDNA and *nirK* gene by qPCR

Three replicates of each soil sample (approximately 0.25 g) were taken for DNA extraction. For the isolation of genomic DNA from soil, the Power Soil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) was used according to manufacturer's instructions with some modification. A Mini Bead-Beater (BioSpec Products; speed of 6 m s^{-1} for 45 s) was used for better disruption of Gram-positive bacterial cell walls and spores. DNA was aliquoted and stored in 1.5-ml Eppendorf microtubes in a freezer (−20°C).

The quantitative polymerase chain reaction (qPCR) methods used in this study have been described

elsewhere (Barta et al., 2010a). Briefly, 20 ng of extracted DNA was used for SYBRGreen qPCR with 16S rDNA general and *nirK*-specific primers (Henry et al., 2006); qPCR assays were performed with an ABI Step One (Applied Biosystems, USA). Two independent qPCRs were performed for each gene and soil replicate. Standard curves were obtained with serial 10-fold plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rDNA and *nirK* gene.

Activities phenoloxidases and peroxidases

Peroxidase (Perox) and phenoloxidase (PhOx) activities were determined in enzymatic assays using the methodology of Hendel et al. (2005) adapted for 96-well microplates. In this procedure, 200-µl aliquots of soil homogenate (soil:water, 1:10 w/v, 4 min of sonication) were distributed into four technical replicate wells. For phenoloxidase, 50 µl of 25 mM L-DOPA (dihydroxyphenylalanin) was added to each sample well. Peroxidase assays received 50 µl of 25 mM L-DOPA plus 10 µl of 0.3% H_2O_2. Negative control wells for PhOx contained 200 µl of acetate buffer and 50 µl of L-DOPA solution; blank control wells contained 200 µl of sample suspension and 50 µl of acetate buffer. For peroxidase, negative and blank control wells also received 10 µl 0.3% H_2O_2. The microplates were incubated in the dark at 20°C for 5 h. Enzyme activities were measured spectrophotometrically at 460 nm and expressed in international enzyme units (IEU). One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of reaction product per hour per gram of sample using a micromolar extinction coefficient of enzymatically oxidized L-DOPA product of 7.9.

Statistical evaluation of data

The effect of locality (mineral and organic soil) was tested by one-way analysis of variance (ANOVA). Factorial ANOVA followed by the Tukey HSD test ($P < 0.05$) (Statistica 8.0; StatSoft, USA) was used to evaluate the effects of fertilization and block on soil properties at each locality for each sampling time. The effect of block was significant for all tested parameters and thus could not be excluded. The effect of sampling time was further assessed by regression. The results from three laboratory replicates were included in all of the aforementioned analyses.

The effect of fertilization and sampling site on microbial community composition (PLFA fingerprint based on mol% of individual PLFA within total PLFA or relative abundance of notional groups of microorganisms) was tested using redundancy analysis (RDA) (ter Braak & Smilauer, 1998). Potential differences between sampling sites and treatments were further tested by ANOVA.

Results

Soluble N and C pools

The organic soil contained a significantly higher concentration of SOC and SON versus the mineral soil ($P < 0.001$). Concentrations of inorganic N forms were comparable in both soils and their proportions within TSN were low, on average 20 and 23% in the organic and mineral soils, respectively (Table 2).

In both soils, the concentrations of SOC and TSN were lower in spring and summer versus autumn sampling ($P < 0.01$, detailed data not shown). Concentrations of SOC and TSN significantly increased with fertilization in both soils ($C < L < H$, $P < 0.05$). This occurred in both spring and summer samplings in the organic soil (the effect of fertilization $P < 0.01$) and in the summer sampling in the mineral soil (the effect of fertilization $P < 0.001$). Concentrations of organic and ammonium N followed the same pattern as TSN in both soils, due to a close correlation among these parameters ($r = 0.95$ and $r = 0.49$, respectively, for the organic soil, and $r = 0.95$ and $r = 0.66$, respectively, for the mineral soil). No effect of fertilization on the parameters was found in the autumn sampling in any soil. In contrast to other N forms, the amounts of nitrates were highest in the control plots and lowest in the low-fertilization treatment in both soils (the effect of fertilization $P < 0.01$), independent of sampling time (Table 2). The above-described effects of fertilization were also apparent in annual means of the measured parameters (Table 2). In the organic soil, concentrations of SOC and ammonium N significantly increased with fertilization, but this trend was not significant for total and organic soluble N. In the mineral soil, SON and ammonium N increased with fertilization, with TSN being the highest in the

Table 2 Concentrations of SOC, TSN, soluble organic N (SON), nitrates, and ammonium in organic and mineral soils with different fertilization treatments

Site	Treatment	SOC (μg C g^{-1})	TSN (μg C g^{-1})	SON (μg N g^{-1})	NO_3-N (μg N g^{-1})	NH_4-N (μg N g^{-1})
Organic soil	C	318.08 a ± 49.62	34.56 ± 4.74	27.94 ± 3.78	2.55 c ± 0.78	4.07 a ± 2.04
	L	333.95 b ± 46.59	35.19 ± 3.56	29.19 ± 2.85	1.69 a ± 0.61	4.30 a ± 1.86
	H	348.94 c ± 50.81	35.63 ± 4.24	28.80 ± 3.77	1.96 b ± 0.66	4.87 b ± 2.12
ANOVA	Block	$P < 0.001$	$P < 0.001$	$P = 0.002$	$P < 0.001$	$P < 0.001$
	Treatment	$p < 0.001$	–	–	$P < 0.001$	$P < 0.001$
	B × T	$P < 0.001$	–	–	$P < 0.001$	$P < 0.001$
Mineral soil	C	153.84 ± 33.20	27.51 b ± 7.16	20.97 a ± 4.94	2.18 c ± 1.45	4.36 a ± 1.95
	L	151.51 ± 25.27	25.97 a ± 3.78	20.46 a ± 2.78	1.14 a ± 0.42	4.37 a ± 1.17
	H	155.15 ± 34.16	29.20 c ± 7.15	22.51 b ± 5.97	1.91 b ± 0.95	4.93 b ± 1.90
ANOVA	Block	–	$P < 0.001$	$P = 0.001$	$P < 0.001$	$P < 0.001$
	Treatment	–	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
	B × T	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Means of the three sampling dates in 2008 ($n = 12$) and standard deviations are given together with results of factorial ANOVA with a block (B) and treatment (T) as parameters. In cases of significant effects of treatment, different letters show significantly different values within the particular soil (Tukey HSD test, $P < 0.05$)

C control, *L* low, *H* high fertilization

high-fertilization treatment. No significant differences were found for SOC (Table 2).

N and C transformations

Both soils showed negligible net N mineralization rates and comparable mean values of microbial N and C immobilization and DEA. The rates of nitrification and C mineralization were significantly higher in the organic versus mineral soil ($P < 0.001$) (Table 3). While net N mineralization rate did not change during the season in any soil, nitrification rate decreased significantly from spring to autumn ($P < 0.001$) in both soils. Microbial N immobilization increased significantly from spring to autumn in the mineral soil ($P < 0.001$) and similarly, C immobilization increased from spring to autumn in the organic soil ($P < 0.01$).

In the organic soil, microbial C immobilization rates decreased significantly (the effect of fertilization $P < 0.05$) versus the control in summer and autumn samplings, but the resulting effect of fertilization on annual means was not significant (Table 3). Similarly, the nitrification rate significantly increased in the high-fertilization treatment versus control in summer and autumn samplings (the effect of fertilization $P < 0.01$), but the resulting effect of fertilization on annual means was not significant (Table 3). Nitrogen immobilization tended to decrease in both fertilized treatments in summer and autumn, with this non-significant decreasing tendency being found in the annual means (Table 3). A tendency for lower C immobilization in the high-fertilization treatment versus the control and low-fertilization treatments was also found in the mineral soil in all samplings and also in the annual means, but the effect of fertilization was not significant at any sampling time (Table 3). Otherwise, the low-fertilization treatment had the lowest net nitrification and C mineralization, but the highest N immobilization, rates from all treatments in the mineral soil (Table 3). The DEA was highest in the high-fertilization treatment versus the control and low-fertilization treatments in the annual means (Table 3).

Microbial biomass C and N

Higher amounts of microbial biomass C and N (C_{mic} and N_{mic}) were found in the organic versus mineral soil ($P < 0.001$). C_{mic} and N_{mic} were significantly the lowest in summer and the highest in spring in both soils, with autumn values being in the middle ($P < 0.001$). The C:N ratio of biomass was comparable between both soils (Table 4), with the highest values in summer and the lowest in spring ($P < 0.01$). C_{mic} decreased in the fertilized treatments in both soils. In the organic soil, this trend was consistent in all

Table 3 Microbial N transformations (nitrification, N-mineralization, N-assimilation, N-DEA) and C mineralization and microbial C immobilization in organic and mineral soils with different fertilization treatments

Site	Treatment	Nitrification (μg N g^{-1} d^{-1})	N mineralization (μg N g^{-1} d^{-1})	N immobilization (μg N g^{-1} d^{-1})	N-DEA (μg N g^{-1} d^{-1})	C mineralization (μg C g^{-1} d^{-1})	C immobilization (μg C g^{-1} d^{-1})
Organic soil	C	1.13 ± 0.25	0.03 ± 0.01	1.08 ± 1.49	17.1 ab ± 9.7	48.19 ± 11.26	139.25 ± 70.67
	L	1.14 ± 0.20	0.03 ± 0.01	0.75 ± 1.72	18.9 b ± 11.6	48.67 ± 12.49	98.74 ± 63.28
	H	1.21 ± 0.30	0.02 ± 0.04	0.38 ± 1.36	16.8 a ± 9.5	45.04 ± 12.78	72.35 ± 62.41
ANOVA	Block	$P < 0.001$	–	–	$P < 0.001$	$P = 0.002$	–
	Treatment	–	–	–	$P = 0.036$	–	–
	B × T	$P = 0.008$	–	–	$P < 0.001$	$P = 0.009$	$P = 0.026$
Mineral soil	C	0.97 b ± 0.27	0.001 ± 0.02	0.85 ± 1.89	20.4 b ± 10.3	27.00 c ± 5.37	147.70 ± 65.34
	L	0.66 a ± 0.30	0.02 ± 0.04	1.21 ± 1.88	18.5 a ± 9.4	23.86 a ± 4.40	145.42 ± 55.54
	H	0.90 b ± 0.29	0.002 ± 0.03	0.94 ± 1.93	22.6 c ± 14.4	25.53 b ± 4.89	112.54 ± 61.84
ANOVA	Block	$P < 0.001$	–	$P = 0.016$	$P < 0.001$	$P < 0.001$	–
	Treatment	$P < 0.001$	–	–	$P < 0.001$	$P < 0.001$	–
	B × T	$P < 0.001$	–	$P = 0.014$	$P < 0.001$	$P < 0.001$	–

Means of the three sampling dates in 2008 ($n = 12$) and standard deviations are given together with results of factorial ANOVA with a block (*B*) and treatment (*T*) as parameters. In cases of significant effects of treatment, different letters show significantly different values within the particular soil (Tukey HSD test, $P < 0.05$). N-DEA means denitrifying enzyme activity—denitrification under optimal conditions measured in soil amended with nitrates and glucose

C control, *L* low, *H* high fertilization

three samplings and also occurred in the annual mean values (Table 4). In the mineral soil, this was observed in the spring and autumn samplings, while in summer, the highly fertilized treatment had the highest C biomass (detailed data not shown). This resulted in significantly lower C_{mic} in the low-fertilization treatment, but non-significantly lower C_{mic} in the highly fertilized treatment versus control in the annual means (Table 4). Microbial N decreased with fertilization in the organic soil only in spring, but the effect of fertilization was also significant in the annual means (Table 4). An opposite tendency was found in the mineral soil (Table 4). The biomass C:N ratio was not affected by fertilization in the organic soil. In the mineral soil, a decreasing trend occurred at all three sample times, with a significant effect of fertilization occurring during the summer and autumn samplings (the biomass C:N ratio in the control was significantly higher than both fertilized treatments, $P < 0.05$) and also in the annual mean values (Table 4).

PLFA analysis

The effect of locality explained 60% of the variability in the PLFA fingerprint (data not shown) and 29% in the proportion of notional groups within soil microbial communities (Fig. 1). The soil microbial community in the mineral soil contained a higher proportion of Gram-positive bacteria ($P < 0.001$), but lower proportions of Gram-negative bacteria and fungi ($P < 0.001$ and $P = 0.001$, respectively), in comparison to the organic soil. This led to a significantly higher bacteria to fungi ratio (B/F) in the mineral soil ($P = 0.010$). The mineral soil further showed lower MUFA/STFA and higher *cyclo*/*prec* ratios than the organic soil ($P < 0.001$ in both cases).

Fertilization significantly affected the composition of the soil microbial community. Its effect explained 7.3% of the variability in the PLFA fingerprint (data not shown) and 7.5% in the proportion of notional groups within soil microbial communities (Fig. 1). Fertilization led to a significant increase in the proportion of actinomycetes ($P = 0.031$) and G+ bacteria ($P = 0.007$). This was caused mainly by the positive effect of fertilization on the following PLFA: 10Me-16:0 (actinomycetes), a15:0 and a17:0 (G+ bacteria). The proportion of bacteria in the microbial community also tended to increase ($P = 0.080$), while fungi and G− bacteria were not affected. Therefore, the B/F ratio was not changed in fertilized plots.

Table 4 Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) and biomass C:N ratio in organic and mineral soils with different fertilization treatments

Site	Treatment	C_{mic} (mg C g^{-1})	N_{mic} (μg N g^{-1})	Biomass C:N
Organic soil	C	2.49 b ± 0.52	138.69 b ± 45.63	19.27 ± 4.13
	L	2.46 b ± 0.38	133.26 b ± 39.69	19.54 ± 3.73
	H	2.20 a ± 0.40	125.73 a ± 32.70	18.54 ± 3.14
ANOVA	Block	$P = 0.001$	$P = 0.001$	$P < 0.001$
	Treatment	$P < 0.001$	$P = 0.017$	–
	B × T	$P < 0.001$	$P = 0.027$	–
Mineral soil	C	1.86 b ± 0.46	100.16 ± 39.17	21.35 b ± 9.30
	L	1.69 a ± 0.25	103.45 ± 26.25	17.43 a ± 4.29
	H	1.78 b ± 0.26	106.79 ± 30.67	18.14 a ± 5.04
ANOVA	Block	$P < 0.001$	$P < 0.001$	$P = 0.001$
	Treatment	$P < 0.001$	–	$P < 0.001$
	B × T	$P < 0.001$	$P < 0.001$	$P = 0.020$

Means of the three sampling dates in 2008 ($n = 12$) and standard deviations are given together with results of factorial ANOVA with a block (*B*) and treatment (*T*) as parameters. In cases of significant effects of treatment, different letters show significantly different values within the particular soil (Tukey HSD test, $P < 0.05$)

C control, *L* low, *H* high fertilization

Further, the MUFA/STFA ratio tended to decrease in fertilized plots of the organic soil while the *cyclo/prec* ratio increased significantly ($P = 0.02$) in fertilized plots of the mineral soil.

Activities of phenoloxidases and peroxidases

The activity of phenoloxidases (PhOx) was higher in organic than mineral soil (Fig. 2). On both localities, fertilization led to a significant decrease in PhOx activities ($P < 0.001$). In the organic soil, both fertilization treatments decreased PhOx activity significantly, while in the mineral soil, only the high-fertilization treatment decreased PhOx activity significantly as compared to the control. A different situation was found for peroxidase (Perox) activity in the soils. In the organic soil, the Perox activity was 10 times lower than in the mineral soil, and it decreased significantly only in the high-fertilization treatment compared to the control (Fig. 3). In the mineral soil, significantly lower Perox activity, as compared to the control, was found in both low- and high-fertilization treatments (Fig. 3).

Quantification of 16S rDNA and *nirK* gene

Significantly higher numbers of copies of the bacterial 16S rDNA gene were found in the mineral compared

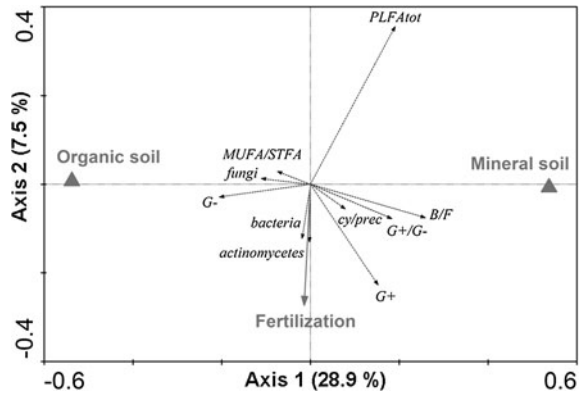

Fig. 1 The effect of fertilization and soil type on total phospholipid fatty acid amount ($PLFA_{tot}$), relative abundance of notional groups within soil microbial community (*bacteria* total; *G+* Gram-positive; *G−* Gram-negative; *actinomycetes*; *fungi*; *B/F* the Bacteria/Fungus ratio) and its metabolic status (*cyclo/prec* cyclopropane PLFA to its monoenoic precursor; *MUFA/STFA* the ratio of monoenoic to saturated PLFA) (RDA analysis) ($n = 4$)

to the organic soil ($P < 0.001$). In addition, their amount increased significantly in both fertilization treatments in the mineral soil as compared to the control ($P = 0.046$) (Fig. 4). This was not the case for the organic soil, where the numbers of copies of the 16S rDNA gene were the same in all treatments (Fig. 4). The organic soil contained a larger number of copies of the *nirK* gene than the mineral soil

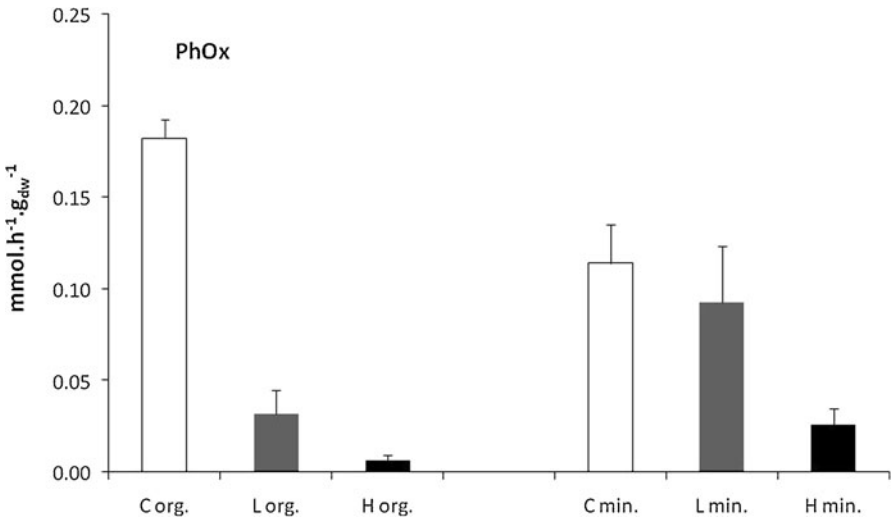

Fig. 2 The effect of fertilization (*C* control, *L* low fertilization, *H* high fertilization treatments) on phenoloxidase (*PhOx*) activity in organic (*org.*) and mineral (*min.*) soils (means ± standard deviation, $n = 4$)

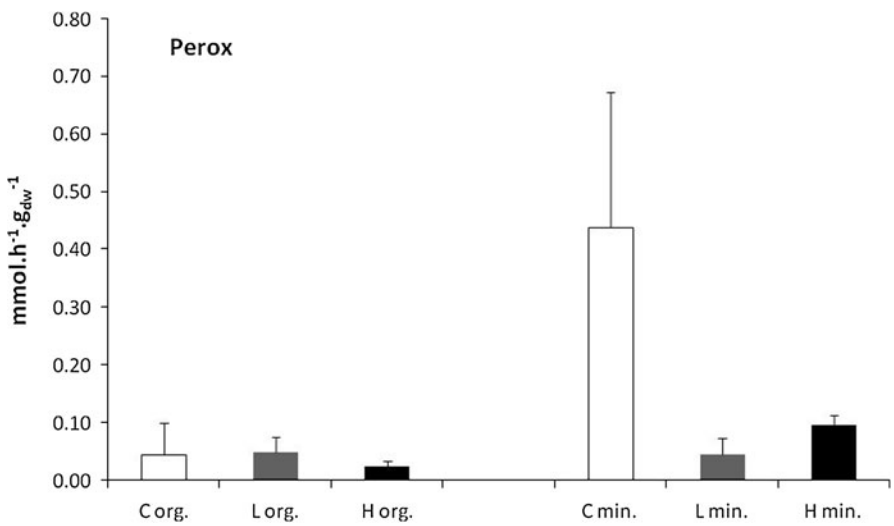

Fig. 3 The effect of fertilization (*C* control, *L* low fertilization, *H* high fertilization treatments) on peroxidase (*Perox*) activity in organic (*org.*) and mineral (*min.*) soils (means ± standard deviation, $n = 4$)

($P < 0.001$) (Fig. 5). In both soils, fertilization led to an increase in their amounts ($P = 0.001$); significantly higher numbers of copies of the *nirK* gene were always found in high-fertilization treatments at both localities (Fig. 5).

Discussion

The amount of added N

During each fertilization event, ca. 3.5 and 23 kg N ha^{-1} were added into the grassland ecosystems in the low- and high-fertilization treatments, respectively. These values correspond to ca. 7 and 45 kg N ha^{-1} $year^{-1}$. Total input of N after 3 years of application of the NPK fertilizer to the low- and high-fertilization treatments was ca. 25 and 110 kg N ha^{-1}, respectively. The actual N deposition in the Czech Republic in the area of the Trebon basin reached 5–15 kg N ha^{-1} $year^{-1}$ in 2008. Thus, the fertilization treatments represent a rather significant N input, especially in the high-fertilization treatment, in both grasslands.

