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EFFECTS OF SOIL ALTERATION ON NITROGEN AND CARBON CYCLING

KOMBINOVANÝ VLIV ACIDITY A ŽIVINOVÉ ÚROVNĚ LESNÍCH PŮD NA KOLOBĚH C A N

Doctoral dissertation

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Prohlašuji, že jsem předloženou závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyly předloženy k získání jiného nebo stejného akademického titulu.

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List of Abbreviations

BC	base cations
CLRTAP	Convention on the Long-range Transboundary Air Pollution
EMEP	European Monitoring and Evaluation Programme
DOC	dissolved organic carbon
DON	dissolved organic nitrogen
DOM	dissolved organic matter
F/B ratio	fungi-to-bacteria ratio
SOM	soil organic matter

Abstract

Forest ecosystems are facing changing environmental conditions induced by anthropogenic pressures, including atmospheric deposition. Elevated sulfur (S) and nitrogen (N) depositions have led to changes in forest soil conditions, inducing acidification and N eutrophication. However, the consequences of atmospheric deposition for the functioning of forest ecosystems may be tree species specific. To assess the effects of S and N inputs on soil functioning of two forest stands, a soil chemistry manipulation experiment took place in two adjacent forest stands – Norway spruce (*Picea abies*) and European beech (*Fagus sylvatica*) – in the Ore Mts. Over six years, separated and combined additions of S and N compounds (50 kg $\cdot ha^{-1} \cdot year^{-1}$) were added to experimental plots in both stands to mimic enhanced atmospheric deposition. The aim of this research was to compare and investigate the soil carbon (C) and N stocks and fluxes, and their responses in relation to acid and N treatments. We found that under ambient conditions, the beech stand contained more C and N in both the forest floor and the mineral soil compared to the spruce stand. Nitrogen cycling in the beech stand was less tight, with periodical soil N leaching. The manipulated increase of soil acidity (decrease of soil solution pH) reduced the availability of dissolved organic C (DOC) and changed the microbial community structure, resulting in suppressed litter decomposition and C fluxes in both stands, with stronger impacts in the spruce stand. Surprisingly, experimental N addition showed no consistent responses in the microbial community composition or soil nutrient cycling, likely because of forest stand adaptation to ambient long-term enhanced N deposition. Our results have implications for the understanding of controls on both N saturation and C sequestration in forests subject to current and/or historic acid deposition. Considering that many European forests have experienced acid deposition, it is possible that recent measurements of C fluxes from these ecosystems might not be representative of systems in long-term balance with climatic conditions, but of systems subject to additional anthropogenic pressures, and in many cases undergoing transient changes as deposition levels decline.

Key words: tree species, sulfur, nitrogen, soil, carbon cycling, nitrogen cycling, C sequestration

Abstrakt

Lesní ekosystémy čelí měnícím se přírodním podmínkám vyvolaným lidskou činností, jako je atmosférická depozice. Zvýšené deposice síry (S) a dusíku (N) způsobily acidifikaci a eutrofizaci N lesních půd. Nicméně, důsledky atmosférické depozice pro fungování lesní ekosystémů mohou být závislé na druhovém složení. K odhalení účinků S a N na půdní procesy byl proveden manipulační experiment ve dvou sousedních lesních porostech tvořeného smrkem ztepilým (*Picea abies*) a bukem lesním (*Fagus sylvatica*), které se nachází v Krušných horách. Po dobu šesti let byly samostatně nebo kombinovaně přidávány látky obsahující S a N (50 kg $\cdot ha^{-1} \cdot rok^{-1}$) na experimentální plochy v obou porostech za účelem simulace zvýšené atmosférické depozice. Cílem tohoto výzkumu bylo porovnat a zjistit zásoby a toky uhlíku (C) a N v půdě a jejich odezvy v reakci na kyselé vstupy a vstupy N. Naše zkoumání ukázalo, že za přirozených podmínek bukový porost obsahoval více půdního C a N jak v organickém horizontu, tak v minerální půdě v porovnání se smrkovým porostem. Půdní cyklus N v bukovém porostu byl více otevřený a umožňoval ztráty N vyluhováním. Experimentální nárůst v půdní aciditě (pokles pH v půdní roztoku) omezil dostupnost rozpustného organického C a změnil půdní mikrobiální strukturu, což vedlo v potlačení rozkladných procesů a toků C v obou porostech, přičemž více ovlivněný byl smrkový porost. Experimentální vstupy N překvapivě neukázaly jasnou odezvu na půdní mikrobiální složení a půdní koloběh živin, pravděpodobně kvůli adaptaci lesních porostů na dlouhodobě zvýšenou depozici N. Díky výsledkům výzkumy můžeme pochopit kontrolní mechanismy saturace N a sekvestrace C v lesích, které byly nebo jsou vystaveny kyselé depozici. Pokud zohledníme fakt, že většina evropských lesů kyselé depozici byla vystavena, je možné, že současné měření toků C z takových ekosystémů nemusí odrážet reakci porostů na klimatické změny. Místo toho mohou porosty procházet změnami v reakci na klesající depozici.

Klíčová slova: druhové složení stromů, síra, dusík, půda, koloběh uhlíku, koloběh dusíku, sekvestrace uhlíku

List of publications and manuscripts included in the doctoral thesis

Publication I: **Růžek, M.**, Myška, O., Kučera, J., Oulehle, F., 2019. Input-output budgets of nutrients in adjacent Norway spruce and European beech monocultures recovering from acidification. Forests 10, 22. https://doi.org/10.3390/f10010068 (IF_{2019} = 2.221)

Publication II: Oulehle, F., **Růžek, M.**, Tahovská, K., Bárta, J., Myška, O., 2016. Carbon and Nitrogen Pools and Fluxes in Adjacent Mature Norway Spruce and European Beech Forests. Forests 7, 282. https://doi.org/10.3390/f7110282 (IF_{2016} = 1.951)

Publication III: Oulehle, F., Tahovská, K., Chuman, T., Evans, C.D., Hruška, J., **Růžek, M.**, Bárta, J., 2018. Comparison of the impacts of acid and nitrogen additions on carbon fluxes in European conifer and broadleaf forests. Environ. Pollut. 238, 884–893. https://doi.org/10.1016/j.envpol.2018.03.081 (IF_{2018} = 5.714)

Publication IV: Choma, M., Tahovská, K., Kaštovská, E., Bárta, J., **Růžek, M.**, Oulehle, F., 2020. Bacteria but not fungi respond to soil acidification rapidly and consistently in both a spruce and beech forest. FEMS Microbiol. Ecol. 96, 174. https://doi.org/10.1093/femsec/fiaa174 (IF_{2019} = 3.675)

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Author's contributions

Publication I: MR performed data processing, statistical analysis and prepared the manuscript. Author's contributions: 85%

Publication II: MR performed the experiment, analysed data, and contributed on statistical analysis and the manuscript writing. Author's contributions: 40%

Publication III: MR performed field work including CO_2 measurement, soil water and soil sampling, analysed the data and contributed on the manuscript writing.
Author's contributions: 30%

Publication IV: MR performed soil sampling and manuscript editing.
Author's contributions: 10%

Publication V: MR designed and performed the field work, performed the laboratory analyses except the CNS measurement and led the writing of the manuscript.
Author's contributions: 90%

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Mgr. Filip Oulehle, Ph.D.

1. Introduction

Forests play an important role in the global carbon (C) cycle and provide wide range of ecosystem services for humankind. Globally, forest ecosystems cover 4.0 – 5.0 billion ha, representing 30.6 % of the continental area (FAO 2019). From a biogeochemical perspective, forests store large amounts of organic matter (861 Pg C) in two major pools – the live biomass (363 Pg C) and soils (383 Pg C to 1-m depth; Pan et al. 2011). Towards the poles, the amount of soil organic matter (SOM) stored in forest soils increases (Pan et al. 2011). Therefore, forest soils in temperate or boreal zones of the Northern Hemisphere represent major a C reservoir, with an additional 1176 Pg C in deeper permafrost soils (Tamocai et al. 2009). Compared to the plant biomass, soils are long-term sink for organic matter, with some SOM persisting for millennia (Schmidt et al. 2011) and significantly contributing to C sequestration. An integral part of SOM is nitrogen (N), which is often a limiting nutrient together with phosphorus. Mechanisms controlling the fate of SOM reside largely in the soil-microbe-plant root complex, which regulates litter decomposition, nutrient uptake, and stabilisation mechanisms. However, the fate of SOM also relies on forest vitality and the stability of environmental conditions. Current anthropogenic activity has created strong pressures on biogeochemical cycles. Fossil fuel combustion emits large amounts of C and other compounds into the atmosphere resulting in a greenhouse effect, climate change and altered atmospheric deposition (Ciais et al. 2013). These processes accelerate environmental changes, which can threaten the stability of forest ecosystems, including SOM stability. Potential soil C release into the atmosphere would further accelerate climate change processes.

The atmospheric depositions of sulfur (S) and N in particular have affected Central European forests since the 2nd half of the last century. Air pollution was recognized as a major threat in industrialized Europe and North America in the past, and is now an increasing problem in developing countries (Galloway and Cowling 2002; Gao et al. 2018). The main sources of atmospheric S and N are industrial and agricultural activities. The deposition of these compounds drives various and diverse responses within forest stands, including changing forest C and N dynamics. This may alleviate nutrient limitation and lead to increased forest growth (Du and de Vries 2018), eutrophication (Aber et al. 1998) and accelerate soil acidification (Johnson and Reuss 1984), with the mobilization of toxic aluminium (Al) into the soil solution (Cronan et al. 1989). Some of

these processes may cause both positive and negative effects on forest dynamics (e.g., tree growth). However, long-term S and N inputs significantly alter the soil environment, with effects on both soil chemistry and microbial communities, which in turn affect forest stability and its ability to store nutrients (nitrogen, base cations) and capacity for C sequestration.

A currently widely discussed topic is the unfavourable tree species composition of forests in Czechia. The majority of our forests are used for forestry and timber production. Especially in Central Europe, original forest stands dominated by *Fagus sylvatica*, *Abies alba* and *Picea abies* have been almost fully converted to even-aged Norway spruce monocultures. The long-term sustainability of this silvicultural practice is questionable because coniferous monocultures slow down soil formation, alter the understory vegetation composition and have a limited ability to face environmental problems (Augusto et al. 2002). The combination of high atmospheric deposition, unfavourable tree species composition and nutrient-poor soils led to decline of mountainous forests during the 2nd half of 20th century in Central Europe, with the "black triangle" region at the Czech-German-Polish borders suffering the most (Grubler 2002; Kolář et al. 2015). Although S depositions markedly decreased since in the 1990s and soil chemistry is undergoing recovery (Johnson et al. 2018), the deposition of N remains significant and exceeds the natural deposition by an order of magnitude.

Since the 1990s, a combination of multiple factors (intensive drought, prolonged vegetation season, increasing temperature, atmospheric deposition) directly or indirectly linked to human activity have induced further physiological stress in forests (Cienciala et al. 2016; Kolář et al. 2020), which has resulted in the current widespread decline of spruce monocultures in Czechia. Beside forest decline, each forest disturbance leads to the release of a substantial amount of C from the biomass and soil to the atmosphere. It is clear that the current tree species composition of forests is no longer sustainable in adequately tackling ongoing climate change and the future tree species composition must change. An appropriate forest composition should be resilient to the changing environment, including atmospheric deposition. Furthermore, suitable tree species should be efficient in long-term soil C sequestration, maintain biodiversity and contribute to sustainable timber production.

This doctoral thesis aims to unravel the impacts of altered soil chemistry on soil organic matter dynamics, using a complex ecosystem comparison of two forest stands

subjected to simulated atmospheric deposition. The selected forest stands represented the most common coniferous and deciduous trees in Central Europe – European beech (*Fagus sylvatica)* and Norway spruce (*Picea abies*) – located in the Ore Mts.

2. Background

2.1 Carbon and Nitrogen cycles in forest soils

Carbon and nitrogen are main the components of all living bodies. Plant biomass contains ca. 45% of C mass, while N is essential component of amino acids, proteins, and nucleic acids. In the soil, both elements occur in organic forms as parts of living bodies, dead organic matter, their dissolved fractions (dissolved organic carbon and nitrogen DOC; DON), and in inorganic forms (carbon dioxide – CO_2, ammonium – NH_4^+, nitrite and nitrate – NO_2^-; NO_3^-, gaseous forms – N_2 and N_2O).

All C within an ecosystem is incorporated to the biomass through plant photosynthesis from atmospheric CO_2. Soil receives organic C by two pathways, either in the form of dead tissues of organic bodies or in the form of plant assimilates supporting mycorrhizal partners and rhizosphere. Organic C serves as source of energy for decomposers and other heterotrophic organisms. As organic C is processed by decomposers, some C is assimilated into their bodies to support microbial growth and some is respired back to the atmosphere as CO_2. Microbial growth depends on carbon-use efficiency, which is defined as the ratio of growth over C uptake (Manzoni et al. 2012). Roughly half of uptake C is invested into microbial biomass and half mineralized to CO_2 (Fig. 1). However, soil respiration C consists of two sources: C directly allocated belowground by trees (autotrophic respiration) and respiration by free-living heterotrophs from decomposed organic matter (Kuzyakov 2006). About half of the C delivered by plant roots contributes to soil respiration (Högberg et al. 2001). The incomplete decomposition of soil organic matter and leaching of dissolved organic compounds controls the production of DOC. This C form percolates with seepage water downward, but only little DOC reaches the mineral subsoil (Fröberg et al. 2011). The major fraction is either mineralized or retained in the soil. DOC interacts with soil mineral particles, contributing to C accumulation in the subsoil (Schmidt et al. 2011; Kaiser and Kalbitz 2012), even though soil C accumulation is not infinite (Guggenberger and Kaiser 2003).

Nitrogen enters temperate and boreal forest ecosystems mainly through atmospheric deposition and biological fixation (Högberg et al. 2017). In unpolluted regions and in forests without N-fixing trees, both pathways of N inputs are comparable, at about 1-3 kg N $ha^{-1} \cdot year^{-1}$ (Sponseller et al. 2016). Besides external inputs, reactive N is also (and dominantly) relieved during SOM decomposition. N-containing compounds

are cleaved by extracellular enzymes to monomers that are bioavailable (DON; Schimel, Bennett 2004). In cases of low N availability, trees compete with free-living microbes in N acquisition (Kuzyakov and Xu 2013). If N enters the plant biomass, it returns to the soil as litter or root exudates. The microbial utilisation of N can be either incorporation into the biomass or conversion to mineral form to gain energy (Fig.1). Processes of N mineralisation transform the organic N firstly to ammonium, then, if not immobilized, to nitrate. Further, nitrate might be reduced and emitted (N_2O, N_2) to the atmosphere or converted back to ammonium. Both ammonium and nitrate can also be incorporated into the living biomass and again returned to the soil environment. Nitrate is an easily mobilizable N form, and thus can be leached out from soil via percolated soil water.

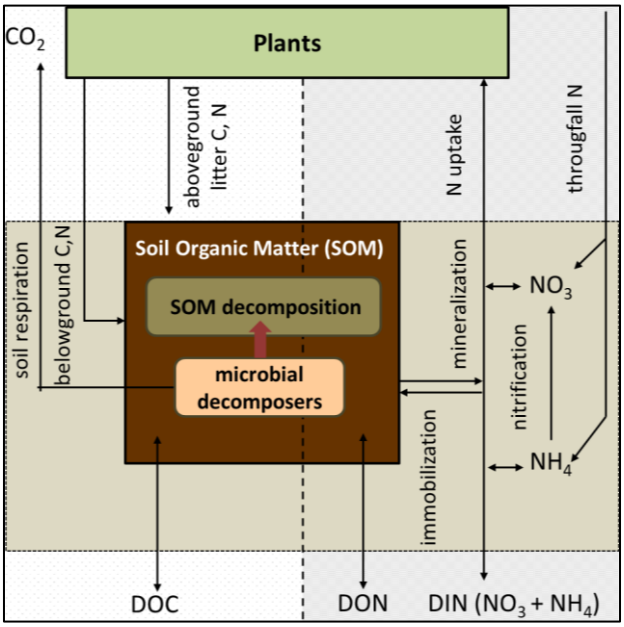


Figure 1: Main C and N pools, processes, and fluxes in forest soils

Both C and N cycles are tightly coupled. The relation between C and N is stoichiometrically constrained in living biomass by the C/N ratio. In plants, the proportionally highest N content can be found in leaves and fine roots (C/N ≈ 20 – 60), whereas tree stems, coarse roots and branches have the highest C/N ratio (C/N ≈ 150 – 250; de Vries, Du, Butterbach-Bahl 2014). If plant production is limited by a lack of N, elevated N inputs may increase C accumulation in trees by 5 – 25 kg C kg N^{-1} (de Vries et al. 2014), and subsequently elevate litter inputs to the soil. When litter falls on the ground, organic litter C mineralizes, leading to a decrease in the soil C/N ratio that usually parallels increasing soil depth. The soil C/N ratio is controlled by two stoichiometrically distinct groups of soil decomposers – fungi and bacteria. Fungi are

characterized by a higher C/N ratio, while bacteria have a lower C/N ratio (Sterner and Elser 2002). To acquire necessary nutrients, microbial decomposers transform the forest floor C/N ratio towards their stoichiometric biomass ratio (Zechmeister-Boltenstern et al. 2015).

2.2. Anthropogenic S and N deposition

Human demands for food and energy have resulted in a large production of atmospheric N (NO_3^-, NH_4^+) and S (SO_4^{2-}) compounds, originating from the production of fertilizers, livestock production, fossil fuel combustion, industry, and traffic (Kopáček and Posch 2011). In the 2nd half of 20th century, S and N deposition was recognized as an ecological threat, causing widespread acidification of soils and surface waters and forest declines across parts of Europe and North America. Since the adoption of the international Convention on Long-Range Transboundary Air Pollution (CLRTAP) and implementation by individual countries, substantial reductions of S and N emissions occurred throughout the developed world (Reis et al. 2012). Current global S deposition on continents is 58.9 Tg S·year^{-1} (Vet et al. 2014) and N deposition is between 63 and 74 Tg N·year^{-1} (Ciais et al. 2013; Tan et al. 2018). Despite reductions of S and N emissions in North America and Europe, global future projections show that S deposition will remain stable and N deposition will double by 2050 (Galloway et al. 2004; Fowler et al. 2013), because of industrial activity in southwest and eastern Asia (Vet et al. 2014).

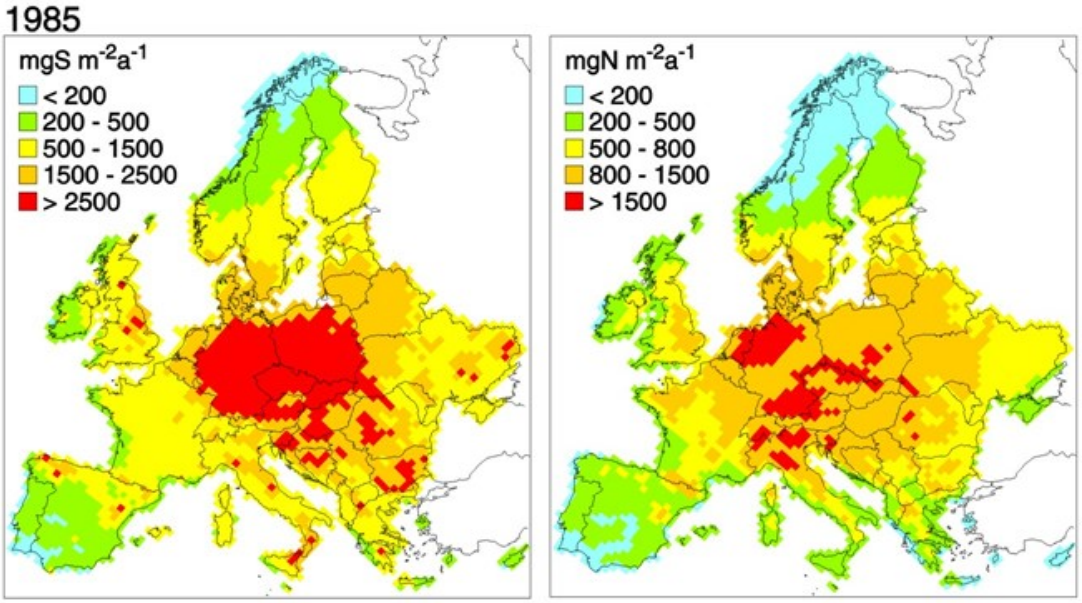


Figure 2: Bulk deposition of S (left) and N (right) over Europe in 1985. Source: Oulehle et al. (2011).

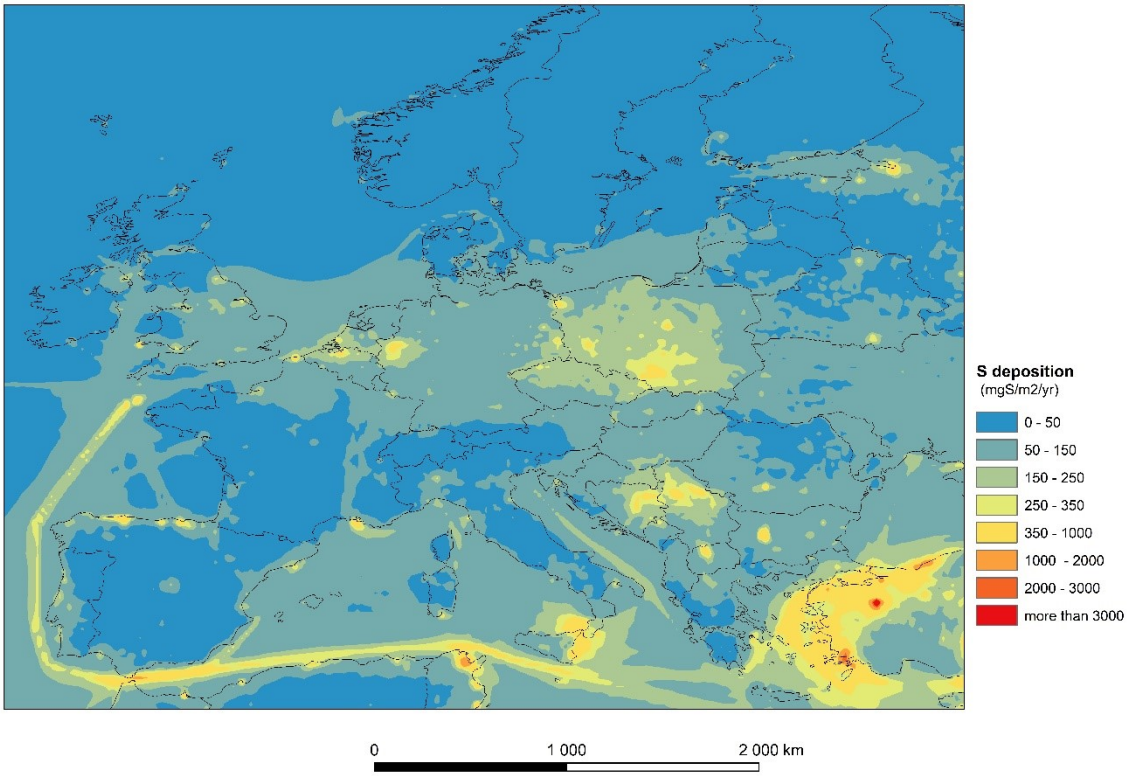


Figure 3: Deposition of sulfur over Europe in 2018. Source: EMEP

In Western Europe, S deposition peaked in the 1970s, while Central and Eastern Europe received high S amounts until the 1990s (Engardt et al. 2017). Reductions in S deposition in the former Eastern Bloc were related to political changes in the late 1980s (Fig. 2). Since then, S deposition has decreased by more than 80% over the past 30 years (Schöpp et al. 2003; Engardt et al. 2017). Currently, the highest bulk S deposition over Europe occurs in central Europe (Fig. 3), while other regions receive less S (Fischer et al. 2010). Over the Czech Republic, mean bulk S deposition was 11.6 kg $S \cdot ha^{-1} \cdot year^{-1}$ in the late 1970s and decreased to the current 3.8 kg $S \cdot ha^{-1} \cdot year^{-1}$ (Oulehle et al. 2016).

After a peak in the 1980s, emissions of N compounds (NH_3 and NO_x) decreased in Europe (Fig.2; Kopáček, Posch 2011). However, while the deposition of nitrate has decreased by 50%, the ammonium form has dropped by only 25% (Engardt et al. 2017). Currently, the N deposition in the NE USA and Europe is about 10 kg $N \cdot ha^{-1} \cdot year^{-1}$ (Fig. 4), which is still five times higher than prior to the industrial revolution (Waldner et al. 2014; Sullivan et al. 2018; Vuorenmaa et al. 2018). In the Czech Republic, current bulk depositions of $N\text{-}NO_3$ and $N\text{-}NH_4$ are 3.3 kg $N \cdot ha^{-1} \cdot year^{-1}$ and 3.9 kg $N \cdot ha^{-1} \cdot year^{-1}$, respectively (Oulehle et al. 2016).

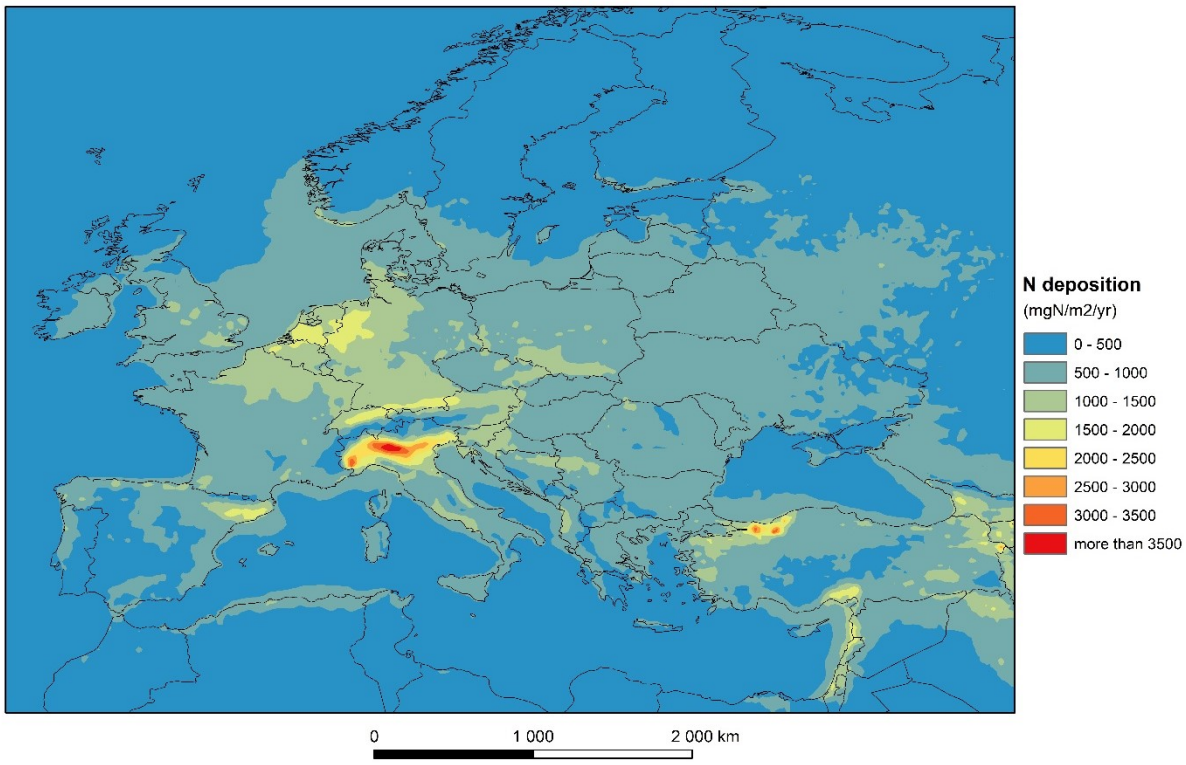


Figure 4: Deposition of nitrogen over Europe in 2018. Source: EMEP

2.3. Anthropogenic N deposition effects on forest ecosystems

Atmospheric N deposition has the potential to change forest ecosystem functioning. Under unpolluted conditions with negligible atmospheric N deposition, N is usually the most limiting nutrient (Vitousek and Howarth 1991; Högberg et al. 2017), and constrains the net primary production of temperate ecosystems (LeBauer and Treseder 2008). Most of N is acquired from decomposing litter and SOM. Strong competition for N between plants and decomposers ensures effective N immobilisation connected with negligible N leaching. The N cycle is essentially closed. Hydrologic N losses are primarily organic N (DON) (Hedin et al. 1995) and in long-term balance with net N inputs from fixation and deposition.

Anthropogenic N inputs increase ecosystem N availability. External N alleviates ecosystems from N limitation and stimulates NPP (LeBauer and Treseder 2008). With increasing N availability, an N surplus stimulates N mineralization and nitrification (Nave et al. 2009; Cheng et al. 2019) and available mineral N forms increase in the system. The ecosystem thus switches from N limitation towards N saturation (Ågren and Bosatta 1988). However, the state of N saturation differs with individual models of N enrichment. Several models of N saturation have been proposed (Aber et al. 1989, 1998; Lovett and

Goodale 2011; Kopáček et al. 2013). Aber´s conceptual model postulates a set of different stages from a N limited (stage 0) to N fully saturated (stage 3), and in the long-term perspective, an ecosystem continually moves towards stage 3 while being N enriched. N saturation is defined as the state, when plant and microbial N demands are fulfilled, and available N is in excess. Then, unused N might be lost through leaching or gas emissions. In contrast, Lovett and Goodale (2011) allowed several simultaneous N fates. N is incorporated into the sinks, which are controlled by their capacity and incorporation rate. If either the capacity of an N sink or its incorporation rate is exceeded, the N sink is saturated and N flows by other pathways. This model better explains the observed rapidity of ecosystem responses to elevated N availability (e.g. forest disturbance). Kopáček et al. (2013) linked N saturation with cycles of other nutrients. This theory states that N (and S) deposition induces C limitation through a set of processes, including lower C belowground allocation, DOC immobilisation and enhanced DOC mineralisation. In general, the principal manifestation of ecosystem N saturation is a state when an ecosystem is not able to efficiently immobilize N. N mineralization and nitrification exceed ecosystem demands for N, the immobilisation capacity of the system is exhausted, and mineral forms are lost from the ecosystem (Stoddard 1994). The soil N cycle is thus opened. The threshold when N deposition brings an ecosystem to N saturation is different for every ecosystem and depends on many factors, such as the ecosystem carbon pool, initial N limitation of the site, colimitation of other nutrients, and the intensity of N inputs (Aber et al. 1998). An increase in N availability is accompanied by set of ecological changes in ecosystem functioning, which are described as follows:

Low N addition alleviates ecosystem N limitation and competition between microbes and plants vanishes as more N is available. Additional N stimulates litter production and microbial activity (Gallardo and Schlesinger 1994; Schimel and Bennett 2004); in addition, plant tissues become enriched by N (Aber et al. 1989). These processes increase litter decomposition and heterotrophic respiration (Knorr et al. 2005; Vivanco and Austin 2011; Forsmark et al. 2020) to fulfil enhanced N demands.

As soil becomes N enriched (i.e., soil C/N decreases), trees take up N more readily and may change their strategy in N acquisition. Instead of C allocation belowground into root nutrient-acquiring system, trees may invest more C aboveground (Liu and Greaver 2010) stimulating forest growth (Etzold et al. 2020). Subsequently, this shift induces changes in the distribution and quality of delivered C to the soil, when a larger amount of

easier decomposable C is deposited on the soil surface. With lower C belowground investment, the plant-microbe interaction weakens (Treseder 2004), and total microbial and mycorrhizal biomass is often reduced (Treseder 2008; Lilleskov et al. 2019). The decrease of soil C/N alters the system towards microbial C/N which is reflected in a shift of the microbial community composition and decrease of the fungi/bacteria ratio (Hobbie et al. 2012). Nitrophilic organisms, which are more efficient in decomposition (production-to-assimilation ratio), replace original nitrophobic and low-efficient communities (Ågren et al. 2001). Together with changes in microbial composition, alterations in the activity or expression of enzymes degrading SOM are also reported. The expression of lignin-degrading enzymes decreases (Carreiro et al. 2000; DeForest et al. 2004), while the activity of enzymes degrading cellulose are reported to increase (Magill and Aber 1998). Changes related to microorganisms and litter C quality have an impact on litter decomposition. Many studies have shown that N deposition stimulates the decomposition of high-quality litter, while inhibiting the decay of low quality (lignin-rich) material (Knorr et al. 2005). The same pattern has been revealed during the decomposition process, when N stimulates decomposition in the early decomposition phase and inhibits lignin degradation in the later phase (Berg and Matzner 1997; Berg and McClaugherty 2009). Beside the biotic stabilisation mechanisms of decomposition, abiotic interactions of N with organic matter may also contribute to SOM stabilization (Nommik and Vahtras 1982).

Altogether, under enhanced N availability ecosystems usually increase the aboveground C litter flux at the expense of belowground C allocation and reduce litter decomposition which results in supressed soil respiration (Janssens et al. 2010) and increased C storage (Nave et al. 2009; Lu et al. 2011). However, the destination of allocated C differs among studies, with some reporting C accumulation in the forest floor (Pregitzer et al. 2008; Lovett et al. 2013; Forsmark et al. 2020) and others in the mineral soil (Nave et al. 2009). Concentrations of DOC and DON have been found to increase in seepage water (Pregitzer et al. 2004) with a more intensive DON flux, resulting in a decrease of the DOC/DON ratio (Brookshire et al. 2007). Yet, the mechanism responsible for the DON increase is not known, but may reflect a shift in plant uptake favouring inorganic N (Brookshire et al. 2007). Due to the higher production of mineral N forms in the soil (excess of N mineralization over N immobilization), higher nitrate leaching, and

gaseous N emissions have been reported (Stoddard 1994; Dise and Wright 1995; Papen and Butterbach-Bahl 1999).

2.4. Acidifying effect

In addition to the ecological impacts of deposited N as a nutrient, inorganic S and N compounds may have also acidifying consequences for soils and may affect forest ecosystem functioning by altering soil chemistry (Hruška and Cienciala 2005). The contact of acid compounds with foliage can cause direct damage to plant tissues (Hantschel et al. 1988). In the soil environment, deposited S and N induce chemical changes stimulating adverse effects on plants and soil microorganisms. Acid deposition increases the soil solution concentration of protons (H^+) and acid anions such as sulfate (SO_4^{2-}), nitrate (NO_3^-) and chloride (Cl^-). In temperate forests on pH neutral or slightly acid soils, free H^+ in the soil solution is neutralized by base cations (BC = Ca^{2+}, Mg^{2+}, K^+, Na^+), which are released from the sorption complex during bedrock weathering. Acidification occurs when BCs are continuously lost from the soil (losses > inputs from deposition and weathering), mostly by solute leaching and via biomass harvesting. From a mass balance perspective, if H^+ inputs exceed the BC weathering rate, soil pH and the acid neutralisation capacity decrease. It is important to note that soil acidity can also originate from other reasons, not only from acid deposition. Internal processes such as nitrification or cation uptake are responsible for H^+ production in soils (Breemen et al. 1984). Further decreases of soil pH dissolve mineral Al and other metals, adding them to the soil solution. As a result, further soil acidification increases H^+ and Al^{3+} concentrations in soil solutions.

Plants and microbes are usually adapted to natural ambient conditions, and significant soil acidity increases may pose additional stress. Plants suffer from high soil solution H^+ concentrations and low soil base cation saturation, especially Mg. Soluble inorganic monomeric Al in the soil solution may induce tree stress either directly from Al poisoning, or indirectly by preventing nutrient uptake (Cronan and Grigal 1995). Reduced base cation uptake causes a nutrient imbalance and higher susceptibility to physical and pathogenic stressors (Cronan et al. 1989). Taken together, these mechanisms may lead to crown yellowing, tree defoliation, reduced forest growth, loss of mycorrhiza and an increase of tree mortality (Roelofs et al. 1985).

In soils, acid deposition controls SOM solubility via physico-chemical mechanisms. At low pH levels, organic acids (DOC and DON in humic substances) are

protonated, which suppress their mobility in soil (Evans et al. 2012). In addition, organic matter solubility may be influenced by other acidity-related processes, including ionic strength (Hruška et al. 2009), complexation with metal ions, such as Al (Clark et al. 2011), and anion adsorption (Borken et al. 2011). A reduced availability of organic C has negative effects on microbial activity since microbes use DOC as an easily available source of energy (Kuzyakov et al. 2000). Moreover, acidity itself stresses soil microbes, decreases the soil microbial biomass, and alters microbial community composition towards more acidotolerant species (Rousk and Bååth 2011) as well as reducing C and N mineralization processes (Persson et al. 1989). Bacteria have a lower tolerance to acidity, which relatively increases fungal biomass and the fungi/bacteria ratio (F/B) in acidified soils (Rousk et al. 2009). Shifts in soil microbial structure can significantly change metabolic processes, and thus C and N cycles (Persson et al. 1989; Pennanen et al. 1998). Reduced C availability and microbial composition changes may result in lower litter decomposition (Berg 1986; Wu et al. 2016). As a consequence, the accumulation of C and N in topsoils (Oulehle et al. 2011; Wu et al. 2016) caused by supressed C decomposition due to acid deposition may occur. These results suggest that S deposition has the potential to greatly alter ecosystem C and N cycles.

2.5. Norway spruce vs. European beech forests and their responses to air pollution

The tree species composition of European temperate forests largely reflects their dominant economic purpose, which is timber production. Evenly aged monocultural conifer stands have been planted for silvicultural reasons far beyond their presumed natural ranges, where otherwise broadleaved deciduous species would dominate. Until recently, the most preferred species by foresters – Norway spruce (*Picea abies*) – was largely planted even in lower altitudes. With ongoing climate change and consequent alteration of environmental conditions, the optimal growth conditions of individual tree species and spatial tree species distribution will change. In Central Europe, the natural expansion of Norway spruce is expected to move towards higher altitudes, replaced by mixed or pure broadleaf stands, including European beech (*Fagus sylvatica*; Treml, Ponocná, Büntgen 2012; Pretzsch et al. 2014). Norway spruce monocultures are considered to be unstable and susceptible to disturbances. Thus, the planting (and growing) of spruce forests in low- and mid- altitudes will likely be even more difficult in the future. In addition, forest ecosystems are currently valued not just for economic production but also according to their ecological and social functions: soil, water, and

climate protection, scavenging of air pollutants, maintaining biodiversity and leisure activities. The introduction of larger proportions of beech trees to forests would increase the stability of forests and alter them back towards the natural tree species composition. Representing two major ecological groups of tree species – evergreen conifers and deciduous broadleaves – beech and spruce forests affect forest functioning through differences in physiological traits (Binkley and Giardina 1998; Augusto et al. 2015). A comprehensive comparison of evergreen conifers and deciduous broadleaves has been conducted (Augusto et al. 2015). They found drier soil conditions under conifers, but biomass and litter production were rather similar between the tree species groups, and evidence of a higher accumulation of soil C stocks under deciduous species was lacking.

In regions with low atmospheric deposition, tree species-driven differences in C and N fluxes are negligible (Rothe et al. 2002; Augusto et al. 2015). Total C and N soil stocks are similar in spruce and beech stands, while higher forest floor C and N have been found in spruce compared to beech forests with the opposite trend in the mineral soil (Vesterdal et al. 2008, 2013). A different SOM distribution results from litter quality and belowground C allocation. Spruce needle litter is enriched in phenolic compounds, which produce organic acids, hampers decomposition process and results in a thick forest floor (Hagen-Thorn et al. 2004). In contrast, the deeper rooting system of beech delivers more C to the mineral soil than shallow-rooting spruce (Oostra et al. 2006).