Changes in soluble C and N pools and their transformations

We found significant temporal changes in soluble C and N pools in both soils. Biomass production of

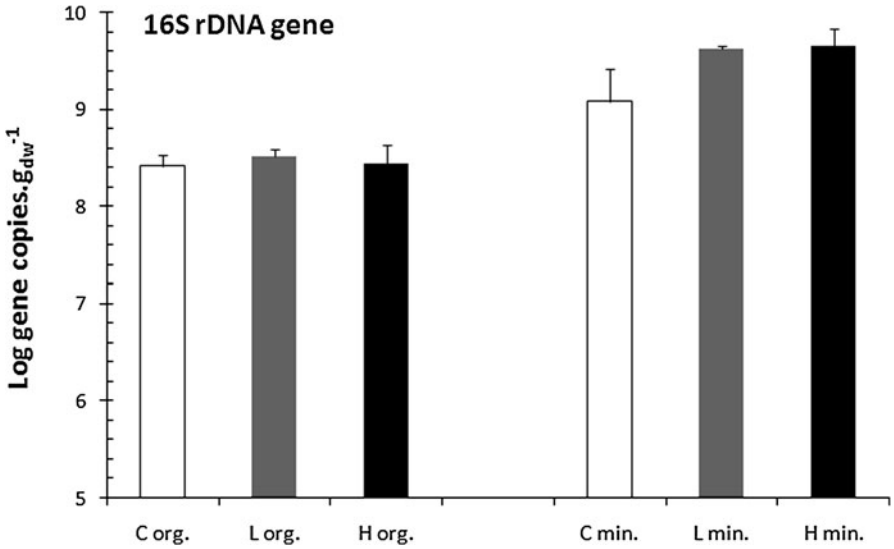

Fig. 4 The effect of fertilization (*C* control, *L* low fertilization, *H* high fertilization treatments) on abundance of bacterial *16S rDNA gene* in organic (*org.*) and mineral (*min.*) soil. Gene copies are shown using a log scale (means ± standard deviation, $n = 4$)

dominant plants in our wet grasslands is highest in late spring–early summer (Picek et al., 2008). In this period, high plant N demand is supported by a high nitrification rate, maintaining low concentrations of soluble N in the soil and low microbial N immobilization. Plant biomass senesced from late summer until autumn (Picek et al., 2008) resulting in C and N being released to the soil during its decomposition, contributing to soluble C and N pools in the soil. Due to low plant N demand, concentrations of soluble N in soil were the highest in autumn. At that time, the nitrification rate decreased and microbial N immobilization was high, which prevented N loss from the system. Such different roles of plants and soil microorganisms in soil N cycle and N immobilization within an ecosystem during the vegetation season were also found in high arctic ecosystems (Bardgett et al., 2007), the Scottish Highlands (Bardgett et al., 2002), and an arctic sedge meadow (Edwards et al., 2006).

Changes in soluble C and N pools due to fertilization were rather transient, occurring in the spring and summer samplings conducted always a week after fertilization. Fertilization led to significant increases in concentrations of SON, ammonium N, and SOC in both soils (in both samplings in the organic soil and in the summer sampling in the mineral soil). No such trends were found in any soil in autumn. The extra N and C had to result either from promoted microbial processes (priming effect), increased plant exudation following fertilization, or from the reaction of fertilizer ammonium N with aromatic components in the soil organic matter (Fog, 1988). The increase in the amount of soil soluble N, lasting more than a week after fertilization, would mean not only increased availability of N for plants and microbes in both rather N-poor ecosystems but also a risk of enhanced N losses from the systems during regular flooding of both grasslands. Also, enhanced losses of SOC through leaching may be expected. Interestingly, we found no tendency for an increasing amount of nitrates, although nitrate N represented half of the added fertilizer N. Nitrate N concentration was higher in the control than the fertilized treatments, which indicates its fast depletion from the soil solution, presumably by plant uptake. This explanation can be supported by several observations. Fertilization led to an increase in aboveground plant biomass on both localities (Picek et al., 2008), which should be connected with enhanced immobilization of N. Moreover, dominant plant species at both localities (*Carex acuta* and *Glyceria maxima*) were found to prefer nitrates versus ammonium as a source of N (Kastovska & Santruckova, 2011).

Net microbial C immobilization rate was a quite sensitive indicator of changes in microbial processes after fertilization. It significantly decreased in the fertilized treatment in the organic soil in summer and autumn and tended to also decrease with fertilization in the mineral treatments in all samplings. In the organic soils, decreased microbial C immobilization was further connected with a decreasing tendency of microbial N immobilization. Decreased microbial C immobilization

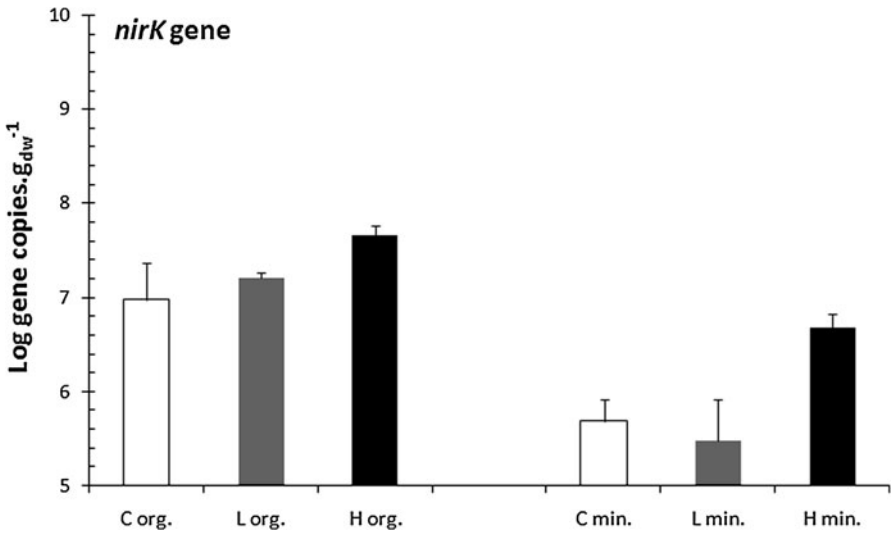

Fig. 5 The effect of fertilization (*C* control, *L* low fertilization, *H* high fertilization treatments) on abundance of denitrification *nirK* gene in organic (*org.*) and mineral (*min.*) soils. Gene copies are shown using a log scale (means ± standard deviation, $n = 4$)

and C transformation may eventually lead to lower C stabilization in soil; it has been found that C stabilized by an association with fine soil particles is very likely of microbial origin, due to a much greater extent of decomposition (Golchin et al., 1996). Microbial N immobilization is an important process in maintaining soil fertility and plant nutrition, because the immobilization and remineralization of N across the ecosystem retards the loss of N (including fertilizer N) via leaching or volatilization of gaseous compounds. This phenomenon is one of the most important factors controlling resilience of the ecosystem (Yevdokimov et al., 2005, 2008). Decreased N immobilization in microbial biomass, namely if it occurs in autumn when the amount of soluble soil N increases, could cause a problem in the future N balance of the system. Further indicators of an accelerated soil N cycle in the fertilized treatments could indicate a slight increase in net nitrification rate in the organic soil, which was also found by Zhong et al. (2007) and Fortuna et al. (2003) in other fertilized soils. These might also include the observed slight increase in the activity of denitrifying enzymes in the highly fertilized treatment of the mineral soil. Both processes could enhance N losses from grasslands in the future.

Changes in microbial biomass and composition of soil microbial community

We found a significant decrease in the amount of microbial biomass C and N due to fertilization in the highly fertilized treatment in the organic soil. In the mineral soil, microbial C tended to decrease with fertilization, while microbial N tended to increase. Microbial biomass is considered to be a robust parameter, which remains unchanged even after long-term management (Hassink & Neeteson, 1991; Bending et al., 2000). According to Yevdokimov et al. (2008), soil is capable of retaining high amounts of nitrates with unchanged size of microbial biomass N. Moreover, Bardgett et al. (1999) found that N addition had no consistent effect on soil microbial biomass.

We further observed a decrease in the biomass C:N ratio in fertilized treatments in mineral soils, which could indicate changes in the soil microbial community. The decreased C:N ratio of microbial biomass in eutrophied soils could be connected with a shift in microbial community composition towards bacteria, as found by Högberg et al. (2003), de Vries et al. (2007), Allison et al. (2007), and Yevdokimov et al. (2008). We also found an increasing tendency in bacterial proportion within the microbial community, but this was non-significant. As fertilization did not affect the proportion of fungi, the bacteria/fungi ratio did not change in our soils. However, there were significant changes within the bacterial community. Fertilization led to increases in the relative abundance of Gram-positive bacterial PLFAs, as also found by Yevdokimov et al. (2008), and Denef et al. (2009), and actinomycetes PLFA.

In fertilized soils, decreasing MUFA/STFA ratio (found in the organic soil), as well as increasing *cyclo*/*prec* ratio (found in the mineral soil) implied a possible

metabolic stress, indicating a possible substrate limitation (Guckert et al., 1986; Bossio & Scow, 1998; Navarrete et al., 2000). However, we have no other indices to specify any putative stress conditions in our fertilized soils.

The effect of fertilization on activities of phenoloxidases and peroxidases

The activity of both groups of lignin-degrading enzymes (phenoloxidases and peroxidases) differed significantly between organic and mineral soils. This difference could be caused mainly by the different C:N ratios of both soils and also content of soluble N. In organic soil, a higher C:N ratio and higher amount of SON could repress the activity of peroxidases in comparison to mineral soil. In both soils, increasing concentration of N through fertilization showed an overall repression of both ligninolytic enzyme activities, only with different quantitative responses for organic and mineral soils. The repression of lignin-degrading enzymes by higher amounts of mineral N was first described by Fog (1988). She observed that high loads of N stimulated cellulose degradation, but suppressed ligninolytic activity. Waldrop et al. (2004) also found that higher mineral N loads suppressed phenoloxidases and peroxidases, but only in soil with a higher C:N ratio. Microbes in such soils can be N-limited, obtaining N by the slow decomposition of lignin. When they receive easily available mineral N, they switch from energy-demanding lignin degradation to more efficient cellulose degradation as a C source (Fog, 1988). This is also likely in our case. In our study, even a low dose of fertilizer caused a dramatic repression of phenoloxidase activity in the organic soil (higher C:N ratio), while in the mineral soil (lower C:N ratio), a much higher N dose was needed to obtain a similar response. Activity of phenoloxidases in the organic soil was more sensitive to fertilization and responded faster than in the mineral soil. These differences in response to fertilization can be caused by (1) different levels of N limitation (see above), (2) different biochemical functions of phenoloxidases (Weintraub et al., 2007; Barta et al., 2010b), and (3) different composition of fungal communities. Most observations of a negative effect of N on ligninolytic enzymes have come from studies of white-rot basidiomycetes, while non-white-rot fungi degraded lignin more rapidly after N addition (Fog, 1988). Therefore, in our case, we propose that organic soil can have a higher proportion of white-rot fungi, because of the higher repression of phenoloxidases after fertilization.

The effect of fertilization on 16S rDNA and *nirK* genes

The mineral soil contained a higher amount of *16S rDNA*, and therefore a larger bacterial community, than the organic soil. This is in accordance with a higher bacteria/fungi ratio in the mineral versus organic soil, shown by PLFA analysis. This likely results from the different quality and C:N ratio of soil organic matter in both soils. Fertilization caused an increase in the amount of *16S rDNA* in the mineral soil, which also coincided with the PLFA results, showing an increasing tendency in bacterial proportion within the microbial community.

The amount of *nirK* genes was higher in the organic versus mineral soil, which can be explained by the higher content of soluble N in the organic soil (Henderson et al., 2010). Fertilization increased the abundance of *nirK* denitrifiers in the highly fertilized treatment of both soils. In accordance with this, the potential activity of denitrifying enzymes (DEA) increased in the highly fertilized treatment of the mineral soil. An absence of a similar response in the organic soil could be caused by the fact that the dynamics of *nirK* genes may not directly correlate with DEA. Another indicator of transiently increased denitrification could be the lower amounts of nitrates, which serve as a source for the denitrifiers, in fertilized treatments versus controls in both soils, measured a week after NPK application.

The response of different soil types to fertilization

Generally, microbial activity was more consistently affected by fertilization in the organic versus mineral soil. A stronger effect of fertilization on microbial processes and parameters in the organic soil could be explained by a combination of several factors. First, the same amounts of fertilizer per area were applied on both localities; however, the amount of applied nutrients was ca. 2.5 times higher in the organic soil than in the mineral one, when calculated per gram of soil due to the much lower bulk density of the organic soil (see Table 1). The organic soil was more N

limited than the mineral soil, which was indicated by the dominant plant species and also higher soil C:N ratio versus the mineral soil. Therefore, N loading into the organic soil should have a larger effect in relieving the system from N limitation. Second, the higher nutrient availability in the organic soil after fertilization was further enhanced by its higher ability to retain more added nutrients in comparison to mineral soils, due to the more developed sorption complex. Therefore, fewer nutrients were leached from the organic soil than from the mineral one, meaning that more nutrients were available for plants and soil microbes in the organic than mineral soil. And, third, the two soils also differed in composition of the soil microbial communities, which could be the reason for the different extent of changes in the PLFA profile, enzyme activities and amounts of *nirK* denitrifiers.

The absence of more significant effects of fertilization on soil N and N cycling in both soils could be explained by several observations. The nitrate and ammonium pools are known to be very dynamic, turning over one to several times per day (Jackson et al., 2008). Thus, transformation pathways and plant uptake of the added N from the soluble soil N pool is rapid, as was also noticed by Németh et al. (1988) and Mengel et al. (1999). We also expect that the majority of N added into the system was immobilized in plant biomass, especially aboveground biomass, the growth of which was supported by fertilization (Picek et al., 2008); plants are known to be better competitors for N versus soil microbes over the longer term (Hodge et al., 2000). Large amounts of added N are removed from the system with the cut biomass, as both localities are regularly mown once or twice a year. All of this likely retards the effect of the fertilization on soil N cycling and also N saturation of the grasslands.

Conclusions

Microbial biomass C, microbial community composition (determined by PLFA analysis), amount of *nirK* denitrifiers, microbial C immobilization, and activities of lignin-degrading enzymes were parameters sensitive to potential eutrophic conditions in wet meadow ecosystems (affected by 2 years of fertilization with an NPK fertilizer, with total N load of 25 and 110 kg N ha^{-1} into the low- and high-fertilization treatments, respectively). Lower microbial biomass C, higher proportions of actinomycetes and other Gram-positive bacteria, a higher amount of *nirK* denitrifiers, and lower microbial C immobilization and activity of lignin-degrading enzymes were found in fertilized soils as compared to non-fertilized ones. Increases in the soluble C and N pools occurred only transiently after fertilizer application into the soils. There were also other indications of changes in the soil microbial community and an acceleration of soil N cycling with fertilization: a tendency for higher net nitrification and lower microbial N immobilization in the organic soil and significantly lower biomass C:N ratio in the mineral soil. Microbial biomass C, microbial C immobilization and the microbial biomass C:N ratio seem to be especially promising parameters for studying the effects of fertilization on soil functioning.

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PAPER 9

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ECOSYSTEMS

Linking Above- and Belowground Responses to 16 Years of Fertilization, Mowing, and Removal of the Dominant Species in a Temperate Grassland

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Abstract

Species-rich oligotrophic meadows are affected by a wide range of management interventions that influence their functioning and capacity to deliver ecosystem services, but long-term studies on the above- and belowground adaptations to different management tools are still scarce. We focused on the interactive effects of NPK fertilization, mowing, and removal of the initially dominant species (*Molinia caerulea*) on plant, soil, and microbial responses in wet oligotrophic grassland in a 16-year full-factorial manipulative experiment. Changes in vegetation composition, soil pH, and nutrient availability were accompanied by altered microbial phospholipid fatty acid (PLFA) composition, whereas treatment effects on soil microbial biomass and carbon (C) mineralization were mainly related to changes in soil organic matter (SOM) content and nutrient availability. Fertilization decreased plant species richness aboveground and lowered SOM storage and microbial activity belowground. Mowing preserved high plant diversity and led to more efficient recycling of N within the grassland, whereas *Molinia* removal significantly affected only plant community composition. Mowing combined with fertilization maintained high species richness only in the short term. Belowground, mowing reduced N leaching from the fertilized system but did not prevent SOM depletion, soil acidification, and concomitant adverse effects on soil microbes. We conclude that annual mowing is the appropriate type of extensive management for oligotrophic species-rich meadows, but the concomitant nutrient depletion should not be compensated for by regular NPK fertilization due to its adverse effects on soil quality.

Key words: microbial community structure; PLFA; grassland; mowing; fertilization; dominant removal; pH.

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Author contributions All authors conceived the study; PK, HS, and EK analyzed the data; PK performed the PLFA analysis; MC analyzed the physico-chemical soil properties and performed microbial biomass measurements; JL designed and managed the long-term experiment and monitored the plant community; PK wrote the paper with editorial assistance from all authors.

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Introduction

Traditionally managed oligo- and mesotrophic species-rich meadows are among the ecosystems most endangered by current land use changes throughout Europe (Kaligaric and others 2006). For economic reasons, their traditional management of mowing once or twice a year is either intensified by fertilization and increasing mowing frequency, leading to significant changes in the above- and belowground components of the ecosystem (Bardgett and others 1999; Hedlund and others 2003), or the management is completely abandoned.

The two compartments of the system closely interact via top-down effects through plant-litter inputs (Bardgett and Shine 1999; Fornara and others 2011) and rhizodeposition (Hartmann and others 2009; Doornbos and others 2012), and bottom-up feedback effects on plant nutrient supply and resource partitioning via the microbial community associated with roots (van der Heijden and others 2008; Friesen and others 2011). These plant–microbe interactions are particularly strong in oligotrophic ecosystems (Bardgett and others 1999; Paterson 2003; van der Heijden and others 2008), while nutrient enrichment weakens the co-determinacy of plants and microbes (Wei and others 2013; Liu and others 2014). Therefore, the type of management with its specific impacts on the above- and belowground compartments of the system exerts an important influence on the functioning and services provided by the ecosystem.

Traditional regular mowing is a convenient management system for maintaining the species richness of grasslands (for example, Silvertown and others 2006; Galvánek and Lepš 2008). Long-term hay harvesting affects nutrient availability by decreasing soil potassium (K) and nitrogen (N) contents (Venterink and others 2009) and increases C allocation belowground (Bardgett and others 1998; Kuzyakov and others 2002). Enhanced belowground C inputs commonly stimulate the biomass and activity of the soil biotic community (Guitian and Bardgett 2000; Uhlířová and others 2005; Zhang and others 2013) and affects N cycling within the ecosystem (Hamilton and Frank 2001; Kaštovská and others 2014). Grassland fertilization potentially has the opposite effects of mowing in many cases. Application of mineral N and phosphorus (P) improves forage production and its nutritional quality but commonly causes loss of plant species richness (Hejcman and others 2014; Lepš 2014). Direct impacts of fertilization on soil include acidification (Liu and others 2014), increased N (Bardgett and McAlister 1999), and decreased base cation availability (Clark and others 2007). These changes are usually accompanied by reduced soil microbial biomass (Treseder 2008; Sillen and Dieleman 2012) and shifts in microbial community composition (Rousk and others 2010a; Zhang and others 2013; Legay and others 2016) including decreased fungal abundance (Bardgett and McAlister 1999; Bååth and Anderson 2003; Treseder 2008). It has been suggested that simultaneous application of fertilization and mowing may partly balance the negative effects of fertilization on plant diversity in semi-natural meadows (Lepš 2014). Whether mowing also compensates for the negative fertilization effects belowground is, however, unknown.

Management effects on the structure and functioning of species-rich wet meadows are dependent on the temporal scale (Hedlund and others 2003; Lepš 2014). Short-term experiments might underestimate the response of vegetation and soil and could thus provide erroneous conservation recommendations. Therefore, long-term studies combining aboveground–belowground approach to study ecosystem responses to management practices are desirable, but rare. To increase our understanding of plant–microbe–soil linkages, we examined a wet temperate grassland subjected for 16 years to mowing, fertilization, and dominant plant species removal in a full-factorial design. Changes in plant community composition in this experiment have previously been described (Lepš 1999, 2004, 2014). We complemented the existing data by studying plant productivity, stoichiometry, and soil microbial and physico-chemical properties. Our aim was to document the adaptation of this grassland to long-term management and any coupling or uncoupling of above- and belowground responses. We hypothesized that (1) fertilization would exert the strongest influence on plant and soil properties due to its direct effect on nutrient availability and soil acidity; the influence of mowing would be less pronounced than the influence of fertilization and mediated through regular biomass removal and a concomitant decrease in nutrient availability; dominant removal would have the weakest effect on belowground characteristics. (2) Combined application of fertilization and mowing would balance the effects of the individual treatments. Fertilization of mown plots would replenish the nutrients withdrawn with the harvested biomass, while mowing would reduce the amount of available nutrients and concomitant soil acidification compared to plots that are only fertilized. We expected that mown fertilized plots would have

intermediate nutrient availability and pH compared to mown-only and fertilized-only plots. (3) The resulting changes in microbial biomass, activity, and community composition in response to management would be mainly driven by the treatment effects on SOM content, nutrient availability, and soil acidity.

Materials and Methods

Experimental Site and Sampling

The study site is a species-rich oligotrophic wet meadow situated in south Bohemia, Czech Republic (48°57′11″ N, 14°35′34″ E, at 510 m a.s.l.). Mean annual temperature and precipitation are 7.8°C and 620 mm, respectively (nearby meteorological station České Budějovice, 400 m a.s.l.). The meadow was traditionally mown once or twice a year until the end of the 1980s and then mowing was re-introduced at the start of the experiment in 1994. Before the treatments were imposed, the plant community was highly similar among plots and characterized as Molinion with some elements of Violion caninae (Chytrý 2012) (dominant species *Molinia caerulea* with 35% cover). The soil is a cambisol with pH 4.1 ± 0.02, total soil C (C_{tot}) and N (N_{tot}) concentrations of 28 ± 0.6 and 2.5 ± 0.05 mg g^{-1}, respectively, and a bulk density of 1.4 ± 0.05 g cm^{-3} (mean values ± SEM for the upper 20 cm of soil in mown, unfertilized, and non-removal plots).

The experiment combined mowing, fertilization, and dominant species removal in a full-factorial design giving eight treatment combinations, each in three replications (plots 2 m × 2 m square with central 1 m^2 used for sampling (see Lepš 1999, 2004 and Table 6 in Online Appendix A for more details). Briefly, fertilization was conducted using commercial NPK fertilizer (78 kg N ha^{-1} y^{-1}, 123 kg P ha^{-1} y^{-1}, and 123 kg K ha^{-1} y^{-1}) applied annually in spring. Mowing was performed annually in late June, and the harvested biomass was removed. *Molinia* individuals were manually removed in April 1995, new individuals were removed annually when needed. The soil samples were collected in October 2010 after plant senescence to eliminate the direct influence of root exudation on the soil microbial community and to assess only permanent changes in soil characteristics. From each of the 24 plots, composite samples were prepared from four subsamples gathered with a soil corer (3-cm diameter) to a depth of 20 cm. Fresh soil samples were homogenized and sieved through a 2-mm mesh. Sample aliquots for enzyme activities and PLFA analyses were stored at −80°C. Samples for the determination of available nutrients and microbial biomass and activity were stored at 4°C for a maximum of 14 days. Subsamples for CEC, C_{tot}, and N_{tot} determinations were air-dried.

Abiotic Soil Properties

The pH of fresh soil samples was measured in 1 M KCl with a pH meter (1:5, w:v; pH 315i, WTW, Germany). The cation exchange capacity (CEC) was determined as the sum of base cations (BC: Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) and exchangeable acidity (Al^{3+} and H^{+}). BC content in soil was measured by atomic absorption spectrophotometry (Varian, Australia) in a 1 M NH_4Cl extract, and Al^{3+} and H^{+} ions were extracted in 1 M KCl and determined by titration according to Thomas (1982). C_{tot} and N_{tot} were measured using an elemental analyzer (vario MICRO cube, Elementar GmbH, Germany); total soil P (P_{tot}) was determined according to Kopáček and others (2001). Soluble organic C and N (C_{ex} and N_{ex}), representing pools of dissolved organic matter available for microorganisms (Wagai and Sollins 2002), were extracted in duplicates from fresh soil (0.5 M K_2SO_4, 1:4, w/v; Rennert and others 2007) within 48 h after sampling and determined with a LiquiTOC II analyzer (Elementar, Germany). The concentrations of NH_4^+ and NO_3^- in sulfate extracts were determined with a Flow Injection Analyzer (FIA Lachat QC8500, Lachat Instruments, USA). Extractable P (P_{ex}) was determined in 0.5 M $NaHCO_3$ (1:5, w/v) and measured with an absorption spectrophotometer (Genesys 10S, UV–Vis, Thermo Scientific, USA).

Plant Biomass and Stoichiometry

To measure the amount of aboveground plant biomass, three non-contiguous 20 cm × 20 cm squares were cut with scissors on the edges of the central 1 m^2 of each plot in June 2010. The litter was separated first, and then the biomass was sorted into species, dried, and weighed. The biomass mean C, N, and P contents were calculated as community-weighted averages of individual species (see Lepš and others 2011 for detailed description and analysis).

Microbial Biomass and Activity

Microbial C (C_{mic}), N (N_{mic}), and P (P_{mic}) were determined by chloroform fumigation-extraction

using $k_{EC} = 0.45$ (Vance and others 1987), $k_{EN} = 0.54$ (Brookes and others 1985), and $k_{EP} = 0.4$ (Brookes and others 1982). Net N mineralization rate (sum of nitrification and ammonification; N_{min}) was measured as the difference in concentrations of NH_4^+ and NO_3^- that occurred between day 7 and day 21 of incubation at 20°C, divided by the number of days. C mineralization (C_{min}) was measured with a gas chromatograph (Agilent 6850 Series, Agilent, USA) as the increase in CO_2 concentration during 48 h of soil incubation at 20°C in bottles sealed with rubber covers.

To characterize the microbial potential to release C, N, and P from organic substrates, activities of six hydrolytic enzymes responsible for the acquisition of C (β-glucosidase, cellobiosidase), N (Ala-aminopeptidase, Leu-aminopeptidase), and P (phosphatase, phosphodiesterase) were determined (Bárta and others 2013; Sinsabaugh and others 2009). Fresh soil subsamples (1 g) were homogenized in distilled water (100 ml) and enzyme activities were measured with standard fluorometric techniques (Marx and others 2001). Based on the preliminary measurements of saturation concentration of each 4-methylumbelliferone/7-amino-4-methylcoumarin-labeled substrate, we used 50 µl of 50 µM substrate solutions for the determination of β-glucosidase and cellobiosidase activities, 200 µM substrate solution for determination of phosphatase activity and 300 µM substrate solutions for determination of phosphodiesterase, Ala- and Leu-aminopeptidase activities. All fluorescence measurements were carried out using the microplate reader INFINITE F200 (TECAN, Germany) at an excitation wavelength of 365 nm and emission wavelength of 450 nm.

Microbial Community Structure: Phospholipid Fatty Acid Profile

The determination of phospholipid fatty acids (PLFAs) was based on the method of Frostegård and others (1993). Briefly, 2 g of soil (dry weight) was extracted twice with a single-phase extraction mixture consisting of chloroform, methanol, and phosphate buffer and consequently purified on silica columns (SPE-SI 1 g/6 ml; Phenomenex®, CA, USA) using chloroform, acetone, and methanol. The polar fraction was trans-esterified to the fatty acid methyl esters (FAME) through mild alkaline methanolysis (Bossio and Scow 1998). All PLFAs were quantified by an internal standard calibration procedure using methyl nonadecanoate (19:0) as the internal standard and six calibration levels of the following FAME standards: Bacterial Acid Methyl Ester (BAME), the 37-component FAME Mix, PUFA-2 and PUFA-3 (Supelco, Bellefonte, USA), BR3 Mixture, methyl nonadecanoate, 10-methyloctadecanoic acid and methyl 9(R),10(S)-methyleneoctadecanoate (Larodan lipids, Malmö, Sweden). To identify the FAMEs, retention times and mass spectra were compared with those obtained from standards. The PLFA profile comprised 27 individual PLFAs and was used to compare the microbial community composition between experimental plots. The fatty acids i15:0, a15:0, i16:0, i17:0, a17:0, i18:0 were used as markers of gram-positive bacteria (*G*+) and 16:1n11, 16:1n9, 16:1n7, 16:1n5, cy17:0, 18:1n7, cy19:0 as markers of gram-negative bacteria (*G*−) (Kaiser and others 2010); 10Me16:0, 10Me17:0 were considered as actinobacterial (Kroppenstedt 1985) and 18:1n9, 18:2n6,9 as fungal markers (Frostegård and Bååth 1996). Total bacterial biomass is represented by the sum of general bacterial markers 15:0, 17:0, 18:1n5, 18:1n11 and markers for *G*+ and *G*− bacteria. The total amount of PLFAs was calculated as the sum of all lipid markers mentioned above and non-specific PLFAs 14:0, 16:0, 17:1n6, 18:0, 20:0 and 22:0 (Kaiser and others 2010). The fungi-to-bacteria ratio (F/B) was calculated from the respective sums of the above bacterial and fungal PLFA markers. The PLFA profiles were compared as mol% of PLFA for detection of structural changes in the microbial community.

Statistical Analysis

We measured numerous biotic and abiotic soil variables, some of which were significantly correlated. To achieve the best statistical power while keeping the global Type I error low, we carried out separate multivariate analyses for logical groups of variables, allowing us to describe the treatment effect on (a) soil physico-chemical properties, (b) plant biomass and stoichiometry, (c) plant community composition, (d) microbial activity, (e) microbial biomass and stoichiometry, (f) microbial community structure (the PLFA profile), and (g) activity of C, N, and P hydrolyzing enzymes. We used Redundancy Analysis (RDA) to test the effect of all treatments together and partial RDA to test three main effects (that is, the significance of fertilization, mowing or dominant removal using the remaining treatments as covariates) without standardization by samples, centering and standardization by variables (because the variables were not always measured at the same scale, see Šmilauer and Lepš 2014) and a Monte Carlo simulation with 4999 permutations. We then examined each variable separately with three-way ANOVA with

interactions, followed by Tukey HSD tests. Principal component analyses (PCA) on the physico-chemical properties, plant community composition, and PLFA profile were used to identify particular trends connected with the applied treatments. To find the best candidates among soil physico-chemical properties for explaining shifts in the PLFA profile, RDA with forward selection of explanatory variables was used. We further correlated the sample scores of the first three principal components of PCA on the microbial PLFA composition and plant community composition (see Table 8 in Online Appendix C) to determine whether the microbial PLFA profiles were related to plant community composition. Consequently, the decomposition of explained variation (Šmilauer and Lepš 2014) was used on selected parameters to quantify their unique and shared effects on the PLFA profile.

Before applying the statistical analyses, data were checked for normality and log-transformed if necessary. All C:N:P stoichiometric ratios were calculated on a molar basis. Statistical tests with a *P* value lower than 0.05 were considered significant. Multivariate statistical analyses were performed with CANOCO for Windows version 5.0 (Ter Braak and Šmilauer 2012). ANOVA and Tukey HSD tests were performed with Statistica 10 (StatSoft, USA).

Results

Comparison of Treatment Effects on Ecosystem Properties

Annual spring application of mineral NPK fertilizer and regular mowing once a year were associated with significant shifts in ecosystem properties (Table 1). Both treatments significantly affected plant community composition, productivity and biomass C:N:P stoichiometry, with the largest proportion of data variability explained by fertilization. Belowground, the soil physico-chemical properties and mineralization rates were significantly influenced, again more by fertilization than by mowing (Table 1), but the microbial biomass and its C:N:P stoichiometry remained unchanged. Fertilization was the only treatment that affected the microbial PLFA profile. Removal of the dominant plant species explained only a small fraction of the variability in plant and soil data (Table 1) and its effect on the examined ecosystem parameters was not significant (shown by three-way ANOVAs with interactions). Therefore, we present below the results of two-way ANOVAs, comprising the effects of fertilization, mowing, and their interactions.

Influence of Treatments on Soil Physico-Chemical Properties

Fertilization significantly acidified soil and decreased soil CEC and C_{tot} content, while significantly increasing soil P_{tot} and P_{ex}. It also lowered C/P and N/P ratios of both bulk SOM and the pools of available nutrients (Table 2). The pronounced fertilization effect is shown on the PCA ordination diagram, separating unfertilized (left) from fertilized (right) plots along the first principal component (PC1) (Figure 1). Unfertilized plots were further separated along the second principal component (PC2) according to the presence or absence

Table 1. The Response of Grouped Plant, Soil Physico-Chemical and Microbial Variables to Experimental Treatments

Group of response variables	Percentage of variation explained by all treatments	Percentage of overall explained variation explained by individual treatments		
		Fertilization	Mowing	Dominant removal
Soil physico-chemical properties	**42.4*****	**68.7*****	**26.7****	4.6
Plant biomass and stoichiometry	**56.4*****	**54.3*****	**37.7*****	8.0
Plant community composition	**30.8*****	**57.6*****	**38.5*****	3.9
C and net N mineralization	**31.1***	**55.1***	**44.7***	0.2
Microbial biomass and stoichiometry	7.7	75.3	24.7	0.0
Microbial community composition—PLFA profile	17.2	**59.1***	27.3	13.6
Activity of C, N, P hydrolyzing enzymes	12.2	30.9	48.8	20.3

The figures shown are proportions of data variability explained by all treatments together and by individual treatments separately (that is, using the remaining two treatments as covariates; values represent the portion of total explained variability). Results of RDA.
*Statistically significant tests are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001.*

Table 2. Soil Physico-Chemical Characteristics (pH, CEC, Contents of C, N, and P and Their Molar Ratios), Contents and Molar Ratios of Extractable C, N, and P Pools and Contents of NO_3^-, NH_4^+ in Plots Under Different Experimental Regimes

Treatment F	Treatment M	pH	CEC (meq g^{-1})	C_{tot} (mg g^{-1})	N_{tot} (mg g^{-1})	P_{tot} (mg g^{-1})	C_{tot}/N_{tot}	C_{tot}/P_{tot}	N_{tot}/P_{tot}
No	No	$^{b}4.0 \pm 0.03$	$^{b}63 \pm 2.8$	$^{b}29 \pm 0.9$	$^{b}2.6 \pm 0.1$	$^{a}0.48 \pm 0.02$	$^{a}13.3 \pm 0.2$	$^{c}157 \pm 9.2$	$^{b}12.1 \pm 0.7$
No	Yes	$^{b}4.1 \pm 0.02$	$^{b}66 \pm 4.2$	$^{b}30 \pm 1.0$	$^{b}2.6 \pm 0.1$	$^{b}0.58 \pm 0.03$	$^{a}13.7 \pm 0.2$	$^{bc}135 \pm 4.3$	$^{a}9.9 \pm 0.3$
Yes	No	$^{a}3.9 \pm 0.03$	$^{a}53 \pm 4.5$	$^{a}27 \pm 0.7$	$^{a}2.4 \pm 0.1$	$^{b}0.62 \pm 0.02$	$^{a}13.2 \pm 0.3$	$^{ab}113 \pm 4.9$	$^{a}8.6 \pm 0.3$
Yes	Yes	$^{a}3.9 \pm 0.02$	$^{a}53 \pm 2.1$	$^{a}26 \pm 1.3$	$^{a}2.3 \pm 0.1$	$^{b}0.64 \pm 0.03$	$^{a}13.2 \pm 0.2$	$^{a}106 \pm 4.5$	$^{a}8.1 \pm 0.4$

Treatment F	Treatment M	C_{ex} (μg g^{-1})	N_{ex} (μg g^{-1})	P_{ex} (μg g^{-1})	C_{ex}/N_{ex}	C_{ex}/P_{ex}	N_{ex}/P_{ex}	NO_3^- (μg g^{-1})	NH_4^+ (μg g^{-1})
No	No	$^{a}86 \pm 8.0$	$^{b}36 \pm 3.1$	$^{a}2.6 \pm 0.2$	$^{a}2.8 \pm 0.2$	$^{b}92 \pm 14$	$^{b}33 \pm 4.3$	$^{b}0.43 \pm 0.03$	$^{a}3.8 \pm 0.1$
No	Yes	$^{a}80 \pm 4.5$	$^{a}22 \pm 1.5$	$^{a}1.5 \pm 0.2$	$^{b}4.3 \pm 0.3$	$^{c}153 \pm 18$	$^{b}38 \pm 6.7$	$^{a}0.31 \pm 0.07$	$^{a}3.6 \pm 0.2$
Yes	No	$^{a}75 \pm 3.3$	$^{b}33 \pm 3.0$	$^{b}11.9 \pm 1.8$	$^{a}2.7 \pm 0.2$	$^{a}18 \pm 2$	$^{a}6.5 \pm 0.6$	$^{b}0.38 \pm 0.05$	$^{a}3.7 \pm 0.3$
Yes	Yes	$^{a}80 \pm 4.9$	$^{ab}30 \pm 2.2$	$^{b}13.4 \pm 1.3$	$^{a}3.2 \pm 0.2$	$^{a}16 \pm 2$	$^{a}5.2 \pm 0.6$	$^{a}0.24 \pm 0.03$	$^{a}3.2 \pm 0.3$

Treatment	d.f.	pH	CEC (meq g^{-1})	C_{tot} (mg g^{-1})	N_{tot} (mg g^{-1})	P_{tot} (mg g^{-1})	C_{tot}/N_{tot}	C_{tot}/P_{tot}	N_{tot}/P_{tot}
F	1	**20.03****	**8.9***	**8.43***	4.64	**12.93***	0.55	**30.65*****	**27.39*****
M	1	0.50	0.20	0.01	0.22	4.66	2.15	5.11	**7.42***
F × M	1	0.58	0.14	1.08	0.05	2.43	1.78	1.30	2.89

Treatment	d.f.	C_{ex} (μg g^{-1})	N_{ex} (μg g^{-1})	P_{ex} (μg g^{-1})	C_{ex}/N_{ex}	C_{ex}/P_{ex}	N_{ex}/P_{ex}	NO_3^- (μg g^{-1})	NH_4^+ (μg g^{-1})
F	1	0.91	0.69	**78.94*****	7.18	**69.66*****	**45.51*****	1.35	1.12
M	1	0.01	**9.16***	0.02	**17.11****	**5.71***	0.23	**6.48***	2.87
F × M	1	0.87	3.77	1.11	**5.25***	**6.36***	0.62	0.05	0.39

Results of two-way ANOVAs (F values) of the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns (P < 0.05). Statistically significant differences are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001 (lower part of the table).*

of mowing (Figure 1; left part of the diagram), pointing to the significant interaction of fertilization and mowing. Mowing of unfertilized plots significantly lowered N_{ex} and increased the C_{ex}/N_{ex} and C_{ex}/P_{ex} ratios (Table 2; Figure 1), but this negative effect of mowing on N_{ex} and P_{ex} was compensated when mown plots were fertilized. The fertilized mown and fertilized unmown plots thus overlaid in the right part of the PCA diagram (Figure 1). The soil NO_3^- content was reduced by mowing, regardless of fertilization (Table 2).

Plant Response to Treatments

Fertilization stimulated aboveground plant productivity, while mowing decreased the amount of litter on experimental plots due to biomass removal (Table 3). Mowing was the only management, which significantly affected plant N content. It decreased N concentration in plant biomass, with concomitant increase of plant C/N ratios independent of fertilization (Table 3). Mowing and fertilization further significantly interacted in their effects on plant stoichiometry. Fertilization of unmown plots increased plant N and P concentrations compared to unfertilized plots; therefore, vegetation in fertilized unmown plots had the lowest C/N ratio from all treatments and lower C/P and N/P ratios than non-fertilized plants. Fertilization combined with mowing lowered plant C content and increased plant P but not N content, resulting in the lowest plant biomass N/P ratio of all treatments. The highest C/P ratio was determined in plant biomass from unfertilized mown plots (Table 4).

Microbial Response to Treatments

Neither the microbial biomass nor the microbial C:N:P stoichiometry reflected significant changes in the elemental stoichiometry of SOM, available nutrients, and plant biomass caused by the treatments. Among other microbial variables, only C mineralization rate decreased in fertilized versus unfertilized plots. Mowing strongly decreased net N mineralization rate in unfertilized plots, but this was balanced by fertilization of mown plots (Table 5). Generally, the net N mineralization was negatively correlated with the soil C_{ex}/N_{ex} ratio ($r = -0.69$, $P < 0.001$) and positively related with soil N_{ex} and NH_4^+ contents ($r = 0.62$ and 0.43, $P = 0.001$ and 0.035, respectively). The potential activities of extracellular enzymes for the acquisition of C, N, and P were spatially variable and not significantly influenced by the management interventions.

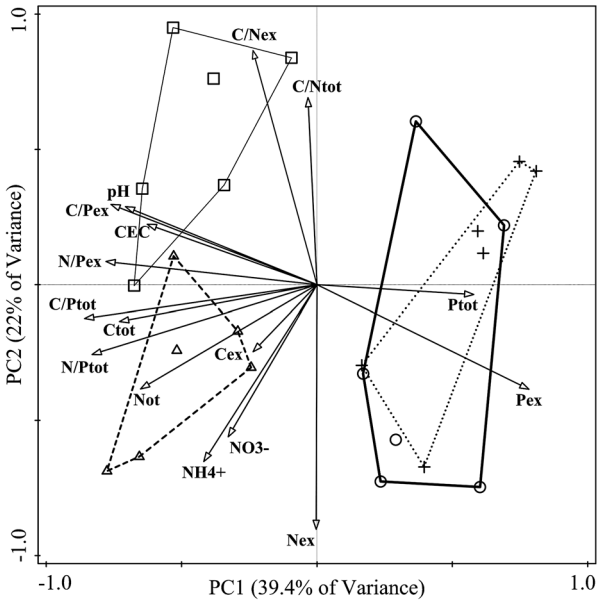

Figure 1. Separation of treated plots by the PCA based on soil physico-chemical properties. The *thin solid* and *dashed lines* represent unfertilized mown (*square*) and unmown (*triangle*) plots, the *bold solid* and *dotted lines* represent fertilized unmown (*circle*) and mown (*plus*) plots, respectively ($n = 6$). The portion of variance explained by the respective axis is given in the axis title.