Differences also arise when forests are exposed to elevated atmospheric pollution. Spruce enhances atmospheric deposition inputs, via dry deposition, to the soil surface more effectively compared to beech (Rothe et al. 2002; Oulehle and Hruška 2005; Augusto et al. 2015), because of the persistent foliage and higher leaf area index (Schrijver et al. 2007). Even taking into consideration the lower throughfall precipitation under conifers, the throughfall S and N fluxes under spruce stands are greater and more concentrated compared to beech forests (Bergkvist and Folkeson 1995; Augusto et al. 2002; Schrijver et al. 2007; Berger et al. 2009b). Coniferous canopies can uptake deposited NH_4^+ in exchange to DON, resulting in lower throughfall NH_4^+ fluxes compared to deciduous trees (Schrijver et al. 2007; Kopáček et al. 2011). On the contrary, beech stemflow fluxes may conduct water and acidify soils surrounding the tree trunk (Falkengren-Grerup 1989). Combining elevated deposition inputs and poor litter quality, soils under spruce stands tend to be more subjected to soil acidification, accumulate more organic matter in the forest floor, and thus increase soil C and N pools (Rothe et al. 2002;

Berger et al. 2009a; Cremer et al. 2016). Therefore, naturally acidic spruce ecosystems give negative feedback via higher acid deposition under the tree canopy and further accelerated soil acidification. Spruce stands are thus more stressed, and have a lower biomass increment and litter production making them more prone to pest infection and/or natural damages (Hlásny and Turčáni 2013).

In the case of N deposition and subsequent alterations to N saturation status, higher N deposition in spruce stands results in spruce stands tending to reach saturation and nitrate leaching faster than beech stands (Rothe et al. 2002). The annual high litter accumulation in beech forests favours high nutrient turnovers and external N inputs are then more rapidly incorporated into new biomass. Thus, beech stand should be able to incorporate more mineral N into organic form compared to spruce. Such incorporation of N into biomass protects N from potential leaching (Persson et al. 2000).

2.6. Concluding remarks

Even though, the general effects of acid and N deposition on forest ecosystems functioning have been extensively studied, a comprehensive conceptual understanding of acidity/N availability effects on C and N processes is still lacking.

Our knowledge of ecosystem responses to elevated acidity and N availability originates from three research approaches – long-term monitoring, large-scale gradient studies, and manipulation experiments. Each approach has specific strengths and weaknesses (see Lovett and Goodale 2011). Monitoring of a specific forest stand is long-lasting and gives high temporal resolution, but spatial representativeness is often limited. In contrast, spatially extensive gradient studies may cover variable environmental conditions (e.g., a deposition gradient), but many other variables co-vary (climate, soil properties, stand age, stand composition), which hinder proper evaluations of a sole factor of interest. Assessments of experimental manipulations depend on their design, duration, applied form and intensity of application. Despite rather advanced experimental designs, spatial representativeness is often limited.

Effects of N deposition have received more attention than S deposition, likely due to the widespread occurrence of N deposition and comprehensive set of mechanisms affecting forest functioning (e.g., nutrient limitation in ecology studies). Many regions exposed to high N depositions were also associated with currently or formerly high S deposition. Ecosystem fluxes in these regions might not be responding only to N inputs

but could be undergoing transient change as S inputs change. also reflect concurrent changes associated with S deposition.

3. Motivation, aims and objectives

3.1. Motivation

This doctoral thesis expanded on long-term and comprehensive biogeochemical research at Načetín site, located in the Ore Mts. The research site Načetín, managed by the Czech Geological Survey, has witnessed profound changes in forest biogeochemistry as a result of declining acid deposition since 1990s (see Chapter 4.1.). Results from continuous precipitation chemistry monitoring (Oulehle et al. 2006), reconstructed historical pollution impacts in the area (Oulehle et al. 2007) and repeated soil surveys (Oulehle et al. 2011) provided evidence about recovery of forest ecosystems after deposition reduction. Between the period 1994 and 2010, that the Oa horizon at one studied site Načetín lost 47% of its C pool, and 42% of its N pool. Concurrently, inorganic N leaching from the ecosystem declined from 1.28 g N m^{-2} yr^{-1} to near-zero. These profound changes in ecosystem biogeochemistry appear to be the result of suppressed decomposition, and consequent organic matter accumulation, during the peak of acidification, followed by the remobilisation of this pool as S deposition has declined. Of the lost N, most has been translocated from the organic to the mineral soil, with a smaller amount incorporated into tree biomass as growth rates have increased. Most of the C lost appears to have been respired to CO_2, with a smaller amount translocated to the mineral soil or lost to surface waters as DOC.

These results from the Načetín monitoring program motivated us to propose field manipulative experiment to provide insights into internal soil processes involved in C and N cycling under changing soil chemistry. The main goal was to test whether soil processes that are altered by experimental treatments respond consistently with monitoring observations, i.e., acidification or N availability are the principal drivers of C accumulation in acidified soils.

3.2. Aims and objectives

The general aim of this thesis was to investigate the effect of artificial S and N additions on soil C and N cycling in two distinct forest stands (*Picea abies* vs. *Fagus sylvatica*). Based on a literature review and results of previous studies (Oulehle and Hruška 2005; Oulehle et al. 2006, 2007, 2011), the following objectives and hypotheses were formulated:

(I.) To describe and compare current soil C and N fluxes and pools, in each of the forest stand (**Publication I**, **II**). We hypothesised higher biomass nutrient pools in beech stand and comparable soil C and N pools in both stands. The higher extent of current soil acidification will be assessed in the spruce stand compared to the beech stand.

(II.) To quantify the effect of acidity/N availability changes on the amount of C and N lost from the forest soil via organic (DOC, DON) and inorganic (NO_3, CO_2) pathways, in two major forest types (**Publication III**). We hypothesised that SO_4 addition, by altering soil acidity, causes a shift from organic C and N loss pathways (DOC, DON) at higher pH, to inorganic (CO_2, NO_3) loss pathways at lower pH. Further, we hypothesized that the ecosystem C balance is sensitive to both N and S deposition, with N deposition leading to increased C accumulation and DOC production/leaching, and S deposition leading to higher C accumulation, greater DOC retention, and suppressed decomposition.

(III.) To measure how acidity/N availability affects a range of key internal N and C transformations, the microbial community and "*in situ*" SOM decomposition (**Publication IV, Manuscript V**). We hypothesised the suppression of microbial activity under acid treatments in both stands, due to the decrease of organic C-availability for microorganisms. A shift towards a higher production of C-mining enzymes was expected. Further, we hypothesised that N availability will reduce litter decomposition by the alleviation of N limitation. Increasing N availability will decrease the fungi/bacteria ratio and qualitatively transform the microbial community structure towards N-tolerant taxa.

4. Site description and methodological approach

4.1 Experimental site – Načetín

The experimental site is in the Ore Mts. close to Czech-German border (Fig. 5). Mean annual temperature is 7.1°C and mean annual precipitation is 1110 mm (2005-2017). The site consists of two forest stands – Norway spruce (*Picea abies*) and European beech (*Fagus sylvatica*). The proximity of stands (700 m) ensures the same abiotic conditions. Both stands lay on mild slope oriented to the northwest. The prevailing soil type is Cambisol dystric (FAO 2014) developed on paragneiss bedrock. The spruce site (50°35'26''N; 13°15'14''E), formed by the 2nd generation of an 80-years-old Norway spruce monoculture, is located at 784 m asl. The beech site (50°35'22''N; 13°16'07''E; 825 m asl.) is composed of solely European beech, aged approx. 150 years (Fig. 6).

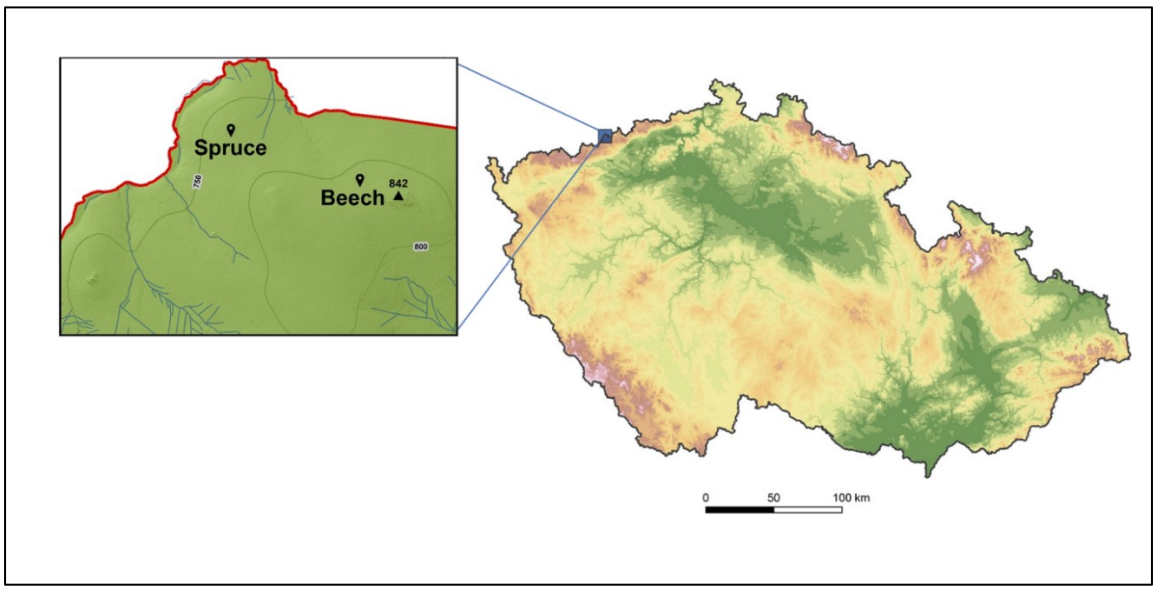


Figure 5: Localization of research site Načetín

Historically, both stands were covered by a mixed fir-spruce-beech forest. While the current spruce stand (2nd generation) was converted to an even-aged monoculture after 1850, the beech stand continued as a mixed forest of dominantly beech and spruce (based on aerial photographs from 1953). During the 1970s and 1980s, however, mature spruce trees continuously died off due to air pollution, and the stand became a pure beech monoculture. The onset of industrial activity with subsequent power-plant brown coal combustion in nearby coal basins has caused increases in pollutant emissions (SO_2, NO_x, ash). Moreover, local brown coal contained a high amount of S, which caused high S emissions. The first study from 1989 reported spruce throughfall depositions of 150 kg

$S \cdot ha^{-1} \cdot yr^{-1}$ and 46 kg $N \cdot ha^{-1} \cdot yr^{-1}$ (Dambrine et al. 1993). The political changes after the 1990s resulted in S emissions abatement and a substantial decrease in acid deposition (Oulehle et al. 2006). The research infrastructure of the Czech Geological Survey in the Načetín research station was established in the spruce stand in the early 1990s. Long-term monitoring was extended to the adjacent beech stand in 2003 (Oulehle and Hruška 2005).



Figure 6: The beech stand(left) and Norway spruce monoculture (right)

4.2 Methods

The research combined approaches from the long-term monitoring and the manipulation experiment. All methods used are described in detail in the publications.

Briefly, both stands in the Načetín research site were established to monitor biogeochemical responses of forested ecosystems to declining acid deposition. Bulk precipitation from the open atmosphere has been collected into two funnels, located in an open area between the two stands. Water percolating through tree canopies (throughfall precipitation) has been sampled into nine collectors in a 15 × 15 m grid in each stand. In addition, the beech stand was equipped with a stemflow collector. Soil leachates were collected below the organic horizon (forest floor), and in the mineral soil at depths of 30, 60, and 90 cm. The soil solution from the organic horizon was collected by zero-tension lysimeters as a composite sample of three replicates. All precipitation and soil water samples were taken monthly. Available precipitation chemistry and soil solution chemistry data covered the period since 1994 in the spruce stand and since 2003 in the beech stand.

The second approach was experimental, with soils of both stands subjected to elevated S and N additions. At each stand, 16 square plots (3 x 3 m) were established in a block-randomized design, to which 4 experimental treatments were applied – control (collected ambient rainwater), the addition of sulfur (S) – 50 kg $S \cdot ha^{-1} \cdot yr^{-1}$ (in form of H_2SO_4), nitrogen addition (N) – 50 kg $N \cdot ha^{-1} \cdot yr^{-1}$ (in form of NH_4NO_3) and combined S+N addition – 50 kg $S \cdot ha^{-1} \cdot yr^{-1}$ and 50 kg $N \cdot ha^{-1} \cdot yr^{-1}$ (in form of H_2SO_4 and NH_4NO_3). Treatments were applied monthly during the vegetation season (April – November). Treatments were diluted in 10 l of ambient rainwater and poured using watering cans on the plots. The experiment was set up in 2013 and soil water collectors were installed, and thus this first year served as a pre-treatment control. This period was then followed by the application of treatments beginning in spring 2014.

A variety of approaches including biogeochemical, soil ecological and microbiological methods were used to obtain information about C and N dynamics. Depending on the approach used, differing subsequent laboratory or computing processing methods were required. Sampling and analytical methods have been specifically described in the individual papers and all analytical procedures were conducted in laboratories of the Czech Geological Survey in Prague, the Global Change Research Institute CAS in Brno, the University of South Bohemia in České Budějovice, and Leibniz University in Hannover. Computational and statistical processing was performed using Microsoft Office package, the R programme and NCSS statistical software.

5. Major findings

The main results from manipulation experiments were published in four papers in international peer-reviewed journals with impact factors, and one manuscript, which is currently undergoing major revisions, is expected to be published in the journal Plant and Soil. **Publication I** presented and discussed the main element and water fluxes in both stands. **Publication II** presented and discussed a comparison of the current C and N fluxes and pools in the spruce and the beech stands. **Publication III** investigated the effects of treatment additions on soil C fluxes (element fluxes and internal soil processes) in the spruce and in the beech stands. **Publication IV** described major changes in soil microbial communities as a consequence of treatment additions. **Publication V** examined the effects of treatment additions on "in situ" litter decomposition and its dynamic over a two-year period.

Publication I (IF_{2019} = 2.221): Růžek, M., Myška, O., Kučera, J., Oulehle, F., 2019. Input- output budgets of nutrients in adjacent Norway spruce and European beech monocultures recovering from acidification. Forests 10, 22. https://doi.org/10.3390/f10010068

Publication I described major changes in nutrient fluxes, including C and N, within both stands during acidification recovery. In the period 2005-2017, bulk precipitation, throughfall precipitation and soil leachates were measured in monthly intervals. Over the investigated period, soil water pH increased from 4.25 to 4.47 under the spruce and from 4.42 to 4.69 pH units in the beech stand. Increases in DOC concentrations by 25% in the spruce forest floor and in the deep mineral soil were related to improvements of soil conditions in the forest floor (stimulation of DOC production by soil microbes) and by a mineral soil solution acidity decline (especially Al concentration), which stimulated a higher solubility of DOC in the mineral soil solution.

Despite no trend in bulk and beech throughfall inorganic N deposition, the spruce stand, with higher canopy-scavenging ability, showed a decrease in throughfall NO_3^- concentration, which may be interpreted as sign of decreasing dry N deposition. However, average N deposition on the soil surface was similar in both forests. Forest floor N losses were dominated by DON export in both stands but the magnitude of inorganic N leaching differed. A higher NO_3/NH_4 ratio in the forest floor soil solution in the beech stand pointed to the higher nitrification rates in this broadleaf forest compared

to the spruce stand. A decreasing trend in throughfall NO_3^- was followed in the mineral soil of the spruce stand, where inorganic N leaching diminished after 2006. Soil inorganic N concentrations in the beech stand showed no trends. Since 2014, large N fluctuations in the beech mineral soil occurred, likely because of nearby forest clear-cutting and seasonal processes. Higher NO_3 concentrations in the deep soil solution (-90 cm) in the beech stand suggested a more open N cycle compared to the spruce stand. Water fluxes through the soil profile were approx. 40% higher in the beech stand in the top mineral soil (up to 30 cm depth), owing to the higher precipitation (stem flow) and deeper rooting system of beech trees.

Decreases in soil solution acidity, increases of DOC concentration and a cessation of NO_3 leaching in spruce stand were reported, and all these changes were connected to the declining acid deposition. Both forest ecosystems are slowly moving towards conditions in which nutrient fluxes will become less controlled by acidity constraints and likely more responsive to climatic or/and forest disturbance variations as seen in the most recent changes in the soil solution inorganic N concentrations in the beech stand.

Publication II (IF_{2016} = 1.951): Oulehle, F., Růžek, M., Tahovská, K., Bárta, J., Myška, O.,2016. Carbon and Nitrogen Pools and Fluxes in Adjacent Mature Norway Spruce and European Beech Forests. Forests 7, 282. https://doi.org/10.3390/f7110282

Publication II examined the main current C and N fluxes and pools in both stands under ambient conditions. The total soil C and N stocks in the beech stand (199 t C·ha^{-1}; 8.6 t N·ha^{-1}) were significantly higher compared to the spruce stand (114 t·ha^{-1}; 4.5 t N·ha^{-1}). This difference was consistent for both the organic and mineral soil layers (0-40 cm). Moreover, soil C and N decreased with depth more rapidly under the spruce stand compared to the beech stand. Both stands differed in the main pathway of C delivered to the soil. While the spruce stand allocated more C directly belowground (9.6 t C·ha^{-1}·year^{-1}) than the beech stand (6.5 t C·ha^{-1}·year^{-1}), in opposite the beech stand delivered more C to the soil through litterfall flux (2.9 t C·ha^{-1}·year^{-1}) than the spruce stand (1.7 t C·ha^{-1}·year^{-1}). The cumulative annual CO_2 soil efflux was similar between the stands, i.e., 9.87 t C·ha^{-1}·year^{-1} and 9.01 t C·ha^{-1}·year^{-1} in the spruce and beech stands, respectively. DOC fluxes in the forest floor were also comparable (≈ 180 kg C ·ha^{-1}·year^{-1}) for both stands and decreased similarly in the mineral soil by roughly 80%. The higher soil C pool under the beech stand was likely a result of more effective long-

term C accumulation in the mineral soil. Deep-rooting beech delivers belowground C directly throughout the mineral soil compared shallow-rooting spruce. In addition, the beech mineral soil contained more oxalate-extractable Fe and Al (Fe_o, Al_o), which might indicate a higher sorption capacity, favouring soil C sequestration compared to the spruce stand. Our results suggest that in the spodic horizons of forested soils, organic matter was preferentially protected by interaction with poorly crystalline minerals represented by the oxalate soluble Al and Fe fraction. The occurrence of Spodosols generally increases with elevation, and so do Al_o+Fe_o stocks. Thus, soils with high rates of mineral weathering, e.g., those with high water availability and DOM leaching (higher DOC and water fluxes in beech stand, see Paper I), are predisposed to accommodate relatively large quantities of organic C (and N) due to the development of soils with high sorption capacities.

Combining N precipitation and litterfall fluxes, the beech stand received higher N input to the forest floor (56 kg $N \cdot ha^{-1} \cdot year^{-1}$) compared to the spruce stand (39 kg $N \cdot ha^{-1} \cdot year^{-1}$). Forest floor N losses were dominated by DON in both stands, and inorganic N leaching occurred in the beech stand. Calculated tree N demands showed that beech trees are more N-demanding - to satisfy annual biomass production, 116 kg $N \cdot ha^{-1} \cdot year^{-1}$ needs to be taken up in beech forest compared to 45 kg $N \cdot ha^{-1} \cdot year^{-1}$ in the spruce forest. However, the beech stand is therefore more efficient in N resorption from senescing foliage. In contrast, spruce litter is relatively rich in foliar N, thus trees need to take up N derived directly via SOM mineralization processes. The higher internal N cycle in the beech stand, combined with a microbial community adapted to high N availability (Bárta et al. 2017), makes the beech forest more prone to N inorganic losses.

Publication III (IF_{2018} = 5.714): Oulehle, F., Tahovská, K., Chuman, T., Evans, C.D., Hruška, J., Růžek, M., Bárta, J., 2018. Comparison of the impacts of acid and nitrogen additions on carbon fluxes in European conifer and broadleaf forests. Environ. Pollut. 238, 884–893. https://doi.org/10.1016/j.envpol.2018.03.081

Effects of experimentally elevated acid and N inputs simulating atmospheric deposition on intrnal nutrient fluxes and processes were examinated in Publication III. We examined whether there has been a shift in the ecosystem C fluxes in either (dissolved) organic or (gaseous) inorganic forms, as a function of altered acidity and N availability. Measurements of microbial characteristics and enzyme activity provided further insights into C metabolism under changing acidity and N availability. Microbial biomass C and N

under the beech stand were higher compared to the spruce stand. In contrast, the microbial C/N ratio was higher in the spruce stand (12.7) compared to the beech stand (10.9). Microbial communities also differed qualitatively, with higher F/B ratio in the spruce stand (0.045) than in the beech stand (0.031). Enzymatic activity expresses microbial requirements for nutrients. While enzymes produced in the spruce stand were relatively more involved in C acquisition, microorganisms in the beech stand largely produced N-acquiring enzymes instead, suggesting a natural C/energy limitation in the spruce stand and high N demands in the beech stand.

Acid treatments immediately decreased soil water pH, induced the suppression of soil water DOC concentrations and increased the F/B ratio in both forest stands. Soil water pH in the forest floor declined by the same magnitude (0.4-0.5 pH units) in both stands, although H^+ increases were significantly higher in the spruce soil solution due to the initial lower pH (3.8) compared to the beech forest floor soil solution (4.2). Reductions in DOC concentrations in forest floor soil solution ranged between 30–39% and 45–53% in the spruce stand and the beech stand, respectively. In the spruce stand, acid treatments decreased soil respiration by 6–10%, accompanied by reductions in microbial biomass and increases in enzymatic C acquisition. In the beech stand, equivalent effects in soil respiration and microbial activity were not observed.

Nitrogen addition treatments had no effect on soil water pH, DOC, soil respiration efflux or microbial activity in either stand, except for a decrease in the expression of N-acquiring enzymes in the beech stand.

Our results provide evidence that soil acidification alters C fluxes in both deciduous and conifer forests. Dissolved organic carbon concentrations in the soil solution were suppressed by acidification in both forest types, and a suppression of soil respiration was detected in the spruce stand. These responses have significant implications for long-term soil C cycling. On the other hand, nitrogen addition alone did not affect either DOC concentrations or the soil CO_2 efflux in either forest type.

Publication IV (IF_{2019} = 3.675): Choma, M., Tahovská, K., Kaštovská, E., Bárta, J., Růžek, M., Oulehle, F. (2020): Bacteria but not fungi respond to soil acidification rapidly and consistently in both a spruce and beech forest. FEMS Microbiology Ecology, 96. https://doi.org/10.1093/femsec/fiaa174

The main driver controlling soil C and N internal and leaching fluxes is the activity and structure of the soil microbial community. In Publication IV, treatment-induced responses of soil microbial communities in both stands and consequent changes in the activity of extracellular enzymes were investigated four years after the onset of treatment additions.

Regardless of the initial dissimilarities in soil chemistry and microbial composition, experimental acidification induced the retreat of the same bacterial groups in both stands, which were replaced by acidophilic and oligotrophic groups. These shifts in the bacterial community resulted in changes of enzymatic activity. In the beech stand, the magnitude and the action of hydrolytic activity was linked to the bacterial biomass and community structure. Contrarily, in the spruce stand enzyme activity was uncoupled from the bacterial community and instead correlated with DOC concentration in the soil solution. We hypothesised that the acidity-induced low bioavailability of SOM (DOC) resulted in microbial limitations in C, energy, and nutrients in the spruce stand. The microbial response to these limitations was the stimulation of hydrolytic enzyme production regardless of their taxonomic composition. Acidity-induced changes in the bacterial community composition were related to a decrease of enzymes degrading lignin-containing compounds (phenol oxidase). Nitrogen additions led to a much weaker bacterial response. Fungi were not influenced by any treatment during manipulation experiments in either stand.

Experimental S and N additions created artificial soil pH gradients across both stands, and the bacterial community composition varied along with changing pH. These results suggest that changes in bacterial composition were tightly linked to changes in soil acidity. As such, the altered bacterial composition caused changes in the observed soil C and N dynamics, while fungal activity remained unresponsive to acid inputs. The low response to N additions by both bacteria and fungi likely arose from the fact, that both stands had been subjected to long-term elevated N inputs from deposition.

Publication V (IF$_{2019}$ = 3.299): Růžek, M., Tahovská, K., Guggenberger, G., Oulehle, F. (under review). Litter decomposition in European coniferous and broadleaf forests under experimentally elevated acidity and nitrogen. Plant and Soil

Publication V investigated the effects of experimental S and N inputs on decomposition processes in both forests. In addition, an in-situ litter bag experiment tested the effects of treatments and stand identity on decomposition processes of various litter materials. Litters of various quality were used to simulate different substrates for decomposers (green tea, rooibos tea, beech leaves, spruce needles). In 2017, litter bags placed between the forest floor and mineral soil of treated plots, were left to decompose for up to 24 months. Results were analysed within into the broader context of observed changes in soil water pH and soil respiration fluxes in the years 2017–2019.

The beech stand supported more effective litter decomposition over the 24 months (higher litter mass loss) compared to the spruce stand. However, beech leaves were not more easily decomposed compared to spruce needles. Acidity suppressed the decomposition of easily degradable material in both stands regardless of soil water pH. Mass loss was reduced under acid treatments by 8–11% in the spruce stand and by 7–8% in the beech stand after 24 months. In the spruce stand, acid treatments significantly reduced the decomposition of lignin-rich litter, whereas no effect was observed in the beech stand. Compared to previous study (Oulehle et al. 2018), acid treatments significantly decreased soil respiration efflux in the spruce stand (by 13%) as well as in the beech stand (by 15%). Nitrogen additions led to no consistent response for any litter decomposition type or soil respiration. However, N treatments induced a soil water pH decrease in 2019, probably as result of N-induced acidification. The litter bag decomposition study further contributed to the main aim of our project, and confirmed, that acidity-induced changes to the soil environment can shift the C soil balance in the soil.

6. Synthesis and discussion

This thesis and the experiments within provide evidence that altered soil chemistry may have profound implication for soil C and N dynamics. We clearly demonstrated that the increase of soil acidity led to the slowdown of soil C cycling (suppression of dissolved organic and gaseous C losses), reduced microbial diversity (mostly bacteria), and suppressed the decomposition of litter in a controlled field experiment. Our results

supported the original hypothesis, that the loss of soil carbon following a decline in acid deposition appears to be the result of suppressed decomposition, and consequent organic matter accumulation, during the peak of acidification. Furthermore, S deposition to European forests has changed more rapidly and more dramatically over the last 50 years than N deposition, implying that S may have had a greater influence on the soil C cycle during this period. This is also raising the possibility that some observed changes may have been incorrectly attributed to N deposition.

6.1 Soil C and N dynamics of spruce and beech stands under ambient conditions

Since the studied forest stands were closely located to each other, the confounding effects of environmental variables (climate, parent material, deposition, land use) on our results (i.e., the effects of tree species on forest biogeochemistry) were minimized. However, forests in the Načetín research site in the Ore Mountains have been undergoing rapid recovery from historical acid deposition, as recorded in 25 years of monitoring data. It was evident that the spruce stand had been more impacted by air pollution, due to more efficient scavenging of air pollutants from the atmosphere (Šantrůčková et al. 2019). The spruce site showed a more pronounced chemical recovery of the soil environment (cessation of inorganic N leaching, decrease of Al soil solution concentrations) compared to less acidified beech forest. However, the beech stand was monitored for a shorter time (since 2003 vs. since 1994 for the spruce stand). The forest stands also differ in stand age (beech: 150 years vs. spruce: 80 years), stand density (beech: 340 trees·ha^{-1} vs. spruce: 540 trees·ha^{-1}), and thus also standing biomass (beech: 258 t C·ha^{-1} vs. spruce: 192 t C·ha^{-1}). All these factors must be kept in mind during when evaluating the results.

Soil C pools were found to be larger under the beech stand in both the forest floor, and the mineral soil. The finding of a higher C stock in the forest floor of the beech stand was in sharp contrast with other paired studies (Vesterdal et al. 2008, 2013; Cremer et al. 2016), that have all reported higher spruce forest floor C stocks. Such larger forest floor C stocks in spruce forest floors are usually ascribed to 1) indecomposable litter, producing a thick mor-type layer of litter in various stages of decomposition (Rothe et al. 2002) and 2) shallow-rooting spruce, delivering C to the topsoil only (Oostra et al. 2006). The higher forest floor C stock in the beech stand of our study was probably related with the higher litterfall C flux, which was almost twice as high as the spruce litterfall. Furthermore, as our results indicated, the decomposition of beech leaves and spruce needles were

comparable, thus the higher production of aboveground litter in the older beech stand may have led to a higher forest floor stock compared to the mature (but younger) spruce stand. Higher mineral soil C stocks in the beech stand were in line with other studies (Vesterdal et al. 2013), and might be explained by the deeper rooting of beech trees allocating C directly to the mineral soil and the higher content of oxalate Fe and Al ensuring SOC stabilisation (Navrátil et al. 2016). Direct C delivery by roots to the mineral soil is thought to be more stable (Rasse et al. 2005). Soil N stocks followed the pattern of soil C stocks, with higher soil N stocks under the beech stand. The spruce forest floor C/N ratio was constantly higher compared to the beech forest floor. Soil C/N usually decreases with soil depth due to preferential C mineralization. This pattern was violated in the mineral soil of the beech stand, probably as result of deeper root C allocation. The tree species impact on the distribution of C allocation within the soil profile may stimulate discussion on the effects of forest species composition on future C sequestration potentials. Carbon allocation to the mineral soil, where belowground litter and rhizodeposits are in tight contact with mineral particles, is generally accepted as a long-term C sink (Fontaine et al. 2007). The association of organic matter with surfaces of mineral particles has been proven to be the main interaction securing a stable C pool (von Lützow et al. 2006). Although recalcitrant litter (such as spruce litter) decomposes for a longer time, it does not contribute to a stable C pool, and its accumulation on topsoil thus represents a relatively vulnerable C pool (Cotrufo et al. 2013).

Soil microorganisms process SOM thus maintain nutrient availability (mineralization) and C fluxes (decomposition). The microbial community composition of both stands reflected the soil chemistry and substrate quality arising from tree species identity. Established microbial communities stoichiometrically followed the forest floor C/N ratio with larger fungi abundance in the spruce stand. Microbial communities in both stands differed in life strategy (Bárta et al. 2017). Analyses of enzyme stoichiometry revealed a stronger connection of metabolism to C acquisition in the spruce stand compared to the beech stand, where the high N demand of the beech forest drove the microbial community towards effective N acquisition.

The larger standing biomass and deciduous character of beech trees caused a higher annual litterfall C flux (2.9 t $C \cdot ha^{-1} \cdot year^{-1}$) to the soil compared to the spruce litterfall C flux (1.6 t $C \cdot ha^{-1} \cdot year^{-1}$). Although the throughfall DOC flux was higher in the spruce stand, it was a negligible C source in comparison to litterfall (Fig.7). In the spruce

stand, larger belowground C allocation (9.6 t C·ha^{-1}·year^{-1}) suggests a larger root biomass investment, root respiration and cooperation with mycorrhizal fungi compared to the beech stand (6.6 t C·ha^{-1}·year^{-1}). Litter decomposition was faster in the beech soil, regardless of litter type. Other observations (Rothe et al. 2002) support that litter decomposition is reduced in spruce forests; however, the main constraint is not the litter itself, but the forest floor or microbial activity (Albers et al. 2004). Despite the fact that spruce needles contain more N and have a comparable lignin/N ratio as beech leaves, needles during the decomposition produce polyphenols, which further retard decomposition (Gallet and Lebreton 1995). The microbial breakdown of relatively recalcitrant spruce litter requires sufficient energy, which can be gained in the form of simple C such as rhizodeposits and DOC. Carbon dissolved in the soil solution is the product of either partial decomposition or litter leaching. Surprisingly, both DOC forest floor fluxes were comparable (180 kg C·ha^{-1}·year^{-1}) despite larger beech litter C inputs. This might suggest more complete litter decomposition in the beech stand or incomplete spruce litter decomposition. With depth, the DOC flux diminishes as organic C is either consumed by microorganisms or stabilized by adsorption on soil mineral particles (Guggenberger and Kaiser 2003). Calculated belowground C allocation was markedly higher in the spruce stand. This calculation assumes unchanging conditions, however, which might be partly violated by the non-steady state decomposition of historically accumulated litter (Oulehle et al. 2011). Since data about the further partitioning of belowground C were not measured, belowground C investment may be only indirectly inferred. One of the main purposes of belowground C supply might be the acquisition of limiting nutrients from organic compounds of decomposing litter, which requires substantial energy for enzymatic production. Thus, despite comparable soil respiration effluxes, the forest stands differed in mechanisms of C cycling and sources of emitted C.

Soils receive N through two major fluxes – litterfall and throughfall precipitation. The litterfall N flux was higher in the beech stand (56 kg N·ha^{-1}·year^{-1}) compared to the spruce stand (39 kg N·ha^{-1}·year^{-1}); however, a lower C/N litterfall ratio was measured in the spruce litter (42) compared to the beech litter (52). Total throughfall N fluxes were similar for both stands (≈ 18 kg N·ha^{-1}·year^{-1}). In the spruce stand, throughfall fluxes were high in DON but low in NH_4^+, which pointed to canopy N transformation processes of Norway spruce (Kopáček et al. 2011). Beech trees had a significantly high stemflow

water flux, which accounted for 10% of the total throughfall N input. Combining both sources of N input, the beech stand, in total, received more N (74 kg $N \cdot ha^{-1} \cdot year^{-1}$) than the spruce stand (57 kg $N \cdot ha^{-1} \cdot year^{-1}$). However, in times of higher air pollution, the spruce stand received more throughfall N through canopy scavenging. Based on our measurements in 2005-2017, the spruce throughfall inorganic N flux decreased from 21 kg $N \cdot ha^{-1} \cdot year^{-1}$ to 11 kg $N \cdot ha^{-1} \cdot year^{-1}$, while the throughfall N flux in the beech stand did not change (16.3 kg $N \cdot ha^{-1} \cdot year^{-1}$). In the recent past, total input soil N fluxes were thus comparable for both stands. Lower N demands in the spruce stand may partly explain the rapid decrease of inorganic N leaching following acid deposition declines. Recently, nitrogen outputs from forest soils were dominated by DON in both stands. However, cessation of the DON flux with depth emphasized stand differences in nitrate leaching in the subsoil. In the spruce stand, nitrate leaching from the forest floor followed the decrease in throughfall N input and almost diminished in subsoil. In contrast, nitrate leaching in the beech stand varied until the end of observations, and seasonal as well as annual dynamics likely reflects changes in weather and disturbance dynamics.

Since N is a limiting nutrient, it is interesting to compare the N economy of trees in both stands, because it partly controls the openness/tightness of the soil N cycle. Annual forest N requirements for foliage development were calculated as 116 kg $N \cdot ha^{-1} \cdot year^{-1}$ and 45 kg $N \cdot ha^{-1} \cdot year^{-1}$ in the beech stand and the spruce stand, respectively. Beech N requirements were much higher than N available through N inputs (throughfall + litterfall). Therefore, N resorption from beech foliage prior to senescence secures enough N to build-up new biomass and partly prevent N losses following the mineralization of fresh beech litter. We may hypothesise that beech trees regulate the ecosystem N availability through N resorption – under N limited conditions, resorption is more effective compared to N saturated conditions. Accounting for resorbed N in the beech stand, nitrogen uptake is then similar for both stands. However, calculated tree N uptake was higher than soil N input in the spruce stand, while both N input and requirements were similar in the beech stand. This further indicates that the soil N cycle in the spruce stand was tighter with no inorganic N leaching, while the N cycle in the beech stand was more open and inorganic N leaching could occur, especially during episodes of high N availability and low uptake. This was demonstrated by N leaching that occurred periodically in the early spring in relation to the flushing of percolating water through the soil by melting snow (Fig.7).

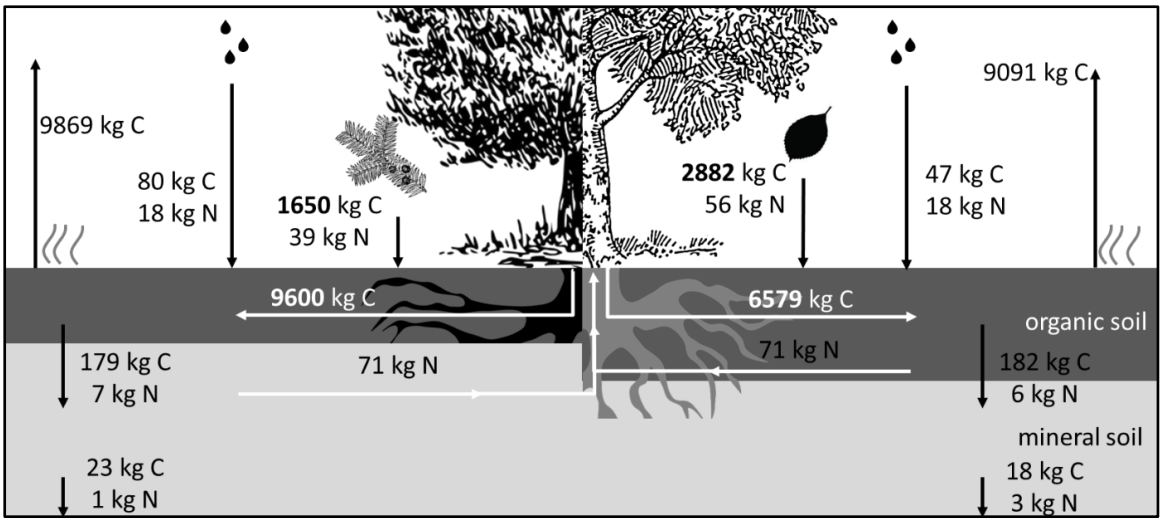


Figure 7: C and N (kg· ha^{-1}·yr^{-1}) fluxes in the spruce (left) and the beech stand (right)

6.2 Experimental manipulations and acidity constraints on SOM dynamics

Over the six years of experiments, plots treated with sulfuric acid received in total 300 kg S ha^{-1}. Both treatments (S and S+N) immediately acidified soil solutions in both stands. The pH of soil water from the beech plots under acid treatments declined more (by 0.44–0.55 pH units) than in the spruce stand (by 0.34–0.37 pH units). Nevertheless, the increase of H^+ concentration in acid treatments was greater in the spruce stand (209 µeq·l^{-1}) because of the overall lower soil solution pH compared to the beech stand (116 µeq·l^{-1}). In mineral soils, soil neutralization capacity mitigated the effects of acid inputs. The reduction of soil solution pH caused a suppression of DOC solubility, and thus a reduction of soil solution DOC concentration, a reduction in microbial biomass, a shift in microbial composition structure, a suppression of high-quality litter decomposition and a reduced soil respiration efflux. Moreover, acidity reduced the decomposition of low-quality litter in the spruce stand. To conclude, elevated acidity led to the reduction of C dynamics in soils, potentially resulting in soil C accumulation.