The relative abundance of *G*+ and *G*− bacteria, actinobacteria, and fungi within microbial community as well as the F/B ratio was unaffected. All experimental manipulations accounted only for 17.2% of the variation in the microbial PLFA profile and their combined effect was not significant (Table 1; the alignment of PLFAs with experimental manipulations shown in Figure 3 in Online Appendix D). Therefore, the first two PCA components of the microbial PLFA composition did not depict any trends connected with the treatments (PC1 and PC2 accounted for 34.6 and 18.7% of variation, respectively; see Table 8 in Online Appendix C). However, the third principal component (PC3, 13.8% of data variation) significantly separated microbial PLFA profiles in fertilized and unfertilized plots (Figure 2A), which corresponds with a significant fertilization effect on the microbial PLFA profile (Table 1, results of the partial

Table 3. Plant Aboveground Productivity, Litter Amount, and Plant Biomass C, N, P Stoichiometry in Plots Under Different Experimental Regimes

Treatment F	Treatment M	Productivity (g m^{-2} DW)	Litter (g m^{-2} DW)	C (mg g^{-1} DW)	N (mg g^{-1} DW)	P (mg g^{-1} DW)	C:N	C:P	N:P
No	No	$^{b}414 \pm 52$	$^{b}650 \pm 134$	$^{b}435 \pm 2$	$^{a}20.1 \pm 0.4$	$^{a}1.9 \pm 0.1$	$^{b}25.4 \pm 0.4$	$^{b}611 \pm 25$	$^{c}24.1 \pm 1.1$
No	Yes	$^{a}270 \pm 22$	$^{a}134 \pm 18$	$^{b}436 \pm 2$	$^{a}18.3 \pm 0.4$	$^{a}1.4 \pm 0.0$	$^{b}27.9 \pm 0.5$	$^{c}788 \pm 12$	$^{c}28.3 \pm 0.6$
Yes	No	$^{b}504 \pm 47$	$^{b}735 \pm 154$	$^{b}438 \pm 1$	$^{b}23.3 \pm 1.0$	$^{b}2.9 \pm 0.2$	$^{a}22.2 \pm 1.0$	$^{a}407 \pm 31$	$^{b}18.5 \pm 1.6$
Yes	Yes	$^{b}500 \pm 50$	$^{ab}299 \pm 74$	$^{a}427 \pm 2$	$^{a}18.7 \pm 0.7$	$^{c}3.3 \pm 0.1$	$^{b}26.9 \pm 0.9$	$^{a}335 \pm 11$	$^{a}12.5 \pm 0.7$

Treatment	d.f.	Productivity (g m^{-2} DW)	Litter (g m^{-2} DW)	C (mg g^{-1} DW)	N (mg g^{-1} DW)	P (mg g^{-1} DW)	C:N	C:P	N:P
F	1	**10.5****	1.10	3.03	5.91	**136.41*****	**6.86***	**194.61*****	**84.77*****
M	1	2.25	**15.88****	**7.53***	**18.16****	0.01	**19.99*****	4.90	0.60
F × M	1	2.02	0.11	**10.99****	3.65	**12.74****	1.81	**28.24*****	**19.22*****

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns (P < 0.05). Statistically significant differences are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001 (lower part of the table).*

Table 4. Microbial Biomass C, N, P Stoichiometry and Soil PLFA Content in Plots Under Different Experimental Regimes

Treatment F	Treatment M	C_{mic} (μg g^{-1})	N_{mic} (μg g^{-1})	P_{mic} (μg g^{-1})	C_{mic}/N_{mic}	C_{mic}/P_{mic}	N_{mic}/P_{mic}	PLFAtot (nmol g^{-1} DW)
No	No	$^{a}896 \pm 41$	$^{a}113 \pm 8$	$^{a}44 \pm 6$	$^{a}9.4 \pm 0.4$	$^{a}56.5 \pm 5.3$	$^{a}6.2 \pm 0.7$	$^{a}152 \pm 8$
No	Yes	$^{a}1003 \pm 74$	$^{a}139 \pm 11$	$^{a}45 \pm 4$	$^{a}8.5 \pm 0.5$	$^{a}60.3 \pm 5.9$	$^{a}7.3 \pm 1.0$	$^{a}157 \pm 14$
Yes	No	$^{a}822 \pm 47$	$^{a}104 \pm 14$	$^{a}35 \pm 2$	$^{a}10.1 \pm 1.3$	$^{a}62.0 \pm 4.2$	$^{a}6.6 \pm 0.9$	$^{a}148 \pm 14$
Yes	Yes	$^{a}827 \pm 70$	$^{a}102 \pm 15$	$^{a}42 \pm 5$	$^{a}10.1 \pm 0.8$	$^{a}52.5 \pm 3.0$	$^{a}5.3 \pm 0.3$	$^{a}139 \pm 13$

Treatment	d.f.	C_{mic} (μg g^{-1})	N_{mic} (μg g^{-1})	P_{mic} (μg g^{-1})	C_{mic}/N_{mic}	C_{mic}/P_{mic}	N_{mic}/P_{mic}	PLFAtot (nmol g^{-1} DW)
F	1	3.69	2.77	1.45	1.65	0.05	0.86	0.01
M	1	0.72	0.76	0.72	0.23	0.31	0.01	0.64
F x M	1	0.60	1.03	0.38	0.20	1.65	2.25	0.12

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns (P < 0.05). Statistically significant differences are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001 (lower part of the table).*

Table 5. The C and Net N Mineralization Rates and Activities of C, N, P Hydrolyzing Enzymes in Plots Under Different Experimental Regimes

Treatment F	Treatment M	C mineralization (μg C-CO_2 g^{-1} day^{-1})	Net N mineralization (μg N-NH_4^+ + N-NO_3^- g^{-1} day^{-1})	C hydrolysing enzymes (nmol g^{-1} h^{-1})	N hydrolysing enzymes (nmol g^{-1} h^{-1})	P hydrolysing enzymes (nmol g^{-1} h^{-1})
No	No	$^{ab}7.9 \pm 0.5$	$^{b}0.184 \pm 0.035$	$^{a}191 \pm 46$	$^{a}107 \pm 13.0$	$^{a}548 \pm 90$
No	Yes	$^{b}8.8 \pm 0.4$	$^{a}0.051 \pm 0.013$	$^{b}426 \pm 119$	$^{a}168 \pm 29.9$	$^{a}794 \pm 94$
Yes	No	$^{a}7.2 \pm 0.3$	$^{b}0.223 \pm 0.040$	$^{b}458 \pm 159$	$^{a}121 \pm 19.9$	$^{a}971 \pm 323$
Yes	Yes	$^{a}7.3 \pm 0.5$	$^{b}0.167 \pm 0.028$	$^{b}448 \pm 106$	$^{a}158 \pm 29.8$	$^{a}798 \pm 46$

Treatment	d.f.	C mineralization (μg C-CO_2 g^{-1} day^{-1})	Net N mineralization (μg N-NH_4^+ + N-NO_3^- g^{-1} day^{-1})	C hydrolysing enzymes (nmol g h^{-1})	N hydrolysing enzymes (nmol g h^{-1})	P hydrolysing enzymes (nmol g h^{-1})
F	1	**5.61***	**5.22***	1.31	0.01	1.23
M	1	1.19	**7.82***	0.79	3.45	0.04
F × M	1	0.73	1.33	0.94	0.21	1.19

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns (P < 0.05). Statistically significant differences are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001 (lower part of the table)*

RDA). Loadings of individual PLFAs revealed that particularly the bacterial markers were aligned along the PC3 (Figure 2B). The fungal PLFAs 18:2n6,9 and 18:1n9 were among the specific markers least correlated with PC3 and thus their abundances were not directly associated with experimental manipulations and changes in soil properties.

The RDA with forward selection of explanatory physico-chemical soil variables highlighted the influence of soil acidification and C/P_{ex} ratio on the PLFA profile ($P = 0.004$ and 0.015, respectively). These parameters affected mainly abundances of bacterial and actinobacterial PLFAs—relative abundance of markers 16:1n9 and 10Me17:0 significantly increased in acidified plots, while the bacterial PLFAs a15:0, i17:0, 16:1n5, cy17:0, and 18:1n7 were more abundant in unfertilized plots with higher soil pH. The PLFAs showing a relative increase with higher C/P_{ex} ratios were bacterial

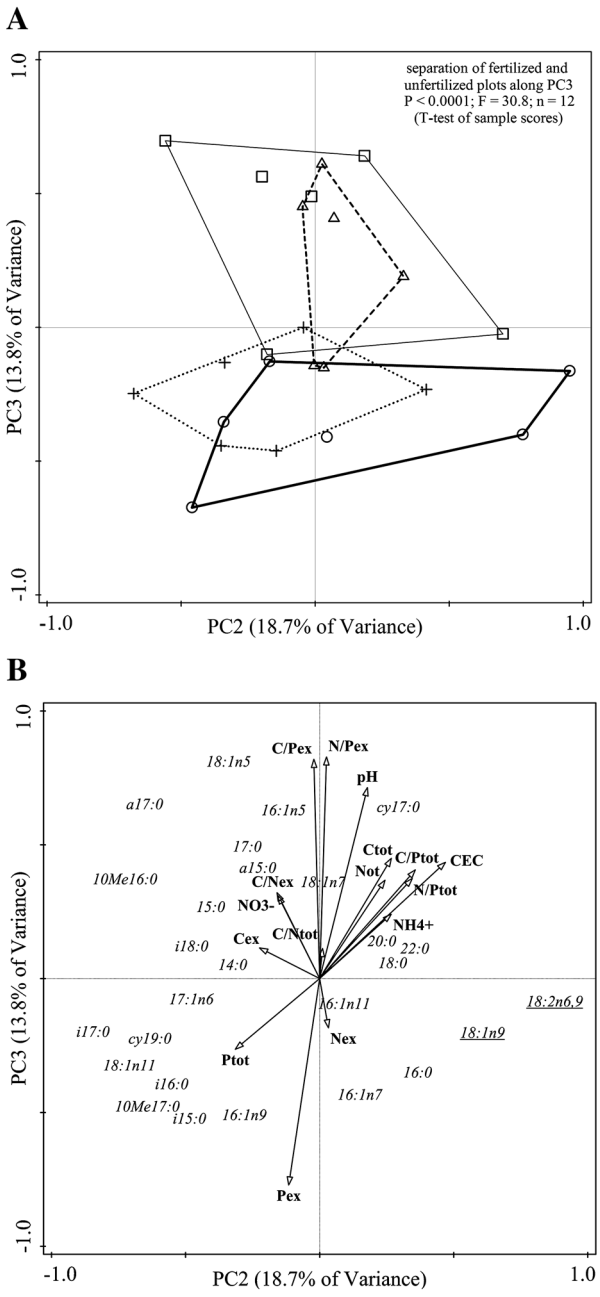

Figure 2. Sample scores on second and third axes from a PCA on the microbial PLFA composition (**A**) and loading of individual PLFAs (*italics*) together with loading of physico-chemical soil properties (*bold*) (**B**). Both figures display the same ordination space. The *thin solid* and *dashed lines* represent unfertilized mown (*square*) and unmown (*triangle*) plots, the *bold solid* and *dotted lines* represent fertilized unmown (*circle*) and mown (*plus*) plots, respectively ($n = 6$; **A**). *Arrows* indicate the direction in which the respective soil parameter value increases. Fungal PLFAs are underlined (**B**). The portion of variance explained by the respective axis is given in the axis title.

markers 16:1n5, a17:0, 17:0, and 18:1n5, whereas i15:0 and 16:1n7 were more abundant in fertilized soils with lower C/P_{ex} ratios (see Table 7 in Online Appendix B).

Microbial PLFA profiles were also related to plant community composition. This was indicated by significant correlations between sample scores on PC1 and PC3 of the microbial PLFA profiles and the corresponding sample scores on PC3 and PC1 of the plant community composition ($r = 0.6$ and 0.69, $P = 0.027$ and 0.003, respectively). The markers responsive to altered plant community composition were saturated, mainly non-specific PLFAs (15:0, 16:0, 17:0, 18:0, 20:0, 22:0), both fungal (18:1n9, 18:2n6,9) and bacterial PLFAs (16:1n5, 18:1n7, a15:0, a17:0) (see Table 7 in Online Appendix B). The plant community PC1 and PC3 together with soil pH and C/P_{ex} explained 22% of the variability in the PLFA profiles ($P < 0.001$). The results from variation partitioning show that 7.5% of the total variation in the PLFA profiles was explained by pH and the C/P_{ex} ratio ($P = 0.017$) and 5.5% solely by PC1 and PC3 of the plant community composition ($P = 0.034$). The rest of the explained variability (9%) was shared by these variables.

Discussion

Fertilization Effects on Vegetation, Soil, and Microbial Characteristics

In agreement with our first hypothesis, regular spring application of NPK fertilizer (78 kg N ha^{-1} y^{-1}, 123 kg P ha^{-1} y^{-1} and 123 kg K ha^{-1} y^{-1}) for 16 years exerted the strongest influence on both above- and belowground ecosystem characteristics of all treatments. Long-term fertilization brought about negative changes in basic soil physico-chemical properties such as decreased soil pH and CEC, as commonly reported in grassland fertilization experiments (for example, Silvertown and others 2006; Clark and others 2007; Liu and others 2014), and increased nutrient availability in the soil. This was reflected aboveground in the vegetation composition, where nutrient loading increased the abundance of competitive species like *Scirpus sylvaticus* L. and nitrophilous *Urtica dioica* L. in unmown plots, and of grasses (for example, *Agrostis capillaris* L., *A. canina* L., *Festuca rubra* L.) in mown plots. These plants outcompeted other species, including the initially dominant *Molinia*, and caused a sharp decrease of species richness (see Lepš 1999, 2004, 2014 for details).

Even though the new plant communities were more productive, the amount of soil C_{tot} decreased, indicating an overall loss of SOM. The only element found in greater amounts in the fertilized soil was P, but its presence mainly in exchangeable forms indicated physico-chemical sorption rather than incorporation into stabilized SOM. Our data showing a negative fertilization effect on SOM content are in agreement with the results of a meta-analysis of fertilization effects on grasslands by Sillen and Dieleman (2012). However, other studies have shown varying effects of N fertilization on SOM content, from the absence of any significant change in C content in mineral soils across biomes (Liu and Greaver 2010) to an increase in soil C sequestration (Conant and others 2001; Rousk and others 2011). The ambiguous image arising from these studies indicates that the fertilization effect on SOM content depends on many factors, such as the fertilization dose, the type of fertilizer, the longevity of fertilization, the ecosystem type, and its initial conditions. While an increase in SOM after grassland fertilization is commonly explained by enhanced input of organic matter due to increased primary production (Nyborg and others 1997; Rousk and others 2011), a decrease in SOM used to be ascribed to an enhanced decomposition of new, nutrient-rich organic matter and its priming effect on the old SOM (Bengston and others 2012; Kuzyakov 2010) as well as a declining plant belowground allocation (Bardgett and others 1999; Eisenhauer and others 2012). In our case, exoenzymatic activity and microbial biomass were not changed and microbial respiration even decreased, which indicated a decrease rather than an increase in potential SOM decomposition after 16 years of NPK fertilization. We thus suggest that the decrease in soil C_{tot} should be ascribed to lower root production, which is commonly observed after fertilization (Bardgett and others 1999; Eisenhauer and others 2012; Kaštovská and others 2012). The significantly increased aboveground productivity could not avert the loss of SOM, because the aboveground litter did not significantly contribute to soil C sequestration in this grassland. This was shown by the absence of differences in soil C_{tot} contents between mown plots, where the aboveground biomass is removed, and unmown plots with significantly more litter left on site (see Tables 2, 3), independent of fertilization. A likely explanation is the significantly faster decomposition of aboveground litter compared with belowground litter (Freschet and others 2013). Our results show that the effect of long-term fertilization on SOM content is a function of belowground rather than aboveground plant production. These findings are in line with the prominent role of belowground plant production in SOM formation suggested by Freschet and others (2013).

We further hypothesized that the resulting changes in microbial biomass and activity in response to management would be mainly driven by the treatment effects on SOM content and nutrient availability (Hypothesis 3). This was supported by the significant correlations across treatments between the SOM content and microbial biomass C as well as C mineralization ($r = 0.754$ and 0.674, respectively, both $P < 0.0001$). Similar relationships were found in other fertilization studies (Treseder 2008; Sillen and Dieleman 2012). Furthermore, C mineralization was also controlled by the relative C availability, as shown by its significant correlations with C_{ex}/N_{ex} and C_{ex}/P_{ex} ratios ($r = 0.512$ and 0.433, $P = 0.011$ and 0.035, respectively). Across all plots, C mineralization decreased together with decreasing availability of C relative to N and P, whereas absolute C availability (C_{ex} content) was not affected. This decrease cannot be ascribed to more efficient microbial metabolism when nutrient availability is higher, as suggested, for example by Agren and others (2001), because the ratios of mineralized to total C and mineralized to microbial C did not significantly change (data not shown). Instead, we conclude that long-term nutrient loading decreased C availability relative to N and P and thus strengthened the C limitation of microbial metabolism, as suggested by Kamble and others (2013). In summary, we suggest that fertilization significantly not only decreased the inputs of belowground plant litter to the soil but also decreased mineralization of preexisting SOM due to a stronger C limitation of microbial activity. In the long-term, however, the lower microbial activity could not compensate for the lower input of root-derived compounds, which resulted in reduced C sequestration in the soil of this grassland.

Mowing Effects on Vegetation, Soil, and Microbial Characteristics

In agreement with our first hypothesis, mowing had a weaker influence on ecosystem properties than fertilization. In contrast to fertilization, mowing increased plant species richness and spatial homogeneity of species composition (Lepš 2014). The long-term annual removal of aboveground plant biomass decreased soil nutrient availability compared with soil from unmown and fertilized

grasslands, which corroborates our hypotheses. This was indicated by lower availability of N and P relative to C, lower concentration of soil-available N (both N_{ex} and NO_3^-) and increased C/N and C/P ratios in plant biomass in comparison with unmown plots. Nutrient depletion was further indicated by significantly decreased plant aboveground productivity in plots that were only mown. Besides soil N availability, net N mineralization also significantly decreased in mown plots, suggesting more efficient N recycling between plants and microbes (Kaštovská and others 2014) and thus tighter plant–microbe interactions in the mown grassland (Bardgett and others 1999; Paterson 2003; van der Heijden and others 2008).

Some grassland studies have reported increased C allocation belowground and stimulation of soil microbial biomass and activity following mowing (Bardgett and others 1998; Guitian and Bardgett 2000; Uhlířová and others 2005; Zhang and others 2013). We found no permanent mowing effect on the biomass or activity of microbes in this long-term experiment, although such an effect could potentially be considerable in the short term following plant defoliation. In summary, mowing (on its own) decreased nutrient availability in the system, leading to more closely coupled plant–microbe relations driving a more efficient N cycling. These changes did not affect soil microbial biomass and its activity and had no effect on the SOM content in the long term. Considering the definitely positive effects on plant community composition and diversity, annual mowing is the most convenient management for sustaining species-rich wet grasslands.

Combined Effects of Mowing and Fertilization

It was recently shown that mowing can partially prevent the decline of plant species richness caused by fertilization (Lepš 2014). This led us to formulate Hypothesis 2 that combination of mowing and fertilization could compensate for a slow depletion of soil nutrients connected with long-term biomass removal (Venterink and others 2009). In support of our hypothesis, we found that fertilization of mown plots enhanced relative nutrient availability, as evidenced by significantly lower C/N and C/P ratios of soil extractable pools. The avoidance of nutrient depletion was further documented by significantly higher plant aboveground productivity and strongly increased plant P content in comparison with plots that were only mown. We also documented a positive effect of mowing on N recycling within the fertilized system, similar to that described above in mown-only plots. In plots under combined management, mowing decreased soil nitrate concentration as compared with fertilized-only plots and thus decreased the potential for N losses from the fertilized grassland.

However, mowing did not counterbalance the soil acidification and decrease in CEC directly connected with the application of mineral NPK fertilizer. Mowing also did not change the trend of decreasing SOM content and concomitant decrease in C mineralization and soil microbial biomass. These soil characteristics are among soil attributes adversely connected with maintaining soil quality and sustainable ecosystem productivity (Tóth 2008). Therefore, we summarize that mowing combined with annual application of NPK fertilizer at the given dose compensated only for nutrient deficiency caused by long-term grassland mowing but did not balance out negative changes in the soil caused by fertilization. Combination of these two management forms thus does not preserve soil functioning and the existing diversity of plant community in the longer term.

Effects of Dominant Species Removal on Vegetation and Soil Characteristics

The removal of *Molinia caerulea* had a positive effect on plant species richness, but the changes were much less pronounced than the effects of fertilization and mowing (Lepš 2014). However, *Molinia* removal did not cause any significant changes belowground. The evaluation of removal effects on soil properties was further complicated by the fact that *Molinia* was found to retreat spontaneously after fertilization and mowing, being replaced by other species in a way similar to the plots where it was selectively removed (Lepš 2004). Even though *s*tudies of microcosms (for example, Bardgett and others 1999; Kaštovská and others 2014; Legay and others 2016) and complex plant communities (Lange and others 2015) demonstrated regulation of microbial abundance and activity as well as C and N turnover by plant species traits, we found no evidence that removal of a single plant species, although dominant, induced a permanent effect on the soil environment in species-rich grassland.

Shifts in Microbial Community Composition as an Adaptation to Soil and Vegetation Changes

Microbial community composition was significantly affected only by NPK fertilization. The

changes we found within PLFA profiles were related mainly to soil acidification and decreasing C/P ratio of the soil solution, which corroborates with our third hypothesis. Soil pH was previously noted as a key factor driving microbial community composition (Fierer and others 2009; Rousk and others 2010a,b; Rousk and others 2011) and fungi-to-bacteria ratio (Bååth and Anderson 2003). In agreement with this, soil acidification significantly altered the microbial PLFA composition even though the overall pH range in our experiment stretched only from 4.18 to 3.85. These findings emphasize the need to consider carefully even very slight changes of soil pH when evaluating fertilization effects on microbial community composition. In accordance with observations by Bååth and Anderson (2003) and Rousk and others (2010a), only PLFA specific to bacteria were sensitive to changes in soil physico-chemical properties (see Table 7 in Online Appendix B), but these changes within bacterial PLFA had no effect on the fungi-to-bacteria ratio. High sensitivity of bacteria to soil pH was previously demonstrated also by gene-based molecular techniques (Fierer and others 2009; Rousk and others 2010a) and can be ascribed to the narrow pH ranges for optimal growth of bacteria (Fernández-Calviño and Bååth 2010). The specific bacterial PLFAs 18:1n7, 16:1n7, 16:1n5, cy19:0, and i16:0 have been noted previously as sensitive indicators of direct pH effects on the microbial community (Bååth and Anderson 2003; Rousk and others 2010b; Rousk and others 2011). Among those, only 18:1n7 and 16:1n5 responded significantly to soil pH changes in our study and we can thus recommend them as widely applicable sensitive indicators of the microbial response to changing pH. The likely explanation of the relatively weak response could be the narrow range of acidification in our experiment.

Shift in microbial PLFA profiles was not only related to soil pH and soil solution C/P ratio but also to plant community composition. The proportions of variability in the PLFA profiles explained by soil characteristics (7.5%) and by altered plant community composition (5.5%) were comparable. However, unlike the pH-related changes, shifts in vegetation composition affected the whole PLFA profile, including specific bacterial and fungal markers and many non-specific PLFAs, with the only exception of actinobacterial markers (see Table 7 in Online Appendix B). Such a widespread response of the microbial community to shifts in plant species composition is likely connected with potential variation in the quantity and quality of biochemical compounds provided by different plants (De Deyn and others 2008) and varying responses of different microorganisms to the altered plant inputs into the soil (Orwin and others 2010). The relation between plant and microbial community compositions indicates mutual adaptation of plant and microbial communities to particular grassland management types and supports the suggestion of specific plant–microbe interactions (for example, Bardgett and others 1999; Hartmann and others 2009; Orwin and others 2010; Doornbos and others 2012).

Conclusions

Long-term annual NPK fertilization of oligotrophic species-rich grasslands had a negative effect on the whole system. Aboveground, plant species richness decreased, and belowground, the soil was acidified and depleted of cations, and although C mineralization decreased, total SOM storage was reduced. Mowing as the single form of management has the potential to preserve plant diversity and decrease nutrient losses through microbial activity and leaching. Management combining fertilization with mowing effectively maintained the high plant species richness only in the short term. In the long term, it significantly reduced N leaching from the system but did not prevent SOM depletion, acidification and concomitant adverse effects on soil microbes. Our results show that making decisions about sustainable grassland management should be based on long-term studies and take into consideration responses above as well as belowground. Here, our results indicate that belowground plant production, driving an input of organic matter directly into the soil profile, had a more important role in affecting soil C storage in the long term than aboveground plant production. The conservation efforts on species-rich oligotrophic meadows should target extensive use by low-frequency mowing but the concomitant nutrient depletion should not be compensated for by regular NPK fertilization due to its adverse effects on soil quality.