In accordance with other "acid rain simulating" experiments (Persson et al. 1989), these results show that experimental acidity additions substantially modified soil microbial functioning in both stands, regardless of the initial pH values. However, initial higher soil water pH in the beech stand ensured that acid additions induced effects that were delayed or not significant compared to the initially more acid spruce stand (to get a similar response, more time and/or higher cumulative dose would be needed under initially less acidic conditions). Consistent declines in DOC and DON concentrations were expected, and were in line with published results from monitoring and experiments

(Monteith et al. 2007; Hruška et al. 2009; Evans et al. 2012). Soil pH controls the dissociation of organic acids, which in turn affect their mobility in the soil-soil solution interface. A lower solubility (availability) of DOC might explain reductions in other observed C-related processes, for instance higher investments to C-mining enzymes and the suppressed decomposition of high-quality litter observed in both stands. In our litter bag experiment, high-quality litter consisted of a high content of water-soluble C compounds (Keuskamp et al. 2013) and an increase of soil acidity might have directly supressed C solubility and therefore the observed lower mass loss under acid treatments. The distinct suppression of decomposition of low-quality litter in the spruce stand was likely caused by multiple factors. Besides lower C availability, low pH itself adversely affected the soil microbial biomass, enzymatic production, and changed the microbial community structure to a higher degree in the spruce stand. While the response of fungi was weak, bacteria strongly responded to increasing acidity either by retreating or by the selection of acidophilic and oligotrophic taxa (Pennanen et al. 1998). These organisms, characterized by higher C demands, must cope with decreased C availability using a higher production of C-mining enzymes. Moreover, declining pH and bacterial biomass coincided with a lower production of enzymes degrading lignin compounds. These shifts in microbial and enzymatic structures, which were more highly pronounced in the spruce stand, might explain the differences in the decomposition of lignin-rich litter (with suppression in the spruce stand). The soil respiration efflux is a composite flux that consists of various sources and determining the main causes and detecting changes can be difficult. Nevertheless, in accordance with other studies (Feng et al. 2017), acidity consistently reduced soil respiration in both stands. The design of our study suggests that acidity affected only heterotrophic part of respiration (trees were largely unaffected by treatments), which is consistent with our measurements of internal soil processes. However, effects of acidity differed in duration of response. After the start of the experiment in 2014, the more acidic soil in the spruce stand significantly responded already in 2015, whereas soils in the beech stand responded two years after, in 2017. (Fig.8).

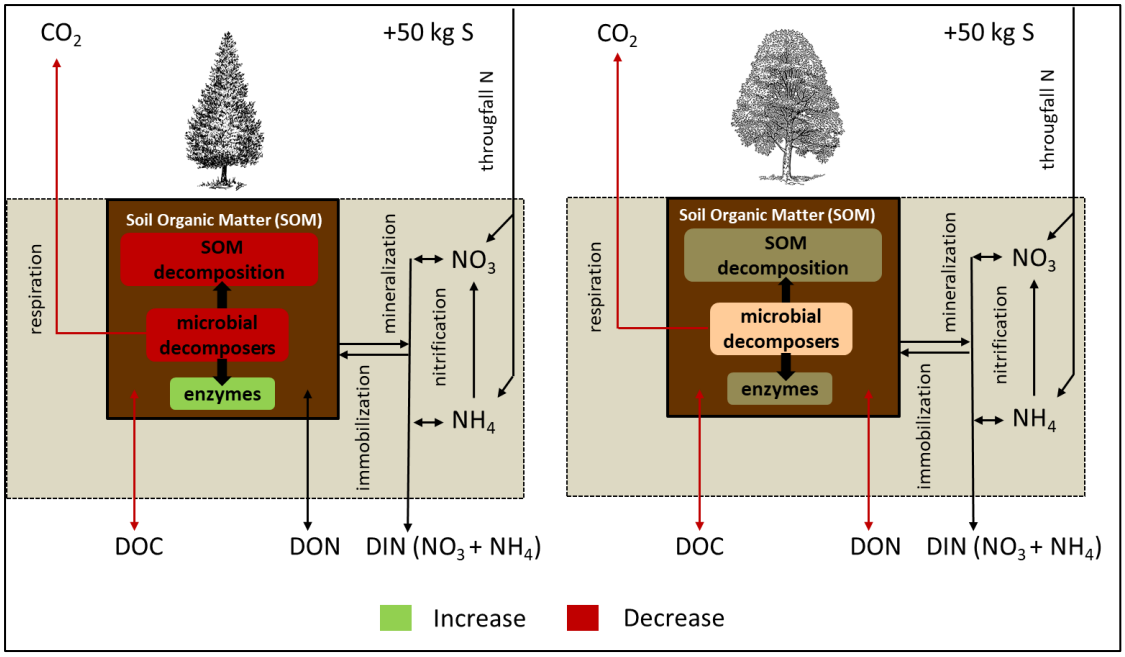


Figure 8: Effects of S addition on soil processes in the spruce (left) and the beech stand (right). Green and red colours refer to relative increase and decrease of fluxes and processes compared to control.

6.3 Experimental manipulation and N availability constrains SOM dynamics.

In contrast to the experimental acid treatments, N addition did not induce consistent effects on ecosystem functioning in either forest stand (Fig.9). There was only a stoichiometric shift in enzymatic activity in the beech stand and a reduced decomposition of needle litter in the spruce stand. In both stands, no changes attributable to enhanced N inputs were observed in soil solution DOC, DON, NH_4^+, NO_3^- concentrations, microbial biomass, microbial community composition, or any other process (soil respiration, litter decomposition). In the sixth year of N additions, soil pH significantly decreased by ≈ 0.2 pH units in the spruce stand, suggesting acidification processes that could have originated from excess inorganic N (onset of nitrification).

Our observations are different than other similar N fertilization studies from Europe, North America, and China (Magill et al. 2000; Moldan et al. 2018; Wang et al. 2018; Forsmark et al. 2020), where N additions have often led to significant (positive or negative) responses in soil functioning. The lack of pronounced effects of N additions in our experiments are likely due to historical higher N deposition, or experimental design. First, historically elevated N deposition, peaking in the 1980s and currently averaging ≈ 10 kg $N \cdot ha^{-1} \cdot year^{-1}$ in bulk deposition, might have already changed the microbial community structure, adapting them to higher N availability. Second, potential limitation may have arisen from the experimental design (treatment plots 3 × 3 m) thus avoiding

trees as a principal component regulating the delivery and uptake of N in the forest ecosystem. Nitrogen saturation is a final step in complex responses of the whole ecosystem, when all compartments (soil, standing biomass) gradually reach N retention limits. Since the capacity is reached, elevated N availability starts to modify the nutrient economics of the ecosystem and shifts in soil processes would be observed. The local surplus of experimental N inputs may have caused a redistribution N away from out the experimental plots by hyphae and root systems. The majority of N added was immobilized in the system, and no substantial N leaching occurred. However, the observed pH decrease in the spruce N-treated plots in 2019 may signify emerging N leaching, which would acidify the soil environment though the onset of nitrification and NO_3 leaching.

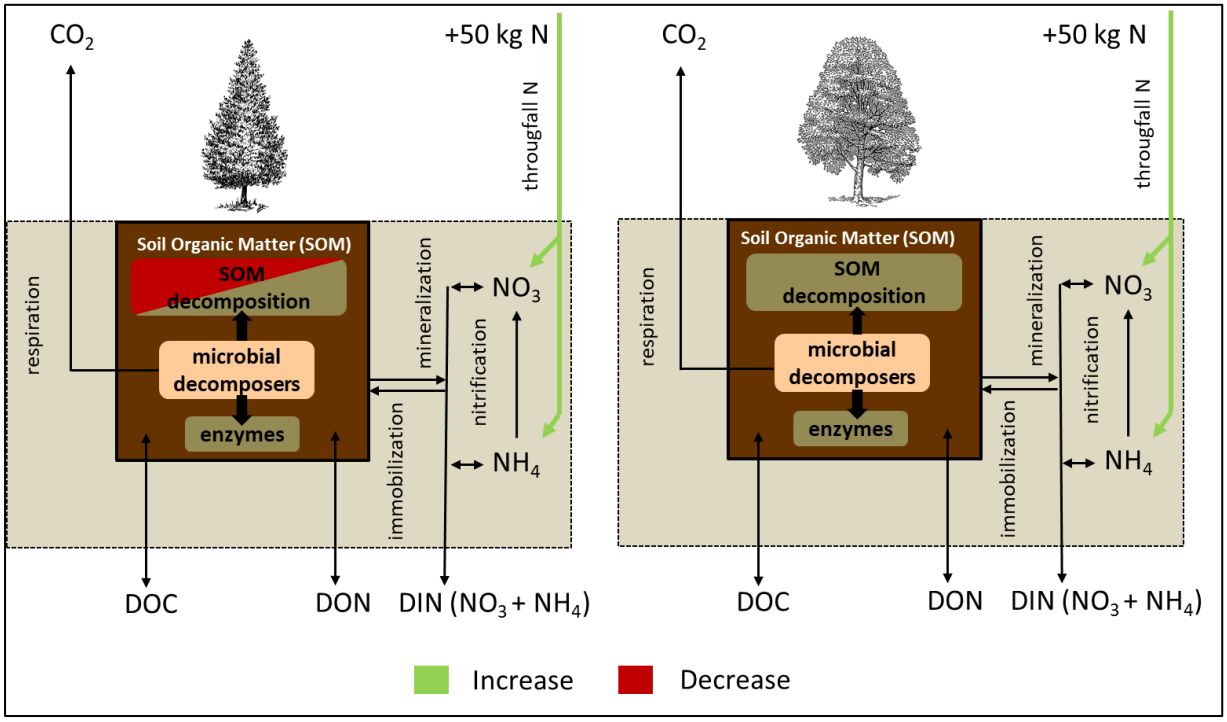


Figure 9: Effects of N addition on soil processes in the spruce (left) and the beech stand (right). Green and red colours refer to relative increase and decrease of fluxes and processes compared to control.

7. Conclusion

Here we emphasize the importance of our work and its significance for the future understanding of forest ecosystem functioning.

Comparisons of ecosystem C and N budgets may help quantify the effects of tree species identity on landscape C and N retention. This is particularly important, as managed forests are currently facing unprecedented declines caused by drought and associated bark beetle outbreaks.

Controlled S and N additions enabled us to determine the principal component (acidity vs N availability), that had a decisive effect on SOM turnover during historical air pollution. Furthermore, our research systematically described and quantified induced responses and underlying mechanisms. To correctly interpret existing records (i.e., results from monitoring programs), and to accurately predict future changes in ecosystem biogeochemistry in response to continuing environmental change (i.e., climate, deposition, land use), we argue that a more holistic approach to the impacts of multiple environmental drivers is needed.

Our results originated from one locality only. The strength of this approach is the elimination of other confounding factors. Generalizing results to other regions and tree species may be questionable. However, we studied generally valid processes that are similar across regions. Key parameters that would modify responses are climate, soil properties and deposition history. Central European mountainous forests on acidic bedrocks have been subjected to comparable air pollution (Oulehle et al. 2016). Study conducted across the entire Czech Republic (Šantrůčková et al. 2019) showed that 31% of investigated soils, particularly mountainous, were classified as strongly acidic due to the combination of bedrock quality, forest management practice and acid deposition. Thus, we infer, that our results are likely to be applicable to these and other similar sites.

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9. Supplements

9.1 Publication I

Růžek, M., Myška, O., Kučera, J., Oulehle, F., 2019. Input-output budgets of nutrients in adjacent Norway spruce and European beech monocultures recovering from acidification. Forests 10, 22. https://doi.org/10.3390/f10010068 (IF_{2019} = 2.221)

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Article

Input-Output Budgets of Nutrients in Adjacent Norway Spruce and European Beech Monocultures Recovering from Acidification

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Abstract: Soil acidification has constituted an important ecological threat to forests in Central Europe since the 1950s. In areas that are sensitive to acid pollution, where the soil buffering capacity is naturally low, tree species can significantly modulate the extent of soil acidification by affecting throughfall deposition and the composition of litter. A principal difference can be expected between coniferous and broadleaf tree species. The aim of our study was to compare long-term trends in element cycling in two stands representing the main types of forest ecosystem in the region (*Picea abies* vs. *Fagus sylvatica*). In the period of 2005–2017, we continually measured element concentrations and fluxes in bulk precipitation, throughfall precipitation, and soil leachates. A continuous decline of acid deposition was detected in both bulk precipitation and throughfall. Declining deposition of S and N in both forests has led to the recovery of soil solution chemistry in the mineral soil, manifested by rising pH from 4.25 to 4.47 under spruce and from 4.42 to 4.69 in the beech stand. However, soil water in the spruce stand was more acidic, with higher concentrations of SO_4^{2-} and Al when compared to the beech stand. While the acidity of soil leachates from organic horizons was driven mainly by organic anions, in lower mineral horizons it was controlled by inorganic acid anions. NO_3^- concentrations in deeper horizons of the spruce stand have diminished since 2006; however, in the beech plot, episodically elevated NO_3^- concentrations in mineral horizons are a sign of seasonal processes and of nearby perturbations. Higher output of S when compared to the input of the same element indicates slow S resorption, delaying the recovery of soil chemistry. Our results indicate that, although forest ecosystems are recovering from acidification, soil S retention and the ability to immobilize N is affected by the dominant tree species.

Keywords: acid deposition; acidification; European beech; Norway spruce; nitrogen; sulfur

1. Introduction

In the second half of the twentieth century, atmospheric deposition of sulfur (S) and nitrogen (N) has caused the acidification and eutrophication of forest soils in Europe and North America, leading to the degradation of forest stands. Initially, the dominant component of acidic deposition was sulfate (SO_4^{2-}) and the influence of nitrogen compounds (NO_3^- and NH_4^+) became important only later. Since the 1990s, S deposition declined rapidly in Central Europe, but N deposition has decreased only slightly [1]. However, in Europe, concentrations are still declining, although the decline is not so sharp as that observed during the 1990s [2].

Sulfate and nitrate anions are neutralized by cations in soil and deplete the cation exchange complex. Due to their increased mobility, nutrients are lost from the rooting zone, and soil base saturation decreases. Furthermore, decreases in pH result in the dissolution of Al^{3+}, which has toxic effects on plant roots [3]. Increased input of atmospheric N may lead to the saturation of ecosystems with N, manifested by subsequent leaching of NO_3^- [4]. In response to air pollution abatement policies, ecosystems are gradually undergoing a phase of recovery, but due to deterioration of soils the return to the pre-industrial state will take a long time [5]. Decreases of acid inputs led to the partial improvements of stream [6,7] and lake chemistry [8,9], and the consequent amelioration of the soil solution chemistry take place slower than was expected [10,11]. Although some positive changes in soil environment were observed i.e. declining trend in soil solution NO_3^- concentrations [12] or decline in total Al concentrations [13], soil solution pH, and acid neutralization capacity (ANC) showed nonuniform trends [11,14].

The tree species composition of forest stands plays a key role in the intensity of processes causing acidification and N saturation. Many studies have shown that conifers are able to more efficiently (higher leaf area index, evergreen character) scavenge atmospheric compounds when compared to deciduous species, which is reflected in the different composition of both throughfall precipitation and of the soil solution [15]. Moreover, conifers are characterized by higher interception, so throughfall under them is less diluted and more efficient in the changing of soil chemistry [16,17]. Other factors, such as the distribution of the root system in the soil profile, the quality/degradability of organic matter, or the amount of topsoil, also play a role in the overall effect on soil chemistry [15]. It is generally argued that species composition is manifested mainly in the topsoil [18]. However, S and N deposition can shift the effect to deep soil through the processes of leaching and nutrient transport. The influence of tree species composition has been detected in many sites in Europe previously affected by acid deposition [19–21], generally showing an adverse effect of conifers on the soil acid-base status. Thus, the greater intensity of acidification, caused by forests of an unsuitable type, means that ecosystems will take longer to recover. Many studies have been conducted to compare the effect of tree species on the restoration of forest stands recovering from the effects of increased atmospheric pollution [13,22–24].

Mid-mountain areas of Central Europe, situated mainly on acidic bedrock, were once naturally covered by European beech forests. During the 18th and 19th centuries, most of these forests were replaced with monocultural Norway spruce plantations to meet the demand for timber. Since the 1970s, this region has been subjected to greater atmospheric deposition. The most affected was a region on the Czech-German-Polish border, called the Black Triangle. Since 2003, throughfall precipitation was measured and the soil solution was analysed in spruce and beech stands. A temporal record spanning 13 years was used to assess the trends of recovery from acidification under these two tree species. The aim of this study was to compare the dynamics of solute concentrations and to determine nutrient fluxes under beech and spruce stands recovering from high atmospheric deposition.

2. Materials and Methods

2.1. Site Description

The spruce and beech stands under study are located in the Czech Republic in the Ore Mountains near the German border, close to the village of Načetín (50°35′26″ N, 13°15′14″ E). Both of the stands lie close to each other (ca. 700 m), so they are exposed to the same geological and climatic conditions. The average annual temperature is 7.1 °C and average annual precipitation is 1110 mm (2005–2017). Paragneiss underlies both stands, which are sloped to the north-west. The dominant soil type is dystric cambisol. The spruce stand lies at an elevation of 784 m a.s.l. and it is covered by 80 years old monoculture of Norway spruce (*Picea abies*). The beech stand is situated at an elevation of 823 m a.s.l. and it is composed predominantly of European beech trees (*Fagus sylvatica*) that are approximately 140 years old [25]. Today, both stands are part of a nature reserve, but conventional forest management is still being applied. The spruce stand has been studied since 1980s as part of research into the phenomenon of acid rain [26–28].

2.2. Water Chemistry

Bulk precipitation was collected monthly by two funnels that were located in an open area between the two stands. Throughfall precipitation below the spruce and beech canopy was collected monthly by nine collectors spaced in a 15 × 15 m grid in each of the two plots. The precipitation was gathered into polyethylene funnels (area of 122 cm^2) during the vegetation season (April–October); these were replaced in winter by open plastic vessels (area of 167 cm^2). Precipitation from all collectors were together creating one composite sample. At the beech plot, one stemflow collector was installed to measure stemflow element fluxes in monthly time step.

The soil solution was collected in both stands using PRENART suction lysimeters (Prenart Equipment ApS, Frederiksberg, Denmark) at depths of 30, 60, and 90 cm in the mineral soil. At each depth, a composite sample from 3–9 lysimeters was collated. The soil solution from organic horizons (i.e., the forest floor) was collected by zero-tension lysimeters as a composite sample of three replicates. All of the lysimeters were sampled monthly.

The pH of the collected precipitation and soil water was measured using a Radiometer TTT-85 pH meter with a combination electrode GK-2401C Radiometer (Radiometer Corp., Copenhagen, Denmark). Anion concentrations of Cl^-, NO_3^-, and SO_4^{2-} were determined by high-performance liquid chromatography (Knauer 1000, Hegauer Weg 38, Berlin, Germany). NH_4^+ concentrations were detected by indolphenol blue colourimetry, and concentrations of Ca, Mg, Na, K, and Al were measured by atomic absorption spectrometry (AAS, Analyst Perkin Elmer 100 and 200, Perkin Elmer co., LTD, Waltham, MA, USA). Ionic concentrations of Ca^{2+}, Mg^{2+}, Na^+, and K^+ were assumed to equal total concentrations.

Total monomeric Al (Al_m) and organic monomeric Al (Al_{org}) concentrations were determined by the pyrocatechol violet method that is described in [29] and then modified by [30]. Al_{org} was separated using a strong cation exchange resin (Dowex 50 W; Sigma-Aldrich, St. Louis, MO, USA) in mixed H+ (98%) and Na+ (2%) forms [31]. The difference between Al_m and Al_{org} was assumed to be inorganic monomeric Al (Al_{in}). Acid-soluble Al (Al_{as}), assumed to be mainly in particulate or microcrystalline form, was calculated as the difference between Al_{tot} (AAS) and Al_m.

Samples for the analysis of dissolved organic carbon (DOC) and total nitrogen (TN) were filtrated using a 0.4 µm glass fibre Macherey–Nagel GF 5 filter. DOC was determined by oxidation and CO_2 was detected using a Apollo 9000 (Tekmar-Dohrmann, Mason, OH, USA) device. Analysis of dissolved nitrogen (DN) uses the oxidation of the sample to NO and emission of light after its reaction with O_3. The light emitted is detected by a chemiluminescence detector and correlated to a specific amount of nitrogen. The difference between TN and the sum of N-NO_3 and N-NH_4 is denoted as dissolved organic nitrogen (DON).

For bulk and throughfall, volume-weighted mean annual concentrations were calculated from the monthly values. The sum of SO_4^{2-}, NO_3^-, NH_4^+, and non-salt Cl^- concentrations in atmospheric deposition was denoted according to Meesenburg et al. [23] as potential acidity (AC_{pot}), which indicates the potential acidity of the soil environment. Annual aboveground element input fluxes were obtained by summarizing the monthly input fluxes. Also incorporated in beech throughfall deposition fluxes were stemflow fluxes. Belowground fluxes were calculated multiplying element concentration by month water fluxes, which were than summarized into annual fluxes. The molar ratio of base cations $K^+ + Ca^{2+} + Mg^{2+}$ to the inorganic form of Al (Bc/Al_{in}) was used as an indicator of potential soil toxicity to tree roots [32]. Critical limits for spruce (1.2) and beech (0.6) were derived from literature [32].

2.3. Data Handling

For the calculation of element fluxes through the soil profile, it is necessary to calculate the volume of passing water. We modelled water percolation outflowing from four horizons (forest floor, 30 cm, 60 cm, and 90 cm). For these purposes, the model Simpel was used (http://www.hydrology.uni-kiel.de/de/forschung/projekte/simpel). This model is based on water bucket storages and enables the calculation of actual evapotranspiration and water passage through soil horizons with

daily time step resolution. Input climatic data (daily sum of precipitation, main air temperature, main relative humidity, duration of sunshine, and wind speed) were obtained from a 15 km distant meteorological station (Nová Ves). Since 2017, on-site meteorological data (air temperature and humidity, photosynthetic active radiation—PAR, precipitation, soil moisture, and soil water potential) were measured. Input data about the forest stands (albedo, geographic position, height of the stand, leaf area index time series, and soil physical parameters) were acquired during previous research or derived from the literature [27]. The Simpel model computes water outputs as actual evapotranspiration (i.e., the sum of evaporation and transpiration), the amount of water in snowpack, and infiltration of water through the soil profile. The Simpel model was further calibrated according to measurements of actual transpiration and soil moisture during the vegetation season of 2017.

Since 2017, in each forest stand, eight trees representing stand DBH variation were selected and equipped with continuously measuring sap flow devices. Tree sap flow was measured by the tissue heat balance method with constant heating power (EMS; Brno, Czech Republic). The scaling up of sap flow from sample trees to the stand level was based on the DBH distribution of the trees in each forest stand [33].

2.4. Statistical Analysis

For the determination of significant trends over the period of 2005–2017, the non-parametric seasonal Mann-Kendall (MK) test was used [34]. This model can determine the statistical significance of a trend, despite its seasonal variability. The data were aggregated into three-month groups representing the four seasons (January–March, April–June, July, September, October–December). If a linear trend was present, then real slope (change per unit time) was estimated using Sen's slope [35]. To estimate differences between stands, a Shapiro–Wilk normality test was conducted. If the data had a normal distribution, the ANOVA test was used, otherwise the non-parametric Kruskal-Wallis test was applied. All statistical tests were performed with a significance level of 5% ($\alpha \leq 0.05$).

3. Results

3.1. Atmospheric Deposition Chemistry

A significant increase of bulk precipitation pH from 4.5 to 5.5 was detected between 2005 and 2017. Similarly, throughfall precipitation pH increased significantly in both stands. Throughfall pH under the spruce canopy increased from 4.3 to 5.0, whereas beech throughfall pH increased from 4.7 to 5.8 (Figure 1).

The decrease of precipitation acidity was driven by downward trends of most acidic anions. SO_4^{2-} concentrations in bulk precipitation decreased significantly by 53%, from 36 $\mu eq \cdot L^{-1}$ in 2005 to 17 $\mu eq \cdot L^{-1}$ in 2017. Throughfall spruce SO_4^{2-} decreased from 112 $\mu eq \cdot L^{-1}$ to 75 $\mu eq \cdot L^{-1}$, which is a decrease by 33% over the 2005–2017 period. Beech throughfall SO_4^{2-} concentrations decreased from 71 $\mu eq \cdot L^{-1}$ in 2005 to 44 $\mu eq \cdot L^{-1}$ in 2017, albeit insignificantly. Over the period of 2005−2017, NO_3^- concentrations in bulk precipitation decreased by 30% from 33 $\mu eq \cdot L^{-1}$ to 23 $\mu eq \cdot L^{-1}$. Similarly, spruce throughfall NO_3^- decreased significantly by 29% from 96 $\mu eq \cdot L^{-1}$ to 68 $\mu eq \cdot L^{-1}$. Throughfall NO_3^- concentrations in the beech stand decreased insignificantly from 72 $\mu eq \cdot L^{-1}$ to 63 $\mu eq \cdot L^{-1}$ (decrease of 13%). Annual concentrations of Cl^- varied around 11 ± 3.2 $\mu eq \cdot L^{-1}$ in bulk precipitation, 39 ± 12 $\mu eq \cdot L^{-1}$ in spruce throughfall, and 22 ± 7 $\mu eq \cdot L^{-1}$ in beech throughfall, without any trend detected.

Concentrations of dissolved organic carbon (DOC) decreased significantly in bulk precipitation (from 120 $\mu mol \cdot L^{-1}$ in 2005 to 83 $\mu mol \cdot L^{-1}$ in 2017). No trend in throughfall DOC concentrations was detected in either of the stands. However, DOC concentrations under the spruce canopy were 2.5× greater when compared to beech throughfall (944 ± 279 $\mu mol \cdot L^{-1}$ vs. 393 ± 114 $\mu mol \cdot L^{-1}$).

Among concentrations of base cations ($BC = K^+ + Mg^{2+} + Ca^{2+}$) in bulk precipitation only, K^+ exhibited a significant change, as its concentration increased from 2.1 $\mu eq \cdot L^{-1}$ in 2005 to 2.9 $\mu eq \cdot L^{-1}$

in 2017. Concentrations of other cations varied over the study period around 10 ± 3.3 μeq·L^{-1}, 10 ± 1.6 μeq·L^{-1}, 5 ± 1.4 μeq·L^{-1}, and 40 ± 7.9 μeq·L^{-1} for Na^+, Ca^{2+}, Mg^{2+}, and NH_4^+, respectively, without any temporal change. Sampling of both types of throughfall precipitation revealed higher concentrations in the spruce stand, where BC concentrations were ca. 1.3 × greater than under beech (Na^+ = 1.5×, K^+ = 1.3×, Mg^{2+} = 1.1×, and Ca^{2+} = 1.4×). Only NH_4^+ concentrations were comparable (spruce: 76 ± 17 μeq·L^{-1} vs. beech: 77 ± 17 μeq·L^{-1}). Decreases in throughfall were detected only for Ca^{2+} concentrations in the spruce stand. Throughfall concentration of Na^+ in the spruce stand were 189% greater and in the beech plot 88% greater than in bulk precipitation. As a result, concentrations of potential acidity AC_{pot} decreased since 2005 by about 30% in the spruce and by 20% in the beech stand (Figure 1).

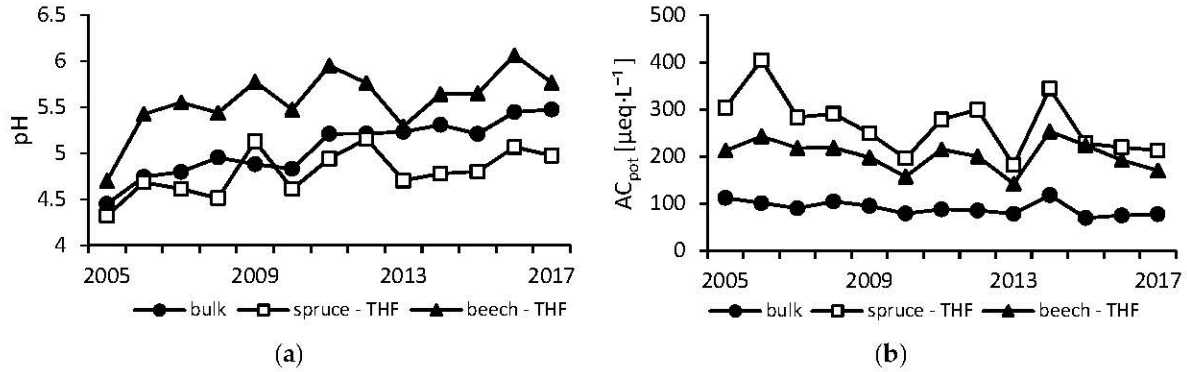


Figure 1. pH (**a**) and mean annual concentrations of potential acidity AC_{pot} (sum of SO_4^{2-}, NO_3^-, NH_4^+, and non-salt Cl^-) (**b**) in bulk, throughfall (THF) spruce, and THF beech precipitation.

3.2. *Soil Solution Chemistry*

3.2.1. Anion Concentrations

SO_4^{2-} concentrations decreased over the course of the study period at all depths in both stands. SO_4^{2-} concentrations in all soil horizons of the spruce stand were greater than in the beech stand (Table 1). The lowest SO_4^{2-} concentrations were measured in forest floor solutions, where they declined from 185 to 81 μeq·L^{-1} under spruce, whereas in the beech they averaged 78 ± 22 μeq·L^{-1} (Figure 2).

Concentrations of SO_4^2 in mineral soil in the spruce stand were similar across the soil profile and significant declines from 494, 467, and 504 μeq·L^{-1} in 2005 to 341, 359, and 289 μeq·L^{-1} in 2017 at 30 cm, 60 cm, and 90 cm, respectively, were detected (Figure 2). The annual average concentrations in the beech stand were 97, 244, and 324 μeq·L^{-1} in 2005 and significantly (except at 30 cm depth) declined to 79, 155, and 206 μeq·L^{-1} in 2017 at 30 cm, 60 cm, and 90 cm depths, respectively (Table 1).

Annual NO_3^- concentrations significantly differed between the plots. Mean NO_3^- concentrations from each horizon of the spruce stand were significantly lower compared to the beech stand. In the forest floor soil solution, NO_3^- concentrations averaged 50 ± 53 μeq·L^{-1} and 65 ± 25 μeq·L^{-1} in the spruce and beech, respectively (Figure 2). In the mineral soil in the spruce stand, NO_3^- concentrations declined with increasing depth (from 8 μeq·L^{-1} in 30 cm to 4.7 μeq·L^{-1} in 90 cm in 2005) and continually declined (at 30 and 90 cm significantly), until they dropped under the detection limits in 2017 (Figure 2). Conversely, in the beech stand, NO_3^- concentrations insignificantly increased over time. Between 2014 and 2017 enhanced NO_3^- concentrations were measured, however without detected trends. NO_3^- concentrations varied around 28 ± 86 μeq·L^{-1}, 52 ± 16 μeq·L^{-1}, and 36 ± 75 μeq·L^{-1} in 30 cm, 60 cm, and 90 cm, respectively (Table 1).

Mean annual Cl^- concentrations in mineral soil increased with depth from 27 to 44 μeq·L^{-1} under spruce and from 37 to 50 μeq·L^{-1} under beech without any temporal trends.

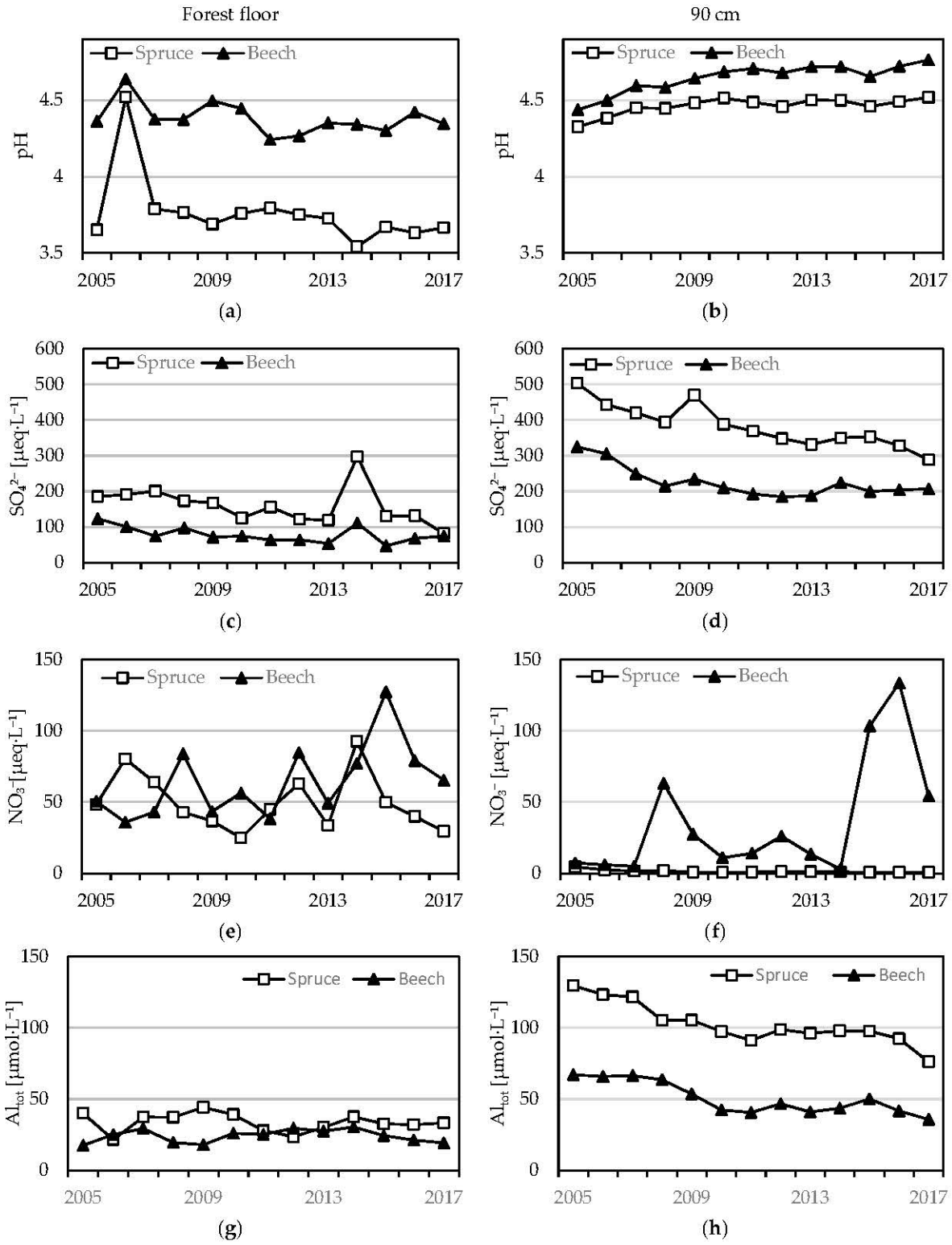


Figure 2. Mean annual pH, SO_4^{2-}, NO_3^-, and total Al (Al_{tot}) concentrations from forest floor (left – **a**, **c**, **e**, **g**) and 90 cm (right – **b**, **d**, **f**, **h**) horizons of beech and spruce soil solutions.

Table 1. Seasonal Mann-Kendall trends for bulk, throughfall and soil solution concentrations, pH and the BC/Al_{in} ratio with mean values in $\mu eq \cdot L^{-1}$ (SO_4^{2-}, NO_3^-, Cl^-, NH_4^+, BC) or $\mu mol \cdot L^{-1}$ (dissolved organic carbon (DOC), Al_{tot}) and Sen's slope with significance (*: p 0.05, **: p 0.01, ***: p 0.001).

Plot	SO_4^{2-}		NO_3^-		Cl		DOC		NH_4^+		BC		pH		Al_{tot}		BC/Al_{in}	
	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope	Mean	Slope
BULK	28	−1.1 *	29		11		165		45		31		5.06	+0.07 ***				
THF																		
Spruce	147		97	−3.0 **	48		1067		95		190		4.79	+0.04 *				
Beech	79		78		29		435		280		148		5.59	+0.04 *				
Forest floor																		
Spruce	160	−6.6 *	50		69		3355	+63 *	32		115	−7.6 *	3.73	−0.01 *	33		42.0	
Beech	78		65		30		2519		17		259		4.38		24		101.6	+4.69 *
Mineral soil																		
30 cm																		
Spruce	400	−11.9 **	2	−0.1 **	27		445		1		68	−6.2 ***	4.43	+0.01 **	109	−3.4 ***	0.4	−0.03 ***
Beech	89		28		37		875	−30 ***	2		152	−5.5 *	4.53		36		5.0	−0.26 *
60 cm																		
Spruce	424	−12.5 ***	2		38		610		1		84	−3.5 ***	4.32	+0.01 **	110	−1.9 ***	0.5	−0.02 **
Beech	166	−8.2 ***	52		37		392	−6 *	3		199		4.62	+0.02 ***	41	−1.4 **	3.0	
90 cm																		
Spruce	386	−13.9 ***	2	−0.1 ***	44		245	+4 *	1		82	−3.4 ***	4.46	+0.01 **	103	−3.2 ***	0.5	
Beech	228	−7.6 ***	36		50		272	+9 ***	1		220		4.64	+0.02 ***	51	−2.5 **	2.6	+0.09 *

3.2.2. pH, Al Concentrations and Al Speciation

In both stands, the measured pH of the soil solution from the forest floor was lower when compared to mineral soil water (Figure 2). The pH of the forest floor solution from the spruce stand decreased significantly from 3.98 to 3.66. The soil solution pH from the forest floor in the beech stand decreased slightly from 4.36 to 4.34, albeit insignificantly. Over the observation period, the soil solution pH in the mineral soil increased in both stands. The pH in the spruce mineral soil solution was similar among all horizons and significantly increased from 4.25 to 4.47. In the beech stand, pH at 30 cm depth remained unchanged (4.53 ± 0.13), but at 60 cm and 90 cm it increased significantly from 4.45 to 4.79 and from 4.43 to 4.76 (Table 1).

Soil solution total Al (Al_{tot}) concentrations from the forest floor of both stands were stable during the observed period (Figure 2). The concentrations of Al_{tot} in the spruce stand were significantly greater (33 ± 15 $\mu mol \cdot L^{-1}$) than in the beech stand (24 ± 8 $\mu mol \cdot L^{-1}$). In both stands, the Al_{tot} concentrations from mineral soil solutions were greater when compared to water draining the forest floor and decreased significantly over the course of the study period. Soil solution Al_{tot} concentrations from all depths in the mineral soil under spruce were similar, ranging between 131 and 121 $\mu mol \cdot L^{-1}$ in 2005, and the concentrations decreased significantly to values ranging from 98 to 76 $\mu mol \cdot L^{-1}$ in 2017. Soil solution Al_{tot} concentrations in the mineral soil in the beech stand solution increased insignificantly at 30 cm from 30 $\mu mol \cdot L^{-1}$ to 36 $\mu mol \cdot L^{-1}$. Al_{tot} concentrations in deeper horizons simultaneously decreased from 51 $\mu mol \cdot L^{-1}$ at 60 cm and 67 $\mu mol \cdot L^{-1}$ at 90 cm in 2005 and then convergated around 32 $\mu mol \cdot L^{-1}$ in 2017 (Table 1).

Out of the Al forms composing Al_{tot}, organically bound Al_{org} concentrations in the spruce stand gradually decreased with depth from 11.8 ± 2.1 $\mu mol \cdot L^{-1}$ in the forest floor to 2.5 ± 0.6 $\mu mol \cdot L^{-1}$ at 90 cm. By contrast, Al_{org} in the beech stand showed similar values in forest floor soil solutions (9.8 ± 2.0 $\mu mol \cdot L^{-1}$) and at 30 cm (10.2 ± 1.5 $\mu mol \cdot L^{-1}$). In deeper horizons, Al_{org} concentrations were similar (5.3 ± 1.2 $\mu mol \cdot L^{-1}$ and 3.7 ± 0.9 $\mu mol \cdot L^{-1}$ in 60 cm and 90 cm). Inverse concentrations as compared to Al_{org} were detected by inorganic monomeric Al_{in} in both stands, with low concentrations in the forest floor solution and high concentrations in mineral soil horizons. Al_{in} concentrations were low in the forest floor solution from both stands (9.0 ± 4.1 $\mu mol \cdot L^{-1}$ and 2.6 ± 1.0 $\mu mol \cdot L^{-1}$ in spruce and beech stands, respectively). Whereas, in the mineral soil under spruce, Al_{in} concentrations were similar, ranging from 100 to 118 $\mu mol \cdot L^{-1}$ in 2005 and converged in 2017 to a roughly similar value across the whole profile ≈ 80 $\mu mol \cdot L^{-1}$, in the beech stand, Al_{in} concentrations continuously increased with depth from 13.8 ± 4.0 $\mu mol \cdot L^{-1}$ at 30 cm, over 28.8 ± 10.6 $\mu mol \cdot L^{-1}$ at 60 cm to 38.1 ± 9.4 $\mu mol \cdot L^{-1}$ at 90 cm. Al_{in} concentrations in the mineral soil at both sites followed the trend of Al_{tot} concentrations. Acid-soluble Al (Al_{as}) concentrations in the spruce stand were stable in the forest floor (14.0 ± 3.3 $\mu mol \cdot L^{-1}$). In mineral soil horizons, they decreased by about 60–70% from 28 $\mu mol \cdot L^{-1}$ at all depths to the range of 3–8 $\mu mol \cdot L^{-1}$. In upper beech horizons, Al_{as} concentrations were stable, and in lower horizons they decreased in the period 2005–2017 from 17 to 6 $\mu mol \cdot L^{-1}$ in 60 cm and from 19 to 5 $\mu mol \cdot L^{-1}$ in 90 cm.

When comparing the distribution of particular Al forms to Al_{tot}, we can conclude that Al_{in} from the forest floor soil solution consisted of 27% of Al_{tot} in the spruce stand and only 11% in the less acidic beech stand. The contribution of Al_{in} to Al_{tot} under spruce was stable (84%, 83%, and 89% at 30 cm, 60 cm, and 90 cm), and in the mineral soil under beech it continuously increased with depth (39%, 67%, and 74% in 30 cm, 60 cm, and 90 cm). Similar proportions of organically-bound Al (Al_{org}) were found in both forest floor solutions (spruce: 35% vs. beech: 40%); however, in the spruce stand, with increasing depth Al_{org} was lower (4%, 6%, and 2% at 30 cm, 60 cm, and 90 cm, respectively) as compared to the beech stand, where Al_{org} accounted for 28%, 12%, and 7% at 30 cm, 60 cm, and 90 cm, respectively (Figure 3).

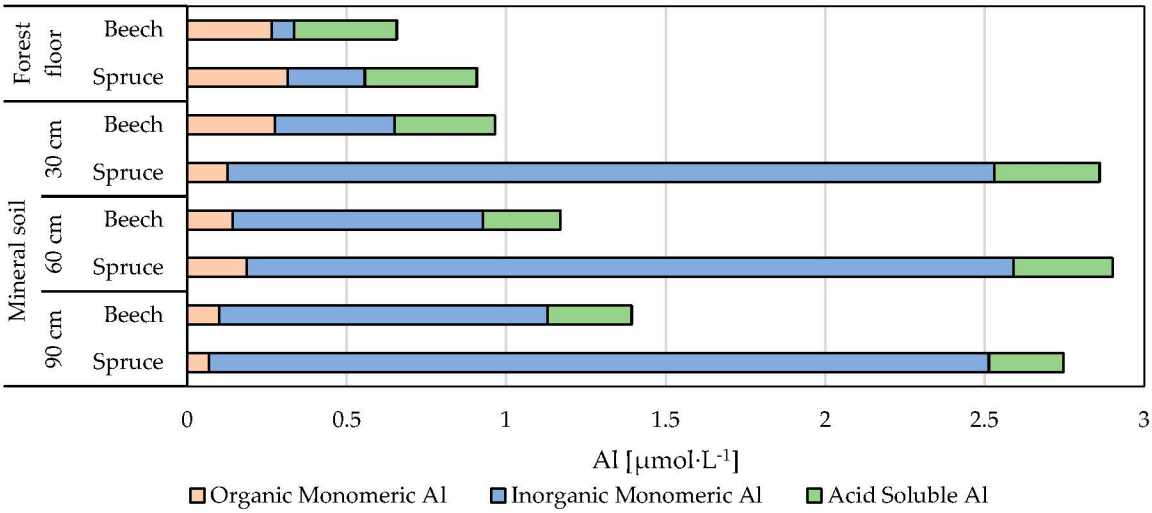


Figure 3. Mean concentrations of Al fractions in the soil solution from the forest floor and from the mineral soil in the soil water in different depths (upper column—beech, lower column—spruce).

3.2.3. NH_4^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, and the Bc/Al_{in} Ratio

In both stands, NH_4^+ concentrations decrease very rapidly with depth. A sharp decrease was measured between the forest floor and the mineral soil. In forest floor leachates, the NH_4^+ concentrations were stable at around 39 ± 52 μeq·L^{-1} and 17 ± 17 μeq·L^{-1} under spruce and beech, respectively (Figure 4). Analysis of variance proved significantly higher NH_4^+ concentrations in mineral soil solutions under beech when compared to under spruce at the depths of 30 cm (1.8 vs. 1.4 μeq·L^{-1}) and 60 cm (2.5 vs. 1.4 μeql·L^{-1}). However, annual concentrations at 90 cm were significantly greater in the spruce stand (1.2 vs. 0.94 μeq·L^{-1}) (Figure 5).

In the soil solution, the Na^+ concentrations increased with depth. The lowest Na^+ concentrations were recorded on the forest floor; in the spruce stand, the concentrations varied around 39 ± 9 μeq·L^{-1}, whereas in the beech stand were around 25 ± 13 μeq·L^{-1} (Figure 4). In the mineral soil under spruce, the concentrations were 86 ± 17 μeq·L^{-1}, 102 ± 15 μeq·L^{-1}, 72 ± 8 μeq·L^{-1} at the depths of 30, 60, and 90 cm, respectively. No trends in Na^+ concentrations were observed in the beech stand, the average concentration being 49 ± 14 μeq·L^{-1} at 30 cm, 51 ± 9 μeq·L^{-1} at 60 cm, and 69 ± 31 μeq·L^{-1} at 90 cm depth (Figure 5).

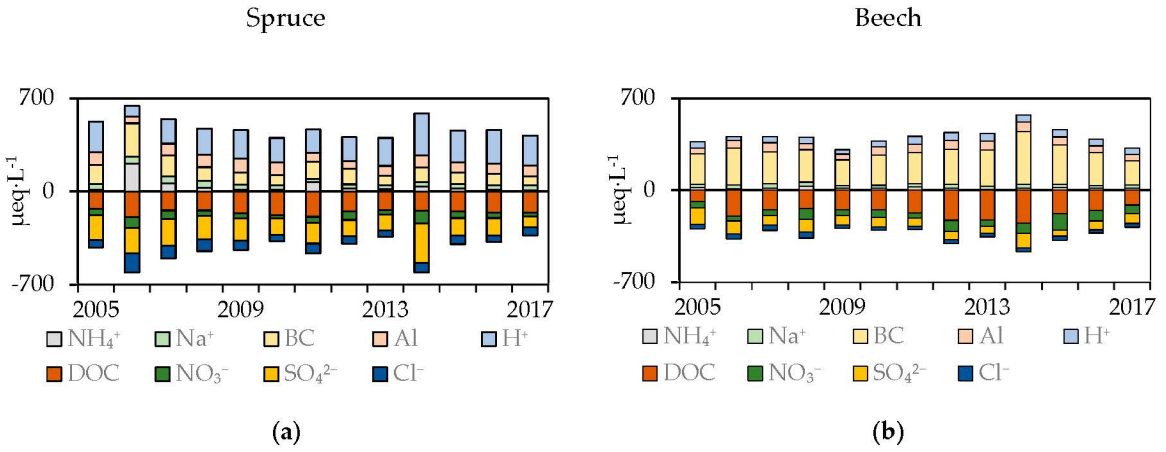


Figure 4. Soil solution concentrations from the forest floor in the spruce (**a**) and the beech (**b**) stand. DOC, NO_3^-, SO_4^{2-}, and Cl^- concentrations were reversed to show how reductions in acid anions were compensated by reductions of base cations, Al and H^+.

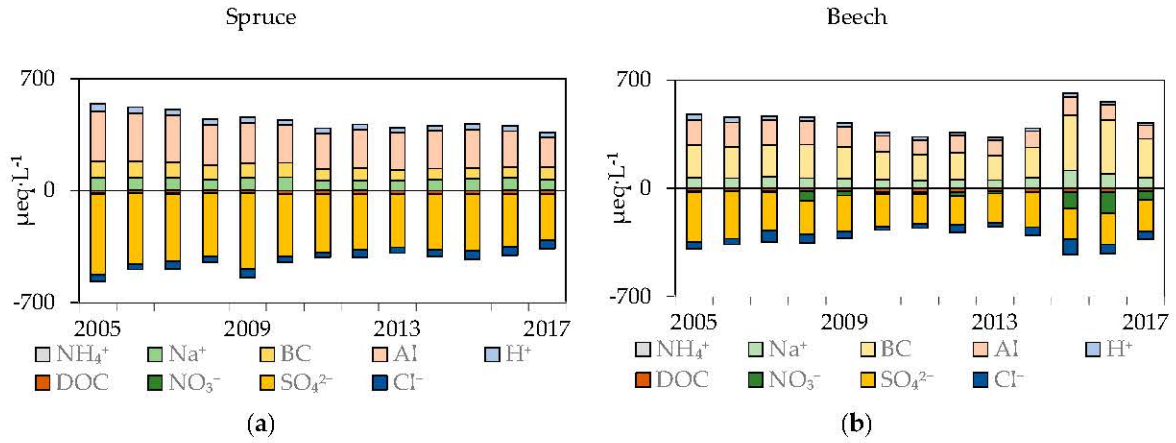


Figure 5. Soil solution concentrations from the depth of 90 cm in the spruce (**a**) and the beech (**b**) stand. DOC, NO_3^-, SO_4^{2-}, and Cl^- concentrations were reversed to show how reductions in acid anions were compensated by reductions of base cations, Al and H^+.

K^+ concentrations in forest floor soil solutions were significantly greater when compared to mineral horizons. While, in the spruce stand, the K^+ concentrations were 49 ± 72 µeq·L^{-1}, in the beech stand they were lower, 36 ± 30 µeq·L^{-1} (Figure 6). Under spruce, K^+ concentrations exhibited a downward trend at 30 cm and 90 cm (from 8.9, and 10.1 µeql·L^{-1} in 2005 to 5.8, and 7.1 µeq·L^{-1} in 2017 at 30 and 90 cm, respectively). Only at 60 cm (4.2 ± 2.5 µeql·L^{-1}) no trend was observed. In the upper mineral soil horizon in beech stand, no change was detected (3.2 ± 1.8 µeql·L^{-1} and 2.7 ± 1.0 µeql·L^{-1} at 30 and 60 cm, respectively). Only at 90 cm a decreasing trend was recorded (from 3.1 µeq·L^{-1} in 2005 to 2.11 µeq·L^{-1} in 2017).

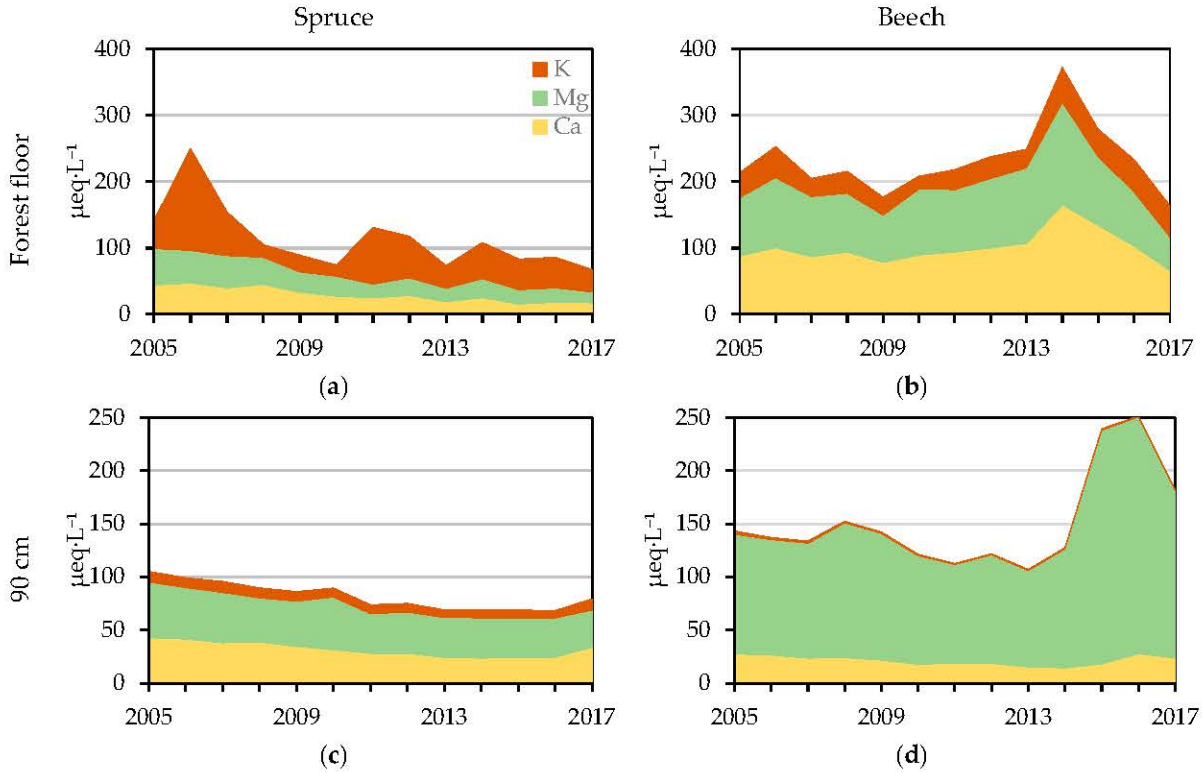


Figure 6. Mean annual BC (K^++Mg^{2+}+Ca^{2+}) concentrations in forest floor (**a**,**b**) and 90 cm (**c**,**d**) soil solution in spruce (left) and beech (right) stand.

Spruce stand Ca^{2+} concentrations in the forest floor soil solution were significantly lower when compared to the beech stand. Although no significant trend in the beech stand was observed, the Ca^{2+} concentrations (101 ± 50 μeq·L^{-1}) were three times greater when compared to the spruce stand, where a significant decrease was detected—decrease from 44 μeq·L^{-1} in 2005 to 18 μeq·L^{-1} in 2017. Differences in the mineral soil of both stands were not large. In the spruce stand, the Ca^{2+} concentrations increased with depth but Ca^{2+} concentration decreased with time (from 24, 31 and 43 μeq·L^{-1} in 2005 to 8, 16, and 34 μeq·L^{-1} in 2017 at 30 cm, 60 cm, and 90 cm, respectively). Ca^{2+} concentrations in all mineral horizons in the beech stand were similar. The main decrease was detected at 30 cm, where the Ca^{2+} concentrations decreased significantly from 43 μeq·L^{-1} in 2005 to 13 μeq·L^{-1} in 2017. In other horizons, the decrease was not pronounced; only at 90 cm an insignificant decline from 27 μeq·L^{-1} in 2005 to 24 μeq·L^{-1} in 2017 was detected (Figure 6).

A similar course was detected for Mg^{2+} concentrations (Figure 6). In the spruce stand, a significant decline was detected in all horizons—from 55, 87, 61, and 53 μeq·L^{-1} in 2005 to 16, 32, 45, and 35 μeq·L^{-1} in 2017 in the soil solution from the forest floor at 30 cm, 60 cm, and 90 cm, respectively. In contrast to the spruce stand, no decline was observed in Mg^{2+} concentrations in anyone horizon in the beech stand. Average Mg^{2+} concentrations were 96 ± 23 μeq·L^{-1}, 77 ± 19 μeql·L^{-1}, 119 ± 32 μeq·L^{-1}, 129 ± 42 μeq·L^{-1} in the forest floor and at 30 cm, 60 cm, and 90 cm, respectively.

The sum of BC cations (K + Ca + Mg) differed significantly between the stands. At all depths in the spruce stand, BC declined significantly from 142, 120, 104, and 105 μeq·L^{-1} in 2005 to 68, 46, 67, and 79 μeq·L^{-1} in 2017 in the soil solution from the forest floor and at the depths of 30 cm, 60 cm, and 90 cm, respectively (Figures 4–6). In the beech stand, BC concentrations decreased between 2005 and 2013, but in the period of 2013–2016, higher concentrations in all mineral horizons were recorded. Therefore, a significant decrease (from 180 μeq·L^{-1} in 2005 to 136 μeq·L^{-1} in 2017) was detected only at the depth of 30 cm. Mean BC concentrations were the highest in the forest floor soil solution (233 ± 102 μeq·L^{-1}), followed by those at the depth of 90 cm (151 ± 60 μeq·L^{-1}), and then those at 60 cm (147 ± 44 μeq·L^{-1}).

The molar ratio of base cation concentrations to Al_{in} (Bc/Al_{in}) fluctuated in the forest floor of spruce stand around 37 ± 32 and significantly increased from 34 to 142 in the beech stand (Table 1). In the mineral soil of the spruce stand, Bc/Al_{in} ratios were below the critical limit of 1.2 (Figure 7), but significant declines were observed at the depths of 30 cm and 60 cm (from 0.7 to 0.3 at 30 cm and from 0.6 to 0.4 at 60 cm) between 2005 and 2017; at 90 cm, the ratios varied around 5.3 ± 0.9 during this period. Bc/Al_{in} ratios in the mineral soil solutions in the beech stand decreased significantly from 11.3 to 3.1 at 30 cm, levelled off around 3.1 ± 1.1 at 60 cm, and significantly increased from 2.3 to 3.4 at 90 cm, which is above the critical limit for beech roots 0.6 (Figure 7).

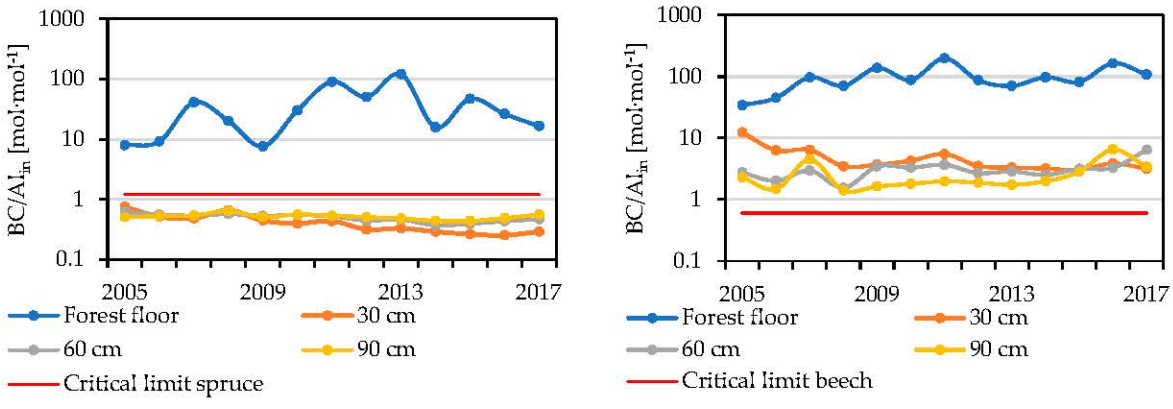


Figure 7. Annual molar Bc/Al_{in} ratios in the soil solution below the forest floor and at 30 cm, 60 cm and 90 cm of the mineral soil in the spruce (**a**) and the beech (**b**) stand. Critical limits are presented according to Sverdrup and Warfvinge [32].

3.2.4. DOC, DON Concentrations, and the DOC/DON Ratio

Soil solution DOC concentrations that were collected from the forest floor were greater in the spruce stand when compared to the beech stand (Figure 8). Significant change was detected only at spruce stand (from 2900 to 3600 $\mu mol \cdot L^{-1}$), at the beech no change was observed (2500 $\pm$ 730 $\mu mol \cdot L^{-1}$). Soil solution DOC concentrations in the mineral soil were lower at both stands when compared to forest floor leachates. Significant increases in the spruce stand were observed only at 90 cm (Figure 8). DOC concentrations in the mineral soil under beech decreased with soil depth; however, the observed temporal trends differed across soil depths. In the 30 and 60 cm horizons, concentrations declined significantly (from 1040 to 680 $\mu mol \cdot L^{-1}$ at 30 cm and from 420 to 360 $\mu mol \cdot L^{-1}$ at 60 cm), but in the 90 cm (Table 1), a significant increase was detected (from 220 to 250 $\mu mol \cdot L^{-1}$).

Concentrations of dissolved organic nitrogen (DON) in both forest floor solutions showed no change and they levelled off around 79 $\pm$ 18 $\mu mol \cdot L^{-1}$ in the spruce stand and 59 $\pm$ 14 $\mu mol \cdot L^{-1}$ in the beech stand between 2005–2017. In the mineral soil, DON concentrations were lower when compared to the forest floor soil solution in both stands. Under the spruce canopy, the DON concentrations remained stable in the whole profile (13 $\pm$ 3 $\mu mol \cdot L^{-1}$, 16 $\pm$ 2 $\mu mol \cdot L^{-1}$ 6 $\pm$ 1 $\mu mol \cdot L^{-1}$ at 30 cm, 60 cm, and 90 cm). The same trend was observed under beech stand (24 $\pm$ 6 $\mu mol \cdot L^{-1}$, 18 $\pm$ 8 $\mu mol \cdot L^{-1}$, and 15 $\pm$ 9 $\mu mol \cdot L^{-1}$ at 30 cm, 60 cm, and 90 cm).

Average values of the DOC/DON ratio in the forest floor solution in the spruce and beech plot were 48 $\pm$ 9 $mol \cdot mol^{-1}$ and 52 $\pm$ 18 $mol \cdot mol^{-1}$, respectively, without any significant temporal trend. Whereas, the mean DOC/DON ratio under spruce at 30 cm depth was comparable with that at the beech plot (spruce: 40 $\pm$ 9 $mol \cdot mol^{-1}$ and beech: 42 $\pm$ 12 $mol \cdot mol^{-1}$), in lower horizons, this ratio was greater under spruce (39 $\pm$ 7 $mol \cdot mol^{-1}$ and 48 $\pm$ 12 $mol \cdot mol^{-1}$ at 60 cm and 90 cm, respectively) as compared to beech (27 $\pm$ 9 $mol \cdot mol^{-1}$ and 31 $\pm$ 12 $mol \cdot mol^{-1}$ at 60 cm and 90 cm, respectively).

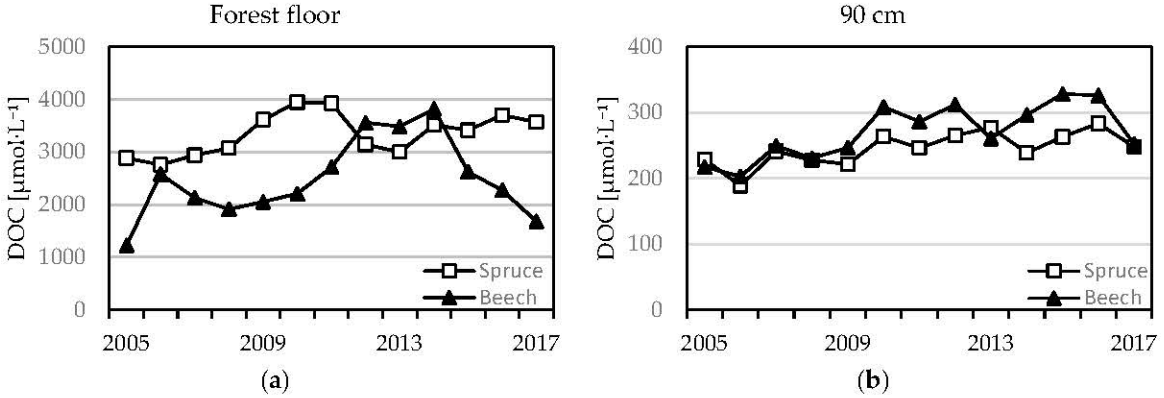


Figure 8. Mean annual molar DOC concentrations from forest floor (**a**) and the 90 cm (**b**) horizons of beech and spruce soil solutions.

3.3. Water Balance

At both stands, no significant temporal trend in annual bulk and throughfall precipitation was detected over the period of 2005–2017. Mean annual bulk precipitation was 1122 $\pm$ 117 mm, and throughfall precipitation was 941 $\pm$ 95 mm and 756 $\pm$ 109 mm under the beech and spruce canopy, so interception accounted for 17% and 33%, respectively. Stemflow was an important part of beech throughfall, accounting for 20% of bulk precipitation. In the spruce stand, the Simpel model calculated a large decrease in seepage water flux below the organic horizon. In the beech stand, a main water seepage flux decrease was calculated between 30 cm and 60 cm of soil depth. The water flux draining soil in the 90 cm horizon was 430 $\pm$ 130 mm in the spruce stand (38% of bulk precipitation) and 511 $\pm$ 101 mm in the beech stand (45% of bulk precipitation). Annual average bulk and throughfall precipitation, calculated seepage water flux and actual transpiration are presented in Table 2. Calculated mean annual actual transpiration was 207 $\pm$ 31 mm in the spruce stand and

345 ± 27 mm in the beech stand. Measured transpiration during the vegetation season of 2017 well represented the modelled data from the Simpel model. During 2017, actual measured transpiration was 165 mm and 337 mm in the spruce and beech stand, respectively.

Table 2. Mean annual water percolating through particular layers of forest stands and actual transpiration. THF—throughfall, STEM—stemflow.

Horizon (mm)	Spruce	Beech
Bulk	1122 ± 117	1122 ± 117
THF (STEM)	756 ± 109	721 ± 79 (220 ± 21)
Organic horizon	632 ± 110	834 ± 91
30 cm	520 ± 113	742 ± 93
60 cm	462 ± 120	591 ± 101
90 cm	430 ± 130	511 ± 101
Actual transpiration (modelled average 2005–2017)	207 ± 33	345 ± 27
Actual transpiration (measured in 2017)	165	337

3.4. Mass Balance

The BC flux in precipitation did not change since 2005 and averaged 4.2 ± 0.8 kg·ha^{-1}·year^{-1} in bulk precipitation, 30 ± 10.5 kg·ha^{-1}·year^{-1} in beech throughfall (including stemflow), and 30 ± 4.1 kg·ha^{-1}·year^{-1} in spruce throughfall. Furthermore, no temporal changes were detected in the bulk fluxes of individual cations (average deposition: Na = 2.5 ± 1 kg·ha^{-1}·year^{-1}, K = 1.4 ± 0.5 kg·ha^{-1}·year^{-1}, Ca = 2.2 ± 0.4 kg·ha^{-1}·year^{-1}, and Mg = 0.64 ± 0.2 kg·ha^{-1}·year^{-1}). Measured fluxes in throughfall were greater when compared to bulk fluxes, especially for K (beech: 14.5 ± 6 kg·ha^{-1}·year^{-1} vs. spruce: 19.8 ± 3.1 kg·ha^{-1}·year^{-1}). The throughfall flux of Ca exhibited a significant decrease under spruce (from 9 to 6 kg·ha^{-1}·year^{-1}), whereas under beech the Ca throughfall flux remained unchanged (6.8 ± 1.5 kg·ha^{-1}·year^{-1}). Mg and Na fluxes in throughfall were 2.3 ± 0.3 kg Mg·ha^{-1}·year^{-1} and 4.9 ± 1.3 kg Na·ha^{-1}·year^{-1} under spruce and 2.1 ± 0.5 kg Mg·ha^{-1}·year^{-1} 3.1 ± 0.9 kg Na·ha^{-1}·year^{-1} under beech, without any temporal change.

Within the soil profile of both stands, almost all K was retained (Figure 9). K flux decreased from 7 kg·ha^{-1}·year^{-1} in the forest floor to 1 kg·ha^{-1}·year^{-1} in 90 cm in the spruce stand, whereas under beech there was a rapid decline from the average of 12.5 kg·ha^{-1}·year^{-1} in the forest floor to values around 1 kg·ha^{-1}·year^{-1} in deep mineral soil. The Na flux increased at both stands from 4.5 kg·ha^{-1}·year^{-1} in the forest floor to 8 kg·ha^{-1}·year^{-1} at 90 cm. Significant differences were observed in Ca and Mg soil flux between the stands. The Ca flux from the forest floor in spruce varied around 2–3 kg·ha^{-1}·year^{-1} within the whole soil profile, whereas under beech it decreased from 15 kg·ha^{-1}·year^{-1} in the forest floor to 3 kg·ha^{-1}·year^{-1} in 90 cm. The Mg flux was unchanged within the soil profile in both stands; however, in the spruce stand, the flux of Mg was lower when compared to the beech stand (spruce: 3 kg·ha^{-1}·year^{-1} vs. beech: 7 kg·ha^{-1}·year^{-1}). Values of Al flux were lowest in the forest floor (5.7 kg·ha^{-1}·year^{-1} and 5 kg·ha^{-1}·year^{-1} under spruce and beech, respectively) and increased with depth to 13 kg·ha^{-1}·year^{-1} and 7 kg·ha^{-1}·year^{-1} in 90 cm in the spruce and beech stand, respectively.

Sulfur deposition in bulk precipitation decreased significantly from 5.8 to 2.9 kg·ha^{-1}·year^{-1} between 2005 and 2017 (Table 3). The same trend was measured in the throughfall (+ stemflow) of both stands; in the spruce stand, the S flux decreased from 15.2 to 8 kg·ha^{-1}·year^{-1} and in the beech stand from 10.9 to 5.5 kg·ha^{-1}·year^{-1}. Values of S fluxes within the soil profile were similar. In the beech stand, the flux of S increased continuously with depth (8.6 ± 1.7 kg·ha^{-1}·year^{-1} in the forest floor, 10 ± 2.3 kg·ha^{-1}·year^{-1} at 30 cm, 14.6 ± 4.4 kg·ha^{-1}·year^{-1} at 60 cm, and 17.9 ± 3.7 kg·ha^{-1}·year^{-1} at 90 cm, respectively); in the spruce stand, the increase was more pronounced when compared to the beech stand (15 ± 4.5 kg·ha^{-1}·year^{-1} in the forest floor, 32 ± 11 kg·ha^{-1}·year^{-1} at 30 cm, 30 ± 12 kg·ha^{-1}·year^{-1} at 60 cm, and 26 ± 7.6 kg·ha^{-1}·year^{-1} at 90 cm). When comparing throughfall

inputs to 90 cm outputs, the S soil solution budget had negative balance under both stands (Figure 9), thus soil was the source of S.

Table 3. Selected mean annual element fluxes in spruce and beech stands. Base cation (BC) is the sum of K + Ca + Mg.

Spruce	Na	K	Ca	Mg	Al	S-SO_4	N-NH_4	N-NO_3	DON	DOC	BC	Cl
							kg·ha^{-1}·year^{-1}					
Bulk	2.5	1.4	2.2	0.6	0.1	4.3	5.9	3.8	–	19.0	4.2	4.1
THF	4.9	19.8	8.2	2.3	0.3	12.5	8.0	8.0	3.2	85.0	30.3	10.2
Forest Floor	5.6	12.5	3.6	2.3	5.7	14.5	3.4	0.3	6.9	253.2	18.4	12.8
Mineral Soil												
30 cm	10.8	1.2	1.5	2.9	15.1	32.2	0.1	0.0	1.0	29.1	5.6	4.7
60 cm	10.3	0.8	2.1	3.1	13.4	29.6	0.1	0.0	1.0	33.5	6.0	6.0
90 cm	7.2	1.5	2.6	2.2	12.0	25.9	0.1	0.0	0.4	12.7	6.3	6.8
Beech	**Na**	**K**	**Ca**	**Mg**	**Al**	**S-SO_4**	**N-NH_4**	**N-NO_3**	**DON**	**DOC**	**BC**	**Cl**
							kg·ha^{-1}·year^{-1}					
Bulk	2.5	1.4	2.2	0.6	0.1	4.3	5.9	3.8		19.0	4.2	4.1
THF	3.1	14.5	5.7	2.1	0.2	7.0	7.8	6.3	2.3	34.6	22.3	5.7
STEM	0.8	3.2	1.1	0.3	0.1	1.7	1.3	0.9	0.7	11.1	4.6	1.4
Forest floor	4.3	10.9	14.8	8.6	5.1	8.6	1.8	6.4	6.9	234.8	34.4	7.8
Mineral soil												
30 cm	8.3	0.9	3.1	6.8	7.0	10.0	0.2	2.4	2.6	78.2	10.8	9.0
60 cm	7.0	0.7	3.1	8.0	6.3	14.6	0.2	4.0	1.4	26.7	11.8	8.2
90 cm	7.6	0.3	2.1	7.5	6.8	17.9	0.1	2.2	1.0	15.8	9.9	8.7

Annual deposition of nitrate (N-NO_3^-) in bulk precipitation decreased significantly from 5.1 to 3.4 kg N·ha^{-1}·year^{-1} (Table 3). N-NH_4^+ deposition remained unchanged (5.8 ± 0.9 kg·ha^{-1}·year^{-1}). In spruce throughfall, a significant decrease in N-NO_3^- flux was observed—from 11 kg·ha^{-1}·year^{-1} to 6 kg·ha^{-1}·year^{-1} between 2005 and 2017, whereas beech throughfall (+ stemflow) flux did not change since 2005 (7.2 ± 1.6 kg·ha^{-1}·year^{-1}). Leaching of N-NO_3 from the forest floor was observed in both the spruce and beech stands. Under spruce, the mean annual flux was lower (3.9 ± 1.8 kg N-NO_3·ha^{-1}·year^{-1}) when compared to beech (6.4 ± 2.0 kg N-NO_3·ha^{-1}·year^{-1}). The N-NO_3 flux in the mineral soil under spruce was 0.08 ± 0.05 kg N-NO_3·ha^{-1}·year^{-1} in 90 cm, whereas in the mineral soil under beech, leaching was higher with a mean N-NO_3 flux at 90 cm of 2.2 ± 2.1 kg N-NO_3 ·ha^{-1}·year^{-1}. Therefore, 98% and 42% of deposited N-NO_3^- in bulk precipitation was retained in the soil environment in both stands (Figure 9). The N-NH_4^+ flux in spruce throughfall declined significantly from 10.3 to 5.1 kg·ha^{-1}·year^{-1}, but in beech throughfall (+stemflow) no change was detected (9.1 ± 2.5 kg·ha^{-1}·year^{-1}). The leaching flux of N-NH_4 from the forest floor was greater under spruce (3.4 ± 4.8 kg N-NH_4 ·ha^{-1}·year^{-1} under spruce and 1.8 ± 0.6 kg N-NH_4 ha^{-1}·year^{-1} under beech). A low NH_4 flux (mostly below the detection limit) was measured in the mineral soil of both stands.

The mean DOC flux in bulk precipitation was 19 ± 6 kg·ha^{-1}·year^{-1}. Throughfall (+stemflow) was 85 ± 27 kg·ha^{-1}·year^{-1} in the spruce stand and 46 ± 15 kg·ha^{-1}·year^{-1} in the beech stand. DOC fluxes from forest floor were comparable in both stands (spruce stand: 253 ± 51 kg·ha^{-1}·year^{-1} vs. beech stand: 235 ± 72 kg·ha^{-1}·year^{-1}). In the mineral soil, the DOC flux decreased sharply to 29 ± 12 kg·ha^{-1}·year^{-1} at 30 cm, 33 ± 12 kg·ha^{-1}·year^{-1} at 60 cm, and 12 ± 4 kg·ha^{-1}·year^{-1} at 90 cm in the spruce stand, whereas in the beech stand the fluxes were 78 ± 14 kg·ha^{-1}·year^{-1}, 27 ± 9 kg·ha^{-1}·year^{-1}, and 16 ± 4 kg·ha^{-1}·year^{-1} at 30 cm, 60 cm, and 90 cm, respectively (Table 3).

The annual DON flux in throughfall (+stemflow) deposition was 3.2 ± 2.2 kg·ha^{-1}·year^{-1} in the spruce stand and 3.0 ± 1.6 kg·ha^{-1}·year^{-1} in the beech stand. In the mineral soil, the values of

DON flux were comparable between the stands; in the spruce stand, the DON flux decreased from 6.9 ± 1.8 kg·ha^{-1}·year^{-1} in the forest floor to 0.4 ± 0.1 kg·ha^{-1}·year^{-1} at 90 cm depth, whereas in the beech stand, the DON flux was highest in the forest floor (6.9 ± 2.1 kg·ha^{-1}·year^{-1}) and declined to 1.0 ± 0.4 kg·ha^{-1}·year^{-1} at 90 cm depth (Table 3).

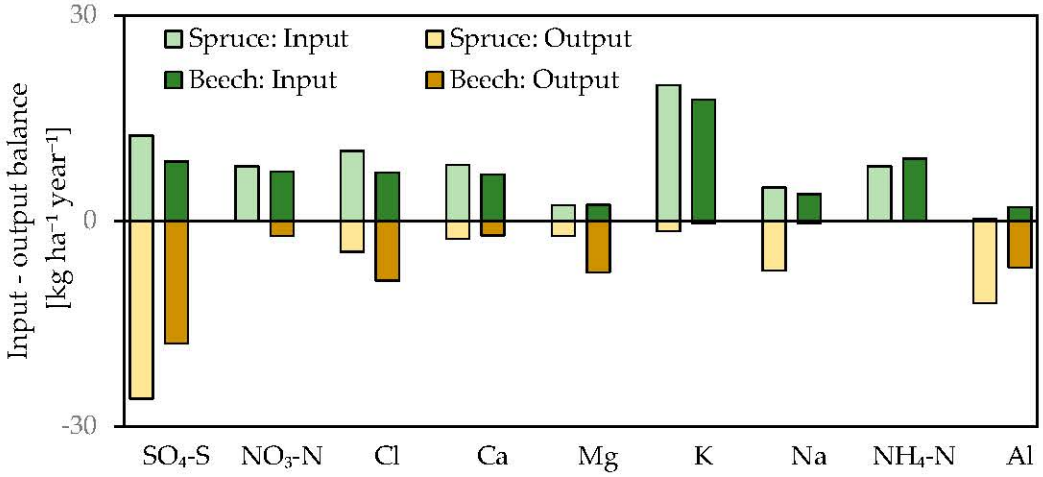


Figure 9. Input–output element budgets for the spruce and the beech stand. Inputs are represented by THF (+STEM) deposition, and outputs are calculated as fluxes passing the 90 cm horizon. Outputs were reversed to show element balances.