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Appendix A

See Table 6.

Table 6. Spatial Arrangement of Experimental Plots

1 mown removed	2	3 mown removed	4
5 removed	6 mown	7 removed	8 mown
9 mown	10 removed	11 mown	12 removed
13	14 mown removed	15	16 mown removed
17 removed	18 mown	19 removed	20 mown
21 mown removed	22	23 mown removed	24

Grey – fertilized, white - unfertilized, "mown" - mown, "removed" - dominant species removed

Appendix B

See table 7.

Table 7. Correlation of PLFA Markers with pH, C/P_{ex} and Plant Community Composition PC1 and PC3 Sample Scores

PLFA indicator	microbial group	pH	C/P_{ex}	PC1	PC3
			r		
i15:0	*G+*	-0.27	**-0.42**	-0.35	-0.22
a15:0	*G+*	**0.61**	0.12	0.39	**-0.58**
i16:0	*G+*	0.19	-0.33	-0.10	-0.03
i17:0	*G+*	**0.51**	-0.04	-0.11	0.35
a17:0	*G+*	0.38	**0.49**	**0.56**	-0.19
i18:0	*G+*	-0.21	0.21	0.02	0.32
16:1n5	*G-*	**0.71**	**0.49**	**0.52**	**-0.46**
16:1n9	*G-*	**-0.41**	-0.33	-0.35	0.12
16:1n7	*G-*	0.03	**-0.42**	-0.33	-0.28
cy17:0	*G-*	**0.61**	0.40	0.21	-0.28
18:1n7	*G-*	**0.43**	0.25	0.19	**-0.54**
cy19:0	*G-*	-0.38	-0.08	0.04	-0.05
18:1n9	*fungi*	-0.23	-0.03	-0.08	**0.42**
18:2n6,9	*fungi*	-0.19	-0.14	-0.24	**0.41**
10Me16:0	*actinobact.*	0.11	0.29	0.32	-0.18
10Me17:0	*actinobact.*	**-0.55**	-0.34	-0.34	0.29
15:0	*gen. bact.*	-0.18	0.35	0.16	**0.41**
17:0	*gen. bact.*	0.06	**0.48**	**0.45**	**0.55**
17:1n6	*gen. bact.*	-0.29	-0.19	-0.21	-0.05
18:1n5	*gen. bact.*	0.38	**0.75**	**0.59**	-0.02
14:0	*nonspec.*	-0.32	0.13	-0.14	0.20
16:0	*nonspec.*	-0.31	-0.13	-0.26	**0.45**
16:1n11	*nonspec.*	0.40	-0.21	0.00	**-0.69**
18:0	*nonspec.*	-0.29	0.18	-0.08	**0.58**
18:1n11	*nonspec.*	-0.37	-0.30	-0.12	-0.18
20:0	*nonspec.*	-0.23	0.23	-0.50	**0.46**
22:0	*nonspec.*	-0.24	0.14	-0.16	**0.48**

Bold values indicate significant correlation between PLFA and the respective variable ($P < 0.05$).

Appendix C

See Table 8.

Table 8. Sample Scores from the First Three Axes from the PCAs on Microbial PLFA Profile and Plant Community Composition.

	experimental design			microbial community composition			plant community composition		
plot	fertilization	mowing	removal	PC1 (34.6%)	PC2 (18.7%)	PC3 (13.8%)	PC1 (21.4%)	PC2 (10.6%)	PC3 (10.2%)
1	0	1	1	-1.572	-1.409	1.759	1.473	0.407	1.005
2	0	0	0	-1.080	-0.119	1.141	-0.158	-0.171	0.116
3	1	1	1	-1.147	-0.851	-0.330	-0.430	-1.501	0.387
4	1	0	0	-1.356	-0.859	-0.890	-0.497	0.382	0.420
5	0	0	1	-0.347	0.064	1.541	0.130	1.780	-1.695
6	0	1	0	-0.231	0.466	1.617	1.482	0.215	0.462
7	1	0	1	0.256	2.395	-0.410	-0.855	0.575	0.419
8	1	1	0	-1.187	-0.886	-1.114	0.049	-0.641	0.562
9	1	1	0	0.164	-0.110	0.001	-0.615	-0.115	0.966
10	1	0	1	-0.907	0.110	-1.032	-0.888	0.596	1.915
11	0	1	0	-0.651	1.766	-0.061	1.102	0.021	1.542
12	0	0	1	0.955	0.830	0.480	-0.687	1.446	0.011
13	1	0	0	-1.428	1.953	-1.009	-1.233	1.231	0.018
14	1	1	1	-0.683	-0.367	-1.162	-1.048	-1.189	1.437
15	0	0	0	0.793	0.081	-0.379	-0.812	1.558	-0.175
16	0	1	1	0.115	-0.497	1.420	1.723	1.187	-0.228
17	0	0	1	0.924	0.177	1.029	0.371	-0.396	-1.891
18	1	1	0	0.321	1.044	-0.584	-0.348	-0.866	-0.614
19	1	0	1	0.969	-1.158	-1.696	-0.658	-1.031	-1.572
20	0	1	0	1.698	-0.452	-0.254	1.447	-0.642	-0.136
21	0	1	1	0.964	-0.033	1.233	2.253	-0.521	-0.192
22	1	0	0	1.582	-0.429	-0.319	-0.099	-1.395	-1.370
23	1	1	1	0.527	-1.707	-0.623	-0.911	-1.730	-0.127
24	0	0	0	1.320	-0.010	-0.359	-0.792	0.796	-1.256

Appendix D

See Figure 3.

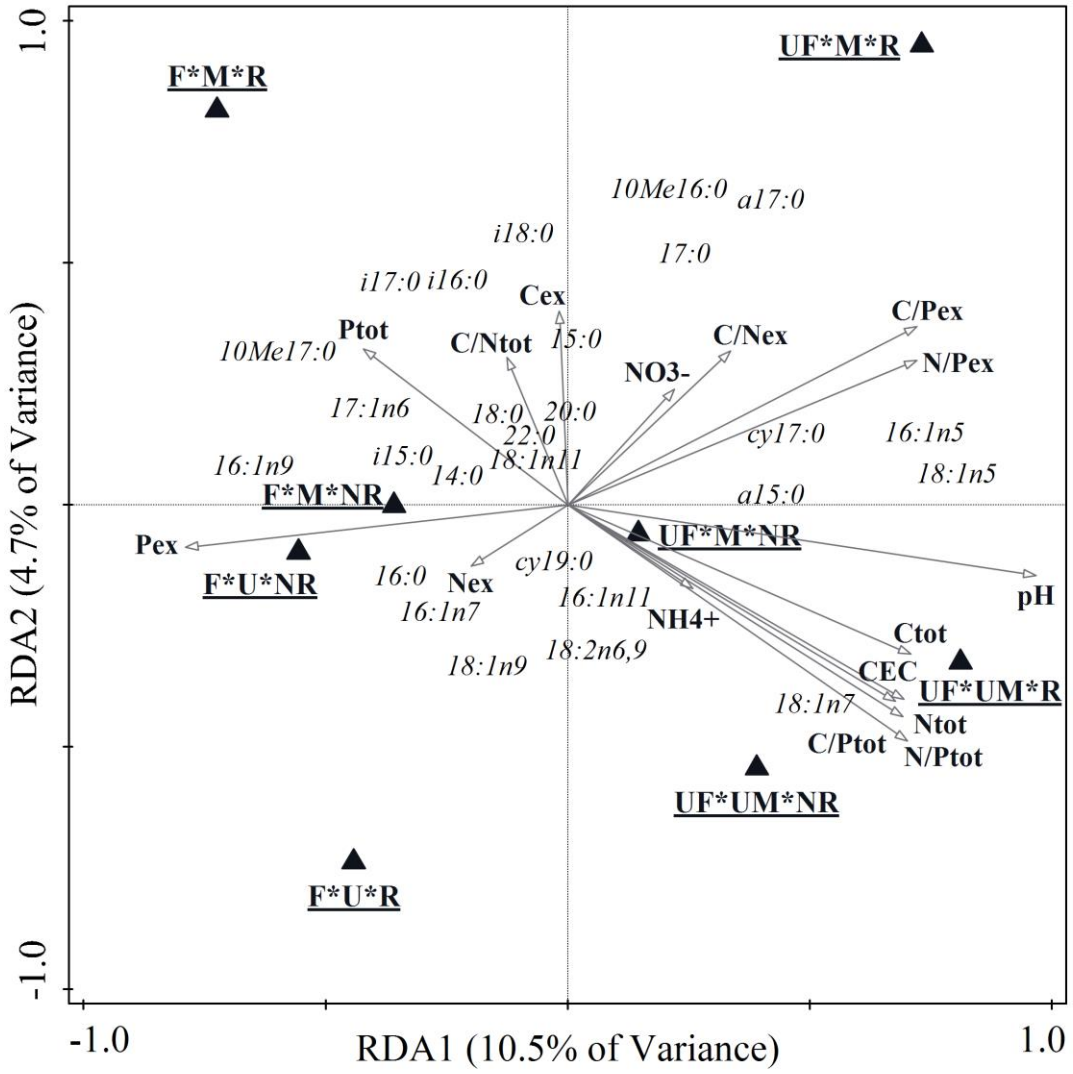

Figure 3. Ordination diagram showing results of the redundancy analysis of PLFA abundance data with management practices combining fertilization, mowing and removal of the dominant plant species (*Molinia cearulea*) as the only explanatory variables. Triangles are centroids (n = 3) of the plots belonging to the same treatment combination (F*M*R – fertilized mown removal, F*M*NR – fertilized mown non-removal, F*UM*R – fertilized unmown removal, F*UM*NR – fertilized unmown non-removal, UF*M*R – unfertilized mown removal, UF*M*NR – unfertilized mown non-removal, UF*UM*R – unfertilized unmown removal, UF*UM*NR – unfertilized unmown non-removal). Arrows indicate loading of physico-chemical soil properties used as supplementary variables. The numbers in parentheses are the proportions of the total variation explained by each axis (both axes are not significant).

PAPER 10

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Comparison of uptake of different N forms by soil microorganisms and two wet-grassland plants: A pot study

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ABSTRACT

Two common plant species of temperate wet grasslands, *Carex acuta* and *Glyceria maxima*, were tested for their preferences in the uptake of different nitrogen (N) sources (amino acid, ammonium, nitrate) and their ability to compete for these sources with soil microorganisms. The experiment was a one-day incubation study with plants growing in soil obtained from the field, which was supplied with a solution containing the three N sources, one at a time labeled with ^{15}N. The bulk of the N demand of both species was covered by nitrate-N, which was the dominant N form in the soil at the time of the experiment. Ammonium-N was taken up less strongly, and organic N formed only a negligible part of their nutrition. The assimilated inorganic N was preferentially transported to apical meristem of the youngest leaf, while organic N remained mostly in the roots. The fast-growing *Glyceria* took up more N and was a better competitor vis-à-vis soil microbes for rarer N forms than *Carex*. However, both plants were poor competitors for N vis-à-vis soil microbes, irrespective of the N form. Microbes took up nitrate ca. five times faster and organic N more than a hundred times faster than plants. Correspondingly, the calculated turnover time of microbial N was 17 days, compared to 40 days for N in plant roots. A significant amount of added ^{15}N was found at non-exchangeable sites in the soil, which points to the importance of microbial N transformation and abiotic N fixation for N retention in soil. In summary, the preferential assimilation of inorganic N by the wetland plants studied here and their poor ability to compete for N with soil microbes over the short term agree with the results of studies carried out with other species from temperate grasslands.

1. Introduction

It has been shown that the concentration of available organic nitrogen (N) in soil solution could be comparable or even exceeds that of inorganic N in many terrestrial ecosystems. Just like the uptake of inorganic N forms by plants, their ability to assimilate intact amino acids has been shown to be ubiquitous (Näsholm and Persson, 2001; Miller and Cramer, 2004; Näsholm et al., 2009). The uptake of organic N may thus represent a substantial fraction of the overall N uptake by plants. Recently, questions about whether this ability is realized under field conditions (with competition between coexisting plant species and soil microorganisms) and how plant uptake of amino acids compares to that of inorganic N compounds have been studied intensively. It was shown that the ability of plants to take up intact amino acids in the field depends on many factors; for instance, amino acid concentration (Sauheitl et al., 2009), microbial activity (Dunn et al., 2006) and related rates of N turnover (Schimel and Bennett, 2004), inter-specific differences between plants in the assimilation of various N forms (McKane et al., 2002; Weigelt et al., 2003, 2005; Bardgett et al., 2003), and soil fertility (Weigelt et al., 2003).

In strongly N-limited ecosystems such as the arctic tundra (Kielland, 1994), boreal forests (Näsholm et al., 1998), alpine and subalpine fen, and shortgrass steppe (Raab et al., 1999), utilization of amino acids was shown to be an important source of N for plants. Plant species-specific differences in uptake of different chemical N forms (both organic and inorganic) have also been shown to occur in strongly N-limited ecosystems (arctic tundra: McKane et al., 2002; alpine communities: Miller and Bowman, 2003), where they help to facilitate species coexistence and the maintenance of plant diversity (McKane et al., 2002; Miller and Bowman, 2003).

A certain importance of the available soil organic N for plant nutrition was also found in temperate ecosystems and agricultural soils. The ability of many grassland plants to take up amino acids was demonstrated in pot studies (Näsholm et al., 2000; Weigelt

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et al., 2003, 2005; Dunn et al., 2006) and in field studies (Bardgett et al., 2003; Harrison et al., 2007, 2008). However, in spite of the fact that the concentration of available amino acids may be comparable to that of inorganic N in temperate grassland soils (Jones, 1999; Bardgett et al., 2003), the primary source of nutrients for grassland plants is thought to be mineral N, produced by microbial mineralization of soil organic matter (Jones et al., 2005; Owen and Jones, 2001). This was attributed to the facts that microbial activity and related N mineralization rates are higher in temperate grasslands compared with the N-limited ecosystems mentioned above (Owen and Jones, 2001; Schimel and Bennett, 2004) and that plants commonly seem to compete poorly with microbes for amino acids (Hodge et al., 2000; Bardgett et al., 2003; Harrison et al., 2007).

The occurrence of inter-species partitioning of the soil N pool in temperate ecosystems is also less than clear. Pot experiments with individual grassland plant species or plants from different functional groups showed species-level differences in preference for different N forms (Weigelt et al., 2003, 2005; Kahmen et al., 2006). However, these differences are not always realized in nature when coexisting species compete for N (Harrison et al., 2007, 2008). Under such conditions, all tested species had similar preferences across different N forms and preferentially took up inorganic N (Harrison et al., 2007, 2008).

Plants are considered to be inferior to soil microbes with regard to N uptake, at least in the short term, irrespectively of the N form (Hodge et al., 2000; Bardgett et al., 2003; Harrison et al., 2007, 2008). This applies in particular to organic N (Bardgett et al., 2003; Harrison et al., 2007, 2008) and also to ammonium, while the difference in uptake rates for nitrate is less pronounced (Jackson et al., 1989). The more effective capture of N by microorganisms is ascribed to their higher ratio of surface area to volume, wider spatial distribution, and higher uptake affinities (Lipson and Näsholm, 2001). Faster turnover of microbial biomass in comparison to roots (Hodge et al., 2000; Bardgett et al., 2003) and microbial predation by protozoa release N back to the soil, where it can be captured and retained by plants. Therefore, plants commonly outcompete microbes and accumulate the majority of N label added into the systems in longer-term experiments (Hodge et al., 2000; Harrison et al., 2008). Furthermore, the N flux through those mineralization–immobilization processes helps to retain N within the ecosystem (Burger and Jackson, 2004; Ledgard et al., 1998).

The aim of this study was to examine whether two coexisting dominant plant species of wet temperate grassland display preferences for different N forms (organic and inorganic) and whether plants and microbes differ in their preferences for these N forms, thereby relaxing competition for N. These objectives were tested in a pot experiment where single plants of *Carex acuta* and *Glyceria maxima* were grown in a mixture of sand and field soil from a mesotrophic wet temperate grassland. After one month, each pot received a mixture of three N forms: ammonium, nitrate and glutamic acid, one of which labeled with ^{15}N. The distribution of ^{15}N in the plant–soil system was determined after a one-day incubation period. Additionally, the distribution of the added N in plant and soil was assessed and plant and microbial N uptake rates and turnover times were estimated. We chose temperate wet grassland because this is a type of ecosystem with specific conditions and plant species differ from those commonly studied. The two co-dominant plant species at the studied site were not infected by endomycorrhiza and differed in their growth rates as well as N demands: the fast-growing *G. maxima* prefers eutrophic soils (Ellenberg indicator value N 9), while the slow-growing *C. acuta* (Ellenberg indicator value N 4) is more typical on oligotrophic–mesotrophic soils. Our predictions were that 1) fast-growing *Glyceria* will take up more N in comparison to slow-growing *Carex*, 2) *Glyceria* will rely mainly on inorganic N forms, while *Carex* will be able to take up more amino acid-N in intact form and thus relax competition with the fast-growing species, and 3) soil microorganisms will be more effective competitors for any of the N forms than plants in the short term but they will preferentially assimilate organic N.

2. Materials and methods

2.1. Site description and sampling

Soil and plants were taken from temperate wet grassland in the floodplain of a small river (Nežárka) in the Třeboň Basin Biosphere Reserve, South Bohemia, Czech Republic. It is a mesotrophic wet meadow, dominated by a mixture of *G. maxima* and *C. acuta*. The soil is silt–loam alluvial substrate (13% clay, 15% sand), classified as Gleysol (FAO-WRB classification). The basic chemical characteristics of the soil are: total C 10.26 ± 1.96%, total N 0.69 ± 0.13%, pH (H_2O) 4.60 ± 0.18, and bulk density 0.70 ± 0.13 g cm^{-3}.

Plants of *C. acuta* and *G. maxima* were sampled using a spade in early May 2008. Soil was washed from the roots and the plants were divided by hand into individuals and used for Experiments 1 and 2 (see below). Availability of the different N forms for plants and microorganisms (i.e., concentrations of exchangeable ammonium, nitrate and amino acids) under field conditions was estimated in soil sieved through a 5-mm mesh immediately after sampling (in May and additionally in October 2008). Concentrations of inorganic N forms were measured in potassium sulfate soil extract (0.5 M K_2SO_4; volume ratio of soil to extractant 1:4; shaken for 30 min; extracts filtered and stored in the freezer) using flow injection analysis (FIA Lachat QC8500, Lachat Instruments, USA). Concentrations of exchangeable amino acids and ammonium were measured in soil extract prepared with a reagent mixture of 0.1 M HCl and 2% thiodiglycol (soil:extractant 1:4, extraction for 1 h at 4 °C followed by centrifugation at 8000 rpm for 10 min at 4 °C; filtration and freeze-drying) and analyzed by HPLC (column Watrex 250 × 4 mm C18 AQ 5 μm) with a fluorescence detector at excitation wavelength 250 nm and emission wavelength 395 nm. The amount of amino acids was recalculated using the ratio of ammonium concentrations in the reagent mixture and in 0.5 M K_2SO_4 to enable a comparison with the ammonium and nitrate pools.

2.2. Experimental design

2.2.1. Plant uptake of amino acids in intact form (Experiment 1)

Direct uptake of amino acids was studied by immersing plant roots ($n = 8$ for each species) in a solution of 1 mM glutamic acid (dual labeled, 99 atom% labeling of C and N; ^{13}C:^{15}N ratio of 4.33, CIL, USA) for 2 h. After immersion, the roots were rinsed three times in 0.5 M $CaCl_2$ and thoroughly washed under running tap water. The plants were immediately separated into roots and shoots, and within shoots, the youngest green leaf and the apical meristem of the youngest leaf (placed on the base of the youngest green leaf) were also separated. All plant parts were placed in the dryer at 60 °C for 60 h. Two other plants of each species were used as a reference (natural abundance control). Dried material was milled and analyzed for C and N contents and isotopic content of ^{15}N and ^{13}C (NC analyzer connected to IR-MS).

2.2.2. Preference in uptake of different N forms by plants and soil microorganisms (Experiment 2)

Plants were grown in field soil in pots (one plant per pot containing an equivalent of 50 g dry soil) under open air conditions for 30 days. Then all pots were supplied with a solution containing a mixture of ammonium (NH_4Cl), nitrate (KNO_3) and amino acid

(glutamic acid). Only one N compound was labeled with ^{15}N (99 atom%; CIL, USA) at a time. A control treatment contained all three N compounds with the natural abundance of their N isotopes (Sigma Aldrich). The solution (10 ml) was spread onto the surface of the soil with a pipette. Any solution leaked out the bottom of the pot (max. 1–2 ml) was collected in a Petri-dish and spread again onto the soil surface. The relatively high volume of applied solution in comparison to the dry weight of the soil and fast occurrence of its leakage out of the bottom should assure relatively fast distribution of the label within the system. The N amount added to each pot was 415 ng N g^{-1} dry soil for each N form (together 1245 ng N g^{-1} dry soil). Three replicates (3 pots with one plant each) were used for each treatment and for the control. All samples were harvested 24 h after N application.

The amount of ^{15}N in plants and its distribution were measured as follows: the aboveground plant biomass was clipped and separated into the apical meristem of the youngest leaf and older non-growing leaves. Roots were separated from the soil manually, rinsed three times with 0.5 M $CaCl_2$ and thoroughly washed under running tap water. All plant parts and ca. 5 g of well-mixed soil from each pot were immediately placed in a dryer (60 °C, 60 h). The dried and milled material was analyzed for C and N contents and content of ^{15}N (NC analyzer connected to IR-MS). The amount and distribution of ^{15}N in the soil was studied as follows: a portion of soil was immediately put into the dryer (60 °C, 60 h), then milled and analyzed for C, N, and ^{15}N contents (NC analyzer connected to IR-MS). One part of fresh soil was immediately extracted with 0.05 M K_2SO_4, while another part was extracted after chloroform fumigation for 24 h. The extracts were filtered, freeze-dried, and analyzed for C, N, and ^{15}N contents. The ^{15}N extracted before the fumigation was considered to be exchangeable ^{15}N (^{15}Nex) and was also characterized for the contents of ammonium, nitrate and amino acid-N as described above to correct the results for ^{15}N labeling for pool dilution. The N bound in chloroform-labile part of microbial biomass ($^{15}Nmic$) was calculated as the difference between ^{15}N in the extract from fumigated soil and ^{15}Nex. The rest of ^{15}N in soil residue ($^{15}Nrest$) was considered to be bound to non-exchangeable positions or microbially transformed to more stable (non-chloroform-labile) chemical forms.