Atmospheric deposition of Cl was 4.1 ± 1.6 kg·ha^{-1}·year^{-1} in bulk precipitation but it increased in spruce throughfall to 10.2 ± 2.8 kg·ha^{-1}·year^{-1} and to 7.1 ± 3.2 kg·ha^{-1}·year^{-1} in beech throughfall (+stemflow). Mineral soil Cl leaching was comparable among horizons (7.8 ± 1.7 kg·ha^{-1}·year^{-1} in the forest floor and 8.6 ± 3.4 kg·ha^{-1}·year^{-1} in 90 cm) in the beech stand; in the spruce stand, leaching from the forest floor (12.8 ± 3.4 kg·ha^{-1}·year^{-1}) was greater than the output flux from 90 cm (6.8 ± 2.1 kg·ha^{-1}·year^{-1}).

4. Discussion

4.1. Water Fluxes

Our results show that, in the spruce forest, less water percolates through the soil profile than in the beech forest. This supports the general assumption that the water regime in coniferous stands is drier when compared to deciduous stands [15]. In the spruce stand, interception was 33%, whereas in the beech stand, it was only 17%. Moreover, stemflow plays a significant role in water input into the soil surface in the beech stand. While in the spruce forest this part of throughfall flux was not significant [18], stemflow in the beech stand reached 19.5% of bulk precipitation. This phenomenon, besides enhanced soil moisture around the trunk, might significantly affect soil chemistry [36]. Despite the same soil physical conditions, different tree species differ in how they use their rooting system and therefore influence the percolation of soil water through the soil profile. The shallow-rooting spruce draws water mainly from the organic and upper mineral soil. By contrast, beech, which roots deeper, also takes up water from horizons 60 and 90 cm beneath the surface. The measured (only in 2017) higher transpiration of the beech forest as compared to that of the spruce forest further highlights the importance of additional water flux via stem flow in the beech stand to maintain optimal soil water content. High interception causes the soil under spruce to be drier than under beech [37]. In total, runoff water from the ecosystem was about 38% and 45% of the bulk deposition in the spruce and beech stand. This is in accordance with data measured in other studies. For the same site, Oulehle et al. [24] modelled a runoff/precipitation ratio 44% for spruce and 42% for beech. Berger et al. [38], for a comparable site in the Alps (700 m a.s.l., 7.7 °C, 1180 mm), calculated a ratio of 46% under spruce.

4.2. Deposition

The Načetín site is located in the area formerly known as the Black Triangle, which had been exposed to heavy atmospheric pollution. At the end of the 1980s, the average annual bulk deposition of S was 15.8 kg·ha^{-1}·year^{-1} [26]. During the 1990s, rapid desulfurization resulted in a radical decrease of SO_4^{2-} in the precipitation [39] and S deposition, which continued until 2017 (2.9 kg·ha^{-1}·year^{-1}). Although N deposition has also decreased since the 1990s, a decline has been observed mainly in the case of N-NO_3. N-NH_4 deposition has been reduced only slightly and it still remains 2–2.5 times greater than during the early 20th century [40]. We have observed a declining pattern of N-NO_3 and stable N-NH_4 in bulk precipitation.

Different properties of the tree canopy modify the concentrations of compounds dissolved in throughfall precipitation, as precipitation solute enrichment in spruce throughfall is usually more pronounced than in beech throughfall. Many studies have shown that conifers are capable of greater atmospheric pollutant scavenging than deciduous species, especially when comparing spruce and beech trees [21]. The main factor affecting higher deposition inputs under spruce is vegetation structure, namely the greater leaf area index, permanent foliage, or higher interception of precipitation [17]. Our results reveal that the concentrations of all measured compounds were higher in throughfall precipitation under spruce as compared to beech. An important role in the solute concentration of throughfall is also played by the internal cycle of elements within the ecosystem, where some elements are released from leaves, for example, K, Mg, and Ca [41]. The stand therefore significantly affects the composition of precipitation and it can partly buffer the acidity reaching the soil, thus affecting the intensity of acidification processes and setting the starting conditions from which soil recovery will take place. As mentioned above, the Načetín study site is located in an area that had experienced a high degree of acidic deposition. At the end of the 1980s, depositions of 150 kg S·ha^{-1}·year^{-1} and 46 kg N·ha^{-1}·year^{-1} were measured in spruce forests [26]. For beech stands, there are no available data from the 1980s; however, it can be assumed that the rate of acid deposition was lower. At the end of the observed period, the annual deposition of S was 7 kg·ha^{-1}·year^{-1} in the spruce stand and 5 kg·ha^{-1}·year^{-1} in the beech stand. As bulk S deposition decreased, the total S deposition in the two stands converged to the similar values [42]. By contrast, the annual average of N (DIN + DON) in the throughfall flux was about 19.2 kg·ha^{-1}·year^{-1} and 19.3 kg·ha^{-1}·year^{-1} under spruce and beech, respectively.

4.3. S, Cl, DOC, and pH

The main cause of anthropogenic acidification of soils is the input of strong inorganic acids that increase H^+ concentrations in soil and ion exchange soil complexes and reduce the soil ANC by reacting with base cations [43]. Since anions other than phosphate, and to a lesser extent SO_4^{2-}, do not interact much with the solid phase of soil, they get rapidly leached out of it. Therefore, anions are what mainly determines the chemical composition of the soil solution. SO_4^{2-} is primarily supplied by atmospheric deposition. The large decrease in soil SO_4^{2-}, as a response to declining S deposition, is a pattern that is consistent across most European forest plots [11]. From the calculated balances for both stands, it is clear that more S got leached out from the ecosystem than entered it. Since the net uptake of S by vegetation is low, the main cause of the higher output of S from the subsoil is the permanent remobilization of S accumulating during the period of high deposition in the past [44]. The soil under spruce, which had been exposed to larger S inputs, has a greater S output (26 kg·ha^{-1}·year^{-1}) as compared to the less affected beech stand (18 kg·ha^{-1}·year^{-1}). SO_4^{2-} is the main acid anion in the mineral soil solutions in both ecosystems, so its decline coincided with increasing soil solution pH. In the mineral soil solution under beech, SO_4^{2-} was not the sole driver of the pH increase because of higher NO_3^- concentrations. Nevertheless, decreasing trends in SO_4^{2-} concentrations in the mineral soil under both stands were the primary cause of increases in pH, despite increasing trends in NO_3^- in some soil horizons under beech. Thus, the discharge of S from the soil ensures recovery from the unfavourable state.

Cl- in deposition were stable over the investigated period as well as Cl^- in soil solution. Cl^- is considered to be a relatively unreactive anion in regions with moderate and high Cl^- deposition [45], thus the inputs should equal the outputs. These mid-mountain central European forests receive relatively low Cl^- in deposition as compared to coastal regions. However, canopies with dry deposition component enhance throughfall Cl^- flux. Moreover, substantial part of Cl in soil can originate from organic matter decomposition [46]. This internal Cl^- cycle, together with additional concentration of throughfall Cl^- via evaporation, could lead to higher Cl^- concentrations leaving spruce forest floor.

Another important soil solution constituent is DOC, which is produced by the decomposition of organic matter. DOC represented the main anion charge in the forest floor solution and substantially decreased with depth. Increasing DOC concentrations coincided with decreasing pH over time in the forest floor at both sites. It seems that the rising production of DOC drives the acidity of the forest floor solution, in contrast to the mineral soil. The rising trend in DOC production was probably a result of a gradual recovery from acidification and the creation of more favourable conditions for microbial activity enhancing organic matter decomposition [28]. Less acidic mineral soil and lower ionic strength of soil solution caused the DOC increase in the deep mineral soil due to the higher solubility of organic acids [47,48].

4.4. N Budget

Mean total N (DIN+DON) fluxes during the study period entering the soil in throughfall were very similar in both stands (19.2 and 19.3 $kg \cdot ha^{-1} \cdot year^{-1}$ under spruce and beech, respectively). Because N is efficiently used in terrestrial ecosystems as a nutrient, under natural conditions main N output from ecosystem is represented by its organic form as DON [49]. However, if the availability of inorganic N exceeds the total combined plant and microbial nutritional demand, ecosystems progress towards N saturation and N leaching occurs, mainly in the form of NO_3^- [50]. The susceptibility of ecosystems towards NO_3^- leaching is usually defined in the literature based on the C/N ratio of the organic soil horizon [51], or it is postulated to occur when dissolved inorganic N concentrations (DIN) in seepage water are greater than 1 $mg \cdot L^{-1}$ [52]. The main leaching constituent is NO_3^-, because NH_4^+ is effectively immobilized in the soil by plants or microbes [53]. However, after N deposition starts to decrease, a change of NO_3^- concentrations in the soil solution can be soon detected [12]. Iost et al. [14] reported an exceedance of critical limits in DIN ($N\text{-}NO_3^- + N\text{-}NH_4^+$) concentrations across most forest monitoring plots in Europe. Also, under both stands were exceeded critical limits in mean annual DIN concentrations in forest floor (spruce: 1.25 $mg \cdot L^{-1}$ vs. beech: 1.13 $mg \cdot L^{-1}$). In the mineral soil under spruce, NO_3^- concentrations decreased during 1990s in the deep soil solution from 175 $\mu eq \cdot L^{-1}$ [39] and diminished further around 2006. Since then, only low NO_3^- concentrations in the top mineral soil have been measured. A higher NH_4^+ flux in the mineral soil is a sign that nitrification processes in the forest floor were retarded, therefore NH_4^+ leaching occurred [38]. At the beech plot, NO_3^- leaching was observed from upper horizons (6.4 $kg \cdot ha^{-1} \cdot year^{-1}$) and from the subsoil (2.2 $kg \cdot ha^{-1} \cdot year^{-1}$). This stands in contrast to studies reporting higher N leaching under spruce [38,42] as a result of higher deposition of N. However, a comparison of the C/N ratio of the two organic horizons [25] revealed a lower C/N ratio in the FH horizon under beech (beech stand: 19.9 vs. spruce stand: 23.3). According to Gundersen et al. [51], when the C/N ratio is less than or equal to 25, there is a high probability that leaching will occur. Under similar N inputs, a lower C/N ratio could be responsible for N leaching from upper mineral horizons in the beech stand. However, the deep-rooting beech is able to retain NO_3^-, so NO_3^- leakage from the subsoil occurs irregularly. NO_3^- leaching from the subsoil occurred mainly during the winter and spring seasons, when deciduous stands are in a dormant phase and unable to retain mineralized N. Accumulation of N during the winter, together with freeze-thaw cycles, can cause N leaching from the soil during the spring snowmelt period [54]. However, this seasonally enhanced N leaching rarely exceeded the critical limit of 1 $mg \cdot L^{-1}$. Higher NO_3^- leaching was detected between 2015 and 2016 (DIN of up to 3.5 $mg \cdot L^{-1}$) in the winter and spring seasons. During this period of higher N leaching, this could have been partially caused by clearcutting at the end of

2014 that took place in the vicinity of the sampling plots. This disturbance could have temporarily released N by altering the environmental conditions (the microclimate, which influences the rates of decomposition and mineralization of organic matter, causing a surplus of unused N). This fact, together with the spring flushing of the soil profile by water from melting snow, could have been responsible for elevated NO_3^- concentrations in the deep soil between 2015 and 2016. Furthermore, the exceptionally dry and hot year of 2015 possibly partly triggered the release of inorganic N from soil organic matter decomposition. In both stands, an increase in DON concentrations in the soil solution was observed. In forest floor solutions at both sites, DON fluxes were reported to be 6.9 $kg \cdot ha^{-1} \cdot year^{-1}$ on average. However, in seepage water from the depth of 90 cm in the spruce stand, DON was the major form of N (0.4 $kg \cdot ha^{-1} \cdot year^{-1}$). Verstraeten et al. [55] reported increasing DON concentrations, accompanied by a decrease in the DOC/DON ratio, as a sign that an advanced process of recovery from acidification is underway. However, no DON increase accompanied by a decrease in the DOC/DON ratio was detected in any of the examined horizons. More likely, the DON increase was related to a DOC increase as a result of lower ionic strength and the greater dissolution of organic acids [47].

4.5. Base Cations, Aluminium and BC/Al_{in}

Base cations concentrations showed a different pattern for each stand. While in the spruce stand, the BC concentration decreased significantly in all horizons, trends in the beech forest were not coherent in comparison to spruce. Moreover, a large decrease in BC concentration was measured between the forest floor and mineral soil solutions in the beech plot. The described reduction in concentrations between the forest floor and the mineral soil is probably related to the different distribution of roots under both stands. The forest floor and the upper mineral topsoil receive large amount of nutrients from throughfall and from decomposing organic matter. Beech, which has deeper roots, receives nutrients from the upper mineral soil, whereas the shallower-rooting spruce acquires them mostly from the transition between the forest floor and the mineral soil. Acidification caused by atmospheric deposition mainly affects upper soil horizons, which contributes to base cations cation depletion (and an increase in Al dissolution). Due to the principle of electroneutrality, anion mobility should be compensated by an equivalent amount of cations. Therefore, the decline in anion concentrations should be followed by an equivalent cation charge. The decline in BC in the soil solution under spruce was connected with the decline of acid anions. This pattern can be seen in air pollution affected acidic soils with small BC pools and low weathering rates [56]. Long-term atmospheric pollution caused the depletion of base cations and then changes in throughfall Ca^{2+} and Mg^{2+} may influence soil solution cations. Connection between BC deposition and BC soil solution was also reported from other monitoring plots [5,11,22]. At the beech plot, a decline was detected between 2005 and 2013; since then, episodic increases of NO_3^- concentrations between 2014 and 2016 caused elevated BC concentrations. Whereas, a distinct reduction in K and Ca concentrations between the forest floor and the mineral soil under beech was observed, Mg concentrations were similar across the whole profile ($\approx$100 $\mu eq\ L^{-1}$).

Dissolution of mineral Al into the soil solution is a pivotal consequence of the soil acidification process. It is well known for its potential toxic effect on tree roots. Al in the soil solution is a composite of several forms of Al ($Al_{tot} = Al_{org} + Al_{in} + Al_{as}$), which differ in their toxicity. The most toxic form is monomeric Al (Al_{in}). Based on our results, Al_{tot} concentrations in the soil solution are determined by soil acidity and the soil horizon. Despite the low pH in the forest floors of both stands, low Al_{tot} values were observed as a result of the absence of an Al source (limited mineral particles). Al inputs via throughfall and litterfall are too low to increase Al_{tot} in the forest floor. Al_{tot} concentrations in forest floor are therefore comparable at both stands. Due to the presence of DOC in the forest floor, Al is bound predominantly to DOC, forming non-toxic Al_{org}.

In mineral horizons, where enough Al can be supplied from mineral soil, Al_{tot} concentrations were driven by soil acidity. There was a noticeable decreasing temporal trend in Al_{tot} associated with reduced atmospheric acid deposition. Identical Al_{tot} concentrations across the soil profile in the spruce stand and increasing Al_{tot} concentrations with depth in the beech stand are related to the

inverse pH pattern. As already mentioned, soil acidification progressed from the top downwards. Analogically, acidification recovery should take place in the same direction. Therefore, the conditions of the beech stand, with its low Al_{tot} together with high pH in the upper soil, can be considered to be more favourable as compared to the spruce stand. The main form of Al in the mineral soil was inorganic monomeric Al (Al_{in}) constituting, on average, 85% and 60% of Al_{tot} under spruce and beech, respectively. The lower Al_{in}/Al_{tot} ratios under beech are a result of high DOC concentrations in upper horizons, as Al binds to DOC.

One parameter often used to describe the chemistry of soil subjected to acidification is the BC/Al ratio [32]. Sverdrup and Warfvinge [32] suggested critical limits under which the roots of particular tree species are more likely to be exposed to toxic Al, adversely influencing the vitality of the trees. Cronan and Grigal [57] recommended the determination of the ratio to the sum of toxic Al (Al_{in} = Al^{3+} + $AlOH^{2+}$ +$Al(OH)_2^+$ + Al_{13}^{7+}), because there might be a large share of other, non-toxic forms. In the beech stand, the Bc/Al_{in} ratio was high above the critical limit of 0.6 in the mineral soil (1.5–1.9), whereas in the spruce stand, the soil solution from all horizons was below the critical limit of 1.2 (0.4–0.5), indicating potential serious root damage or stress. This low ratio should be reflected in the health-related conditions of the spruce stand. Although we did not carry out any measurements of stand vitality, we observed no signs of damage (defoliation, needle yellowing). The question of the practical relevance of the Bc/Al ratio has been asked by Hansen et al. [58] and European-scale comparison has been performed by Iost et al. [14]. Iost et al. [14] rarely observed exceedance of critical limit. However, they used the Al_{tot} fraction, whereas we considered only the toxic form Al_{in}. Hruška et al. [59] found a relationship between the health status of spruce and the Bc/Al ratio only when examining the forest floor soil solution. They did not find any relationship with the Bc/Al ratio in the mineral soil solution. Spruce can perhaps avoid the toxic area by redistributing its roots closer to the forest floor, where the BC/Al_{in} ratio was about 37. However, this shallow rooting very often leads to exposure to other threats, such as drought.

5. Conclusions

Atmospheric SO_4^{2-} deposition has decreased in both stands and a decline in NO_3^- deposition was detected only in the spruce stand. These changes are reflected in the soil chemistry as a decrease in SO_4^{2-} in both stands and a decline in NO_3^- concentrations under spruce. The episode of NO_3^- leaching from the beech stand is a consequence of the ecosystem's greater susceptibility to perturbations. Increasing pH and decreasing Al concentrations are a sign of ongoing successful recovery from acidification. However, low Bc/Al_{in} ratios under spruce in the upper mineral soil still indicate unfavourable conditions in the rooting zone.

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9.2 Publication II

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Article

Carbon and Nitrogen Pools and Fluxes in Adjacent Mature Norway Spruce and European Beech Forests

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Abstract: We compared two adjacent mature forest ecosystem types (spruce vs. beech) to unravel the fate of assimilated carbon (C) and the cycling of organic and inorganic nitrogen (N) without the risk of the confounding influences of climatic and site differences when comparing different sites. The stock of C in biomass was higher (258 $t \cdot ha^{-1}$) in the older (150 years) beech stand compared to the younger (80 years) planted spruce stand (192 $t \cdot ha^{-1}$), whereas N biomass pools were comparable (1450 $kg \cdot ha^{-1}$). Significantly higher C and N soil pools were measured in the beech stand, both in forest floor and mineral soil. Cumulative annual CO_2 soil efflux was similar among stands, i.e., 9.87 $t \cdot ha^{-1} \cdot year^{-1}$ of C in the spruce stand and 9.01 $t \cdot ha^{-1} \cdot year^{-1}$ in the beech stand. Soil temperature explained 78% (Q_{10} = 3.7) and 72% (Q_{10} = 4.2) of variability in CO_2 soil efflux in the spruce and beech stand, respectively. However, the rather tight N cycle in the spruce stand prevented inorganic N losses, whereas losses were higher in the beech stand and were dominated by nitrate in the mineral soil. Our results highlighted the long-term consequences of forest management on C and N cycling.

Keywords: *Fagus sylvatica*; *Picea abies*; carbon; nitrogen; budget; respiration; productivity

1. Introduction

Temperate forests are one of the most important features within the European landscape. They play an important ecological role—providing ecosystem services (e.g., ensuring vital water resources, maintaining biodiversity, scavenging air pollutants)—as well as a social role, with forests being perceived as a "real" natural heritage. Forests are also relevant economic resources (timber production, tourism). Broadleaf forests of lowlands and mixed conifer-broadleaf forests of mid-altitude sites have always been influenced by human activities and therefore have been altered considerably [1–3]. The historical retreat of temperate forests, their considerable fragmentation, changes in woody species composition and soil degradation that resulted from over exploitation caused declines in ecosystem stability and biodiversity of temperate forests [4]. Especially in Central Europe, original forest stands dominated by *Fagus sylvatica*, *Abies alba* and *Picea abies* have been almost completely converted to even-aged Norway spruce monocultures. These changes significantly slow down soil formation [5] and alter understory vegetation composition [6].

The influence of tree species composition on the functioning of temperate forests has received increasing attention in recent years due to efforts to alter the even aged spruce monoculture management by introducing broadleaf tree species. Furthermore, spatial distributions of tree species are

expected to change as a result of ongoing climate change [7]. In natural forests, expansion of European beech has been well documented in different parts of Europe [8–10], and expansion of Norway spruce towards higher altitude was detected in tree-line ecotones in Central Europe [11]. Both natural and human-induced changes in tree species distribution have major impacts on forests functioning.

Augusto and others [12] summarized the effects of evergreen gymnosperms (EG) and deciduous angiosperms (DA) on forest ecosystems, pointing out that EG species generally induce drier soil conditions. However, biomass and litter production were rather similar among tree species groups, and field evidence of higher accumulation of soil carbon stocks under DA species was lacking. Similarly, Vesterdal and others [13] have shown similar carbon (C) and nitrogen (N) soil stocks in spruce and beech stands, while higher forest floor C and N contents were measured in spruce compared to beech stands with the opposite trend in the mineral soil.

A significant fraction of the forest ecosystem C pool is stored in the soil [14]. Carbon allocation from photosynthesis to belowground net primary productivity (NPP) and aboveground NPP might determine the longterm fate of assimilated C as a large proportion of recalcitrant soil C originates from root decomposition [15] and decaying mycorrhizal tissues [16]. Furthermore, beech has deeper roots compared to spruce [17], suggesting different vertical allocation of belowground NPP in the soil profile.

Soil respiration (F_s) represents one of the major fluxes of C to the atmosphere [18] and equals about 55% of C fixed during photosynthesis [19]. It is important to identify environmental factors that control F_s and their effects on CO_2 emission rates when potential impacts of environmental change, including community shifts and alteration of moisture/temperature regimes, are studied. Along with soil temperature, moisture, physical and chemical properties, soil respiration varies with vegetation types. Nonetheless, vegetation type seems to be less important in controlling soil respiration rates compared to abiotic factors [20]. Among temperate forest ecosystems, the effect of different tree species on soil respiration rates has often been found to be minimal [21–24].

Ecosystem productivity depends on various environmental factors, of which nutrient limitation plays an important role due to the stoichiometric constraints of elements in plant tissues [25]. In Europe, N inputs into forest ecosystems accelerated during the 20th century due to human industrial and agricultural activities [26,27]. Higher N availability of this (naturally) limiting nutrient has been demonstrated to be partly responsible for enhanced mountain forest productivity in recent years [28]. Nonetheless, inorganic N leaching has occurred from forested ecosystems across central Europe [29–31]; thus, the factors responsible for ecosystem N retention need to be determined in order to predict future ecosystem responses to global change.

In this paper, we provide an assessment of C and N pools and fluxes for adjacent mature European beech and managed Norway spruce forest ecosystems. We focus on (i) differences in C and N biomass and soil pools; (ii) C and N allocations in tree biomass; (iii) dynamics of soil respiration and (iv) input–output budgets of water, C and N. The similar site conditions and acidic deposition history of both stands provide an excellent opportunity to identify the specific role of tree species (intensively managed spruce vs. extensively managed beech) in the long-term regulation of C and N cycling in forest ecosystems.

2. Materials and Methods

2.1. Site Description

The study sites are situated near the Czech-German border in the Ore Mts. (50°35′25″ N, 13°15′11″ E for the spruce stand and 50°35′20″ N, 13°16′1″ E for the beech stand) at an elevation between 780 and 820 m a.s.l., close to the village of Načetín. The spruce stand consists of Norway spruce (*Picea abies* (L.) Karst.) monoculture planted in the early 1930s, and the beech stand consists of European beech (*Fagus sylvatica* L.) monoculture. The history of the forests can be briefly described as follows: based on maps of the Stabile cadaster from 1842, both sites were dominated by mixed beech-conifer (spruce/fir)

forests. However, soon after this mapping (1850s), the current spruce stand was converted into spruce monoculture, which was first harvested in 1930s. Thus, the current spruce forest is a second generation plantation. Unlike the spruce stand, the beech stand continued as a mixed forest of dominantly beech and spruce (based on aerial photographs from 1953). During the 1970s and 1980s, however, mature spruce trees continuously died off due to air pollution (SO_2 emissions causing acid rain), and the stand became pure beech monoculture. In the spruce stand, spruce trees survived the peak acid deposition as the plantation was relatively young. It is likely that before the 2nd World War, dense settlements in that area caused human exploitation of natural resources (timber harvest) and historical agricultural activities (forest pasture, litter raking, see [3]), which affected the surrounding landscape. After the war, German inhabitants were expelled from the former Czechoslovakia, and human activities largely ceased in this border region.

The distance between stands is approximately 1 km; annual precipitation averages 1012 mm (1992–2015), and annual temperature is 6.5 °C. Both stands are underlain by paragneiss bedrock. The dominant soil type is dystric cambisol. Soils at both sites were acidified by acid deposition during the 20th century, which resulted in low soil pH and low base saturation. More substantial soil acidification was documented in the spruce stand due to the higher acid deposition under the spruce canopy compared to the beech stand [32]. In 2003, the pH of the forest floor was 3.80 in the spruce stand and 4.34 in the beech stand, and soil base saturation was 19% and 70% in the spruce and beech stands, respectively. In the mineral soil (up to 40 cm), soil pH was 4.07 in the spruce and 4.23 in the beech stand, and base saturation was 2.8% in the spruce and 5.5% in the beech stand.

2.2. Deposition, Soil Solution Sampling and Analysis

Bulk deposition has been measured since 1992, and throughfall has been sampled since 1993 in the spruce stand and 2003 in the beech stand. Bulk collectors comprise two samplers, and throughfall collectors comprise nine samplers (for more details see [33]). In 2003, a stemflow collector was installed to analyse stemflow fluxes of one representative (based on DBH distribution) beech tree within the beech stand. All input fluxes of elements were measured and analyzed at a monthly time step.

In 2013, four plots (9 m^2) within each stand were randomly established to monitor the soil solution chemical composition of the forest floor and mineral soil. At each plot, four to six suction Rhizon samplers were installed in the forest floor (L + F + H horizon), comprising 10 cm long, 2.5 mm diameter porous membranes attached to 50 mL syringes, which were used to apply suction and collect samples. Samples were collected by applying suction, and samples were bulked to provide one composite sample per plot. Within the centre of each plot, a porous suction lysimeter (Prenart) was placed at a depth of 30 cm in the mineral soil. The soil solution was collected by applying suction to a 2 L collecting bottle. The soil solution was sampled in the three-week interval during the snow/freeze free period (usually April–November). The soil solution could not be collected when the soil moisture was low.

Samples were stored at 4 °C, and they were analyzed for nitrate (NO_3^-) and chloride (Cl^-) by high-performance liquid chromatography (Knauer 1000); ammonium (NH_4^+) was determined by indophenol blue colorimetry. DOC (dissolved organic carbon) was determined by a nondispersive infrared detector after sample conversion to CO_2 by a combustion furnace. Analysis of dissolved nitrogen (DN) was based on sample combustion to NO and its reaction with O_3 to produce an excited state of NO_2*, which, when it decays to its ground state, emits light. The light is detected by a chemiluminiscence detector and is correlated to a specific amount of nitrogen. DON (dissolved organic nitrogen) was calculated as the difference between the DN and the sum of $N\text{-}NO_3$ and $N\text{-}NH_4$. DOC and DON samples were filtered (glass fiber Macherey-Nagel) before analysis in a Tekmar-Dohrman Apollo 9000 analyzer (Tekmar Dohrmann, OH, USA).

2.3. Water Budget

To assess water fluxes through the soil profiles, we used measurements of the chloride (Cl) mass budget (Cl input = Cl output). Chlorine compounds are highly soluble in water and mobile in soils, so transport of atmospherically derived Cl through terrestrial ecosystems is rapid and conservative if there is active hydrologic flow. Weathering and other internal sources of Cl are generally negligible compared to atmospheric deposition [34], and although some cycling of Cl has been observed in Swedish forest organic horizons [35], the effect of this on the full-profile or catchment input-output budgets appears limited [36]. The calculated water flux was then used to calculate solute fluxes through the soil profile. Canopy interception was defined as the difference between precipitation and throughfall water fluxes. Evaporation from soil and tree transpiration was defined as the difference between throughfall water fluxes and seepage of the 30 cm soil depth. Total evapo-transpiration (ET) thus comprised the total water vapour flux between the canopy and the atmosphere.

2.4. Soil Sampling, Analysis and Temperature and Moisture Measurements

Soils were quantitatively collected in 2003, based on four replicates within each stand. Soil masses were estimated by excavating 0.5 m^2 pits following the method described in [37]. The forest floor was divided into L (litter) and FH (fermentation + humus) horizons. Mineral soil was sampled following the exact depth strata of 0–10 cm, 10–20 cm and 20–40 cm. Forest floor horizons were resampled in 2010 in the spruce stand. In the fine soil fraction (<5 mm for organic horizons and <2 mm in mineral soil), total C and N were simultaneously determined by a Carlo-Erba Fusion 1108 analyzer (Thermo, Torino, Italy).

In 2013, thermometers Pt1000 A class sensor (Environmental Measuring Systems, Brno, Czech Republic) and soil moisture probes Campbell Scientific CS650 (Campbell Scientific, UT, USA) were installed in three replicates in each stand into the boundary layer between the forest floor and mineral soil (to a depth of ca. 7 cm) to continuously monitor the soil temperature and moisture.

2.5. Biomass Measurements and Litterfall Collection

In the spruce stand, two surveys of tree biomass were conducted in 1994 (2500 m^2) and 2013 (1000 m^2). Diameter at breast high (DBH, in cm), tree height and number of tree individuals were measured. Furthermore, in 1994, eleven trees were cut at the spruce site, and different parts of tree biomass were measured to obtain site-specific biomass functions:

$$\text{Stem (kg DW)} = 0.3397 \times (\text{DBH})^2 - 1.786 \times (\text{DBH}) + 10.22 \ (R^2 = 0.99) \quad (1)$$

$$\text{Bark (kg DW)} = 0.018 \times (\text{DBH})^2 - 0.0947 \times (\text{DBH}) + 0.5417 \ (R^2 = 0.99) \quad (2)$$

$$\text{Branches (kg DW)} = 2.53 \times (\text{DBH}) - 29.331 \ (R^2 = 0.88) \quad (3)$$

$$\text{Needles (kg DW)} = 1.55 \ (\text{DBH}) - 16.08 \ (R^2 = 0.87) \quad (4)$$

Estimation of root biomass was based on equation from [37]:

$$\text{LN roots (kg DW)} = -8.35 + 4.57 \times \text{LN(DBH)} - 0.33 \times \text{LN(DBH)}^2 + 0.28 \times \text{LN(Age)} \ (R^2 = 0.97)$$

Annual aboveground net primary production (ANPP) of different biomass components was calculated for the period 1994 to 2013 from the above equations based on DBH growth measurement.

A biomass survey was conducted in the beech stand in 2013 only, and DBH, tree height and number of tree individuals were measured. Biomass increments for the last 20 years were calculated based on ring width series. The diameters were multiplied by a constant of 1.096 to account for bark thickness and water loss [38]. Total tree biomass was calculated according to changes in DBH measurements using models developed by [39].

Litterfall amount has been measured since 2013, based on 5 frame collectors (0.5 m^2) placed randomly within each site. Litterfall was collected bimonthly and divided into needles/leaves, twigs and fruiting parts.

All biomass parts were sampled for total C and N contents. Samples were dried (60 °C), homogenized and analyzed using NCS Flash 2000 (Thermo Scientific, MA, USA). Data on N concentration in roots were obtained from Schulze (2000) [40].

2.6. CO_2 Flux Measurements

In each plot within a stand, four soil collars (286.5 cm^2) were inserted into the forest floor to a 5 cm depth (16 soil collars in each stand). Soil CO_2 efflux (F_s) was measured monthly between 2013 and 2015 during the snow-free period (April–November) using a LiCor infrared gas analyzer LI-8100A (LiCor Biosciences, NE, USA) attached to a LiCor Survey Chamber (8100-103 20 cm survey chamber) (LiCor Biosciences, NE, USA). The chamber was placed on top of open, permanently installed collars for 30 s before measurements. The CO_2 efflux was calculated on the basis of a linear increase in chamber CO_2 concentration over time.

The relationship between soil temperature and soil respiration was modelled according to the van't Hoff equation [41]:

$$F_s = \alpha e^{\beta T} \text{ (where } Q_{10} = e^{\beta 10}\text{)}, \quad (5)$$

where F_s is the respiration rate measured in situ, α and β are fitted parameters, T is measured soil temperature and Q_{10} is the temperature sensitivity of respiration. The equation was fitted for each stand. F_s was calculated on an hourly basis using site-specific soil temperatures and summarised for the entire year to provide an annual CO_2 efflux estimate.

2.7. Belowground C Allocation

Total soil CO_2 efflux is the sum of heterotrophic and root respiration. The former is a product of above- and belowground litter production [42]. These authors proposed, based on a mass balance approach, that the difference between F_s and litterfall C can be used to estimate total belowground carbon allocation (TBCA). TBCA = soil respiration − aboveground litter + changes in root, soil and litter carbon stores [43]. Thus, TBCA comprises root production, root respiration, root exudates and plant-assimilated C used by mycorrhizae. The fraction of TBCA that is not used for root respiration is often termed BNPP (belowground net primary production). BNPP of the coarse root production was estimated according to the biomass functions, and BNPP of fine roots was estimated as 14% of NPP following [44]. Total autotrophic respiration (R_{total}) was calculated as 1.33 × (ANPP + BNPP) [45], and root autotrophic respiration (R_{root}) was calculated as the difference between R_{total} and aboveground autotrophic respiration (R_{above}). R_{above} was calculated as 0.96 × ANPP [45]. BNPP of root exudates and myccorhizae ($BNPP_{ex/mycc}$) was calculated as the difference between TBCA and the sum of R_{root} and BNPP of roots. As such, gross primary production (GPP) and net primary production (NPP) could be estimated as:

$$GPP = ANPP + TBCF + R_{above} \quad (6)$$

$$NPP = GPP - R_{total} \quad (7)$$

Water use efficiency (WUE) was defined as GPP/ET following Tang et al. [46].

2.8. Statistical Analysis

We used one-way ANOVA to compare soil characteristics (soil mass, C and N pools, C and N concentrations, and C/N ratio), litterfall mass and C and N concentrations, soil solution chemistry and solute fluxes and soil respiration rates between stands. We tested whether data were normally distributed using the Shapiro-Wilk test. The Kruskal-Wallis comparison was used to compare average ranks if data distribution was non-normal. The effects of soil temperature and moisture on CO_2

effluxes were modelled using exponential equations. The residuals (CO_2 observed − CO_2 measured) were related to soil moisture measurements to test whether the unexplained variation in CO_2 effluxes could be attributed to soil moisture.

3. Results

3.1. Carbon and Nitrogen Stocks

3.1.1. Carbon and Nitrogen in Vegetation Biomass

In 2013, 540 individual spruce trees and 340 individual beech trees per hectare were present in the stands (Table 1). The age of the trees in the spruce stand was estimated as 80 years, and the age of the beech trees was approximately 150 years.

Table 1. Number of trees (trees·ha^{-1}) and their DBH (diameter at breast height), tree height and crown area (m^2) characteristics in both stands.

	No. of Trees	DBH	Height	Crown Area
Spruce stand	ha	cm	m	m^2
Avg	540	37.9	25.0	29.5
Median		37.7	25.8	24.6
Min		14.7	14.9	4.0
Max		59.2	30.0	105
Beech stand				
Avg	340	40.1	29.9	37.8
Median		42.0	31.2	32.6
Min		16.9	20.2	4.4
Max		55.6	34.8	123

Spruce stand total C biomass was 192 t·ha^{-1}, of which 69% was stored in the stem, 9% in branches, 6% in needles and 24% in roots. The total biomass C in the beech stand was 258 t·ha^{-1}, 1.34× higher than in the spruce stand. The largest part of biomass C was stored in the stem (72%), with 15% in branches, 1% in leaves and 13% in roots (Table 2).

Table 2. Stocks (kg·ha^{-1}) of biomass (dry weight), carbon, nitrogen and the mass ratio of C:N in spruce and beech vegetation.

2013	Stem	Branches	Leaves	Roots	Abovegr	Total
Spruce stand			kg ha^{-1}			
DW	249,344	35,973	23,000	95,518	308,317	403,835
C	118,438	17,087	10,925	45,371	146,451	191,822
N	374	227	276	570	877	1447
C/N	317	75	40	80	167	133
Beech stand						
DW	390,095	79,084	4104	70,611	473,283	543,894
C	185,295	37,565	1949	33,540	224,809	258,350
N	495	543	96	320	1134	1454
C/N	374	69	20	105	198	178

The total biomass N pool in the spruce stand was 1447 kg·ha^{-1}, of which 26% was in the stem, 16% in branches, 19% in leaves and 39% in roots. In the beech stand, the total biomass N pool was 1454 kg·ha^{-1}, of which 34% was in stem biomass, 37% in branches, 7% in leaves and 22% in roots (Table 2). Due to the higher C pool in beech biomass and rather similar biomass N pools, the total biomass C/N ratio was higher in the beech stand. The lowest C/N ratio (g/g) was measured in

needles (40) and leaves (20), whereas the highest C/N ratio was measured in stem biomass (317 in the spruce and 374 in the beech stand).

3.1.2. Carbon and Nitrogen in Soil

The soil carbon concentration was significantly ($p < 0.001$) lower in deeper mineral soil (10–40 cm) in the spruce stand compared to the beech stand. A higher C concentration in the beech stand was measured in the L horizon ($p = 0.034$) compared to the spruce stand. No significant differences were observed in the mass of fine soil throughout the soil profile in both forests, but due to the higher %C in the beech stand, a higher C pool was measured in beech forest soil (199 t·ha^{-1}) compared to spruce soil (114 t·ha^{-1}). In both forests, 33% of soil carbon was present in LFH horizons (Table 3).

Table 3. Pools (kg·ha^{-1}) of fine soil (LFH < 5 mm; 0–40 cm < 2 mm), carbon, and nitrogen and the mass ratio of C:N in spruce and beech soils. Total C and N concentration in soil profiles of spruce and beech forests; standard deviations in parentheses and significant differences ($p < 0.05$) among stands are highlighted in bold.

	Spruce Stand						**Beech Stand**					
	Fine soil kg ha^{-1}	C	N	C/N g·g^{-1}	C %	N %	Fine soil kg ha^{-1}	C	N	C/N g·g^{-1}	C %	N %
L	36,505	16,026	586	27.3	43.2 (0.41)	1.59 (0.08)	28,940	13,135	509	25.8	45.8 (1.85)	1.79 (0.09)
FH	95,838	21,386	916	23.3	31.6 (3.30)	1.35 (0.18)	142,565	52,225	2627	19.9	36.7 (4.30)	1.84 (0.16)
0–10	540,305	36,817	1325	27.8	6.7 (1.74)	0.24 (0.04)	467,559	47,127	2035	23.2	10.0 (2.22)	0.43 (0.09)
10–20	508,009	15,707	638	24.6	3.1 (0.45)	0.13 (0.01)	533,821	34,929	1378	25.3	6.5 (0.36)	0.26 (0.02)
20–40	1,031,098	23,653	999	23.7	2.2 (0.48)	0.09 (0.02)	1,022,629	51,265	1999	25.7	5.1 (0.51)	0.2 (0.02)
Total	2,211,754	113,590	4464	25.4			2,195,514	198,681	8547	23.2		

The soil nitrogen concentration was significantly higher in all horizons in the beech soil compared to spruce soil ($p = 0.016$ for the L horizon, $p = 0.006$ for the FH horizon, $p = 0.01$ for 0–10 cm and $p < 0.001$ for 10–40 cm). The total N pool accounted for 4460 kg·ha^{-1} in the spruce stand and 8550 kg·ha^{-1} in the beech stand. The nitrogen pool in the LFH horizon accounted for 34% of the total soil pool in the spruce stand and 37% in the beech stand. Lower soil C/N was measured in the beech stand, except for the mineral soil from 10 cm to 40 cm.