2.3. Calculations and evaluation of results

Two-way ANOVA (STATISTICA 8) and Tukey HSD tests were used for testing differences in concentrations of N forms in soil exchangeable N pools within and between the samplings (sampling time and N form as parameters), and for a comparison of weights and N contents of the two plant species used in the experiment (plant species and part of tissue as parameters).

Experiment 1: Correlations between ^{15}N and ^{13}C excess in plant tissues of *C. acuta* and *G. maxima*, which would indicate direct uptake of amino acid in intact form, were explored. The ^{13}C:^{15}N ratio of glutamic acid (4.33) was further compared to the measured ratio of newly assimilated ^{15}N and ^{13}C and used to calculate percentages of amino acid assimilated by plants in intact form.

Experiment 2: Data on the distribution of the added ^{15}N in the plant–soil systems (%) comprised the average amount of ^{15}N immobilized in plants (meristem, shoots, roots) and ^{15}N present in soil (exchangeable, microbial and residual) and were expressed as percentages of the added ^{15}N (415 ng ^{15}N g^{-1}). The missing amount of ^{15}N was considered as a loss from the system. Differences between the allocation of particular N forms to the various plant and soil pools were tested by ANOVA followed by Tukey HSD tests. Uptake rates of particular ^{15}N forms by plants and soil microorganisms were calculated from the ^{15}N excess of plant and microbial biomass and expressed per unit biomass (μg N g^{-1} tissue day^{-1} and μg N g^{-1} biomass day^{-1}, respectively) to enable their comparison. All results were further corrected for a dilution of the added ^{15}N in the soil exchangeable N pool to approximate true N uptake rates. Pool dilution for any particular N form (nitrate, ammonium, amino acid) was calculated from the amount added (415 ng ^{15}N g^{-1}) and its actual concentration in the exchangeable soil pool at the time of the experiment. This approach cannot totally substitute a full pool dilution study. Therefore, the uptake rates for particular N forms are only approximate as we cannot say exactly that N was assimilated in the form, which was added or it was transformed before. In spite of this, a comparison of N uptake rates for particular N forms between both plant species and plants versus microbes is relevant is not affected by this fact and the total N uptake rates represents real N uptake of plant and soil microorganisms well.

3. Results

3.1. Characteristics of plants and soil exchangeable N pool

The concentration of exchangeable N, available to microbes and plants, was lower in May than in October (Two-way ANOVA, $p < 0.01$; Table 1). October is at the end of the vegetation period and there will have been low plant demand for N. At both sampling dates, the amounts of exchangeable N in the different forms decreased in the order ammonium-N > nitrate-N > amino acid-N (Table 1), and the compositions of the soil exchangeable N pool were similar, with ammonium-N comprising 46%, nitrate-N 33% and amino acid-N 21%.

The amounts of exchangeable ammonium-N and amino acid-N at the time of label addition (after 30 days of growth in pots) were similar to that in the field in May, while the concentration of nitrate-N was much higher (Two-way ANOVA, $p < 0.001$; Table 1) due to a relatively high nitrification rate and probably lower plant N uptake in comparison to field conditions. This resulted in dilution of the added ^{15}N by factors of 3 for amino acids, 5 for ammonium, and 55 for nitrate-N. These calculated dilution ratios were used to correct the estimates of plant and microbial N uptake of different N forms.

Both plant species had more biomass in aboveground plant parts than (live) roots (Two-way ANOVA, $p < 0.001$; Table 2). Plants of *G. maxima* had a greater biomass than *C. acuta* (Two-way ANOVA, $p < 0.001$), a higher shoot/root ratio (2.3 and 1.7, respectively) and higher N concentration (%) in all plant parts (Two-way ANOVA, $p < 0.001$). The apical meristem of the youngest leaf comprised 6–7% of plant biomass, independent of plant species, and contained the highest percentage of N in the entire plants (Table 2).

3.2. Plant uptake of amino acid (Experiment 1)

Linear relationships between ^{15}N and ^{13}C excess found in the roots of both species and in the apical meristem of *Carex* (Fig. 1)

Table 1
Amounts of exchangeable N in soil under field conditions (May and October 2008) and at the time of the labeling experiment (after 30 days of plant growing in pots – June 2008); mean ± standard deviation; different letters show significant differences in values.

N form (μg N g^{-1})	Field conditions		Laboratory
	May 2008	October 2008	June 2008
Amino acid-N	$1.05^{a} \pm 0.12$	$2.23^{a} \pm 0.08$	$1.25^{a} \pm 0.13$
NH_4-N	$2.31^{c} \pm 0.88$	$4.84^{b} \pm 1.57$	$2.14^{b} \pm 0.27$
NO_3-N	$1.67^{b} \pm 0.15$	$3.36^{b} \pm 1.94$	$20.28^{c} \pm 4.35^{*}$
			$25.38^{d} \pm 5.13^{**}$

* For *Carex acuta*.
** For *Glyceria maxima*.

Table 2
Characteristics of plants used in the pot experiment at the time of labeling; mean ± standard deviation ($n = 12$).

Species	Plant tissue	Weight (mg)	N content (%)
Carex acuta	Meristem	6.93 ± 4.45	4.31 ± 1.22
	Leaves	63.69 ± 29.83	2.62 ± 0.41
	Roots	36.99 ± 12.85	0.83 ± 0.24
	Whole plant	107.60 ± 21.45	2.11 ± 0.53
Glyceria maxima	Meristem	22.98 ± 7.03	7.87 ± 0.81
	Shoot, leaves	225.54 ± 99.92	4.44 ± 0.61
	Roots	97.60 ± 32.90	2.20 ± 0.50
	Whole plant	346.13 ± 97.23	4.04 ± 0.76

indicate that the plants took up at least part of their amino acids directly as glutamic acid. The percentages of amino acid taken up in the intact form were 17.7 ± 3.9% for *Glyceria* and 38.2 ± 10.1% for *Carex*. The remaining N was taken up after amino acid mineralization in soil. No such correlation was found in shoots, which indicates that no or only narrow transport of intact amino acids taken up by roots to other tissues took place, which we were not able to detect by the used method (measuring ^{13}C and ^{15}N excess in the whole-plant biomass).

3.3. Distribution of ^{15}N for different N forms within the plant–soil system

After one-day incubation, plants immobilized only a small amount of the added N, irrespective of the N form. The highest enrichment of plant biomass was found for nitrate-N and the lowest for amino acid-N, as evidenced from the percentages of ^{15}N found in whole plants (Table 3). Much greater amounts of added ^{15}N were found in the soil. In contrast to plants, the chloroform-labile part of soil microbial biomass was highly enriched in amino acid-N and significantly less so in mineral N forms (Table 3). Differences among ^{15}N forms were also found in their allocation into residual more stable soil N (Nres). This soil fraction contained the bulk of the added amino acid-N (>50%), a significant percentage of the added ammonium-^{15}N (30–50%), but only up to 15% of the added nitrate-^{15}N (Table 3). The amount of added ^{15}N that was not immobilized by plants, microorganisms or abiotic fixation after one-day incubation and so remained in the soil exchangeable N pool (Nex) decreased in the order nitrate (ca. 50% of added ^{15}N) > ammonium (ca. 30% of added ^{15}N) > amino acid (ca. 12% of added ^{15}N) and was independent of plant species (Table 3).

Losses from the plant–soil system were the largest for nitrate-^{15}N, which is the most mobile N form and also a substrate for denitrification, and the lowest for amino acid-^{15}N, whose immobilization in soil was the highest among the three N forms (Table 3).

3.4. Plant and microbial uptake of different N forms

To enable a comparison of plants and microbes, N uptake rates were expressed as the amounts of N taken up per unit of N in plant or microbial biomass per day (Table 4).

Both plant species preferentially took up nitrates compared with the other N sources. Amino acid-N was immobilized at the lowest rate (Table 4). In the soil, where nitrates were the prevailing N source (see Table 1, last column), nitrate-N assimilation represented 97.6% of whole-plant N nutrition, ammonium-N 2.2% and amino acid-N only 0.2% in the case of *Carex*. For *Glyceria*, nitrate-N assimilation comprised 94.2% of plant N uptake, ammonium-N 5.2% and amino acid-N 0.6%. The total N uptake rate by roots of both plants was comparable, but, the rates of N transport into above-ground tissues lower for *Glyceria* than *Carex*. Therefore, *Carex* plants assimilated only non-significantly more N per g biomass than *Glyceria* (Table 4). In result, however, *Glyceria* plants took up more than three times more N than *Carex* plants during the same time (ca. 300 μg N g^{-1} day^{-1} and 230 μg N g^{-1} day^{-1}, respectively) due to their significantly greater biomass and N content (Table 2).

We found differences among the N forms with regard to transportation of assimilated N within the plants. While both mineral N forms were preferentially allocated to the apical meristem of the youngest leaf, a larger fraction of organic N remained in the roots and its allocation to meristem was lower. This difference was more evident for *Carex*. The smallest amounts of N taken up were allocated to the shoots of both species (Table 4). Comparing the different plant tissues and organs, the N allocation, expressed as N uptake to %N in tissue per day, decreased in the order roots > meristem > shoots (Table 4). There is probably no point in calculating N turnover times for meristem, which is not in a steady-state but divides actively. The calculated residence times were ca. 40 days for root N and more than 100 days for shoot N (Table 4).

Microbial immobilization of organic N was much higher than in plants. The largest amount of immobilized N originated from nitrate-N (Table 4). The N assimilation by soil microorganisms consisted of 70% nitrate-N, 10% ammonium-N and 20% amino acid-N in soil with *Carex*, and 81% nitrate-N, 8% ammonium-N and 11% amino acid-N in soil with *Glyceria* (Table 4). This partitioning of N uptake was complementary to the respective N demands of the two plant species, i.e., when plants took up more nitrates but less ammonium, microorganisms did the opposite. The percentage of N taken up per unit of existing N in the soil microbial biomass and day was significantly higher than in the plants, corresponding to an N turnover period of 17–18 days (Table 4).

4. Discussion

The concentration of exchangeable N (ammonium, nitrate and amino acids) in the soil of wet grassland, available to microbes and

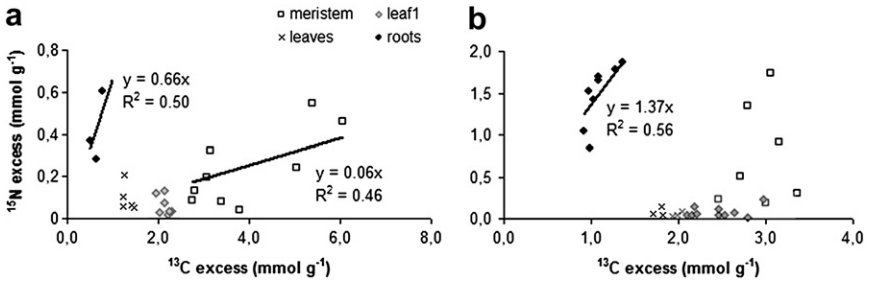

Fig. 1. Relationship between ^{13}C and ^{15}N excess in the unit biomass of meristem, the first youngest leaf (leaf 1), other leaves and root tissues of a) *Carex acuta* and b) *Glyceria maxima*.

Table 3
Distribution of added ^{15}N (%) in the plant–soil system of both plant species for amino acid-N (AA-N), ammonium-N (NH_4-N) and nitrate-N (NO_3-N) after one-day incubation. The partitioning among exchangeable N (Nex), chloroform-labile microbial N (Nmic) and remaining soil N (Nres) is shown in soil. Mean ± standard deviation ($n = 3$). Different letters show significant differences within a column (for each plant–soil system separately).

Species–soil	N form	Plant	Soil			Losses
			Nex	Nmic	Nres	
Carex–soil	AA-N	$0.04^a \pm 0.02$	$12.1^a \pm 1.9$	$20.2^b \pm 1.8$	$50.1^b \pm 17.8$	17.5
	NH_4-N	$0.16^b \pm 0.06$	$30.6^b \pm 4.1$	$5.5^a \pm 4.3$	$47.0^b \pm 12.2$	20.8
	NO_3-N	$0.84^c \pm 0.23$	$46.2^c \pm 7.5$	$3.7^a \pm 2.7$	$14.1^a \pm 5.0$	35.3
Glyceria–soil	AA-N	$0.46^a \pm 0.32$	$12.4^a \pm 2.8$	$17.8^b \pm 5.8$	$51.8^b \pm 22.4$	17.5
	NH_4-N	$2.44^b \pm 1.19$	$32.5^b \pm 1.4$	$5.3^a \pm 3.8$	$31.3^b \pm 8.2$	28.4
	NO_3-N	$2.96^b \pm 1.59$	$54.4^c \pm 4.8$	$4.7^a \pm 4.2$	$0.8^a \pm 2.9$	38.0

plants, accounted for only ca. 1% of total N storage under field conditions. The fractions for the different N forms were comparable (ammonium-N 46%, nitrate-N 33%, and amino acid-N 21% of the soil exchangeable N pool). Our results describe the situation occurring in the wet meadow from late spring to late summer, when the soil is usually not flooded (water table 30–60 cm below soil surface), aerobic conditions prevail and high plant N demand keeps the available N pool small. Generally, the fact that exchangeable amino acids comprised a relatively high proportion of the soil available N in temperate wet grassland indicates that amino acids may play an important role in plant nutrition in this ecosystem. This finding is consistent with a number of recent studies from different ecosystems (taiga – Jones and Kielland, 2002; temperate forest – Yu et al., 2002; vineyard – Christou et al., 2006; temperate grassland – Bardgett et al., 2003; Warren and Taranto, 2010).

The composition of the different available N forms characteristic for field conditions shifted significantly in favor of nitrate-N during the time of the experiment, confirming the high nitrification potential of this soil shown previously (Picek et al., 2008). The fact that nitrate-N does not accumulate in the soil under field conditions probably points to its strong uptake by plants and microorganisms (see also below), and to a very fast turnover of the nitrate pool (daily or even faster) as reported by Jackson et al. (1989), Ledgard et al. (1998) and Burger and Jackson (2004).

To examine whether organic N was an N source of any importance for wet-grassland plants, we tested the two co-dominant plant species, which form more than 90% of plant biomass in situ, for their ability to take up organic N. Glutamic acid was used as a model compound. The linear relationship found between ^{13}C and ^{15}N enrichment in plant roots offered the first piece of evidence that both species were able to take up at least part of the amino acid in intact form. The percentage of assimilated intact amino acid was 17% for *G. maxima* and 38% for *C. acuta*, which could support findings that plants of low productivity grasslands have a better ability to utilize organic N directly than plants of high fertility grasslands (Weigelt et al., 2003, 2005; Bardgett et al., 2003). In spite of this, the main pathway for taking up organic N in both plant species was after its mineralization.

The preferred uptake of inorganic N forms by both species was further confirmed in the pot experiment, where a mixture of three N forms (nitrate, ammonium and amino acid) was applied to the soil. In this case, plant immobilization of organic N was very low in comparison to N uptake from inorganic sources, amounting to less than 1% of plant N demand. A preferential uptake of mineral N forms was also found in the majority of studies with plant species from temperate grasslands (Bardgett et al., 2003; Harrison et al., 2007, 2008). This fact is usually explained by the relatively high rates of N mineralization, which was found in our soil as well (Picek et al., 2008), greater availability of inorganic N in these systems (Schimel and Bennett, 2004), as well as efficient microbial consumption of organic molecules before they reach plant roots (Hodge et al., 2000; Owen and Jones, 2001; Bardgett et al., 2003), which also agrees with our results. The greater uptake of nitrate-N compared with ammonium-N could be explained by the fact that nitrate-N was the most abundant N form present in the soil during the pot experiment. However, this could also apply to the situation in the field. Although the concentration of nitrates in the exchangeable N pool is low, this can be caused by their rapid uptake by plants and microbes. The soil examined here has a high nitrification potential (Picek et al., 2008) and the nitrate pool is known to be very dynamic, being consumed as rapidly as it is produced (Jackson et al., 1989, 2008). Moreover, plants in wet grasslands are usually not limited by water availability, and high plant transpiration rates may ease the competition through induced mass flow in the root environment. This mechanism would favor uptake of mobile N forms that are present in the soil solution (Näsholm et al., 2009). Different N forms were also differently allocated within the plants. While both mineral N forms were preferentially transported to growing plant tissue, organic N remained mostly in roots. This may be caused by differences in the transportation mechanisms for particular N forms (Miller and Cramer, 2004). While many plant species directly allocate a fraction of nitrate-N to shoots, the allocation of amino acid-N is much slower due to trans-amination

Table 4
Plant and microbial N uptake rates for particular N forms (amino acid-N (AA-N), ammonium-N (NH_4-N) and nitrate-N (NO_3-N)) and total N (N bio represents N in plant or microbial biomass), and the turnover rates of plant and microbial N calculated from the results of labeling experiment and corrected for dilutions of particular N pools in soil. Mean ± standard deviation ($n = 3$).

Species	Plant tissue	Uptake of N form			Total N uptake (mg N g^{-1} N bio day^{-1})	N turnover	
		AA-N (μg N g^{-1} N bio day^{-1})	NH_4-N (μg N g^{-1} N bio day^{-1})	NO_3-N (μg N g^{-1} N bio day^{-1})		(% N in biomass day^{-1})	(Day)
Carex	Root	146 ± 67	621 ± 312	24155 ± 7237	24.92 ± 7.62	2.49	40.1
	Leaves	13 ± 5	116 ± 26	7042 ± 2655	7.17 ± 2.69	0.72	139.5
	Meristem	22 ± 7	490 ± 326	15889 ± 5740	16.40 ± 6.07	1.64	61.0
	Whole plant	32 ± 13	233 ± 104	10506 ± 3677	10.77 ± 3.79		
Glyceria	Root	177 ± 87	1019 ± 513	24183 ± 5466	25.38 ± 6.07	2.54	39.4
	Shoot, leaves	16 ± 5	218 ± 31	3359 ± 1547	3.59 ± 1.58	0.36	278.3
	Meristem	35 ± 14	581 ± 278	7114 ± 5274	7.73 ± 5.57	0.77	129.4
	Whole plant	43 ± 19	388 ± 137	7051 ± 2633	7.48 ± 2.79		
Carex–soil microbes		12526 ± 1134	5709 ± 4473	41690 ± 30450	59.93 ± 36.06	5.99	16.7
Glyceria–soil microbes		6354 ± 3141	4782 ± 3430	46141 ± 41556	57.28 ± 48.13	5.73	17.5

reactions. Allocation of ammonium-N was thought to be similar to that of amino acid-N, as it is usually incorporated into amino acids before transportation (summarized in Näsholm et al., 2009). However, our results showed that the transport of ammonium-N, at least to the growing tissue, was much faster than that of amino acid-N.

We found relatively small differences between both plant species in their N nutrition. *G. maxima* immobilized more N in its biomass than *C. acuta*, owing to higher N content within its biomass, greater biomass and faster growth. Similar results were also obtained by Weigelt et al. (2005) and Harrison et al. (2008). *Glyceria* as a fast-growing species was also able to compete more effectively with microbes for ammonium-N and amino acid-N, which made up a slightly larger percentage of its nutrition, while *Carex* was more dependent on nitrate-N, the most mobile and most abundant N form in soil solution.

Soil microbes were more effective competitors than plants for all added N forms during the one-day incubation period, as shown by the immobilization of labeled N in plant and microbial biomass (Table 3). This was especially clear in the case of organic N, where plants took up less than 0.5% of added ^{15}N, while ca. 20% was immobilized in chloroform-labile microbial biomass. We can expect that ca. 50% of ^{15}N in residual soil (i.e., after fumigation–extraction) was also microbially transformed (fixed in non-chloroform-labile biomass (nonextractable microbial fraction, such as cell wall components, polymeric structures and other storage compounds)). The rates of N immobilization by microorganisms were more than a hundred times higher than those for plants in the case of amino acid-N, more than ten times higher for ammonium-N, and ca. five times higher for nitrate-N. The effective microbial competition for N, and especially organic N, corresponds to the findings of the majority of similar short-term studies carried out with grasses in the laboratory or the field (Hodge et al., 2000; Dunn et al., 2006; Bardgett et al., 2003; Harrison et al., 2007, 2008), although contrasting results have also been reported, especially for inorganic N forms (Ledgard et al., 1998). The advantage of microorganisms against plants in short-term competition for N has been attributed to a higher surface area to volume ratio, greater spatial distribution and higher uptake affinities (Lipson and Näsholm, 2001).

There are two things that should be mentioned: when we compare N uptake rates of plants and soil microorganisms in this study. i) Both plant species were non-mycorrhizal, which is characteristic for plants of sedge meadows and of other types of wet grasslands but uncommon for most other grassland plants. Concerning plant N nutrition, arbuscular mycorrhizal fungi might enhance plant capture of inorganic N to some extend but their role in contribution to the direct uptake of organic N is equivocal (Hodge et al., 2000). In summary, it seems unlikely that fungal N capture is an important factor in plant-microbial competition (Hodge et al., 2000; Miller and Cramer, 2004). 2) In many studies, label is distributed in the plant–soil system by an injection on several places using a special needle. We applied a diluted solution containing ^{15}N on the soil surface. Although the solution was well distributed within the system, microorganisms near soil surface were favored in the access to the added N in comparison to plant roots at the beginning. This could contribute to their significantly higher N uptake rates as compared with plants to some extent.