3.2. Carbon and Nitrogen Fluxes

3.2.1. Water, Carbon and Nitrogen Fluxes in Precipitation and Soil Leachate

The average site precipitation depth was 1092 mm·year^{-1} between 2013 and 2015. Interception was 331 mm·year^{-1} in the spruce forest and 194 mm·year^{-1} in the beech forest. Evaporation from soil and plant transpiration was higher in the beech stand (584 mm) compared to the spruce stand (450 mm). Thus, total evapotranspiration (ET, including interception) was comparable among stands.

Precipitation input of DOC in bulk deposition was 21 kg·ha^{-1}·year^{-1} over the period 2013–2015. A significantly ($p < 0.001$) higher concentration of throughfall DOC was measured in the spruce forest compared to the beech forest, representing a flux of 80 kg·ha^{-1}·year^{-1} in the spruce forest and 36 kg·ha^{-1}·year^{-1} in the beech forest. In the beech stand, additional input of DOC via stemflow accounted for 11 kg·ha^{-1}·year^{-1}. Throughfall DOC flux was 1.7× higher in the spruce stand compared to the beech stand. Total nitrogen bulk deposition was 11.1 kg·ha^{-1}·year^{-1}, of which DON accounted for 14%, N-NH_4 for 54% and N-NO_3 for 32%. Only DON concentration in throughfall was significantly ($p = 0.003$) higher in the spruce stand compared to the beech stand. Inorganic N throughfall concentrations did not differ significantly between forests. Spruce throughfall total N

flux was 17.6 kg·ha^{-1}·year^{-1} and DON accounted for 23% of throughfall N flux. Beech total N throughfall flux was 16.3 kg·ha^{-1}·year^{-1} and DON accounted for 9%. Beech stemflow of total N was 1.9 kg·ha^{-1}·year^{-1}. Total N flux in the soil surface of the beech forest was thus 18.2 kg·ha^{-1}·year^{-1} over the period 2013–2015 (Table 4).

Table 4. Fluxes (±standard deviation) of water (mm), DOC and N (kg·ha^{-1}·year^{-1}) in bulk deposition, throughfall and beech stemflow for the period 2013–2015.

2013–2015	H_2O	DOC	DON	N-NH_4	N-NO_3
	mm		kg ha^{-1} year^{-1}		
Bulk deposition	1092 ± 198	21 ± 5.0	1.5 ± 0.6	6.0 ± 1.7	3.6 ± 0.7
Spruce					
Throughfall	761 ± 181	80 ± 13	4.1 ± 0.3	6.7 ± 0.5	6.8 ± 0.4
Beech					
Throughfall	750 ± 104	36 ± 5.0	1.5 ± 0.9	8.4 ± 2.3	6.4 ± 0.8
Stemflow	148 ± 31	11 ± 1.5	0.6 ± 0.2	0.8 ± 0.1	0.5 ± 0.1

The calculated seepage flux of water under the forest floor was higher for the beech stand (443 ± 61 mm) compared to the spruce stand (336 ± 96 mm). Despite significantly ($p < 0.001$) higher DOC concentrations in the forest floor soil water in the spruce stand (53 ± 16 mg·L^{-1}) compared to the beech stand (40 ± 7.6 mg·L^{-1}), the resulting average annual fluxes of DOC were similar. Similar seepage fluxes of water in 30 cm and non-significant ($p = 0.22$) differences in DOC concentrations (7.1 ± 2.4 mg·L^{-1} in spruce and 5.4 ± 1.2 mg·L^{-1} in beech) resulted in comparable DOC leaching at the 30 cm depth (Table 5).

Table 5. Fluxes (±standard deviation) of water (mm), DOC, N (kg·ha^{-1}·year^{-1}) and mass ratio of DOC/DON in seepage water from the forest floor and mineral soil at the depth of 30 cm for the period 2013–2015.

2013–2015	H_2O	DOC	DON	N-NH_4	N-NO_3	DOC/DON
	mm		kg ha^{-1} year^{-1}			g·g^{-1}
Spruce stand						
LFH	336 ± 96	179 ± 59	5.6 ± 1.7	0.34 ± 0.13	0.58 ± 0.18	32 ± 0.59
30 cm	311 ± 103	23 ± 10	0.83 ± 0.18	0.10 ± 0.05	0.12 ± 0.13	28 ± 6.7
Beech stand						
LFH	443 ± 51	182 ± 3.1	5.2 ± 0.49	0.18 ± 0.05	1.1 ± 0.37	36 ± 3.5
30 cm	314 ± 44	18 ± 5.0	0.63 ± 0.16	0.70 ± 1.1	1.2 ± 1.0	28 ± 1.5

The concentration of DON in the spruce forest floor (1.64 ± 0.55 mg·L^{-1}) was significantly ($p < 0.001$) higher compared to the beech forest floor (1.17 ± 0.25 mg·L^{-1}). However, forest floor DON fluxes were comparable among stands. DON fluxes dominated the total N flux in the forest floor of both stands. No significant differences ($p = 0.30$) were measured in NO_3^- concentrations in forest floor soil solution among stands (0.97 ± 1.28 mg·L^{-1} in spruce and 0.88 ± 1.19 mg·L^{-1} in beech). Total N loss from the forest floor was similar (6.5 kg·ha^{-1}·year^{-1}) in both stands. In mineral soil, at a depth of 30 cm, DON concentration was similar in both stands (0.30 ± 0.25 mg·L^{-1} in spruce and 0.20 ± 0.10 mg·L^{-1} in beech). Although DON dominated total mineral soil N flux in the spruce stand, NO_3 was the dominant form of N leaving the mineral soil in the beech stand. A large and significant difference ($p < 0.001$) in NO_3^- concentration was measured between stands (0.12 ± 0.12 mg·L^{-1} in spruce and 1.28 ± 1.40 mg·L^{-1} in beech). DOC/DON ratios were not statistically different between stands for either forest floor or mineral soil (Table 5).

3.2.2. Soil Respiration

Soil efflux (F_s) ranged between 0.95 and 7.3 g·m^{-2}·day^{-1} of C in the spruce stand (average of 3.65 ± 1.67 g·m^{-2}·day^{-1}) and between 0.59 and 8.5 g·m^{-2}·day^{-1} of C in the beech stand (average of 3.69 ± 1.87 g·m^{-2}·day^{-1}). Therefore, no significant differences ($p = 0.81$) in F_s were measured among stands. Strong seasonal variations in F_s were measured, revealing high temperature sensitivity. Soil temperature explained 78% of F_s variability in the spruce stand and 72% in the beech stand (Figure 1). Higher temperature sensitivity of F_s was documented in the beech forest ($Q_{10} = 4.2$) compared to the spruce stand ($Q_{10} = 3.7$). However, R_{10} and R_0 were similar or slightly higher in the spruce stand (Table 6). Cumulative F_s was 9869 ± 598 kg·ha^{-1}·year^{-1} of C in the spruce stand compared to 9091 ± 573 kg·ha^{-1}·year^{-1} of C in the beech stand for the period 2013–2015.

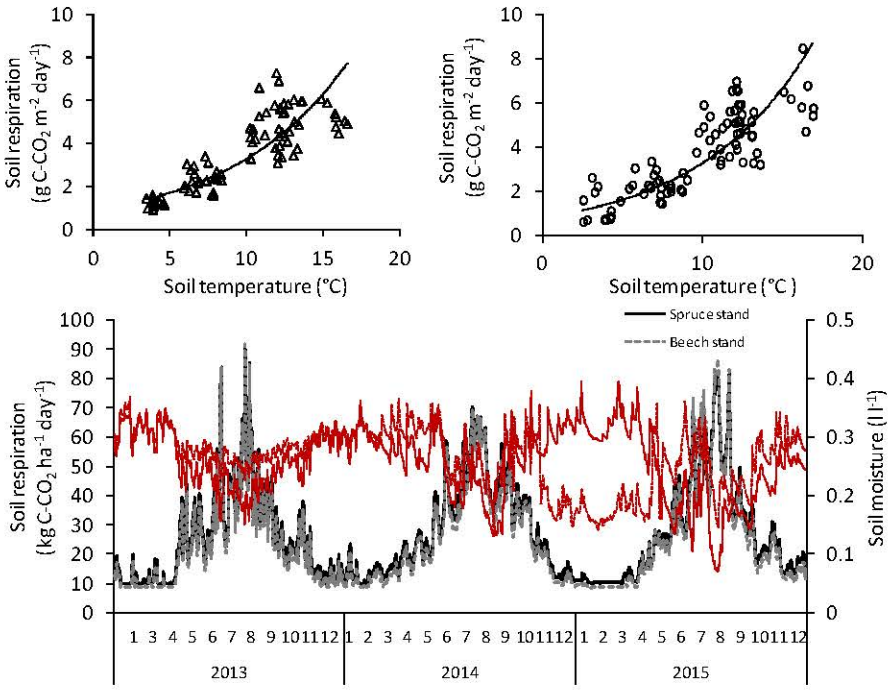


Figure 1. Measured soil efflux (g·m^{-2}·day^{-1}) of C and corresponding soil temperature (°C) in spruce (left) and beech (right) stands, and (lower panel) modelled daily soil respiration (kg·ha^{-1}·day^{-1}) in spruce (black solid line) and beech (grey dashed line) stands for the period 2013–2015. Soil moisture under the forest floor was measured in the spruce stand (solid red line) and in the beech stand (dashed red line) for the period 2013–2015.

Table 6. Stand-specific exponential relationships between soil temperature (°C) and soil respiration (g C·m^{-2}·day^{-1}). Intercept (α), exponential coefficient (β), Q_{10}, R_{10}, r^2, number of measurements (n) and p value.

	Model	$F_s = \alpha^* e^{\beta T}$					
	α	β	Q_{10}	R_{10}	r^2	n	p
Spruce stand	0.89	0.130	3.7	3.3	0.78	84	<0.001
Beech stand	0.78	0.143	4.2	3.2	0.72	84	<0.001

3.2.3. Carbon Allocation and Partitioning in Forest Ecosystems

Carbon allocation of GPP into ANPP was slightly higher in the beech stand (31% of GPP) compared to the spruce stand (27%). However, in the beech stand, allocation to foliage biomass dominated over the woody biomass (Table 7). Litterfall production was higher in the beech stand and 68% of

annual litterfall was comprised of leaves. In the spruce stand, lower litterfall production was measured compared to beech, and needlefall accounted for 78% of total litterfall flux (Table 8). Calculated TBCF was higher in the spruce stand compared to the beech stand and accounted for 48% of GPP in the spruce stand and 38% of GPP in the beech stand. Estimated BNPP of coarse roots (based on allometric equations) and BNPP of fine roots (14% of NPP, [44]) were used to estimate the total autotrophic respiration [45]. Similarly, assuming that above-ground autotrophic respiration can be estimated from ANPP [45], then aboveground respiration accounted for 26% of GPP in the spruce forest and for 30% GPP in the beech stand. Root respiration accounted for 28% of GPP in the spruce stand and for 30% GPP in the beech stand. Thus, total GPP allocated to autotrophic respiration was similar (53%) in both forests. Estimated NPP was higher in the spruce stand compared to the beech stand. Aboveground net primary production accounted for 57% of NPP in the spruce stand and for 68% in the beech stand. Estimated BNPP:TBCA was similar in both stands (0.42 in spruce and 0.39 in beech). The proportion of BNPP of fine roots of TBCA was 0.14 in spruce and 0.17 in beech. Similarly, BNPP of root exudates and mycorrhizae accounted for 13% and 16% of the respective TBCA values. WUE was higher in the spruce stand (2.6 g·C·kg^{-1} H_2O) compared to the beech stand (2.2 g C·kg^{-1} H_2O) (Table 7).

Table 7. Carbon allocation in spruce and beech stands (kg C·ha^{-1}·year^{-1}).

	ANPP Wood	ANPP Foliage	ANPP Litter	BNPP Coarse Root	ANPP	TBCA	F_s	R_{above}	R_{root}	R_{total}	GPP	BNPP Fine Root	BNPP ex/mycc	NPP	WUE
	kg C ha^{-1} year^{-1}														g C kg^{-1} H_2O
Spruce stand	3495	230	1650	1382	5375	9600	9869	5160	5602	10,763	20,136	1335	1281	9373	2.6
Beech stand	2502	18	2882	370	5401	6579	9091	5185	3999	9184	17,165	1135	1075	7980	2.2

ANPP (aboveground net primary production) and BNPP (belowground net primary production) of the coarse roots were derived according to biomass equations. Total belowground carbon allocation (TBCA) equals soil efflux (F_s) − ANPP litter + BNPP coarse roots, BNPP of the fine roots was estimated as a 0.14 fraction of NPP following [44], total autotrophic respiration (R_{total}) was calculated as 1.33 × (ANPP + BNPP root) and aboveground autotrophic respiration (R_{above}) as 0.96 × ANPP following [45]. BNPP ex/mycc accounted for C root exudates and mycorrhizae. Gross primary production is the sum of ANPP, TBCF and R_{above}. Net primary productivity (NPP) equals GPP − R_{total}. WUE (water use efficiency) was calculated as the ratio of GPP and evapo-transpiration (precipitation − seepage at 30 cm depth).

Table 8. Biomass (dry weight), carbon and nitrogen fluxes (kg·ha^{-1}·year^{-1}) and respective mass C:N ratios in individual litterfall compartments in spruce and beech forests.

2014–2015	DW	C	N	C/N
	kg ha^{-1} year^{-1}			g·g^{-1}
Spruce litterfall				
Needles	2589	1295	31	42
Twigs	312	148	4.3	35
Fruits	438	208	3.7	56
Spruce total	3338	1650	39	42
Beech litterfall				
Leaves	4104	1946	37	52
Twigs	554	263	4.2	62
Fruits	1415	672	15	46
Beech total	6074	2882	56	52

3.2.4. Nitrogen Fluxes

The annual requirement of N to sustain ANPP (N_{req} above) was calculated as 60 kg·ha^{-1}·year^{-1} for the spruce stand and 127 kg·ha^{-1}·year^{-1} for the beech stand. Based on allometric equations, higher annual uptake of N to support coarse root growth (N_{req} coarse roots) was calculated for the spruce compared to the beech stand. Fine root growth, estimated as 14% of NPP (McCormack et al., 2015), required 28 kg·ha^{-1}·year^{-1} of N in the spruce and 23 kg·ha^{-1}·year^{-1} of N in the beech stand (Table 9).

Annual litterfall delivered on average 39 kg·ha^{-1}·year^{-1} of N to the forest floor in the spruce stand and 56 kg·ha^{-1}·year^{-1} of N in the beech stand (Table 8). Needlefall N consisted of 80% of total litterfall in the spruce stand, and leaf fall consisted of 73% of total litterfall in the beech stand. The average C/N ratio of litterfall was lower in spruce compared to beech (Table 8). Nitrogen requirements to build up foliage in the beech stand (116 kg·ha^{-1}·year^{-1}) were significantly higher than the annual litterfall N flux; thus, N translocated back to the tree (N_{trans}) from foliage tissues accounted for 60 kg·ha^{-1}·year^{-1}. In the spruce stand, only 5.7 kg·ha^{-1}·year^{-1} of N was translocated during senescence. Therefore, net N uptake was calculated as 71 kg·ha^{-1}·year^{-1} for both forest ecosystem types. Litterfall and throughfall N fluxes provided 57 kg·ha^{-1}·year^{-1} of N, which is 14 kg·ha^{-1}·year^{-1} less than the annual N needs calculated for the spruce stand. On the other hand, a roughly balanced N cycle was calculated for the beech forest (Table 9).

Table 9. Nitrogen allocation in spruce and beech stands (kg N·ha^{-1}·year^{-1}). Annual nitrogen requirements (N_{req}) of the wood and coarse roots were derived according to biomass equations. N_{req} of the fine roots was estimated as a 0.14 fraction of NPP following [44]. Annual N translocation (N_{trans}) is the difference between foliage N_{req} and annual N litter production. Net N uptake (N_{uptake}) was calculated as the difference between the sum of N_{req} of aboveground and coarse root production and N_{trans}. Total flux of N to the forest floor (N_{insoil}) consisted of litterfall N and throughfall N fluxes.

	N_{req} Wood	N_{req} Foliage	N_{req} abovegr	N_{req} Coarse Roots	N_{req} Fine Roots	N_{req} Belowgr	N_{trans}	N_{uptake}	N_{insoil}
	kg ha^{-1} year^{-1}								
Spruce stand	15	45	60	17	28	45	5.7	71	57
Beech stand	12	116	127	3.5	23	26	60	71	74

4. Discussion

Both forest stands have been altered by different forest management strategies since the 1850s and by impacts of acid deposition during the 20th century. Former mixed forests were converted into pure spruce monocultures, with current second rotation of Norway spruce in the spruce stand. The beech stand was transformed by acid deposition to broadleaf monoculture during the 1970s and 1980s by induced selective conifer dieback. It is very likely that in the 1850s, similar site conditions prevailed at both stands. Since then, significantly higher soil C and N stocks have been measured in the beech forest compared to the spruce forest. While standing biomass was higher in the beech stand, due to the relatively high tree density combined with the high wood biomass of the 150 year old beech forest, the biomass N stocks were comparable among stands, resulting in a lower C/N ratio in the spruce stand compared to the beech stand.

Although estimated historical N inputs were higher in the spruce stand due to higher pollutant scavenging by spruce needles [47], current deposition fluxes of N are similar in both forests. On the contrary, DOC throughfall fluxes were higher in the spruce stand. Few differences were measured in the DOC soil solution fluxes in the soil profiles. Nitrogen fluxes were dominated by DON at both stands, with higher NO_3^- losses in the beech stand. Soil respiration dynamics were similar for both forest sites, with slightly higher Q_{10} in the beech stand compared to the spruce stand. As a result, annual soil respiration was similar at both sites. Higher litterfall C flux in the beech forest caused lower total belowground carbon allocation (TBCA) compared to the spruce stand. The estimated fraction of TBCA that accounted for belowground net primary productivity (BNPP) was similar in both forest ecosystems. Due to the lower TBCA, both gross primary productivity (GPP) and net primary productivity (NPP) were lower in the beech stand compared to the spruce stand. Due to the similar evapotranspiration (ET) in both stands, water use efficiency (WUE) was higher in the spruce stand compared to the beech stand. Higher N fluxes were measured in the beech stand compared to the spruce stand, together with higher susceptibility of inorganic N losses in the beech stand compared to the spruce stand.

4.1. Tree Species Effects on C Cycling

Annual litterfall was higher in the beech stand compared to the spruce stand. Spruce litterfall flux was comparable to other studies on temperate forests of similar age, whereas beech litterfall was higher at our investigated stand [48,49]. The relatively high measured litter flux in the beech stand was a result of high crown area and dimensions of beech trees in the plot. Litterfall is an important component of forest ecosystem NPP and nutrient cycling [50]; thus, it is highly important for forest management strategies to sequester C [51]. Our results suggest that old growth broadleaf forest could deliver a substantial amount of assimilated C to the forest floor compared with lower inputs in managed forests.

Surprisingly similar fluxes of the DOC from the forest floor were measured in the two forest stands. DOC flux represented 11% of annual litterfall flux in the spruce stand and 6% of annual litterfall flux in the beech stand. This can be interpreted as showing that spruce litter is an important source of soil solution DOC [52]. As for the forest floor, similar seepage fluxes of DOC were observed in the mineral soil. A significant reduction of DOC flux occurred from top soil to 40 cm depth, by roughly 80%. Whether it is absorbed in the mineral soil or respired needs further research. However, despite comparable fine soil pools in both forest floor and mineral soil, significantly higher C concentration in deeper mineral soil (20–40 cm) caused a significantly higher soil C pool in the beech stand compared to the spruce stand. As we did not observe significantly higher DOC flux from decaying litter in the beech stand compare to the spruce stand, we suggest that higher soil C pool under the beech forest was a result of long-term accumulation of recalcitrant SOM that originated from root and mycorrhizal decomposition [15,16]. Simultaneously, higher contents of oxalate-extractable Fe and Al were found in the beech mineral soil [53], suggesting higher sorption capacity in this stand, favouring C sequestration in the beech stand over the spruce stand. Contrary to previous paired studies (e.g., [13]), we did find a higher C stock in the forest floor of the beech stand compared to the spruce stand. In an earlier study, we documented partial loss of forest floor C in the spruce stand as a result of recovery from acidification [54]. In the early 1990s, forest floor C mass was 58 $t \cdot ha^{-1}$, comparable to the current beech stand, but it has since declined by 47%. We do not know whether a similar proportional decline occurred at the beech stand due to the lack of available historical data. We can only suggest that relatively high N availability at the beech stand (low soil C/N, some inorganic N leaching) may have stimulated greater stabilization into humus through a combination of chemical reactions and enzyme inhibition [55,56], thus preventing SOM loss from the forest floor after acidification retreat.

Soil temperature explained 78% and 72% of temporal variation in soil respiration in the spruce and beech stands, respectively. Soil moisture did not provide additional explanatory power in multiple regression analysis, suggesting low moisture stress in our mountainous forest ecosystems. Q_{10} was slightly higher in the beech stand (4.2) compared to the spruce stand (3.7), but both values were close to the Q_{10} values reported for other temperate ecosystems [23,41,57]. Based on our calculations, potential respiration at 0 °C was higher in the spruce (0.89 $g \cdot m^{-2} \cdot day^{-1}$ of C) than in the beech stand (0.78 $g \cdot m^{-2} \cdot day^{-1}$ of C) and similar at 10 °C. Thus, annual CO_2 soil efflux was higher in the spruce stand compared to the beech stand, due to the calculated higher soil respiration in winter and spring periods.

One of the assumptions when calculating TBCA is that the total soil C pool is in a steady state. This assumption might be violated, given the significant decline of forest floor C in the spruce stand noted above [54]. It is difficult to predict whether the spruce soil is currently in a steady state; thus, further calculation might be biased by the annual net loss of C from soil. Moreover, comparison with published relationships between soil respiration and litterfall presented in [42,58] suggested higher soil CO_2 efflux compared to annual C in litterfall in the spruce stand. The soil respiration rate of the beech stand is in line with similar published global measurements. Based on our data, 42% and 39% of TBCA goes to the BNPP in the spruce and beech stand, respectively. This is less than published results from the Rocky Mountains by McDowell and others [59], but within the range of BNPP:TBCA ratios (0.26–0.53) published by Litton and Giardina [60]. Based on our calculation, 27% and 34% of TBCA (spruce and beech, respectively) goes to the fine roots, mycorrhizal production and root exudates. Our

estimates of fine root production and root respiration are based on literature review data [44,45] with the same partitioning of NPP applied to both stands. Thus, the presented results are more or less indicative. Nevertheless, our estimates are close to a published review by Nadelhoffer and Raich [61], who estimated that approx. 33% of TBCA goes to fine root production.

Calculated ANPP and BNPP were used to calculate autotrophic respiration of both belowground and aboveground components [45] to constrain GPP of both stands. Despite similar ANPP, lower BNPP at the beech stand caused lower GPP compared to the spruce stand. However, both GPP and NPP were lower in the old beech forest ecosystem compared to the younger spruce forest, suggesting continuous decline of both GPP and R_{total} in aging forests [62,63]. Similar evapotranspiration (ET) for both stands and lower GPP for the beech stand resulted in lower WUE for the beech stand compared to the spruce stand.

4.2. Tree Species Effects on N Cycling

In the spruce throughfall, higher DON and lower NH_4 fluxes were measured compared to beech throughfall. Our results are in line with the study of Kopacek and others [64], showing the importance of the Norway spruce canopy in chemical N transformation of precipitation. On average, beech stemflow accounted for roughly 10% of total N input to the forest floor by precipitation. Despite similar current N throughfall fluxes, estimated historical N deposition to the spruce forest was higher compared with the beech stand [32], suggesting higher cumulative N deposition flux in the spruce plantation compared to the beech forest. Litterfall N flux was higher in the beech stand compared to the spruce stand; however, a lower C/N litterfall ratio was measured in the spruce litter (42) compared to beech litter (52). Combined precipitation and litterfall fluxes yielded higher N flux in the forest floor in the beech stand compared to the spruce stand. Dissolved losses of N from the forest floor accounted for 16% of annual N litterfall in both stands. Forest floor N losses were dominated by DON, with higher inorganic N leaching (mainly as NO_3^-) in the beech stand compared to the spruce stand. Nevertheless, long-term measurements at the spruce stand revealed cessation of NO_3^- leaching following reduction of acid deposition [33]. Currently, N seepage losses in the 30 cm soil depth account for ca. 10% and 23% of precipitation inputs in the spruce and beech forests, respectively.

Annual forest requirements to build up canopy foliage accounted for 45 $kg \cdot ha^{-1} \cdot year^{-1}$ of N in the spruce stand and for 116 $kg \cdot ha^{-1} \cdot year^{-1}$ of N in the beech stand. Those values are, with respect to the beech stand, much higher than annual N recycling through aboveground litter production and precipitation deposition. Thus, retranslocation of N from senescing foliage is probably crucial for N retention in the beech stand. We may hypothesise that retranslocation of N before the onset of litterfall prevents the ecosystem from leaching higher amounts of this limiting nutrient, as enhanced SOM mineralization and nitrification in the period of low plant N demand can attribute to the mineral N losses from the ecosystem. Calculated N_{uptake} from soil was, due to N translocation, similar in both forest stands. Moreover, calculated potential N soil requirements were higher compared to N inputs via throughfall and litterfall in the spruce stand and comparable in the beech stand. This suggests more tightly constrained N cycling in the spruce stand compared to the beech stand. Higher susceptibility of the beech stand to inorganic N soil leaching could be due to this less constrained N cycling. However, high gross N mineralization and nitrification rates in old growth forests [65,66] can lead to N losses when availability of labile C declines. Thus, N leaching in an old growth beech stand can be a result of C limitation coupled with a higher N availability due to the higher total N return in litter [50].

5. Conclusions

Forest management activities and the history of acid deposition induced considerable changes in dominant tree species distribution in the study area. We compared second generation of Norway spruce plantation with extensively managed European beech forest in terms of C and N pools and fluxes. Significantly higher C and N soil pools were measured in the beech stand suggesting higher sorption capacity in this stand, favouring long-term C sequestration in the beech stand over the spruce

stand. Soil respiration dynamics were similar for both forest sites, with slightly higher Q_{10} in the beech stand compared to the spruce stand. However, similar fluxes of soil water DOC were measured among both stands. Higher N fluxes were measured in the beech stand compared to the spruce stand, together with higher susceptibility of inorganic N losses in the beech stand compared to the spruce stand. Calculated whole ecosystem C and N budgets serve important input data for ecosystem models, which often aims to quantify long-term consequences of land use changes on landscape C and N retention.

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9.3 Publication III

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Comparison of the impacts of acid and nitrogen additions on carbon fluxes in European conifer and broadleaf forests☆

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ABSTRACT

Increased reactive nitrogen (N) loadings to terrestrial ecosystems are believed to have positive effects on ecosystem carbon (C) sequestration. Global "hot spots" of N deposition are often associated with currently or formerly high deposition of sulphur (S); C fluxes in these regions might therefore not be responding solely to N loading, and could be undergoing transient change as S inputs change. In a four-year, two-forest stand (mature Norway spruce and European beech) replicated field experiment involving acidity manipulation (sulphuric acid addition), N addition (NH_4NO_3) and combined treatments, we tested the extent to which altered soil solution acidity or/and soil N availability affected the concentration of soil dissolved organic carbon (DOC), soil respiration (Rs), microbial community characteristics (respiration, biomass, fungi and bacteria abundances) and enzyme activity. We demonstrated a large and consistent suppression of soil water DOC concentration driven by chemical changes associated with increased hydrogen ion concentrations under acid treatments, independent of forest type. Soil respiration was suppressed by sulphuric acid addition in the spruce forest, accompanied by reduced microbial biomass, increased fungal:bacterial ratios and increased C to N enzyme ratios. We did not observe equivalent effects of sulphuric acid treatments on Rs in the beech forest, where microbial activity appeared to be more tightly linked to N acquisition. The only changes in C cycling following N addition were increased C to N enzyme ratios, with no impact on C fluxes (either Rs or DOC). We conclude that C accumulation previously attributed solely to N deposition could be partly attributable to their simultaneous acidification.

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1. Introduction

Human activities have been altering element cycling for several thousand years (Ruddiman, 2003; Shotyk, 1998). Since the Industrial Revolution, emissions of greenhouse gases, nitrogen (N) and sulphur (S) have been increasing (Kopáček and Posch, 2011; Lamarque et al., 2013) and their negative impacts on ecosystems led to the implementation of various international agreements to abate these emissions (United Nations Framework Convention on Climate Change, Convention on Long-range Transboundary Air Pollution, Kyoto Protocol, Paris Agreement). Besides exerting significant negative pressures on global biodiversity (Butchart et al., 2010), atmospheric pollutant deposition has impacted natural biogeochemical cycles of terrestrial ecosystems, including the ecosystem C balance (Hyvönen et al., 2007; Lamersdorf and Borken, 2004; Thomas et al., 2013).

Nitrogen limitation is an inherent feature of many temperate terrestrial ecosystems, thus fertilization by atmospheric N deposition may have increased C pool in biomass and soils of terrestrial ecosystems and thus stimulate the terrestrial CO_2 sink (de Vries et al., 2009). Increased N availability may affect terrestrial C balances via increased net primary productivity (NPP) and/or decreased litter decomposition rates (Janssens et al., 2010; Liu and

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Greaver, 2010). The potential of a negative effect of increased N availability on decomposition rates was suggested by many experimental N addition studies (e.g. Berg and Matzner, 1997; Olsson et al., 2005; Hobbie, 2008). Stimulation of litter decomposition is expected at sites with low ambient N deposition and high-quality litter (low lignin), whereas suppression of litter decomposition is suggested at sites with higher ambient N deposition or at sites with low quality (high lignin) litter (Knorr et al., 2005). Despite current studies have focused on the impact of N deposition on C balance, there is clear evidence that acidification (either from S or N deposition) may also affects a range of key processes, including slowdown of litter decomposition (Chen et al., 2015; Liang et al., 2013; Oulehle et al., 2011), as well as mobility and leaching of dissolved organic carbon (Evans et al., 2012). Suppression of litter decomposition at low pH may occur through reduction in the availability of C for microbial community (Persson et al., 1989) and/or by direct effect on the microorganisms themselves (Błońska et al., 2017; 2016; Pennanen et al., 1998; Wei et al., 2013). Input of N to the soil, either from deposition or fertilization, can have an acidifying effect, depending on the fate (immobilization vs. leaching, nitrification) of mineral N in the soil. Thus, it is likely that experimentally induced responses to N fertilization might in fact be due to changes in soil acid-base status (Evans et al., 2008; Chen et al., 2016). A better, integrated understanding of soil C and N responses to changing inputs of both S and N is needed to predict whether soils will in future act as C sinks or C sources as atmospheric pollutant loadings change. Accounting for, that much of Europe and North America have experienced high historic depositions of S, and receive high N inputs, whilst other regions including large parts of Asia continue to receive high depositions of both S and N (Duan et al., 2016; Larssen et al., 2006; Posch et al., 2015), it is likely that recent measurements of C fluxes from these regions might not be in long-term balance with climatic conditions, but represent ecosystems subjected to multiple anthropogenic pressures, and in many cases undergoing transient change as deposition levels decline or rise.

Here, we present a set of replicated acidity/N availability manipulation experiments in adjacent mature broadleaved (beech forest) and coniferous (spruce forest) sites. Spruce and beech represent the dominant tree species in the Central European forests. Both stands are influenced by similar abiotic conditions (climate, deposition, bedrock, original soil type) but differ in vegetation cover as a result of long-term differences in land-use. Furthermore, long-term ambient monitoring from spruce site provided evidence of C soil loss as a consequence of acidification recovery (Oulehle et al., 2011) and both sites were intensively studied to assess the rates of acidification recovery in formerly highly polluted areas (Oulehle et al., 2006). We examined whether there has been a shift in the ecosystem C fluxes in either (dissolved) organic or (gaseous) inorganic forms, as a function of altered acidity and N availability. *In situ* decomposition experiments and measurements of microbial characteristics and enzyme activity provided further insights into C metabolism under changing acidity and N availability.

2. Material and methods

2.1. Sites and experimental design

In 2013 two parallel acidity and N availability manipulation experiments were established, at two adjacent forests (50.59 N, 13.26 E) selected to represent major European forest types – mature Norway spruce forest (*Picea abies* (L.) Karst.) and mature European beech forest (*Fagus sylvatica* L.). The spruce stand consists of Norway spruce monoculture planted in the early 1930s, and the beech stand consists of European beech monoculture (Oulehle et al., 2016). The distance between stands is approximately 1 km and both stands are underlined by gneiss bedrock. The dominant soil type is dystric cambisol (Dambrine et al., 1993). Soils at both sites were acidified by acid deposition during the 20th century, which resulted in low soil pH and low base saturation. Despite similar bulk S deposition, total deposition is higher in the spruce stand compared to the beech stand. Throughfall fluxes of inorganic nitrogen are similar in both stands (Table 1; Oulehle et al., 2011, 2016). Ground cover vegetation in the spruce stand (60%) is formed mainly by *Avenella flexuosa* L. Drejer with admixture of *Vaccinium myrtillus* L., *Dryopteris dilatata* (Hoffm.) A. Gray, *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Galium saxatile* L. The ground cover vegetation in the beech stand (1.5%) comprises mainly of *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Avenella flexuosa* L. Drejer.

At each forest stand, soil acidity and N availability were manipulated for 3 years by systematic addition of S and N treatment solutions. At each experimental site, sixteen 3 × 3 m plots were assigned to control (Ctrl), nitrogen (N), acid (S) and acid + nitrogen (S + N) treatments in a randomized blocked design, thus four replicates per treatment were available. In October 2012 plots were established (mineral soil lysimeter installation), and in April 2014 treatments began following a period of pre-treatment measurements (April 2013–March 2014). Treatments consists of monthly additions (April–November) of sulphuric acid (H_2SO_4) and ammonium nitrate (NH_4NO_3), mixed with rainwater collected at sites and applied using 15 l watering cans evenly across the plots, followed by addition of extra 15 l rainwater to properly seep in soil all added chemicals. The treatment dose was equivalent to 50 kg S ha $^{-1}$ year $^{-1}$, while the additional water addition was equivalent to 2.5% of average annual rainfall. Nitrogen treatments gave an input of 50 kg N ha $^{-1}$ year $^{-1}$. NH_4NO_3 addition, albeit ammonium is a weak acid, did not significantly alter pH of added solution (applied solution pH was ≈5.4).

2.2. Soil solution sampling and analysis

Every 3–5 weeks during the snow/ice free period (usually April–November) samples were taken, ideally after rainfall when soil moisture conditions permitted collection of soil water. In every case soil water was collected before treatment application. Soil water samples were collected using Rhizon® suction samplers (Rhizosphere Research Products, Wageningen, NL), comprising 10 cm long, 2.5 mm diameter porous membranes attached to 50 ml syringes. Four to six Rhizon samplers were inserted randomly into the forest floor of each plot, to give one composite sample per plot. In the mineral soil, Prenart Super quartz standard suction samplers (Prenart Equipment, Frederiksberg, DK) were inserted into 30 cm soil depth. Soil solution was collected by applying suction into a 2 l collecting bottle.

Samples were stored at 4 °C, and analysed for pH using Radiometer TTT-85 with a combination electrode, nitrate (NO_3^-) by high-performance liquid chromatography (Knauer 1000); ammonium (NH_4^+) was determined by indophenol blue colorimetry. DOC (dissolved organic carbon) was determined by a Tekmar-Dohrman Apollo 9000 analyzer (Tekmar Dohrmann, OH, USA). Samples for DOC determination were filtered (0.4 μm glass fiber Macherey-Nagel GF 5) before analysis.

2.3. Soil CO_2 flux measurements

Soil CO_2 efflux (Rs) was measured monthly between 2013 and 2016 during the snow-free period (April–November/December) using a LiCor infrared gas analyzer LI-8100A (LiCor Biosciences, NE, USA) attached to a LiCor Survey Chamber (8100–103 20 cm survey

Table 1
Stand characteristics, soil properties (BS – base saturation), biomass properties (net primary productivity – NPP) and measured sulphur (S), dissolved inorganic nitrogen (DIN) deposition and dissolved organic carbon (DOC) and nitrogen (DON) leaching. Data from Oulehle et al. (2016).

	Site description					Soil (Forest floor)				
	Latitude	Longitude	Temperature	Precipitation	Forest age	pH	BS	C/N	C	N
			°C	mm	years		%	g g $^{-1}$	kg m $^{-2}$	
Spruce	50.591	13.253	6.5	1092	85	3.80	19	24.9	3.74	0.15
Beech	50.589	13.267			155	4.34	70	20.8	6.54	0.31

	Biomass				Ambient deposition 2013–2015				Soil leaching (Forest floor)	
	NPP	Litterfall	Litter C/N	Biomass C/N	Bulk S	Throughfall S	Bulk DIN	Throughfall DIN	DOC	DON
	kg C ha $^{-1}$ year $^{-1}$		g g $^{-1}$		kg ha $^{-1}$ year $^{-1}$				g m $^{-2}$	
Spruce	9373	1650	42	133	4.0	14	9.6	14	17.9	0.56
Beech	7980	2882	52	178		7.5		16	18.2	0.52

chamber; LiCor Biosciences, NE, USA). In each plot, four soil collars (287 cm^2) were placed into the forest floor to a 5 cm depth (64 soil collars in each forest stand) in March 2013. The CO_2 efflux was calculated based on a linear increase in chamber CO_2 concentration over time.

We used the van't Hoff equation (Davidson et al., 2006) to model the relationship between soil temperature and soil respiration:

$$Rs = \alpha e^{\beta T} \left(\text{where } Q_{10} = e^{\beta 10} \right) \quad (1)$$

where Rs is the *in situ* measured respiration rate, α and β are fitted parameters, T is *in situ* measured soil temperature and Q_{10} is the calculated temperature sensitivity of respiration. Rs was calculated on an hourly basis using on site measured soil temperatures and summed for the whole year to estimate an annual CO_2 efflux.

2.4. Microbial characteristics and DNA extraction

Soils for microbial characteristics were sampled every year in May. Microbial C (C_{mic}) and N (N_{mic}) were calculated as the difference between the chloroform fumigated and non-fumigated soil samples (sulphate extractable C and N), and amended using extraction efficiency factors of 0.38 for microbial C (Vance et al., 1987), and 0.54 for microbial N (Brookes et al., 1985). Microbial respiration was assessed as CO_2 production after one week incubation of soil in closed vials placed in the dark (10 g of fresh soil, 60% of water holding capacity, 15 °C), using gas chromatography (Agilent GC HP 6850, USA).

Into a FastPrep™ Lysis Matrix E tube (MP Biomedicals, Solon, OH, USA) approx. 0.5 g of soil was added. Then Hexadecyltrimethylammonium bromide (CTAB) extraction buffer, containing 5% CTAB (in 0.7 M NaCl, 120 mM potassium phosphate, pH 8.0) and 0.5 ml phenol-chloroform-isoamylalcohol (25:24:1), was added and agitated at speed 5–6 for 45s in a FastPrep Instrument (MP Biomedicals, Solon, OH, USA). Following bead beating, chloroform extracted samples were precipitated in a PEG 6000/1.6 M NaCl solution. 70% ethanol was used to wash pellets and re-suspended in molecular biology grade water. Quantus™ (Promega, USA) was used for fluorometrical quantification of total DNA.

Quantative PCR (qPCR) of bacterial and fungal genes of ribosomal small subunit rRNA (SSU rRNA) was performed using the FastStart SybrGREEN Roche® Supermix and Step One system (Life Technologies, USA) as described previously (Bárta et al., 2017; Gittel et al., 2014). R^2 values for the standard curves were >0.99. Slope values were -> 3.37 giving an estimated amplification efficiency of >93%.

Detection limits (i.e. lowest standard concentration that is significantly different from the non-template controls) were less than 100 gene copies for each of the genes per assay. All samples, standards and non-template controls were analysed in duplicates.

2.5. Extracellular enzyme activities

Extracellular enzyme activities were assessed in soils sampled in May and October each year. Microplate fluorometric assays under standard conditions were used to determine extracellular enzyme activities. All hydrolytic enzyme activities were determine in 1 g soil which was suspended in 100 ml of distilled water and for 4 min sonicated to allow disruption of the soil particles. Then 200 μL of the soil suspension was added into 50 μL methylumbelliferyl (MUF) of substrate solution for β-glucosidase (BG), exocellulase (cellobiohydrolase - CEL), phosphatase (PME) and N-acetyl-glucosaminidase (NAG) determination or to 50 μL of 7-aminomethyl-4-coumarin (AMC) substrate solution for leucine-aminopeptidase (LEU) determination (Bárta et al., 2014). 50, 100 and 300 μM concentrations of each fluorogenic substrate were tested and the one with the highest enzymatic activity was picked for further analysis. Plates were incubated for 120 min at 20 °C. Excitation wavelength of 365 nm and emission wavelength of 450 nm were used for fluorescence quantification using Infinite F200 microplate reader (TECAN, Germany). To assess enzyme activities stoichiometry we calculated C_{enz}/N_{enz}, C_{enz}/P_{enz} and N_{enz}/P_{enz} ratios, where C_{enz} = BG + CEL, N_{enz} = NAG + LEU and P_{enz} = PME (Sinsabaugh et al., 2009).

2.6. Data analysis

After testing the data for normality (Kolmogorov-Smirnov), skewness and homogeneity of variances using Bartlett's test, pH, DOC and soil respiration were analysed for the pre-treatment period (May 2013–April 2014) and for each full year of manipulation data (2014, 2015, 2016) using repeated measures ANOVA (treatment as a fixed factor) for each of the stands and for each time period. Fisher's LSD multiple comparison test was used at $p < 0.05$ to detect which treatments were different. Effects of forest stand, season (spring and autumn sampling - for enzyme activities only) and treatments on microbial biomass, fungi and bacteria abundances, fungi to bacteria ratios, enzymatic activity and enzyme stoichiometry were determined using a general linear model (GLM) for each time period. We refer only to the treatment effects in depicted figures. All data were analysed using statistical software NCSS v11.

3. Results

3.1. Soil solution chemistry

Pre-treatment soil water chemistry, in both forest floor and mineral soil, was similar among plots and no significant differences were detected in respect of soil water pH and DOC at either the spruce or beech stands (Fig. 1, Suppl. Fig. 1).

From the onset of treatment in April 2014, immediate and significant changes in forest floor soil water pH occurred at both stands. In acid treatments (S and S + N), soil water pH was reduced by 0.41–0.48 pH units at the spruce stand and 0.41–0.51 pH units at the beech stand (based on 2015–2016 means). Nitrogen addition alone did not induce any significant changes in soil water pH. Consistent reductions of mean annual DOC concentrations in response to pH manipulation were detected at both forest stands, although these were not significant at the beech stand. DOC concentrations were reduced in acid treatments by 30%–39% at the spruce stand, and by 45%–53% at the beech stand (based on 2015–2016 means). Nitrogen addition treatments did not lead to significant changes in forest floor soil water DOC concentrations (Fig. 1). Despite the alteration of soil water chemistry in the forest floor due to treatments, soil water chemistry in mineral soil (30 cm depth) revealed only limited changes of pH under acid treatments in the beech stand, and no significant changes in DOC concentrations in mineral soil water in either forest stand (Suppl. Fig. 1).

3.2. Soil respiration

Significant exponential relationships existed between soil respiration and soil temperature thus soil temperature exerted a strong control over soil CO_2 efflux, with coefficients of determination (R^2) ranging between 0.88 and 0.90 at the spruce stand and 0.79 and 0.83 at the beech stand (soil temperature ranged between 1.3 and 9.4 °C during the respiration measurements). Annual mean soil respiration in the spruce stand (3.30 μmol CO_2 m^{-2} s^{-1}) and beech stand (3.39 μmol CO_2 m^{-2} s^{-1}) did not significantly differ. Rather high variability of the soil CO_2 efflux among individual collars, combined with strong (seasonal) temperature dependence made it difficult to detect treatment effects on total soil respiration. Nevertheless, consistent reductions of mean annual soil respiration were observed under the S only treatment in the spruce stand in 2015 and 2016 ($p < 0.05$), by 6%–10%. A non-significant, though consistent, reduction of Rs under combined treatment was measured in spruce stand. No significant effect of N addition on soil respiration was detected in the spruce stand. In the beech stand, neither acidity nor N manipulations affected soil respiration (Fig. 2). Calculated mean annual C efflux was 9.9 t C ha^{-1} $year^{-1}$ in the spruce stand and 9.7 t C ha^{-1} $year^{-1}$ in the beech stand (based on 2013–2016 means for control plots; Eq. 1). Acid treatment in the spruce stand thus reduced C soil efflux by 0.53–0.95 t C ha^{-1} $year^{-1}$.

3.3. Microbial activity characteristics

Microbial biomass C, N and their ratios significantly ($P < 0.05$) differed among forest stands, with higher biomass C and N in the beech forest (324 μmol g^{-1} and 31 μmol g^{-1}, respectively) compared to the spruce forest (290 μmol g^{-1} and 23 μmol g^{-1}, respectively). Microbial C to N ratio was significantly lower in the beech stand (10.9) compared to the spruce stand (12.7) (Fig. 3). Microbial respiration did not significantly differ between stands on average. Microbial biomass C and N were negatively related to soil pH across both forests (Fig. 4), but in the spruce site acidic conditions led to higher respiration rates. No significant treatment effects were detected, although there was a tendency for microbial C and N to decrease under acid treatments (S, S + N) in the spruce forest, thus a shift toward higher microbial C/N was detected. Significantly ($P < 0.05$) higher fungal to bacterial ratios were measured in the spruce stand (0.045) compared to the beech stand (0.031), consistently with the higher microbial C to N ratios. Despite no significant treatment effects on fungi/bacteria ratio in the spruce stand (Fig. 5), significantly lower bacterial gene copies were measured under acid treatments (Suppl. Table 2). Therefore, consistent increases of fungi/bacteria ratio under acid treatments (Fig. 5) appear to be attributable to decreases in bacterial abundance rather than increases in fungi. In the beech stand, a significant treatment effect on fungi/bacteria ratio was detected in 2016 (Fig. 5). This resulted from

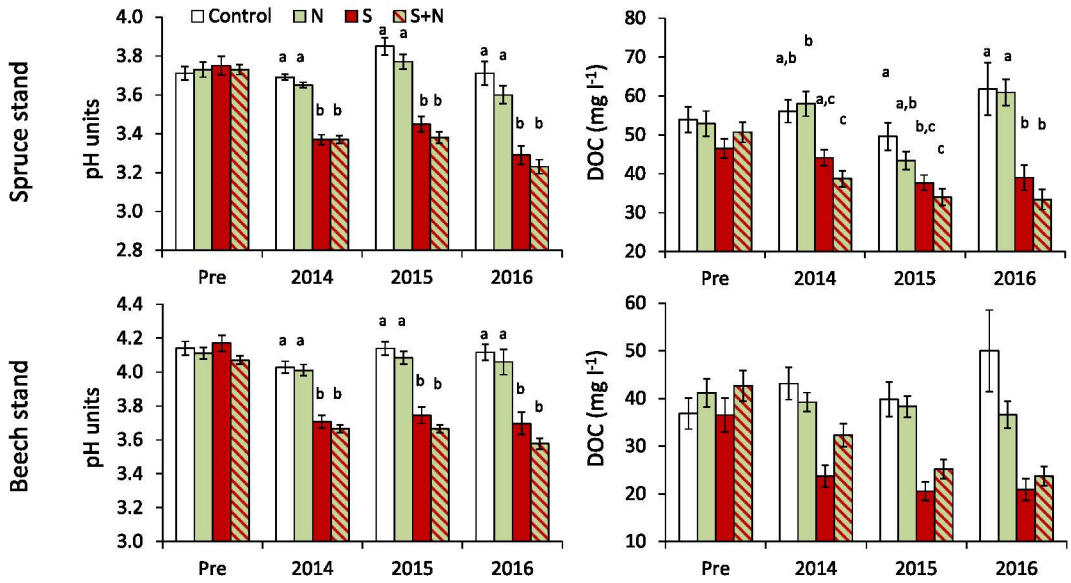


Fig. 1. Mean and standard error of forest floor soil water pH and DOC at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S + N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters ($P < 0.05$).

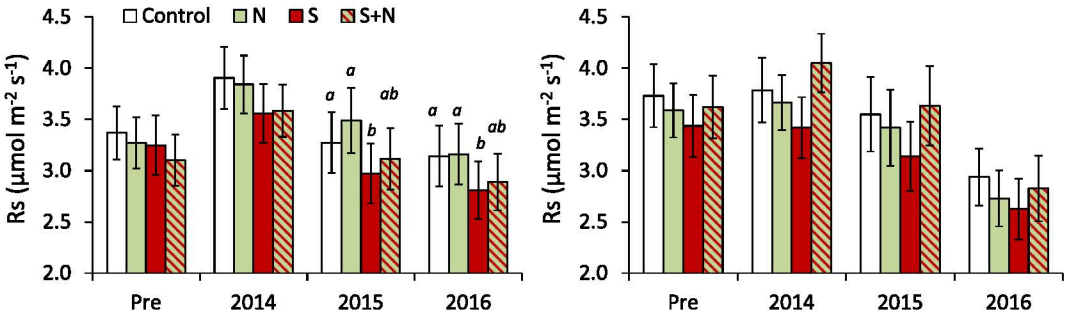


Fig. 2. Mean and standard error of soil respiration at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S + N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters ($P < 0.05$).

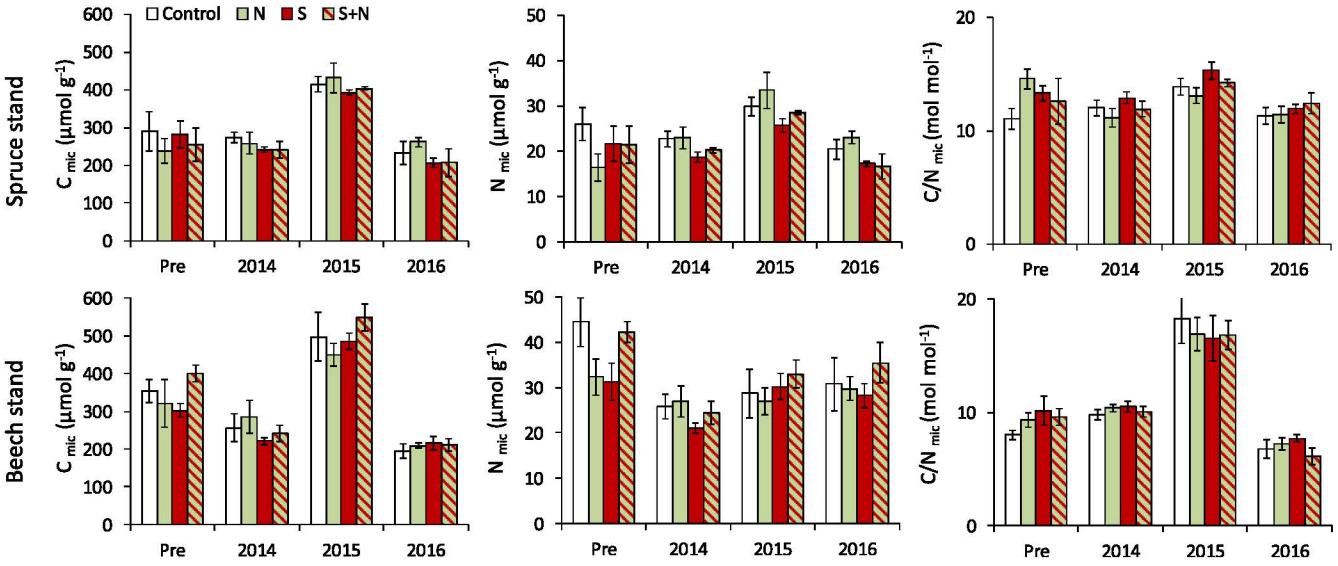


Fig. 3. Mean and standard error of microbial C, N and C/N at each forest stand for control, nitrogen (N), sulphur (S)and sulphur + nitrogen (S + N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters ($P < 0.05$).

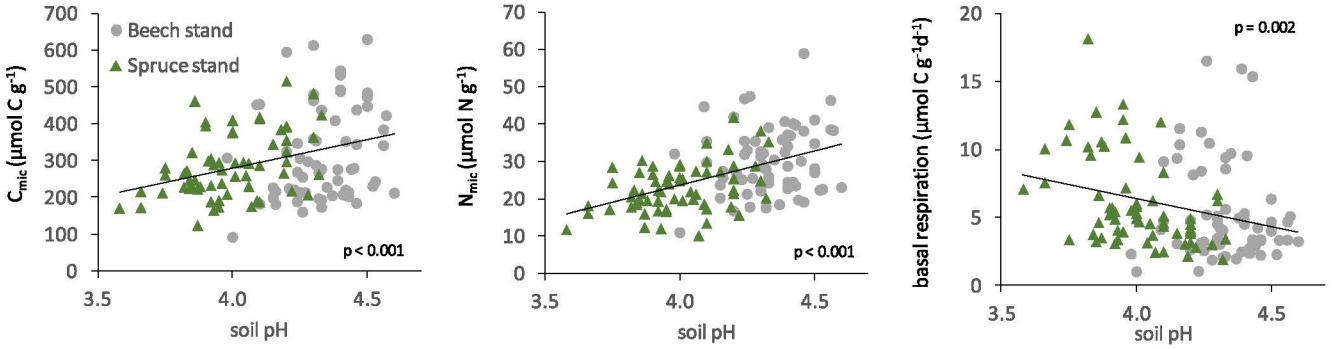


Fig. 4. Soil microbial C, N and microbial respiration related to soil pH among forest stands. Linear regression and its significance (p value) highlighted for all data.

decreases of bacteria and increases (albeit insignificant) in fungal gene copies (Suppl. Table 2).

3.4. Enzymatic activities

Forest type significantly ($P < 0.05$) affected activity of all enzymes except these involved in C cycling (β-Glucosidase - BG and cellobiohydrolase – CEL). Consistently higher activities of enzymes primarily involved in N acquisition (leucine-aminopeptidase - LEU and N-acetyl-β-D-glucosaminidase - NAG) were measured in the beech stand compared to the spruce stand. Similarly, phosphatase (PME) activity was significantly higher in the beech stand compared to the spruce stand. Stoichiometry of enzymatic activity revealed significantly higher C_{enz}/N_{enz} and C_{enz}/P_{enz} ratios in the

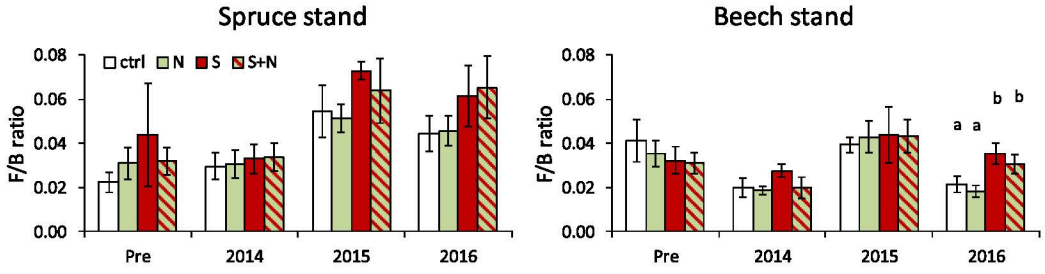


Fig. 5. Mean and standard error of fungi to bacteria ratio at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S + N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects were assessed by GLM analysis for each stand and time period separately.

spruce stand compared to the beech stand ($C_{enz}/N_{enz} = 4.3$ in the spruce stand and 1.9 in the beech stand; $C_{enz}/P_{enz} = 1.0$ in the spruce stand and 0.8 in the beech stand). Conversely, a higher N_{enz}/P_{enz} ratio was measured in the beech stand compared to the spruce stand (0.43 vs 0.23) (based on control means, Fig. 6).

Enzymatic activities among treatments did not significantly differ in the pre-treatment period. After treatment started, a tendency towards higher C enzyme activities under all treatments (N, S and S + N) was detected in the spruce stand (Suppl Table 1, Fig. 6). These changes resulted in significant ($P < 0.05$) alteration of enzyme stoichiometry by treatments. C_{enz}/N_{enz} ratio was significantly higher under all treatments (mean of 5.3) compared to control (3.9), and C_{enz}/P_{enz} ratio significantly increased under all treatments (mean of 1.3) compared to control (1.0). No treatment effect on N_{enz}/P_{enz} ratio was detected. This suggests that treatments primarily affected C enzyme activities in the spruce stand. In the beech stand, a significant reduction of NAG activity was detected under all treatments. This resulted in a significant increase of C_{enz}/N_{enz} ratio under all treatments (mean of 2.4) compared to the control (1.8). The absence of observed change in the C_{enz}/P_{enz} ratio and a consistent (albeit insignificant, $P = 0.097$) change in N_{enz}/P_{enz} ratio suggested that treatment effects at the beech stand primarily affected N acquiring enzymes (Suppl Table 1, Fig. 6). In the spruce stand, C_{enz} and N_{enz} were strongly negatively related to soil pH (Suppl. Fig. 2) while in beech no such dependence was found.

4. Discussion

Our results provide evidence that soil acidification alters C fluxes in both deciduous and conifer forests. Dissolved organic carbon concentrations in soil solution were suppressed by acidification in both forest types, and suppression of soil respiration was detected in the spruce stand. These responses have significant implications for long-term soil C cycling. On the other hand, nitrogen addition alone did not yet affect either DOC concentrations or soil CO_2 efflux in either forest type.

4.1. Acidification alters dissolved organic carbon leaching

Monthly addition of sulphuric acid immediately reduced forest floor soil solution pH by 0.4 units in both stands. Due to higher initial acidity in the spruce stand, this equated to a higher post-treatment hydrogen ion concentration of 270 μeq l^{-1} in the spruce stand, compared to 130 μeq l^{-1} in the beech stand. This "acid forcing" induced a consistent decline in soil solution DOC of around 50%. In line with other lab and field experimental pH manipulations (Clark et al., 2011; Ekstrom et al., 2011; Evans et al., 2012); analyses of long-term monitoring data (Finstad et al., 2016; Monteith et al., 2007); and modelling assessments (Rowe et al., 2014; Sawicka et al., 2017), we therefore found clear and quantitatively significant effects of soil solution acidity on soil solution DOC

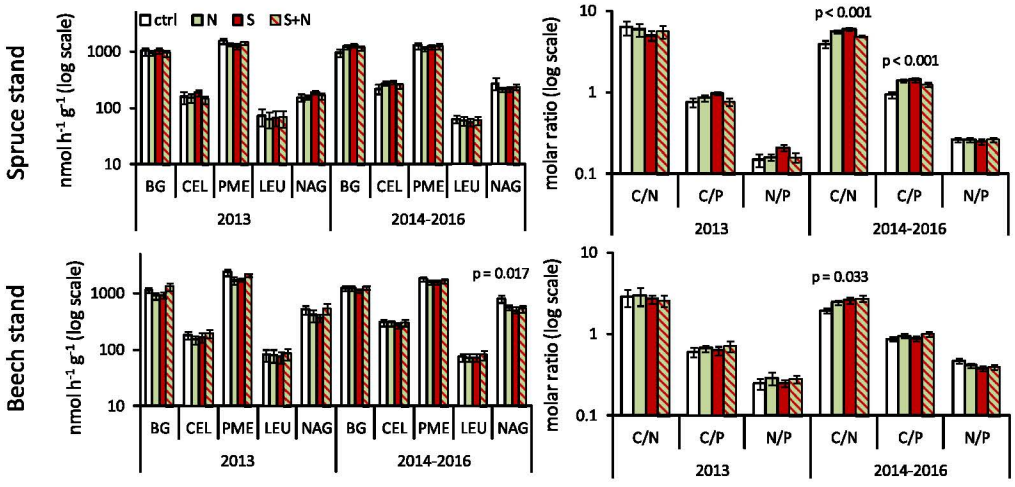


Fig. 6. Mean and standard error of enzyme activities and their ratios at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S + N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by p value. We calculated enzyme C/N, C/P and N/P ratios, where C = BG + CEL, N = NAG + LEU and P = PME.

concentration. Albeit different litter quality the effect of acid treatments on forest floor soil solution DOC concentration was consistent at both sites, suggesting a prevailing chemical (rather than biotic) control over DOC partitioning between soil and soil solution (Oulehle et al., 2013). Whether these effects were mediated by altered dissociation of organic acids in response to changing acidity, or direct effect of changed ionic strength (Hruska et al., 2009) remains unresolved. The neutralization capacity of forest mineral soils at both stands was highlighted by the delayed soil solution pH decrease in deeper depth. This ultimately led to a progressive acidification of mineral soil, due to the leaching of base cations accompanied predominantly by sulphate anion.

The absence of soil solution pH and DOC responses to N addition in our experiment is consistent with the interpretation that chemical processes control DOC mobility. The effect of NH_4NO_3 addition on soil acidity depends on the fate of NH_4 and NO_3; If both NO_3 and NH_4 are taken up by the biota no acidification occurs (regardless of whether nitrification has occurred or not) but if NO_3 is leached in excess of any leaching of NH_4^+ (due to preferential uptake and/or nitrification of NH_4^+) this will cause acidification, and may suppress DOC leaching (Evans et al., 2008). Contrary to previously suggested positive effects of N deposition on DOC leaching (e.g. Pregitzer et al., 2004) we did not detect any substantial DOC increase following N addition in our systems. However, long-term N inputs may stimulate primary productivity (Liu and Greaver, 2010), and thus ultimately increase the supply of ecosystem C available for leaching as DOC (Rowe et al., 2014), provided that other feedbacks such as changes in plant species and litter quality/quantity do not offset the increase in NPP. In forests subjected to long-term elevated N deposition, as in our case, response of soil solution DOC concentrations may differ from N limiting systems where production of DOC may be more responsive to increased N availability.

4.2. Acidification effects on soil carbon dynamics

Measured total soil respiration (Rs) rates were closely related to the seasonal course of soil temperature, and soil temperature was the overriding control on temporal variation in Rs, with limited effects of soil moisture (Oulehle et al., 2016). A large part of Rs in forests is derived from autotrophic respiration (Högberg et al., 2001), thus detection of subtle changes in soil CO_2 effluxes, driven by heterotrophic decomposition of fresh litter and SOM, is difficult. Nevertheless, consistent declines in Rs were detected in the spruce stand under acid treatment. The measured respiration decline of 6%–10% under S treatment is similar to that found in a recent meta-analysis (7%) based on experiments from China (Feng et al., 2017). The reduction of CO_2 effluxes under S treatment corresponds to the 27%–49% of annual litter production (aboveground litterfall of 1937 kg C ha $^{-1}$ year $^{-1}$ between 2013 and 2016, Oulehle et al., 2016). Based on a total S addition of 50 kg S ha $^{-1}$ year $^{-1}$ this corresponds to the suppression of decomposition by 10.6–19 kg C kg S $^{-1}$. The reduction in decomposition per unit S deposition may not be linear, however, as this would require linear relationships between S input and soil water hydrogen ion concentration, and subsequently with Rs. In a previous study (Oulehle et al., 2011) we demonstrated a continuous loss of forest floor C pool, at a rate of 1160 kg C ha $^{-1}$ year $^{-1}$, since precipitation acidity declined in the early 1990s as a consequence of "acid rain" abatement policy (total loss of 18.6 t C ha $^{-1}$ between 1994 and 2010). We proposed that recent loss of soil C carbon was derived from the soil C accumulated during the acidification period. Based on our calculations with the lower estimate of S deposition effect on C decomposition (10.6 kg C ha $^{-1}$ S ha $^{-1}$), a C pool of roughly 20 t C ha $^{-1}$ could have been accumulated between the 1960s and 1990s (the time period during which precipitation acidity fell below 4.3; Kopáček et al., 2016). Our experiment thus provides a plausible explanation for observed rates of C accumulation in forest soils exposed to acidification stress and could account for major changes in forest carbon stocks in the forests of Central Europe and other acidification-impacted regions.

Acid treatments in our experiment induced decreases in soil solution pH and DOC concentration in both forests, but only appeared to suppress Rs in the spruce stand. The two forest ecosystems can be characterized by: i) higher soil pH and base saturation in the beech stand; 2) higher substrate concentration and enzyme C to N ratios in the spruce stand; 3) an apparently higher degree of interactions among the microbial community in the spruce stand (Bárta et al., 2017); and 4) contrasting fungal life strategies, with saprotrophic fungi more abundant in the beech soil, and ectomycorrhizal fungi more abundant in the spruce soil (Bárta et al., 2017). Taken together, these features suggest a much stronger connection of soil metabolism to C acquisition in the spruce stand compared to the beech stand. Consequently, a decrease of soil solution pH and following suppression of labile C availability would be expected to have a much stronger impact on SOM decomposition in the spruce stand compared to the beech stand (Klotzbücher et al., 2011). We found a strong positive relation between soil pH and microbial biomass and high microbial respiration and C mining activity under low pH in the spruce stand. It coincided with a consistent (though non-significant, Fig. 3) decrease in microbial biomass following acid treatments in the spruce stand (roughly 11% based on 2016 data) and observed decreases in Rs. These results support our conclusion that the microbial (or at least prokaryotic) community in the spruce stand was most probably more stressed due to more acidic conditions (from the beginning of the experiment, and intensified by S treatments) than the beech community. The only acid treatment effects which were consistent across both forest types were significant changes in enzyme stoichiometry, and partially significant changes in fungi to bacteria ratios. Acid treatments significantly changed the C_{enz} to N_{enz} ratio, with increases following acid treatments in both stands. However, in the spruce stand the increase in C_{enz}/N_{enz} ratio appeared to be driven by increase of C_{enz} activities, which was further supported by the negative relationship between soil pH and C_{enz} activity. In addition, C_{enz}/P_{enz} ratio increased in response to acid treatments in spruce stand, with no change in the beech stand; this supports the assumption of predominantly enhanced C enzyme activities in the spruce forest (Fig. 6). More specific studies are needed to unravel the feedback between altered soil pH and microbial biomass and enzyme activities. It is also important to link actual qualitative changes among microbial communities caused by altered soil chemistry with corresponding C fluxes.

It has been proposed that microbes adjust their enzymatic activity to gain the limiting element from the substrate that is rich in this element and that the production of enzymes increases with substrate complexity (Mooshammer et al., 2014). As shown previously, acidity affects the solubility and bioavailability of organic matter (Evans et al., 2012; Scheel et al., 2007). We infer that shifts towards enzymatic C mining after acidification were most probably connected with organic matter stabilization and thus reduced availability to microbes, causing them to produce more C-acquiring enzymes to sustain C supply. This interpretation is also supported by observed negative relationships between BG and CEL and soil pH in the spruce stand and found shifts to fungal prevalence in both stands.

Based on our data, qualitative changes in the microbial community under acid treatments can be attributed to decreases of bacteria in both forests, and to an increase of fungi in the beech forest, resulting in higher fungi to bacteria ratios. According to the literature, bacteria are generally more impacted by low pH than

fungi (Rousk et al., 2009; Strickland and Rousk, 2010). In addition, fungi on average have higher C to N biomass stoichiometry, slower growth and turnover (Rousk and Bååth, 2011) and probably also higher carbon use efficiency than bacteria (Allison et al., 2005; Six et al., 2006). It means that they can store more C in their biomass per unit of consumed C than bacteria. Thus, the observed shifts in F/B ratio (either due to bacterial decreases and/or fungal increases) might partly contribute to decreases of soil microbial respiration in the field under acid treatments in both stands.

4.3. N addition effects on soil organic matter dynamics

In contrast to the rather limited number of experimental studies of soil acidity effects on SOM cycling in natural or semi natural ecosystems (Chen et al., 2016; Pennanen et al., 1998; Persson et al., 1989; Wu et al., 2016), many studies have linked enhanced soil N availability to the soil C cycle (e.g. Olsson et al., 2005; Hobbie, 2008; Janssens et al., 2010; Liu and Greaver, 2010). In general, meta-analyses of N addition experiments have shown stimulation of biomass productivity or reduction of soil respiration, leading to potentially increased C sequestration. Although ^{15}N tracing studies have suggested only minor N deposition contributions to C sequestration in temperate forests (Nadelhoffer et al., 1999), many field-scale N addition studies have suggested that N deposition could substantially enhance soil carbon sequestration by both forests and heathlands (Field et al., 2017; Prescott, 2010). Our plot experimental design did not allow testing of N addition effects on plant biomass productivity, therefore allocation of C to below-ground was assumed constant over the duration of our experiment. Based on our data we did not see any significant effects of N addition on Rs (Fig. 2), microbial biomass (Fig. 3) and respiration (data not shown). There was no evidence of N addition effects on fungi to bacteria ratios (Fig. 5), but we did observed shifts in enzyme C to N stoichiometry (Fig. 6), similar to those in the acid treatments, suggesting ongoing qualitative changes in microbial processes yet not detectable on a quantitative basis.

The lack of pronounced effects of N addition on the C cycle at our sites may be attributable to the high pre-existing levels of available N in these systems; both forests have been subjected to elevated N deposition since the 1950s, peaking in the 1980s with bulk deposition estimates of ca. 17 kg ha ^{1} year ^{1} (Oulehle et al., 2016; see Table 1 for current N fluxes). Microbial communities may therefore already be adapted to increased N availability at our sites, whereas systems receiving lower levels of ambient N deposition may be more responsive to additional N inputs (Phoenix et al., 2012). Nevertheless many other temperate forested ecosystems, especially those in Europe and North America, have been exposed to high levels of historic N deposition, and our data suggest that heterotrophic processes in these systems may be relatively unresponsive to additional N inputs. Forest ecosystems in parts of the world (apart of P-limited tropical forests) where N deposition is currently increasing, such as parts of Asia, Africa and South America (Dentener et al., 2006; Lamarque et al., 2013), but where fertility (i.e. soil C/N ratio) remains low may be expected to show greater change, potentially including effects on ecosystem C sequestration (Hyvönen et al., 2008).

With regard to dissolved carbon losses (and to a lesser extent also gaseous C fluxes) it is also possible that the absence of responses to N addition also reflects the lack of resulting changes in soil acidity. Previous work has shown that changes in DOC leaching in many N addition studies can be explained by experimentally-induced shifts in acid-base chemistry (Evans et al., 2008), but in our study the addition of NH_4NO_3 over 3 years was insufficient to alter soil solution pH, because little of the added N was leached as NO_3 (ca. 6% of added N in the spruce forest floor and ca. 11% of added N in the beech forest floor, data not shown). Thus the clear, chemically-mediated changes in DOC concentration observed in the acidification treatments were not replicated in the N-only treatment.

4.4. Implications for understanding of ecosystem responses to multiple drivers

A high proportion of biogeochemical research to date has taken place within areas of Europe and North America that have, as a result of industrialisation and agricultural intensification, been exposed to multiple environmental stressors, including the simultaneous deposition of a range of atmospheric pollutants. Nevertheless, experiments carried out within these ecosystems often focus on a single driver, whilst many analyses of monitoring data have considered only a partial range of potential driving variables. Studies of forest carbon sequestration in response to atmospheric deposition have overwhelmingly focused on N deposition, yet our results suggest a potentially similar rate of soil C accumulation in response to S deposition (>10 kg C kg S ^{1}) as that estimated for N deposition (10–15 kg C kg N ^{1}; de Vries et al., 2009). Furthermore, S deposition to European and North American forests has changed more rapidly and more dramatically over the last 50 years than N deposition, implying that S may have had a greater influence on the soil C cycle during this period, and also raising the possibility that some observed changes may have been incorrectly attributed to N deposition (Kolář et al., 2015). In order to correctly interpret existing records, and to accurately predict future changes in ecosystem biogeochemistry in response to continuing environmental change, we argue that a more holistic approach to the impacts of multiple environmental drivers is needed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.03.081.

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9.4 Publication IV

Choma, M., Tahovská, K., Kaštovská, E., Bárta, J., **Růžek, M.**, Oulehle, F., 2020. Bacteria but not fungi respond to soil acidification rapidly and consistently in both a spruce and beech forest. FEMS Microbiol. Ecol. 96, 174. https://doi.org/10.1093/femsec/fiaa174 (IF_{2019} = 3.675)

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RESEARCH ARTICLE

Bacteria but not fungi respond to soil acidification rapidly and consistently in both a spruce and beech forest

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One sentence summary: The acidity-driven shifts in bacterial community were closely related to changes in the activity of enzymes in forest soils, in contrast to a lack of connection between exoenzymes and fungal community composition.

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ABSTRACT

Anthropogenically enhanced atmospheric sulphur (S) and nitrogen (N) deposition has acidified and eutrophied forest ecosystems worldwide. However, both S and N mechanisms have an impact on microbial communities and the consequences for microbially driven soil functioning differ. We conducted a two-forest stand (Norway spruce and European beech) field experiment involving acidification (sulphuric acid addition) and N (ammonium nitrate) loading and their combination. For 4 years, we monitored separate responses of soil microbial communities to the treatments and investigated the relationship to changes in the activity of extracellular enzymes. We observed that acidification selected for acidotolerant and oligotrophic taxa of Acidobacteria and Actinobacteria decreased bacterial community richness and diversity in both stands in parallel, disregarding their original dissimilarities in soil chemistry and composition of microbial communities. The shifts in bacterial community influenced the stoichiometry and magnitude of enzymatic activity. The bacterial response to experimental N addition was much weaker, likely due to historically enhanced N availability. Fungi were not influenced by any treatment during 4-year manipulation. We suggest that in the onset of acidification when fungi remain irresponsive, bacterial reaction might govern the changes in soil enzymatic activity.

Keywords: soil bacteria; soil fungi; soil enzymatic activity; soil acidification; forest soil; amplicon sequencing

INTRODUCTION

Acidification and enhanced nitrogen (N) availability affect microbial communities and their activity differently. Acidification reduces the availability of dissolved organic carbon (DOC) and base cations in soil, mobilises toxic Al^{3+} and exposes soil microorganisms to physiological pH-induced stress (Prescott, Harley and Klein 2005; Oulehle *et al.* 2011; Evans *et al.* 2012; Kopáček *et al.* 2013). Low pH reduces cell growth rate and thus soil microbial biomass, enhances cell maintenance requirements and favours acidotolerant species (Lauber *et al.* 2009; Rousk *et al.* 2010; Rousk and Bååth 2011; Tedersoo *et al.* 2014;

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Fierer 2017; Glassman, Wang and Bruns 2017). The change in metabolic requirements and capabilities of the adjusted microbial community together with modification of substrate availability alter the secretion of extracellular enzymes that cleave complex organics to release utilisable forms of carbon (C), nutrients and energy (Burns *et al.* 2013; Mooshammer *et al.* 2014), which might alter soil processes.

N input increases soil N availability, which leads to shifts in microbial communities, their activity and further C and N transformations (Aber *et al.* 1998; Janssens *et al.* 2010; Kopáček *et al.* 2013; Gao *et al.* 2015). In forests, the direct effect of enhanced N availability is often combined with decreased tree belowground C allocation (Högberg *et al.* 2003, 2010). The reduced supply of plant-derived C usually causes a decline in microbial biomass (Högberg and Högberg 2002; Wallenstein *et al.* 2006), namely, ectomycorrhizal fungi retreat. Their taxonomic composition changes and the fungi-to-bacteria (F: B) ratio decreases (Kjøller *et al.* 2012; Choma *et al.* 2017; Van Der Linde *et al.* 2018; Lilleskov *et al.* 2019). Saprotrophic fungi and bacteria might benefit from a decrease in ectomycorrhizal fungi and the overall composition of microbial community changes (Tahovská *et al.* 2020). The combination of decreased microbial biomass, altered resource availability and restructuralised microbial community leads to reduced activity of exoenzymes (Frey *et al.* 2004; Gallo *et al.* 2004; Ramirez, Craine and Fierer 2012). This is usually explained by a reduced production of fungal enzymes (Frey *et al.* 2004; Gallo *et al.* 2004), but bacterial enzymatic production might be suppressed as well (Freedman *et al.* 2016). As a result, soil C and N transformations change (Kopáček *et al.* 2013; Gao *et al.* 2015; Kyaschenko *et al.* 2017). In particular, N retention in the microbial biomass is lowered and the risk of excess N leaching increases (Frey *et al.* 2004; Treseder 2004; Högberg, Chen and Högberg 2007; Janssens *et al.* 2010; Bahr *et al.* 2013; Choma *et al.* 2017). Nevertheless, the ecosystem response depends on the nature of N input (dose, duration, regularity and form of N) and on its initial conditions, especially the N limitation stage (Tahovská *et al.* 2020). The negative consequences of N input may likely be connected to accompanying acidification, which results from reactive N transformations (Kopáček *et al.* 2013; Gao *et al.* 2015; Chen *et al.* 2016) and coupled S deposition, rather than the nutritional N effect (Evans *et al.* 2008). Additionally, studies questioning the superiority of fungi in soil enzymatic activity and N retention capacity have emerged (Carrara *et al.* 2018). This fact highlights the need for a more targeted effort in understanding the divergent impact of acidification and nutritional N effects on soil microbial communities and their functioning.

The ecosystem response to acidification and N input further depends on the tree species composition (Rothe *et al.* 2002; Růžek *et al.* 2019). Norway spruce (*Picea abies*) and European beech (*Fagus sylvatica*) are common tree species in Europe. Spruce litter is more acidic and of higher C: N compared with beech (Bauer, Schulze and Mund 1997; Calvaruso, N'Dira and Turpault 2011). Additionally, due to higher leaf area and year-round foliage, spruce is more efficient in atmospheric compound interception (Oulehle and Hruška 2005; De Schrijver *et al.* 2007). As a result, soils under spruce are usually more acidic and thus more susceptible to further acidification, less fertile and, consequently, differ in the composition of soil microbial communities and metabolic potential from beech stands (Augusto *et al.* 2002; Nacke *et al.* 2016; Bárta *et al.* 2017; Asplund *et al.* 2018; Bahnmann *et al.* 2018).

The Načetín experimental site presents an acidity and N input manipulation experiment established in factorial design in adjacent beech and spruce forest stands. After 4 years of treatment, we traced the changes in soil and soil solution biochemical variables, performed amplicon sequencing to describe the composition of soil microbial communities and measured potential exoenzymatic activities. We aimed to disentangle the separated effects of acidity and increased mineral N availability on soil microbial communities and their activities under beech and spruce. We hypothesised that (i) the acidification will change the structure of the soil microbial community and decrease its diversity, with a stronger impact on bacteria than fungi, (ii) the acidification-driven changes in microbial communities will be more recognisable in the spruce stand due to its lower original pH, (iii) together with an acidification-induced decrease in C availability, we expect enhanced activity of C-mining enzymes both in absolute terms and in relation to other (N- and P-acquiring) enzymes and (iv) the effect of N addition will be less dramatic compared with changes induced by acidification as the systems are not strictly N-limited. The most affected microbial group will be ectomycorrhizal fungi.