Several factors have been identified that help plants to relieve the severe competition for N with microbes and fulfill their N demands. These are i) large spatial differences in N availability in soil (Hodge et al., 2000; Schimel and Bennett, 2004), which were not captured in our experiment, ii) temporal differences in turnover times of microbial and root biomass (Hodge et al., 2000), and iii) the partitioning of uptake of different N forms (Weigelt et al., 2003, 2005; Kahmen et al., 2006). Faster turnover of microbial biomass in comparison to plant roots (in our case ca. 17 and 40 days, respectively, but we suppose that the turnover of the active part of microbial biomass will have been even faster), due to higher growth rate and predation, causes a release of microbially immobilized N into the soil, where it becomes available again to plants. Therefore, plants are able to retain captured N to a greater extent in the longer term (Hodge et al., 2000; Harrison et al., 2007). The rapid N fluxes through microbial immobilization–remineralization cycles and plant uptake keep the available N pool in soil small and prevent N loss from the ecosystem (Ledgard et al., 1998; Burger and Jackson, 2004). We found that while soil microorganisms effectively took up organic N, plants preferentially assimilated inorganic N forms, in particular nitrate-N, which was the most abundant available N form in the soil. Furthermore, there was a certain complementarity in N nutrition, in as far as when a particular species took up relatively more nitrates and less ammonium, microorganisms in its rhizosphere behaved in the opposite way.

A significant amount of added N was found remaining in the soil after fumigation–extraction (residual soil). This soil fraction contained the majority of added amino acid-N (>50%), a significant percentage of the added ammonium-N (30–50%) and only up to 15% of added nitrate-N. We suppose that the N in residual soil would be partly bound up in a non-chloroform-labile part of microbial biomass (cell walls, polymeric structures, storage compounds) and partly abiotically immobilized at non-exchangeable positions. An indirect evidence of an existence of an abiotic N fixation in our soil was obtained in additional experiment, where the N was extracted immediately after its addition into the soil. Ca 27% of added glutamic acid, 20% of ammonium and 8% of nitrate were fixed in the soil within minutes (results not shown). This indicated that ca. half of the ^{15}N found in residual soil could be abiotically fixed. Abiotic immobilization of ammonium, but also of nitrate, was also shown by other authors to be a significant mechanism of N stabilization in grassland soils, reaching 20–50% of added N. A large fraction of this N pool was shown to become incorporated into slowly cycling and unavailable forms of organic matter within one year (Barrett and Burke, 2002; Kaye et al., 2002; Barrett et al., 2002).

5. Conclusions

Our results for the competition for N and preferential N uptake by plants and soil microbes, the first for species from temperate wet grasslands, are in agreement with work carried out with other types of temperate grasslands.

The two plant species characteristic for wet grasslands, *C. acuta* and *G. maxima*, were able to take up intact amino acid-N. When other available N sources where present, both plants preferentially assimilated nitrate-N, the dominant N form in the soil at the time of measurement. Both plants preferentially allocated N into the apical meristem of the youngest leaf, with the exception of organic N, which mostly remained in roots. The faster-growing *G. maxima* took up more N into its biomass and was able to compete more effectively with soil microbes for less available N forms (ammonium and organic N) than *C. acuta*. In summary, both plant species were weak competitors for N vis-à-vis microbes, irrespective of N form, in the one-day incubation experiment. For organic N, the N uptake rate was more than a hundred times faster for microbes than for plants, the difference was ca. fivefold in case of nitrate-N. In line with the faster N uptake by microbes, microbial N also underwent a faster turnover in comparison with plant roots (17 and 40 days, respectively). The turnover period of N in plant shoots (mostly non-growing biomass) was more than 100 days. A significant amount of N added into the system was fixed in

non-exchangeable positions, probably partly by abiotic immobilization and partly by microbial transformation.

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PAPER 11

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Direct effect of fertilization on microbial carbon transformation in grassland soils in dependence on the substrate quality

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Abstract

Response of microbial metabolism (growth, substrate utilization, energetic metabolism) to fertilization by N and P and resulting changes in soil-organic-matter (SOM) decomposition (priming effect) were studied in grassland soils with relatively high organic-matter content. Treatments with and without glucose addition were studied to simulate difference between rhizosphere and bulk soil. Our expectation was that fertilization would decrease soil respiration in both treatments due to an increased efficiency of microbial metabolism. At first, fertilization activated microbial metabolism in both treatments. In glucose-nonamended soils, this was connected with a short-term apparent priming effect but if glucose was available, the higher energetic demand was covered by its mineralization in preference against SOM, causing significant SOM savings as compared to unfertilized soils. After a relatively short period of 1–3 d, however, the phase of deprived microbial metabolism occurred in both treatments, which was characterized by lower soil respiration in fertilized than in unfertilized soils. Fertilization further decreased net microbial growth following glucose addition, shortened turnover time of microbial biomass and changed the partitioning of assimilated glucose within microbial biomass (decreased accumulation of storage compounds and increased the proportion of mineralized glucose). As a result, fertilization reduced soil respiration mainly due to a deprivation of microbial metabolism. The rate and range of microbial response to fertilization and also the amount of saved soil C were larger in the soil with higher SOM content, likely driven by the higher content of microbial biomass.

Key words: grasslands / soil fertilization / microbial growth / priming effect / soil respiration

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1 Introduction

Organic-matter (OM) decomposition is a key process, determining ecosystem C storage and nutrient cycling (*Wardle*, 1992), the rate of which is largely dependent on nutrient availability (*Fennesy* et al., 2008; *Manning* et al., 2008). While grassland fertilization by N and P increases primary production (*Saggar* et al., 1997; *Henry* et al., 2005) and input of OM into the soil (*Manning* et al., 2008), it needs not result in soil C sequestration (*Conant* et al., 2001b; *Bradford* et al., 2008). A wide range of responses to fertilization in terms of C sequestration in grasslands was reported, from increased grassland-soil C stocks to negative soil C balance (*e.g., Lovell* et al., 1995; *Conant* et al., 2001a, b), depending on the rate of soil-organic-matter (SOM) decomposition. We expect that the apparent discrepancies in the studies can be connected to soil-C-substrate quality and physiological status of the decomposer community.

Studies to date have shown that SOM-decomposition rate after fertilization is generally connected with C-substrate quality. The decomposition rate of easily hydrolyzable substrates is stimulated by elevated nutrient input (*Melillo* et al., 1982; *Corstanje* et al., 2007), while decomposition of low-quality litter is retarded (*Ågren* et al., 2001; *Waldrop* and *Zak*, 2006). Physiological attributes of the decomposer community affect the resulting decomposition rate after external fertilization (*Hobbie*, 2005). There exists quite a lot of information about shifts in microbial-community structure to external nutrient inputs (*Bardgett* et al., 1999; *de Vries* et al., 2006; *Yevdokimov* et al., 2008; *Denef* et al., 2009, and others). However, studies on physiological (metabolic, anabolic) adaptations of the microbial community are quite scarce, mostly concerning studies on changes in activities of important enzymatic groups (*Waldrop* and *Zak*, 2006; *Manning* et al., 2008), notice of an increase in decomposer efficiency (*Liljeroth* et al., 1990; *Ågren* et al., 2001) or a decrease in decomposer growth rate (*Ågren* et al., 2001; *Yevdokimov* et al., 2008). A more complex study, describing changes in microbial biomass and respiration after addition of glucose and glucose *plus* nutrients (N, P), was conducted by *Dilly* (2001). He found that nutrient amendment to different soils generally stimulated respiration rate and microbial growth. The microbial response was dependent on biomass content, the added concentration and composition of available substrates, and emergent system properties.

Our aim was to investigate the physiological changes of soil microbiota and the fate of added, easily available C in response to NP fertilization. Our focus was on (1) the temporal

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dynamics of microbial biomass and respiration, (2) the direction, magnitude, and duration of the induced priming effect (PE), and (3) the resulting changes in decomposition rates. The treatments with and without glucose addition should simulate a difference between the rhizosphere, where C flow is mainly driven by regular daily input of easily hydrolyzable root exudates, and bulk soil, in which mostly indigenous transformed SOM serves as a substrate. We expected that fertilization would decrease SOM losses from glucose-nonamended soils (based on results of *Fog*, 1988, and others). We further expected that an input of easily degradable glucose would cause higher soil respiration due to a positive priming effect (*Kuzyakov*, 2002; *Blagodatskaya* and *Kuzyakov*, 2008). In fertilized soils, however, the soil C losses could decline or be equilibrated by increased efficiency of microbial metabolism (*Liljeroth* et al., 1990; *Ågren* et al., 2001). As a result, fertilization could support C accumulation in grassland soils.

2 Material and methods

2.1 Soils

Soils from sedge grasslands were used in the experiment. Sedge grasslands with high SOM (≥20%) typically surround rivers and intensively managed fishponds in S Bohemia, the agricultural part of the Czech Republic. They are often affected by nutrient runoff from fields or nutrient-rich floods; some are also directly fertilized. Soils were sampled from two oligo-mesotrophic wet meadows located in the Třeboň Basin Biosphere Reserve. Both sites are irregularly flooded part of the year, mainly during spring and autumn. The soils were sampled in summer (August 2006), when the groundwater level is commonly in the range of –20 to –40 cm and the upper soil layer is aerobic. The Zablati site is a sedge meadow on silt-loam peat soil (Histosol, FAO-WRB classification), dominated by *Carex acuta.* The Hamr site lays on a mineral, silt-loam alluvial substrate, classified as a Gleysol (FAO-WRB classification), dominated by a mixture of *Glyceria maxima* and *C. acuta.* In the past, both localities were fertilized by slurry with high contents of N and P; nowadays, NPK fertilizer is applied in several experimental plots. Basic characteristics of the soils are given in Tab. 1. Soils were sieved through a 5 mm mesh and stored at 4°C until analyzed in February 2007.

2.2 Experimental design and soil analyses

Soils were divided into glucose-nonamended and glucose-amended treatments. Glucose ($\delta^{13}C$ of [–11.79 ± 0.06] ‰; water solution with C concentration of 0.40 mg C [g dry soil]$^{-1}$) was used as a readily available substrate, as it is the monomer of most plant-originated organic polymers and a major component of root exudates (*Nguyen*, 2003), which most soil microorganisms are capable to metabolize (*Anderson* and *Domsch*, 1978). The difference between $\delta^{13}C$ of glucose and SOM enabled us to follow glucose partitioning in microbial metabolism and quantification of PE. Half of the glucose-nonamended and glucose-amended samples were fertilized. The amount of added nutrients was calculated from the NPK fertilization of the studied localities in the field; 300 kg of NPK fertilizer ha^{-1} y^{-1} (containing 15% [w/w] N and 7% [w/w] P) divided into two doses. We used concentrations of N 1 mg g^{-1} (NH_4NO_3) and P 0.45 mg g^{-1} (KH_2PO_4) in our laboratory experiment. Water content of all samples was adjusted to 65% of water-holding capacity by distilled water. Samples (10 g) were incubated at 20°C in aerobic conditions.

Soil CO_2 respiration was measured after 2, 8, 24, 72, 168, and 408 h in air-tight closed glass jars by gas chromatography. Samples were ventilated after each measurement. The $\delta^{13}C$ of CO_2 (in relation to Pee Dee Belemnite as the reference standard material) was measured by an isotope-ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany). The measured $\delta^{13}C$ was corrected for the content and $\delta^{13}C$ of atmospheric CO_2 (measured always in blank samples) by a two-component mixing model (*Balesdent* et al., 1988), to obtain the real amount and $\delta^{13}C$ of microbially derived CO_2 at each sampling time.

We further analyzed the amounts of organic C before (C_{SOL}) and after (C_{FE}) chloroform fumigation (24 h) and extraction by 0.05 M K_2SO_4 at 8, 24, 72, 168, and 408 h in glucose-amended samples and at 8, 168 and 408 h in glucose-nonamended samples. Analyses of C and $\delta^{13}C$ of these fractions were conducted in freeze-dried extracts on an NC Elemental analyzer (ThermoQuest, Germany) connected to the IR-MS.

2.3 Calculations

For each glucose-amended sample, amounts of C derived from glucose and SOM (glucose-C and SOM-C, respective) were calculated by a two-component mixing model (*Balesdent* et al., 1988) in the C_{CO2}, C_{SOL}, and C_{FE} fractions. The $\delta^{13}C$ of C_{CO2}, C_{SOL}, and C_{FE} of glucose-nonamended soils were used as natural-abundance controls and $\delta^{13}C$ of –11.79‰ was used for glucose. The results were used in the following calculations.

(1) Any change in SOM-C-mineralization rates in fertilized treatments, as compared to unfertilized ones in both glucose-nonamended and glucose-amended soils, were denoted as positive or negative priming effects, according to *Kuzyakov* (2002).

Table 1: Basic characteristics of mineral (Min) and organic (Org) soils used in the experiment.

	SOM / %	C_{ORG} / %	N_{TOT} / %	P_{TOT}	pH_{H2O}	Bulk density / g cm^{-3}
Min	20.0 ± 2.0	10.1 ± 0.2	0.7 ± 0.04	0.1 ± 0.03	4.7	0.5 ± 0.04
Org	42.4 ± 8.6	23.0 ± 0.2	1.3 ± 0.04	0.2 ± 0.02	4.8	0.2 ± 0.02

(2) Microbial biomass (C_{MIC}) derived from glucose or SOM was calculated separately always as the difference between glucose-C or SOM-C in C_{FE} and C_{SOL}, using k_{EC} of 0.45 for the part of C_{MIC} derived from SOM (*Wu* et al., 1990) and the calculated k_{EC} for glucose-derived C_{MIC} (see below). Total microbial biomass was the sum of the SOM- and glucose-derived parts. The $\delta^{13}C$ of the chloroform-labile part of C_{MIC} was calculated by a two-component mixing model (*Šantrůčková* et al., 2000). The chloroform-labile part of C_{MIC} (C flush; the difference in C extracted from soils before and after fumigation) contains intracellular metabolites, while chloroform-nonlabile part (nonextractable microbial fraction, the proportion of which from C_{MIC} is equal to $1 - k_{EC}$) contains cell-wall components, polymeric structures, and other storage compounds (*Šantrůčková* et al., 2004).

(3) Partitioning of the added glucose in glucose-amended soils (initial amount 400 μg C g^{-1}) was calculated according to *Nguyen* and *Guckert* (2001) (Tab. 2). We assumed no sorption of glucose on soil particles, as it is uncharged (*Jones* and *Edwards*, 1998).

2.4 Statistics

Effects of glucose amendment and fertilization were tested by either *t*-test (results in figures) or repeated measures ANOVA, when temporal changes were taken into account (STATISTICA 7.0). The Tukey-HSD test was used as a post-hoc test. A level of significance of $p = 0.05$ was used.

3 Results and discussion

3.1 $\delta^{13}C$ of chloroform-labile C_{MIC} and CO_2 in glucose-nonamended soils

Independently of fertilization, the calculated $\delta^{13}C$ of chloroform-labile C_{MIC} was on average –30.5‰ with small temporal changes on the border of significance (Fig. 1). The $\delta^{13}C$-CO_2 exponentially decreased by ≈3‰, reaching an asymptote of ≈–28.5‰ after 3 d (Fig. 1). We suppose that the initial decrease of $\delta^{13}C$-CO_2 could be explained by a gradual equilibration of samples after wetting by distilled water. The water we used was not degassed (contained atmospheric CO_2 with $\delta^{13}C$ of –8‰), thus the equilibration connected with dissolution of soil CO_2 could result in a 1‰–2‰ increase in the produced gaseous CO_2. Further decrease could be explained by microbial isotopic discrimination in the transition period after soil disturbance (*Šantrůčková* et al., 2000). The equilibrated $\delta^{13}C$-CO_2 (≈–28.5‰) corresponded well to the $\delta^{13}C$ of SOM, representing the substrate for microbial respiration (–29.3‰ and –28.5‰ in mineral and organic soil, respectively), which is in agreement with *Šantrůčková* et al. (2000).

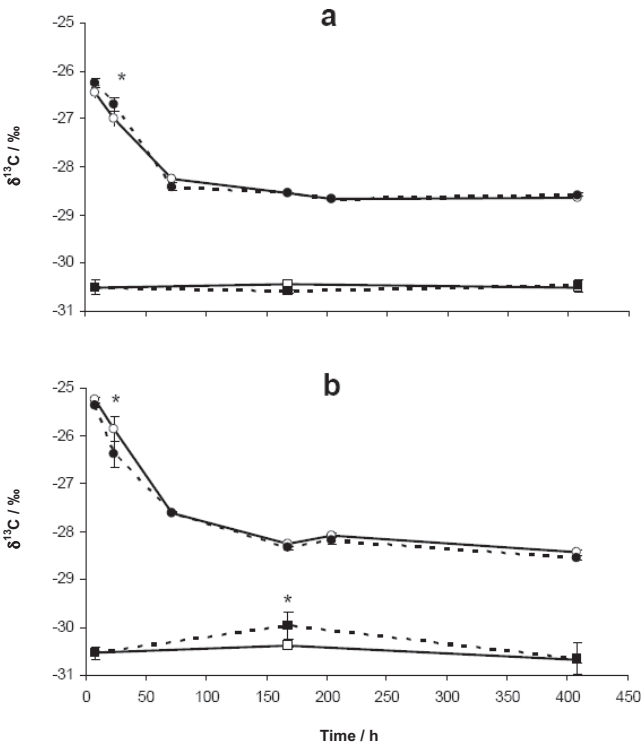

Figure 1: Glucose-nonamended mineral (a) and organic (b) soils: the $\delta^{13}C$ (‰) of chloroform-labile part of microbial biomass (□, ■) and respiration (○,●) in unfertilized (solid line) and fertilized (dotted line) treatments. Means and standard deviations ($n = 3$) are shown. Asterisks indicate statistically significant differences between fertilized and unfertilized treatments at particular times (*t*-test, $p = 0.05$, $n = 3$).

Table 2: Calculation of the partitioning of added glucose-C.

Parameter	Abbreviation	Calculus	Units
glucose-C in the extract of nonfumigated soils	C_{UNAS}(glc)	measured	μg C g^{-1}
glucose-C assimilated by microbes	C_{ASS}(glc)	400 – C_{UNAS}(glc)	μg C g^{-1}
proportion of glucose-C mineralized to CO_2	C_{CO2}(glc)	mineralized glucose-C × 100 / C_{ASS}(glc)	%
chloroform-labile glucose-derived C	C_{FE}(glc)	(glucose-C in the extract after the chloroform fumigation – C_{UNAS}) × 100 / C_{ASS}(glc)	%
microbial glucose-derived C	C_{MB}(glc)	(400 – C_{UNAS}(glc) – C_{CO2}(glc)) × 100 / C_{ASS}(glc)	%
chloroform-nonlabile glucose-derived C	$C_{non\text{-}FE}$(glc)	C_{MB}(glc) – C_{FE}(glc)	%
proportion of microbial C released by the chloroform fumigation	k_{EC}(glc)	C_{FE}(glc) / C_{MB}(glc)	–
glucose-C efficiency		C_{MB}(glc) / (400 – C_{UNAS}(glc))	–

3.2 Microbial growth and respiration in glucose-nonamended soils

Microbial biomass (Fig. 2a, b) and respiration (Fig. 2c, d) were only slightly higher in organic soil than in the mineral one, despite the large difference in SOM content (Tab. 1). Microbial response to the experimental NP fertilization was also similar in both soils. Immediately after fertilizer application, soil CO_2 respiration was enhanced and a transient positive PE occurred in the fertilized treatments of both soils (Fig. 2c, d—embedded figures). This probably occurred as a consequence of accelerated internal microbial metabolism and a higher production of exoenzymes, which increased SOM breakdown and the flow of available C back to the microbes (*Kuzyakov*, 2002; *Schimel* and *Weintraub*, 2003; *Blagodatskaya* and *Kuzyakov*, 2008). The increased substrate availability following fertilization enabled a slight but significant growth of microbial biomass in the mineral soil but was not sufficient to support any microbial growth in the organic soil (Fig. 2a, b).

The activation of microbial metabolism in fertilized treatments lasted only 1–3 d in the organic and mineral soils, respectively. This was followed by a phase of depressed microbial activity, in which the SOM-mineralization rate decreased under the level found in the unfertilized treatments (Fig. 2c, d—embedded figures). This could be due to C starvation of the activated microbial community. The increased energetic demands (faster turnover and elevated maintenance energy) could not be met by the present exoenzyme system due to its slow inactivation and a gradual exhaustion of the products of the increased SOM breakdown (*Schimel* and *Weintraub*, 2003). Repressed microbial activity in the fertilized treatments resulted in a decrease of total soil CO_2 respiration and significant SOM savings in comparison to the unfertilized variants in both soils (Fig. 2c, d).

In summary, NP fertilization of soils without addition of available C had a neutral or only minor effect on microbial biomass, which is an often reported result as reviewed by *Wardle* (1992). The C/energy limitation of microbial metabolism occurring after fertilization as a consequence of higher energetic demands of activated microbial community (see above) led to significantly lower soil CO_2 respiration as

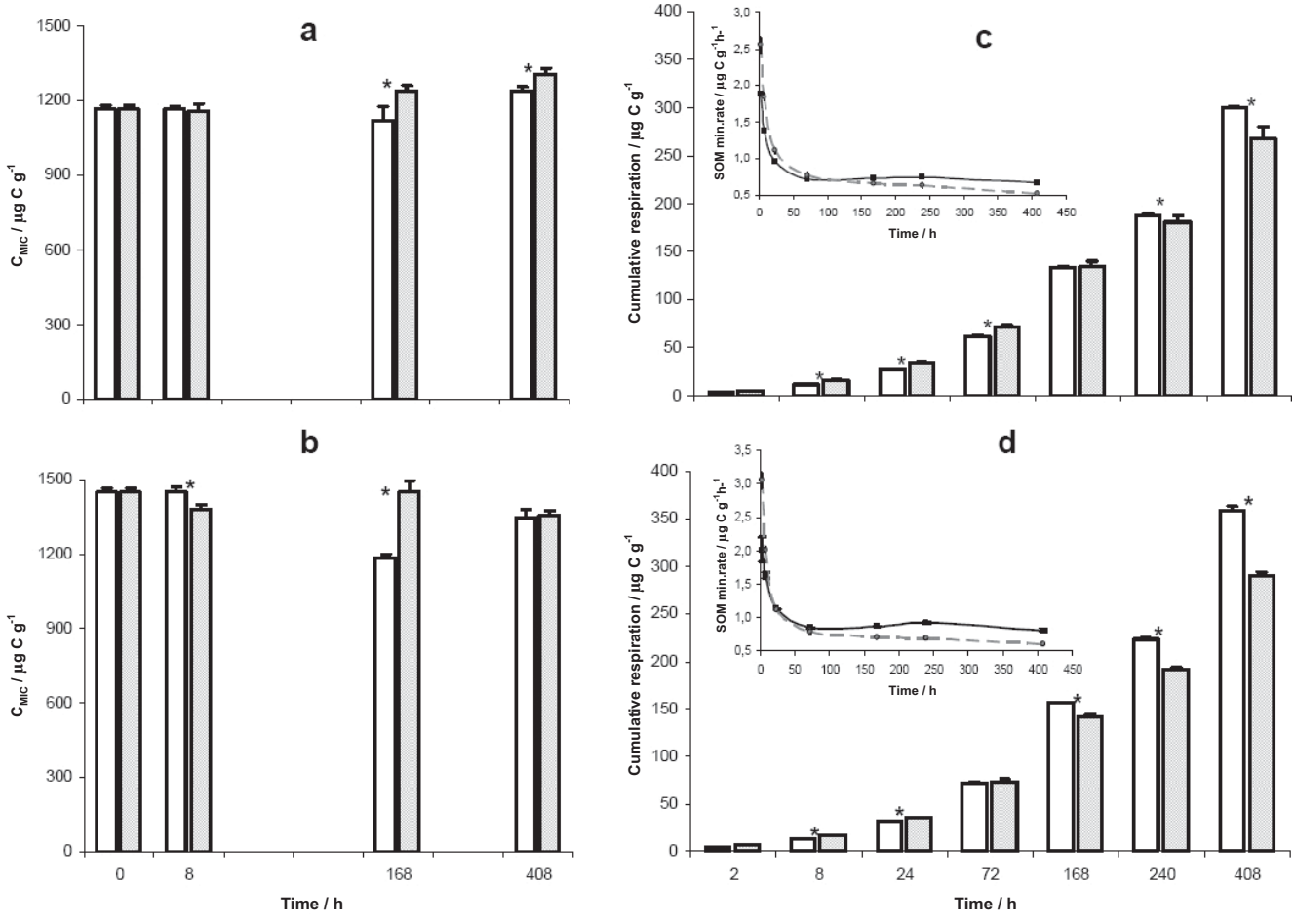

Figure 2: Glucose-nonamended soils: total microbial biomass in mineral (a) and organic (b) soils, cumulative respiration in mineral (c) and organic (d) soils; unfertilized treatments (white bars) and fertilized treatments (gray bars); SOM-mineralization rates in unfertilized (solid line) and fertilized (dotted line) treatments in the embedded figures. Means and standard deviations ($n = 3$) are shown. Asterisks indicate statistically significant differences between fertilized and unfertilized treatments at particular times (*t*-test, $p = 0.05$, $n = 3$).

compared to unfertilized treatments (Fig. 2). This confirms an expectation concerning the negative effect of nutrient addition on mineralization of low-quality SOM (*Fog*, 1988) and agrees with the studies of *Butnor* et al. (2003) and *Bowden* et al. (2004). The response of the microbial community to NP fertilization was larger in the organic soil, as seen from the larger positive PE at the beginning, as well as faster occurrence of the C-starvation phase and greater negative PE at the end of the experiment. This was likely related to the higher microbial biomass and lower quality (degradability) of SOM in this soil, which is in accordance with the results of *Dilly* (2001).

3.3 $\delta^{13}C$ of chloroform-labile C_{MIC} and CO_2 in glucose-amended soils

The $\delta^{13}C$ of chloroform-labile C_{MIC} and CO_2 were higher and more dynamic in comparison to natural soils, reflecting the incorporation of glucose with $\delta^{13}C$ of –11.79‰. The $\delta^{13}C$ increase in chloroform-labile C_{MIC} was slightly higher in fertilized than in unfertilized treatments (Fig. 3). The $\delta^{13}C$ of total C_{MIC} could not be determined or even calculated because we expected that the $\delta^{13}C$ of total biomass would differ from that of chloroform-labile C_{MIC} due to preferential glucose incorporation into chloroform-nonlabile C_{MIC} (Tab. 3). The initial $\delta^{13}C$-CO_2 of the fertilized treatments was 4‰–6‰ higher than in the unfertilized ones and exponentially decreased with time. The $\delta^{13}C$-CO_2 of the unfertilized variants peaked after 8 h and decreased to similar or lower values in comparison with fertilized variants (Fig. 3).

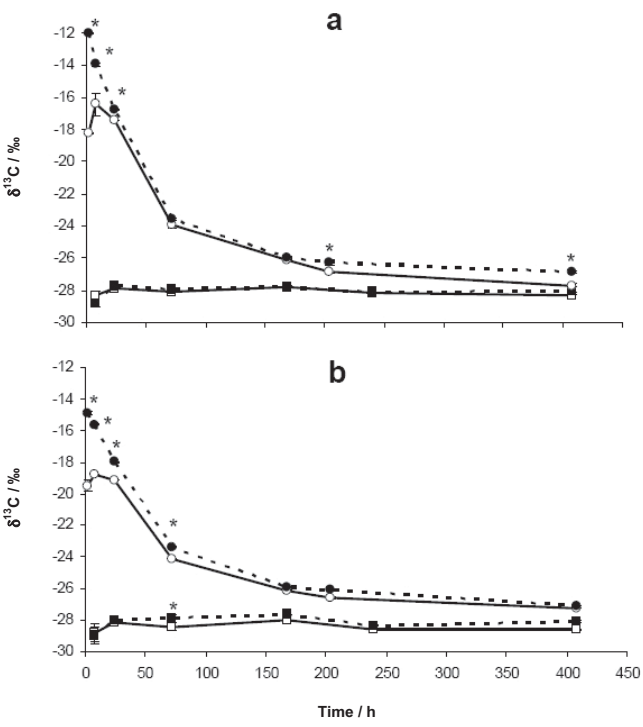

Figure 3: Glucose-amended mineral (a) and organic (b) soils: the $\delta^{13}C$ (‰) of chloroform-labile part of microbial biomass (□, ■) and respiration (○,●) in unfertilized (solid line) and fertilized (dotted line) treatments. Means and standard deviations (n = 3) are shown. Asterisks indicate statistically significant differences between fertilized and unfertilized treatments at particular times (t-test, p = 0.05, n = 3).

Table 3: Microbial utilization of glucose (400 μg C g^{-1}) in unfertilized and fertilized soils 8 h, 3 d, and 17 d after the glucose addition (mean ± standard deviation, n = 3). Within a given line, values with the same superscript letter are not significantly different (Tukey HSD test, p = 0.05, n = 3).

	Mineral soil + glucose			**Mineral soil + glucose + NP**		
	8 h	3 d	17 d	8 h	3 d	17 d
Unassimilated C / % glucose-C added	$13.0^{b} \pm 1.6$	$0.4^{a} \pm 0.1$	$0.2^{a} \pm 0.2$	$26.6^{c} \pm 0.3$	$0.2^{a} \pm 0.2$	$0.0^{a} \pm 0.1$
Glucose-C partitioning / % glucose-C assimilated						
CO_2	$11.0^{a} \pm 0.7$	$22.2^{c} \pm 0.6$	$28.4^{d} \pm 1.1$	$14.9^{b} \pm 0.1$	$29.7^{d} \pm 0.4$	$37.3^{e} \pm 0.5$
C_{FE}	$21.5^{b} \pm 0.4$	$20.5^{ab} \pm 0.4$	$18.1^{a} \pm 0.4$	$17.3^{a} \pm 2.9$	$20.0^{a} \pm 0.4$	$20.2^{a} \pm 0.2$
$C_{non\text{-}FE}$	$67.5^{e} \pm 0.3$	$57.3^{d} \pm 0.2$	$53.5^{c} \pm 0.7$	$67.8^{e} \pm 2.8$	$50.3^{b} \pm 0.7$	$42.5^{a} \pm 0.2$
C_{MB}	$89.0^{e} \pm 0.7$	$77.8^{c} \pm 0.6$	$71.6^{b} \pm 1.1$	$85.1^{d} \pm 0.1$	$70.3^{b} \pm 0.4$	$62.7^{a} \pm 0.7$
k_{EC}	$0.24^{b} \pm 0.002$	$0.26^{c} \pm 0.003$	$0.25^{bc} \pm 0.002$	$0.20^{a} \pm 0.033$	$0.28^{d} \pm 0.004$	$0.32^{e} \pm 0.004$
	organic soil + glucose			**organic soil + glucose + NP**		
	8 h	3 d	17 d	8 h	3 d	17 d
Unassimilated C / % glucose-C added	$14.9^{c} \pm 0.2$	$2.9^{b} \pm 0.9$	$1.8^{a} \pm 0.2$	$23.7^{d} \pm 0.9$	$2.0^{ab} \pm 1.0$	$1.5^{a} \pm 0.5$
Glucose-C partitioning / % glucose-C assimilated						
CO_2	$8.5^{a} \pm 0.3$	$17.3^{c} \pm 0.1$	$25.5^{d} \pm 0.7$	$13.1^{b} \pm 0.7$	$23.3^{d} \pm 0.3$	$30.7^{e} \pm 1.1$
C_{FE}	$15.0^{a} \pm 2.7$	$19.3^{b} \pm 0.6$	$18.0^{b} \pm 1.3$	$16.8^{a} \pm 0.3$	$22.8^{b} \pm 1.9$	$21.6^{b} \pm 1.3$
$C_{non\text{-}FE}$	$76.5^{e} \pm 2.7$	$63.4^{c} \pm 0.8$	$56.5^{b} \pm 0.6$	$70.1^{d} \pm 0.3$	$53.9^{b} \pm 2.1$	$47.7^{a} \pm 0.2$
C_{MB}	$91.5^{e} \pm 0.6$	$82.7^{c} \pm 0.1$	$74.5^{b} \pm 0.7$	$86.9^{d} \pm 0.9$	$76.7^{b} \pm 0.3$	$69.3^{a} \pm 1.1$
k_{EC}	$0.16^{a} \pm 0.030$	$0.23^{c} \pm 0.008$	$0.24^{c} \pm 0.015$	$0.19^{b} \pm 0.004$	$0.30^{d} \pm 0.025$	$0.31^{d} \pm 0.014$

3.4 Glucose partitioning in microbial metabolism

About 20% of assimilated glucose-C was found in the chloroform-labile part of C_{MIC}, while markedly higher amounts of glucose were in the chloroform-nonlabile C_{MIC} fraction (≈70% of assimilated glucose) similar to *Šantrůčková* et al. (2004) (Tab. 3). This resulted in a quite low conversion factor k_{EC} for glucose in comparison to commonly used ones for SOM (Tab. 3). While glucose-derived C in the chloroform-labile part of C_{MIC} varied minimally during incubation, it decreased over time in chloroform-nonlabile C_{MIC} (Tab. 3). This temporal dynamic of the glucose-derived chloroform-nonlabile C_{MIC} fraction indicated that it included not only structural compounds (cell-wall components, polymeric compounds). A majority of the partitioned glucose was stored in compounds, which were gradually used for respiration. This is consistent with the models of short-term glucose utilization proposed by *Bremer* and *Kuikman* (1994) and *Nguyen* and *Guckert* (2001). The storage compounds could be glycogen or poly-β-hydroxybutyric acid, which are accumulated by bacteria (*Martin*, 1992). Microorganisms in fertilized soils preferentially used the glucose-C for respiration (Tab. 3, Fig. 4c, d), which was related to a faster decrease of the glucose-derived chloroform-nonlabile C_{MIC} fraction and ≈5% lower efficiency of glucose assimilation (Tab. 3). This is somewhat contradictory to the higher decomposer efficiency under higher nutrient availability proposed by *Liljeroth* et al. (1990) and *Ågren* et al. (2001). This could be a response to osmotic stress caused by high N input.

3.5 Microbial growth and respiration in glucose-amended soils

Soil microorganisms quickly took up the added glucose, which contributed more than 50% to short-term respiration (1–3 d) (Fig. 4c, d) and induced growth of microbial biomass (Fig. 4a,b). In unfertilized treatments, this activation of microbial metabolism was connected with increased SOM decomposition and uptake. It was displayed by SOM incorporation into the new biomass (only in mineral soil; Fig. 4a) and increased SOM mineralization, causing a commonly observed transient positive PE (Fig. 4c, d—embedded figures) (*Blagodatskaya* and *Kuzyakov*, 2008). However, SOM-C uptake and mineralization was much less in the fertilized treatments of both soils compared to the unfertilized ones, as described before by *Merckx* et al. (1987) and *Liljeroth* et al. (1990). In our study, glucose-C covered the vast majority of the energetic demand of microbial metabolism (Fig. 4c, d) and also the synthesis of all new biomass (Fig. 4a, b). Additionally, the assimilated glucose caused a huge pool substitution within the microbial biomass in the organic soil.

Glucose depletion from the available-C pool within 3 d of addition (Tab. 3) was reflected in a gradual decrease of microbial biomass, mainly the SOM-derived part (Fig. 4 a, b), in both treatments. Finally, total microbial biomass was lower in fertilized treatments, containing a higher proportion of glucose-derived C than SOM-derived C at the end of the experiment (Fig. 4 a, b). Therefore, SOM mineralization as well as total soil respiration was lower in fertilized treatments (Fig. 4 c, d).

Table 4: Amounts of soluble organic C (C_{SOL}) in unfertilized (–) and fertilized (NP) treatments of mineral (Min) and organic (Org) soils at three sampling times during the incubation (mean ± standard deviation, n = 3). Within a given soil, effect of fertilization and time on C_{SOL} was evaluated; values with the same superscript letter are not significantly different (Tukey HSD test, p = 0.05, n = 3).

	C_{SOL} / μg C g^{-1}		
	8 h	3 d	17 d
Glucose-nonamended soils			
Min	62.41^{a} ± 2.23	60.99^{a} ± 1.20	65.58^{a} ± 2.71
Min-NP	72.03^{b} ± 3.10	83.14^{c} ± 2.67	85.83^{c} ± 0.54
Org	129.65^{a} ± 3.49	130.75^{a} ± 0.70	146.44^{b} ± 1.22
Org-NP	150.65^{b} ± 3.49	162.18^{c} ± 10.36	169.27^{c} ± 2.59
Glucose-amended soils			
Min	110.74^{c} ± 11.84	58.89^{a} ± 3.57	63.02^{ab} ± 7.94
– from that GLC	51.82 ± 6.40	1.91 ± 0.27	0.74 ± 1.87
Min-NP	181.22^{d} ± 4.91	76.94^{b} ± 4.05	73.28^{b} ± 4.09
– from that GLC	106.56 ± 1.07	–	–
Org	177.16^{c} ± 3.09	145.65^{ab} ± 3.14	134.59^{a} ± 7.95
– from that GLC	59.67 ± 0.74	11.41 ± 3.63	7.21 ± 0.60
Org-NP	232.37^{d} ± 10.36	177.03^{c} ± 4.78	153.00^{b} ± 3.96
– from that GLC	94.96 ± 3.51	7.86 ± 3.98	6.00 ± 2.06

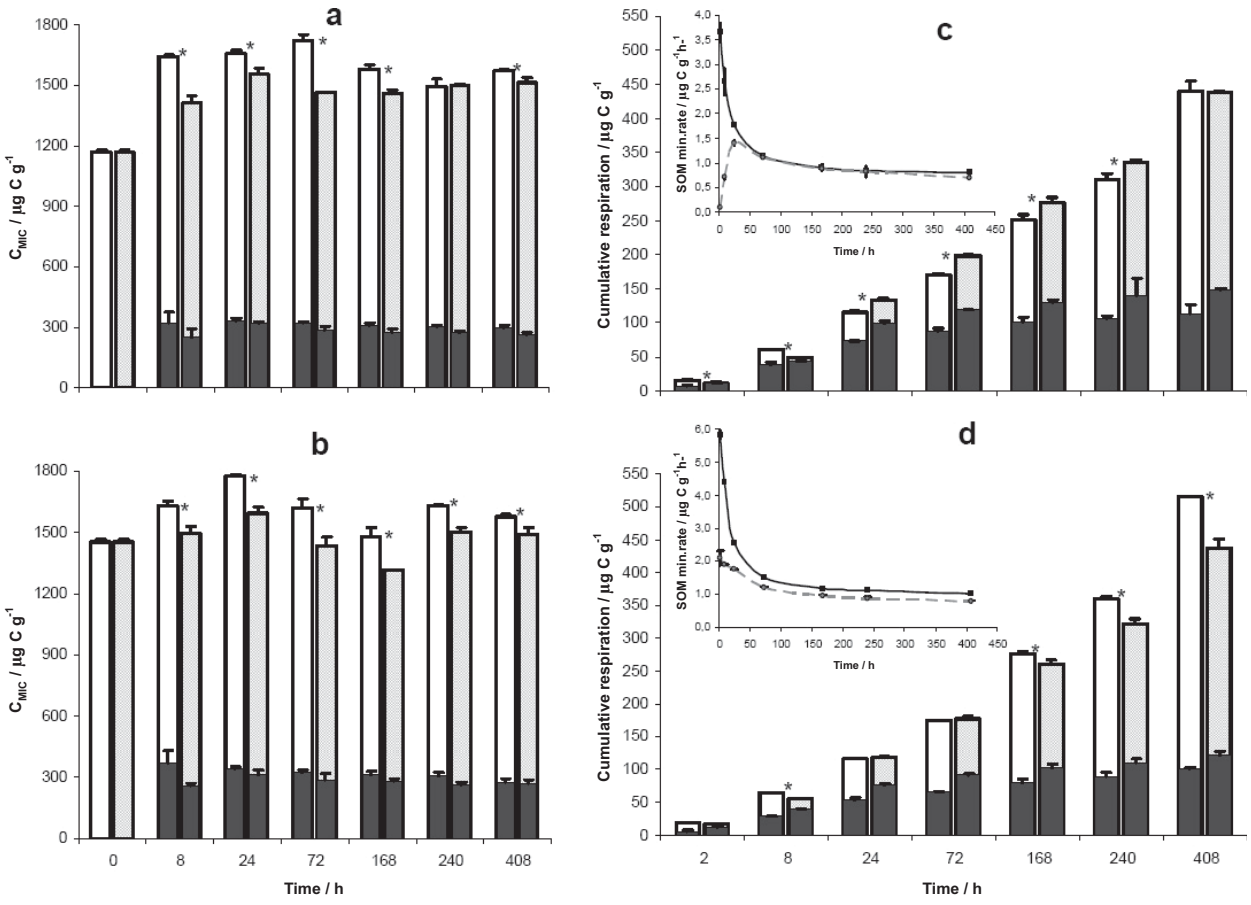

Figure 4: Glucose-amended soils: total microbial biomass in mineral (a) and organic (b) soils, cumulative respiration in mineral (c) and organic (d) soils; unfertilized treatments (white bars) and fertilized treatments (gray bars), black parts represent glucose-derived C in particular fractions; SOM-mineralization rates in unfertilized (solid line) and fertilized (dotted line) treatments in the embedded figures. Means and standard deviations (n = 3) are shown. Asterisks indicate statistically significant differences between fertilized and unfertilized treatments at particular times (t-test, p = 0.05, n = 3).

Fertilization of soils with sufficient available substrate retarded SOM utilization and provoked the preferential use of glucose in anabolic as well as catabolic processes of microbial metabolism. This resulted in the following differences in comparison to unfertilized treatments. (1) A large positive priming effect obviously occurred after glucose addition, but this was suppressed in both fertilized soils (Fig. 4c, d—embedded figures) due to preferential glucose utilization. (2) Net microbial growth in fertilized treatments was covered exclusively by glucose. This growth was slower and smaller than in unfertilized treatments, in accordance with *Ågren* et al. (2001) and *Yevdokimov* et al. (2005), and more energy-demanding. This could be a consequence of osmotic stress caused by high N input (*Yevdokimov* et al., 2005, 2008). (3) The activated microbial community in fertilized soils had lower glucose-assimilation efficiency and different strategy of glucose economy within microbial biomass (Tab. 3). It used higher proportion of glucose in respiration processes and accumulated less as a storage than in unfertilized treatments (Tab. 3). (4) Suppressed SOM transformation and the different physiological status of the microbial community led to significant SOM savings (Fig. 4 c, d—embedded figures) and, in the longer term, also to lower total soil CO_2 flux (Fig. 4 d). The response of the microbial community to NP fertilization was again larger in the organic soil with greater microbial biomass, similarly to glucose-nonamended soils.

It is known that repeated C-substrate additions, which could be the parallel to everyday exudate flow into the rhizosphere, induce repeated positive priming effects (*Hamer* and *Marschner*, 2005). Our results (Fig. 4c, d—embedded figures) indicate that fertilization in the doses we used might suppress SOM losses, which are connected with the apparent positive priming effects following C-substrate pulses, in both studied soils.

3.6 Effect of fertilization on sulfate-soluble C

Fertilization significantly increased the amount of sulfate-soluble OM in both glucose-amended and glucose-nonamended soils (Tab. 4). The increase of the dissolved C pool, similar to our results, has already been observed in forest soils after chronic N depositions (*Pregitzer* et al., 2004; *Sjöberg* et al., 2003) as well as after N additions under laboratory conditions (*Fog*, 1988; *Waldrop* and *Zak*, 2006). In our

study, the source of the majority of extra soluble C cannot be of microbial origin as often suggested (*Kuzyakov*, 2002; *Blagodatskaya* and *Kuzyakov*, 2008), as there was no corresponding decrease in microbial biomass. Therefore, we tend towards the explanation of *Fog* (1988). He suggested that the extra soluble C is of chemical origin; resulting from condensation reactions between the added N and indigenous carbohydrates or lignin-degradation products (browning, Maillard reaction).

4 Conclusions

The present study showed that NP fertilization decreased losses of soil organic C from soils both with and without available-C inputs. Decreased microbial respiration due to C/energy limitation of activated microbial metabolism occurred within a short time period after NP addition in both cases. Additionally, increased immobilization of soil organic C into microbial biomass contributed to lower C losses in the soils without C input. In soils with available-C input, fertilization decreased C immobilization in relation to unfertilized conditions. As a consequence, soil-organic-C savings were caused by suppression of its assimilation, exclusive use of glucose in the synthesis of new biomass, and by the preference of glucose-C against soil organic C in respiration processes.

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