MATERIALS AND METHODS

Sites and experimental design

In 2013, two parallel acidity and N availability manipulation experiments were established at two adjacent forests (50.59 N, 13.26 E) selected to represent major European forest types, mature (80 years) Norway spruce forest (*Picea abies* (L.) Karst.) and mature (140 years) European beech forest (*Fagus sylvatica* L.). Stem density was higher in spruce monoculture (540 stems/ha) compared with the beech forest (340 stems/ha). However, aboveground biomass stock was higher in beech (473 t/ha) compared with spruce (308 t/ha) forests. The distance between forests is ~1 km; annual precipitation averages at 1012 mm and the mean annual temperature is 6.5°C. Both stands have gneiss bedrock. Soils were classified as Dystric Cambisol according to WRB FAO classification (FAO 2014). Ground cover vegetation in the spruce stand is formed mainly by *Avenella flexuosa* L. Drejer with an admixture of *Vaccinium myrtillus* L., *Dryopteris dilatata* (Hoffm.) A. Gray, *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Galium saxatile* L. The ground cover vegetation in the beech stand (1.5%) primarily contains *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Avenella flexuosa* L. Drejer (Oulehle *et al.* 2016b).

At each forest stand, 16 3 × 3 m plots were set up with four treatment variants: control, N, acid (S) and acid + nitrogen (S+N). The experiment was designed in a randomised blocked design, thus four replicates of each treatment variant were present in both stands. Tree stems were always outside the plots to avoid uneven treatment distribution. The manipulative experiment began in April 2014. Treatments consist of monthly additions (April–November) of sulphuric acid (H_2SO_4) and ammonium nitrate (NH_4NO_3), mixed with rainwater collected at sites and applied evenly across the plots using 15 L watering cans, followed by the addition of 15 L rainwater to let all the added chemicals properly seep into the soil. The control plots received 30 L of rainwater. The treatment doses were equivalent to 50 kg S ha^{-1} $year^{-1}$ and 50 kg N ha^{-1} $year^{-1}$, and the additional watering was equivalent to 2.5% of the average annual rainfall. The NH_4NO_3 addition did not significantly alter the pH of the added solution (the pH of the applied solution was ~5.4). The dose represented peak acidification S deposition in the late 1980s (Oulehle *et al.* 2016a). Nitrogen treatments gave an input of 50 kg N ha^{-1} $year^{-1}$ and represented N input within the upper range of N deposition over Europe (Lamarque *et al.* 2013).

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Soil solution sampling and analysis

Soil solution samples were taken every 3–5 weeks during the snow-/ice-free period (usually April–November), ideally after rainfall when soil moisture permitted collection. Soil solution was always sampled before treatment application using Rhizon® suction samplers (Rhizosphere Research Products, Wageningen, the Netherlands), comprising 10 cm long, 2.5 mm diameter porous membranes attached to 50 mL syringes. Four to six Rhizon samplers were inserted randomly into the forest floor at each plot to give one composite sample per plot. The samples were stored at 4°C and analysed within 2 weeks. We measured the pH and concentration of anions (Cl^-, F^-, NO_3^-, SO_4^-), SiO_2, NH_4^+, base cations (Ca^{2+}, Mg^{2+}, Na^+, K^+), forms of Al (monomeric, organic monomeric, inorganic monomeric, acid soluble, total), DOC, dissolved nitrogen (DN), PO_4^- and total phosphorus. Here we list the methodology only for variables that significantly affected the microbial community composition (according to step-wise selected models, see below); for a complete description of methods, refer to the supplementary information. Solution pH (pH) was determined using a Radiometer TTT-85 with a combination electrode. Concentrations of DOC and DN were measured in the filtered solution samples (glass fibre Macherey-Nagel) using a Tekmar-Dohrmann Apollo 9000 analyser. Dissolved organic N (DON) in the solution was calculated as the difference between the DN and the sum of nitrates (high-performance liquid chromatography; Knauer 1000) and ammonium (indophenol blue colorimetry).

Soil sampling and analysis

Soil sampling (composite of F+H horizons) was performed during 2014–2017, always in May and September. For each treatment plot, two soil samples were combined. Soil sampling consisted of ~250 g of fresh soil taken from the whole F+H layer, 5 cm deep in spruce and 3 cm deep in beech forests, randomly distributed within the treatment plots. Soil samples were sieved on site (5 mm mesh size); all roots were gently removed and soils were immediately transported at 4°C for further laboratory analysis. The subsamples for DNA and enzymatic activity analysis were stored in a freezer (-80°C) immediately upon arrival at the laboratory; the other analyses were started within 1 week upon sampling. The soil chemical parameters, microbial biomass, marker gene abundances and enzymatic activities presented in this study come from samples taken in 2017. The soil and soil solution data from other years hasvebeen published (Oulehle *et al.* 2018). Microbial community composition was analysed for all sampling occasions. Nevertheless, only the final experimental data (year 2017) are presented in detail, while only partial results from 2014–2016 are used as supporting information. Total soil carbon (C_{tot}) and N (N_{tot}) were determined in dried (60°C, 48 h), milled soil samples using an elemental analyser (Vario Micro Cube, Elementar, Germany). Soil-extractable NH_4^+ and NO_3^- were measured after the extraction with 0.5 M K_2SO_4 (extractant: soil, 4: 1, v/w; shaken 45 min/150 opm; centrifuged 4000 rpm/15 min; filtered through 0.45 μm glass-fibre filter) by flow injection analysis (QuickChem 8500, Lachat Instruments, USA). Soil microbial biomass carbon (C_{mic}) and N (N_{mic}) were measured by the fumigation extraction method as described previously (Oulehle *et al.* 2018). All data were expressed per gram of soil dry weight (105°C, 5 h). We also determined soil water-extractable DOC, DN, DON, NH_4^+ and NO_3^-. The full methodology for these variables is provided in detail in the supplementary information.

Soil enzymatic activity

The activity of hydrolytic extracellular enzymes was determined in soil suspension by microplate fluorometric assays (1 g soil, 100 mL of distilled water, 4 min sonication) as follows: 200 µL of soil suspension was added to 50 µL of methylumbelliferyl solution for β-glucosidase (BG), cellobiohydrolase (CEL), phosphatase (PME) and N-acetyl-glucosaminidase (NAG) determination or to 50 µL of 7-aminomethyl-4-coumarin substrate solution for leucine-aminopeptidase (LEU) determination (Bárta *et al.* 2014). From three tested concentrations of each fluorogenic substrate (50, 100 and 300 μM), the one with the highest enzymatic activity where the enzyme was saturated was picked. Plates were incubated at 20°C for 2 h. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm using an Infinite F200 microplate reader (TECAN, Germany). The activity of C, N and phosphorus (P) mining enzymes was calculated as EC = BG+CEL, EN = NAG+LEU and EP = PME, respectively (Sinsabaugh, Hill and Shah 2009). Enzymatic stoichiometric ratios EC: EN, EC: EP and EN: EP were accordingly derived. The total hydrolytic activity was calculated as Etot = EC+EN+EP. The potential activity of phenoloxidases (PhOX) was determined photometrically using l-3,4-dihydroxyphenylalanine substrate (Bárta *et al.* 2010). The enzyme assay was immediately measured photometrically (absorbance 450 nm) and after incubation for 13 h at room temperature.

Soil microbial communities

Soil DNA was obtained according to Bárta *et al.* (2017). Quantitative PCR of markers specific for bacteria and fungi, genes for ribosomal small subunit (SSU; 16S rRNA and 18S rRNA genes, respectively), was performed with the use of FastStart SybrGREEN Roche® Supermix (Roche, France) and the StepOne system (Life Technologies, USA) as described previously (Gittel *et al.* 2014; Bárta *et al.* 2017). The fungi-to-bacteria (F: B) ratio was calculated as the count of 18S copies divided by 16S copies. Barcoded amplicon sequencing was performed to assess the microbial community composition. The PCR primers (515F/806R) targeting the V4 region of the SSU rRNA gene were used in the case of bacteria (Caporaso *et al.* 2011) while the primers ITS1f and ITS2 were used for fungi (White, Bruns and Taylor 1990). A detailed description of sequencing library preparation and Illumina sequencing is given in the supplementary material. Bacterial paired-end reads were joined using USEARCH 8.1 (Edgar 2010), while only forward reads were further processed in the case of fungi. Raw reads were quality filtered (min. quality mean 25, no ambiguous bases, trim quality right 25: in case of fungal forward reads) using PRINSEQ-lite 0.20.4 (Schmieder and Edwards 2011). Bacterial sequences were filtered and trimmed to a length of 250 bp. The ITS1 region was extracted from fungal reads using ITSx 1.0.11 (Bengtsson-Palme *et al.* 2013) prior to length filtering at 150 bp. USEARCH 8.1 was used for OTU table construction (similarity cut-off 97% and 98.5% for prokaryotes and fungi, respectively, singletons discarded). Taxonomy was assigned with BLAST (Altschul *et al.* 1990) within QIIME v. 1.9.1 (Caporaso *et al.* 2010). SILVA 132 (Quast *et al.* 2013) and UNITE 7.2 (UNITE Community 2017) databases were used for annotation in the cases of bacteria and fungi, respectively. The detailed workflow of sequence data processing is available at https://github.com/chomic-kbe/nacetin. Further processing of OTU tables was performed with phyloseq package 1.22.3 for R (McMurdie and Holmes 2013). In general, 2000 reads per sample

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is accepted as sufficient to reveal the main pattern in the microbial community (Caporaso *et al.* 2011). Only a single bacterial sample did not reach this limit and was discarded. The fungal samples had overall lower read counts, thus a more relaxed limit of 1300 reads per sample was chosen as a compromise to preserve the reliable portion of samples within the dataset. (Two samples were discarded.) Alpha diversity was estimated from rarefied datasets (rarefaction to the size of the sample with minimal sequencing depth within the respective dataset; 6065 and 1310 for bacteria and fungi, respectively), while the other calculations (beta diversity based on the Bray-Curtis sample dissimilarity matrix, taxa relative abundances, etc.) were performed on non-rarefied data (Mcmurdie and Holmes 2014). Alpha diversity—OTU richness and Shannon index (Magurran 2004)—was calculated at the OTU level. Lifestyle was manually assigned to fungal OTUs. Information for OTUs assigned at the genus or species level was searched for in the FUNGuild database v. 1 (Nguyen *et al.* 2016) and the available literature (e.g. Rinaldi, Comandini and Kuyper 2008; Tedersoo *et al.* 2014; Buzzini, Lachance and Yurkov 2017; Agerer and Rambold 2020). Reference sequences of other OTUs were BLAST-compared with the GenBank NCBI database and for hits with ≥99% identity. Metadata were searched for information implying lifestyle. The effort was taken to cover at least 90% of sequences in each sample. Bacterial genera with members potentially possessing genes for phenol-oxidase production (ppo, ligE, lcc_ascomycetes, lcc_basidiomycetes) were identified by a search in FunGene v. 9.8 (Fish *et al.* 2013). Their relative abundance was summed. The sequence data are stored at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the study accession number PRJEB36421.

Statistical analysis

All the steps of statistical evaluation were performed in R 3.6.1 (R Core Development Team 2019). The considered significance level was $\alpha = 0.05$. The treatment effect on soil and soil solution biochemical variables, activity of hydrolytic enzymes, marker gene abundances, diversity measures and taxa relative abundance were tested using linear mixed-effect models with stand, treatment and sampling time (May and September) as fixed effects and block as a random effect using *lme4* v. 1.1–20 (Bates *et al.* 2015). Relative abundance data were square root-transformed prior to testing. Post-hoc comparison of treatments within respective stands was done using estimated marginal means: *emmeans* v. 1.4.1 (Lenth *et al.* 2019). The stand effect was evaluated by a linear model (stand and sampling time as factors) that included only control plots. Treatment effect on overall bacterial and fungal communities was examined with the help of the package *vegan* v. 2.5–4 (Oksanen *et al.* 2019). PERMANOVA analysis (9999 permutations) of Bray-Curtis dissimilarities at the OTU level was based on a model considering stand, treatment and sampling time as explanatory variables (permutations restricted to treatment blocks). Prior to analysis, the homogeneity of multivariate dispersions was checked. To reveal the stand effect, only data from control plots were evaluated by a model including stand and sampling time as factors. Ordination plots are based on non-metric multidimensional scaling (NMDS). To discover soil and soil solution parameters best explaining the differences between microbial communities, distance-based redundancy analysis (db-RDA) model forward selection was performed for both complete datasets and respective stands. (The variance inflation factors were checked to confirm non-collinearity of selected variables.) Pairwise PERMANOVA comparison of treatments within respective stands was performed using *RVAideMemoire* v. 0.9–72 (Hervé 2019).

RESULTS AND DISCUSSION

Primary stand differences in soil biochemistry, microbial communities and enzymatic activity

Adjacent beech and spruce stands share the same bedrock, soil type, climate and acidification history (Oulehle *et al.* 2018). However, the long-term presence of different tree dominants is significantly reflected in different soil biochemical properties. The spruce stand originally had lower soil solution pH (~3.9 and ~4.3 in spruce and beech, respectively), more C_{tot} with a higher C_{tot}:N_{tot} ratio and higher soil solution DOC concentration than the beech stand (Table 1, Table S1 and Fig. S1). DON and NH_4^+ dominated the dissolved N pool in both stands. Nevertheless, NO_3^- concentration was 7-fold higher in the beech than in the spruce stand (Table 1 and Table S1). C_{mic} and EC were comparable at both sites, while N_{mic} and EN, EP and PhOX were significantly higher in the beech stand (Table 1 and Table S1).

The composition of microbial communities was followed throughout the whole experiment. However, the focal results presented within this study come from the fourth experimental year. The sequencing of the 2017 samples yielded 2044 725 bacterial and 222 477 fungal high-quality sequences with a range of 6065–55 933 and 1310–8342 sequences per sample for bacteria and fungi, respectively. Clustering identified 2154 bacterial and 1168 fungal OTUs across all samples. The beech stand soil bacterial community, with an average of 572 OTUs and mean Shannon diversity of 5.03, was more diverse compared with the spruce stand with 464 OTUs and a Shannon index of 4.78 (Table 1). Fungal OTU richness was higher in spruce than in beech (163 and 141, respectively) as well as Shannon diversity (though insignificantly: 3.80 and 3.47, respectively; Table 1). The two stands did not differ either in bacterial (16S rRNA) or in fungal (18S rDNA) marker gene abundances, as well as their ratios (Table 1). The original soil microbial communities (control treatments only) differed considerably between beech and spruce stands, as shown by the significant stand effect in PERMANOVA analysis explaining 46.1% and 26.2% of variability for bacterial and fungal community, respectively (both $P < 0.001$; Table S2).

NMDS visualisation of all samples from 2017 clearly separated beech and spruce stand soil bacterial communities (Fig. 1A). Generally, Acidobacteria contributed to more than 50% of bacterial sequences, which made them dominant in both stands (Table 2). The following high abundant phyla were Proteobacteria, Actinobacteria, Verrucomicrobia and Planctomycetes (Table 2). Comparing control plots, Acidobacteriales and Actinobacteria reached significantly higher proportions in the spruce stand than in the beech stand. Beech soils were richer in multiple groups of several Alphaproteobacterial orders (Caulobacterales, Micropepsales, Reyranellales and Rhodospirillales), Verrucomicrobia, Planctomycetes and Deltaproteobacteria (Table 2 and Table S5). Fungal community dissimilarity between stands was also clearly presented by an NMDS projection (Fig. 1B). Significant stand differences were found in the relative abundances of the most represented fungal groups. Within Ascomycota, Helotiaceae, Hyaloscyphaceae (both Helotiales), Eurotiales and Capnodiales reached a higher abundance in the spruce stand compared with the beech stand soil (Table 3). Regarding Basidiomycota, spruce soils contained more sequences assigned to ectomycorrhizal genera *Tylospora*

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Table 1. Soil and soil solution physico-chemical parameters, activity of hydrolytic enzymes, bacterial and fungal diversity measures and marker gene abundances (mean ± SD) in respective treatments in both stands in 2017, the fourth year of the experiment. Within each stand, the significant difference ($P < 0.05$) from control treatment is denoted by *. The last column shows stand differences (i.e. the difference between control treatments of both stands) at $P < 0.05$ (+), $P < 0.01$ (++) and $P < 0.001$ (+++); n.s., not significant.

		Beech				Spruce				Stand difference (controls)
		control	N	S	S+N	control	N	S	S+N	
Soil solution pH		4.25 (0.36)	4.19 (0.39)	3.74 (0.28)*	3.65 (0.26)*	3.87 (0.35)	3.62 (0.12)	3.25 (0.3)*	3.24 (0.19)*	++
Soil solution DOC	mg l^{-1}	38.8 (21.8)	41.2 (16.4)	22.5 (12.1)*	22.5 (8.7)*	64.3 (30.3)	79.7 (33.7)	46.4 (12.5)	36.9 (14.9)*	+
Soil solution DON	mg l^{-1}	1.52 (0.60)	1.63 (0.59)	1.01 (0.43)*	1.25 (0.50)	2.16 (0.91)	2.56 (0.65)	2.00 (0.45)	1.99 (0.65)	n.s.
Soil solution DOC:DON	g:g	24.0 (5.3)	24.9 (2.5)	21.4 (3.5)	18.1 (3.4)*	27.4 (6.9)	30.1 (5.4)	23.1 (2.5)	18.4 (4.4)*	n.s.
Soil C_{tot}	mmol g^{-1}	26.6 (3.3)	26.9 (5.5)	25.0 (5.0)	25.6 (4.2)	32.2 (2.3)	30.9 (1.9)	31.5 (2.4)	30.4 (2.4)	++
Soil N_{tot}	mmol g^{-1}	1.17 (0.17)	1.17 (0.25)	1.10 (0.21)	1.10 (0.16)	1.14 (0.11)	1.19 (0.10)	1.17 (0.09)	1.11 (0.10)	n.s.
Soil C_{tot}:N_{tot}	mol:mol	22.8 (0.9)	23 (0.6)	22.8 (1.1)	23.3 (1.6)	28.5 (2.9)	25.9 (0.8)*	26.9 (1.4)	27.5 (0.9)	+++
Soil NH_4^+	μmol g^{-1}	2.05 (1.30)	2.34 (1.39)	1.94 (1.38)	1.93 (1.77)	3.21 (2.23)	3.88 (2.50)	2.42 (0.84)	2.76 (0.61)	n.s.
Soil NO_3^-	μmol g^{-1}	0.96 (0.35)	0.74 (0.54)	0.64 (0.32)	0.64 (0.50)	0.13 (0.02)	0.16 (0.05)	0.12 (0.02)	0.14 (0.02)	+++
C_{mic}	μmol.g^{-1}	271 (44)	271 (59)	233 (63)	272 (43)	265 (28)	256 (38)	209 (41)*	213 (37)*	n.s.
N_{mic}	μmol.g^{-1}	27.2 (4.2)	27.1 (7.6)	23.6 (7.0)	26.9 (6.5)	21.3 (3.6)	21.4 (5.3)	17.1 (3.0)	18.0 (3.1)	+
C_{mic}:N_{mic}	mol:mol	9.97 (0.48)	10.2 (1.0)	9.95 (0.74)	10.3 (0.8)	12.7 (2.3)	12.7 (3.9)	12.2 (0.6)	11.9 (1.2)	++
Etot	nmol g^{-1} h^{-1}	4486 (933)	4371 (1132)	3125 (787)	5183 (2981)	3001 (815)	3767 (974)	4229 (1441)*	4132 (1696)	++
EC	nmol g^{-1} h^{-1}	1803 (427)	1947 (516)	1644 (424)	2413 (1295)	1853 (520)	2118 (616)	2395 (910)	2267 (879)	n.s.
EN	nmol g^{-1} h^{-1}	824 (269)	796 (431)	550 (279)	1046 (992)	319 (104)	262 (80)	345 (90)	389 (164)	+++
EC:EN	mol:mol	2.41 (0.91)	2.85 (1.04)	3.59 (1.74)	3.03 (1.23)	6.39 (3.32)	8.30 (1.96)	6.98 (2.02)	5.89 (0.43)	++
EC:EP	mol:mol	0.99 (0.22)	1.22 (0.24)	3.21 (2.89)*	1.37 (0.31)	3.80 (3.09)	1.52 (0.18)*	1.65 (0.47)*	1.59 (0.21)*	+
EN:EP	mol:mol	0.44 (0.1)	0.49 (0.24)	0.93 (0.95)	0.53 (0.30)	0.59 (0.46)	0.19 (0.05)*	0.25 (0.09)*	0.27 (0.03)*	n.s.
PhOX	nmol g^{-1} h^{-1}	344 (103)	328 (122)	335 (80)	327 (70)	188 (74)	211 (61)	169 (32)	207 (53)	++
Bac. Shannon		5.03 (0.10)	4.91 (0.10)*	4.81 (0.09)*	4.84 (0.08)*	4.78 (0.13)	4.73 (0.12)	4.38 (0.18)*	4.48 (0.11)*	+++
Bac. OTU richness	OTUs	572 (33)	525 (29)*	494 (37)*	493 (20)*	464 (27)	439 (18)	349 (36) *	376 (24)*	+++
Fun. Shannon		3.47 (0.39)	3.07 (0.51)	3.37 (0.62)	3.40 (0.46)	3.80 (0.24)	3.69 (0.29)	3.57 (0.47)	3.98 (0.39)	n.s.
Fun. OTU richness	OTUs	141 (30)	128 (17)	143 (28)	141 (19)	163 (21)	150 (20)	151 (35)	171(18)	+
Bac. 16S rRNA gene	10^{10} copies g^{-1}	3.13 (1.60)	2.59 (1.72)	2.30 (1.31)	2.72 (0.86)	2.48 (0.74)	2.85 (0.59)	2.08 (0.57)	2.20(0.49)	n.s.
Fun. 18S rRNA gene	10^{8} copies g^{-1}	6.85 (2.69)	8.01 (1.74)	6.93 (2.01)	8.00 (2.46)	6.13 (2.09)	5.77 (2.17)	5.26 (1.12)	4.84(1.15)	n.s.
F:B	copy:copy	0.030 (0.023)	0.051 (0.037)	0.040 (0.022)	0.032 (0.014)	0.027 (0.013)	0.021 (0.007)	0.026 (0.006)	0.023 (0.008)	n.s.

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Table 2. The bacterial phyla (and some of their nested groups): their mean relative abundance in the control treatment plots of the beech and the spruce stands in 2017, the fourth year of the experiment. Asterisks denote the significance of stand difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s., not significant). Higher (+) or lower (-) relative abundance in respective treatments compared with control marked for differences at $P < 0.05$ for each stand separately. The phyla were divided into two groups based on their overall reaction to the acid treatments, i.e. the difference in the total phylum relative abundance in S and/or S+N treatments compared with control.

			% mean relative abundance in control			Treatment significant change to control					
						beech			spruce		
Phylum			beech	spruce	stand difference	N	S	S+N	N	S	S+N
Phyla with overall positive or no reaction to acid treatments	Acidobacteria		51.2	52.0	n.s.						
		Acidobacteriales	24.6	33.3	***		+	+		+	
		Subgroup2	14.0	8.2	***					−	−
		Solibacterales	12.2	10.2	n.s.		−	−			−
	Actinobacteria		9.2	17.4	***		+	+		+	+
		Acidimirobiiia	0.7	1.5	***		+	+		+	+
		Catenulisporales	<0.1	<0.1	n.s.		+	+			
		Franikales	5.4	11.4	***		+	+		+	+
		IMCC26256	0.7	1.5	***		+	+		+	+
		Solirubrobacterales	<0.1	<0.1	n.s.		+	+		+	
	Chloroflexi		0.2	0.4	n.s.						+
	Patescibacteria		1.0	0.6	**			+		+	
	Thaumarchaeota		<0.1	<0.1	n.s.						+
	WPS2		1.8	2.0	n.s.		+	+			
Phyla with overall negative reaction to acid treatments	Armatimonadetes		0.2	0.1	n.s.		−	−		−	−
	Bacteroidetes		3.2	2.8	n.s.		−	−		−	−
	Elusimicrobia		0.2	<0.1	***		−	−		−	−
	Fibrobacteres		<0.1	<0.1	**			−		−	−
	Gemmatimonadetes		1.0	0.4	***		−	−		−	−
	Verrucomicrobia		6.5	2.4	***		−	−		−	−
	Planctomycetes		3.3	2.3	**	−	−	−	−	−	−
	Proteobacteria		21.8	19.2	**					−	−
	Alphaproteobacteria		19.3	17.7	n.s.						
		Acetobacterales	4.1	4.0	n.s.			+			+
		Azospirillales	0.1	0.1	n.s.		−	−		−	−
		Caulobacterales	1.7	1.1	*					−	−
		Elsterales	2.6	2.5	n.s.		+	+			
		Micropepsales	2.4	2.1	*					−	−
		Reyranellales	0.1	<0.1	**		−	−		−	−
		Rhizobiales	7.0	6.9	n.s.	+					−
		Rhodospirillales	0.7	0.4	***		−	−		−	−
		Rickettsiales	0.3	0.3	n.s.					−	−
		Sphingomonadales	0.1	0.1	n.s.						
	Gammaproteobacteria		0.4	0.4	n.s.					−	−
		Betaproteobacteriales	<0.1	<0.1	n.s.			−			−
		WD_260	0.1	0.1	n.s.			−		−	−
	Deltaproteobacteria		2.1	1.0	***		−	−	−	−	−
		Bdellovibrionales	<0.1	<0.1	**		−	−	−	−	−
		Myxococcales	1.9	1.0	***		−	−	−	−	−

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Table 3. The selected fungal taxonomic groups: their mean relative abundance in the control treatment plots of the beech and the spruce stands in 2017, the fourth year of the experiment. Asterisks denote the significance of stand differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s., not significant; n.t., not tested).

			% mean relative abundance in control		
			beech	spruce	stand difference
Ascomycota			31.3	38.0	n.t.
	Dothideomycetes				
		Venturiales	2.4	2.1	n.s.
	Eurotiomycetes				
		Capnodiales	0.3	0.7	**
		Chaetothyriales	2.6	4.1	n.s.
		Eurotiales	0.5	1.1	*
	Leotiomycetes				
		Helotiales	18.0	25.0	n.s.
		Hyaloscyphaceae	3.5	7.2	**
		Helotiaceae	2.0	7.9	***
		Thelebolales	0.4	<0.1	***
	Pezizomycetes				
		Byssonectria	2.7	1.3	n.s.
Basidiomycota			35.3	39.6	n.t.
	Microbotryomycetes				
		Leucosporidiales	0.6	0.2	**
	Agaricomycetes				
		Amanita	6.0	0.5	*
		Cortinarius	0.4	0.7	n.s.
		Hygrophorus	0.2	1.7	*
		Inocybe	0.5	<0.1	**
		Atheliaceae	9.7	13.8	n.s.
		Tylospora	1.1	10.2	**
		Piloderma	6.2	0.4	n.s.
		Boletales	7.8	7.8	n.s.
		Sistotrema	0.6	0.2	n.s.
		Phallus	0.6	<0.1	n.s.
		Russula	5.1	<0.1	*
	Tremellomycetes				
		Cystofilobasidiales	0.8	0.5	n.s.
		Solicoccozyma	2.8	2.8	n.s.
Mortierellomycota			30.5	18.2	n.t.
		Mortierellales	30.5	18.2	*
Other lineages			0.2	0.2	n.t.
Unassigned fungal sequences			2.7	4.0	n.t.

and *Hygrophorus* (Table 3). In the beech stand, genera *Amanita*, *Inocybe*, *Russula* and *Piloderma* (marginally insignificant) were more abundant compared with the spruce stand, where they accounted for only fractions of a % (<1.1%) in relative abundance (Table 3). Mortierellomycotina reached higher abundance in the beech stand. Other fungal lineages such as Rozellomycota, Chytridiomycota and Glomeromycota were detected as well, but in negligible amounts (Table 3). Examining lifestyle groups, moulds were more abundant under beech, while spruce soils were richer in ericoid mycorrhizal and root-associated fungi (Table S6).

A step-wise selected db-RDA model has shown that the main drivers of bacterial community composition differences were C_{tot}: N_{tot} ratio and pH, with 26.2% and 12.9% of explained variability, respectively (Table S3). The other significant factors were DOC, C_{tot} and NO_3^- with 4.5%, 2.5% and 2.3%, respectively (Table S3); in the case of the fungal community, C_{tot}: N_{tot} was also the strongest predictor among the soil chemical variables with 16.4% explained variability. The other significant variables, DON, pH and $C_{tot,}$ accounted for a lower portion of explained variability (5.1%, 2.4% and 2.4%, respectively; Table S3). Given that all these variables were discovered as stand-specific, this implies that tree species was the most influential factor shaping the composition of microbial communities. When 'stand' was included in the batch of explanatory variables, most of the remaining part of explained variability was attributed to pH, DOC concentrations and N_{tot} for bacterial, and pH and N_{tot} for fungal community composition (Table S4).

Acidification effect on soil biochemistry, microbial communities and enzymatic activity

Both S and S+N treatments significantly acidified the soil solutions in both stands with immediate effect (Oulehle *et al.* 2018). In the fourth year, the pH was ~0.4 units lower in acidified plots compared with the controls (Table 1 and Table S1). The acidification was accompanied by a 40% reduction in soil solution DOC concentrations on average in both stands and also a DON decrease (of 30%) but only in the beech stand. In the

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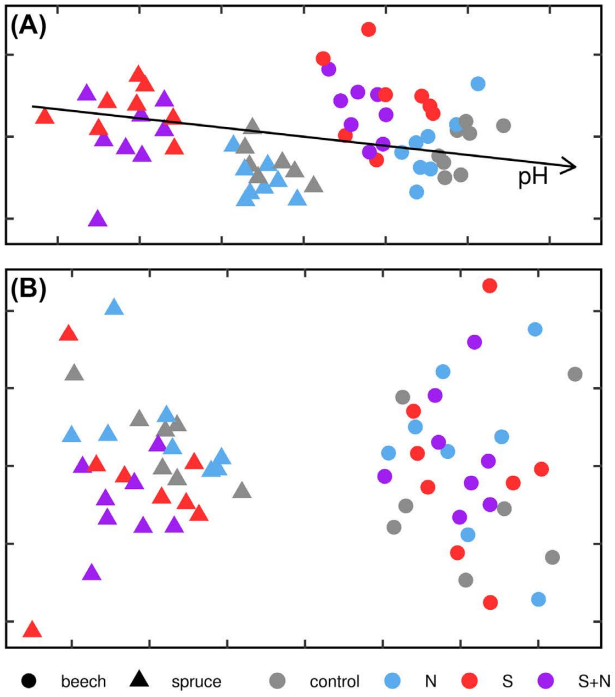


Figure 1. Visualisation of an NMDS ordination based on Bray-Curtis dissimilarity of (A) bacterial and (B) fungal communities' composition (stress = 0.06 and 0.13, respectively). Soil solution pH in panel (A) was passively projected.

spruce stand, C_{mic} was reduced by 20% (Table 1). Acidifying treatments further induced changes in hydrolytic activities. Specifically, both S and S+N treatments increased the total activity of hydrolytic enzymes (Etot) in the spruce stand, mainly due to a stimulation of EP, and decreased EC: EP and EN: EP compared with the control (Table 1 and Table S1). In the beech stand, the S treatment but not the S+N significantly enhanced EC: EN compared with the control (Table 1 and Table S1).

Bacterial community composition changed shortly after the initiation of both acidifying treatments in both stands (Table S7 and S8, Fig. S2). The magnitude of the effect gradually increased over time, from 10% explained variability in bacterial community composition for both stands in 2014 to 24% and 39% in the beech and in the spruce stands, respectively, in 2017 (Table S7, Fig. S2). The treatment-induced decrease in pH and DOC concentrations was the moving force of these shifts, just as these two variables were the most influential for shaping the composition of bacterial communities when the stand effect was previously accounted for (Table S4).

A detailed view of compositional changes showed that, in 2017 after 4 years of treatment, the bacterial communities of acidified (S and S+N) and control treatments were remarkably distinct and clustered separately in the NMDS ordination plot (Fig. 1A). Although starting from different original communities, the acidification-induced changes were analogous in both stands. The majority of responding taxa showed the same positive or negative reactions in both stands (Table 2). After 4 years of treatment, the soils of both acid treatments were still dominated by Acidobacteria (Table 2) and the relative abundance of this phylum commonly found in high abundance in acid forest soils (Baldrian *et al.* 2012; Nacke *et al.* 2016) was not affected by acid treatment. However, within the group, Acidobacteriales known for low pH optima in a range of 3.8–6.0 (Campbell 2014) partly replaced other Acidobacteria, mainly Subgroup 2 and Solibacterales (Table 2). These also occur abundantly in acid soils (Lauber *et al.* 2009), yet pH optima of their cultured representatives (e.g. *Bryobacter* sp., *Paludibaculum* sp., *Holophaga* sp.) are usually higher than 5.5 (Kulichevskaya et al. 2010, 2014; Thrash and Coates 2015). We further observed an increase in relative abundance of Actinobacteria in acidified soils, although they generally do not prefer low pH (Lauber *et al.* 2009; Zhang *et al.* 2017). A large portion of Actinobacterial reads belonged to *Acidothermus* sp. (Table 2) tolerant to low pH (Barabote *et al.* 2009), which is probably the cause of their success in these acidified soils. The majority of other bacterial lineages declined in acidified soils (Table 2), which led to decreased bacterial diversity (OTU richness and Shannon index) in both stands (Table 1). We observed a lower relative abundance of Bacteroidetes, Gemmatimonadetes and Fibrobacteres, which were previously shown to suffer in acid soils, but also Armatimonadetes, Elusimicrobia, Verrucomicrobia or Planctomycetes, for which no obvious pH preference was observed since they prosper uniformly at wide pH ranges (Lauber *et al.* 2009; Rousk *et al.* 2010; Zhang *et al.* 2017). However, these studies barely covered pH lower than 4, while in our soils, the pH dropped down to 2.9, which also probably caused stress to these bacterial generalists. Alongside pH stress, bacteria with a rather copiotrophic lifestyle such as Bacteroidetes and Gemmatimonadetes (Ho, Di Lonardo and Bodelier 2017) were in all likelihood mutually disadvantaged by low C availability in the acidified soils. On the contrary, many acidotolerant Acidobacteria and Actinobacteria are capable of degrading polysaccharides (Barabote *et al.* 2009; Větrovský, Steffen and Baldrian 2014; Lladó *et al.* 2016), which probably contributed to their higher relative abundance in the acid-treated soils of both stands.

In contrast to the fast and complex bacterial reaction, the response of the fungal community composition to treatments was weak and slow. Modest compositional changes occurred in the spruce stand only after 4 years, where fungal communities in acid treatments differed from the control (Tables S7 and S8) due to the relative abundance increase of *Cladophialophora*, *Inocybe* and Capnodiales (in S+N only) and the decline of Filobasidiales and *Cortinarius* (Table S9). The poor response of fungi in contrast to bacteria is in accordance with the generally observed lower susceptibility of fungi to direct pH stress compared with bacteria. Fungal communities are rather shaped by indirect pH effects through changed resource availability and competition with bacteria (Rousk *et al.* 2010; Rousk, Brookes and Bååth 2010; Glassman, Wang and Bruns 2017). In the spruce stand, yeasts (namely, *Solicoccozyma terricola*) decreased under acid treatment. Yeasts usually do not produce enzymes involved in the degradation of complex C compounds and rely on the actual availability of resources (Botha 2011). Therefore, their lower relative abundance in the acidified spruce soils likely stems from decreased C availability.

The changes in microbial communities are in line with our hypotheses (i) and (ii). We observed a dramatic shift in bacterial community composition and a relatively weaker, but significant, response of fungi, and the acidifying effect was stronger in the originally more acid spruce stand.

Relations of enzymatic activity and composition of bacterial community along a generated pH gradient

The sulphuric acid addition created a robust continuous pH gradient, on which the range of pH values in plots in beech and spruce stands overlapped. After 4 years of treatment, this pH gradient ranged from ~2.9 in acidified plots in the spruce stand

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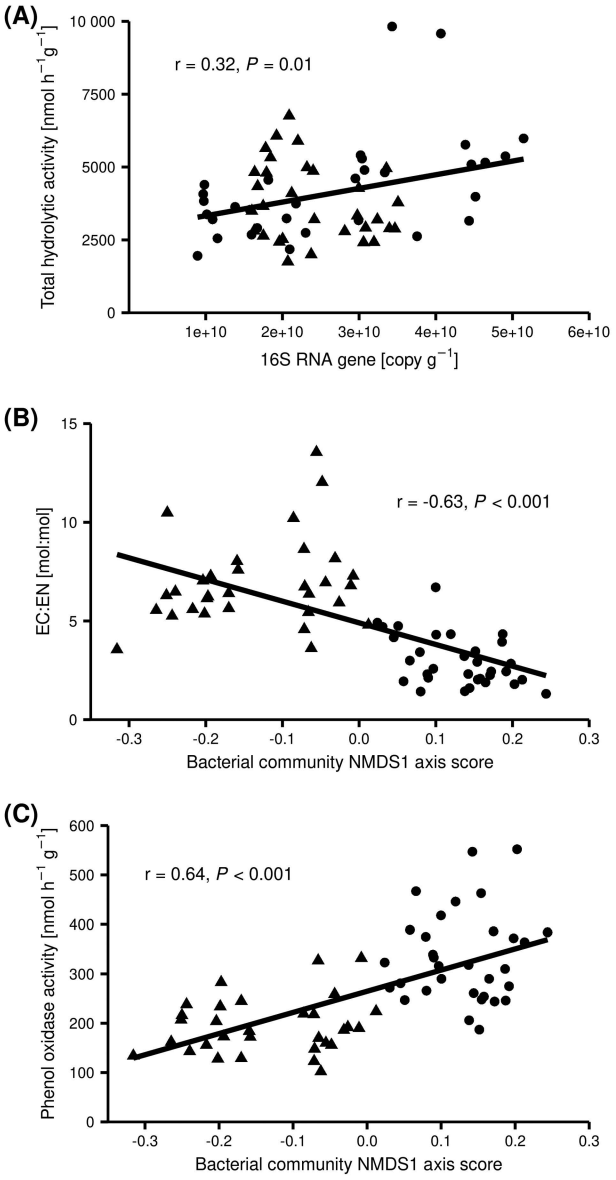


Figure 2. The relationship between **(A)** bacterial marker (16S rRNA) gene abundance and total hydrolytic activity, **(B)** bacterial community composition (NMDS1 axis score) and ratio of C-to-N mining enzymes and **(C)** bacterial community composition (NMDS1 axis score) and phenol oxidase activity among both stands (beech—circles, spruce—triangles). Correlation coefficients and corresponding *P*-values indicated.

to ~5.0 in the control and N-treated plots in the beech stand (Fig. S1). None of the chemical soil and soil solution parameters were correlated to the soil solution pH, but we found a close positive correlation of microbial biomass (C_{mic}, N_{mic}) and bacterial gene marker abundance (16S rRNA) with soil solution pH ($r = 0.42$, $P < 0.001$; $r = 0.52$; $P < 0.001$ and $r = 0.40$, $P = 0.001$, respectively; Fig. S3a–c). The bacterial gene marker abundance was further positively related to microbial biomass ($r = 0.50$ and 0.43, C_{mic} and N_{mic}, respectively, $P < 0.001$; Fig. S3d and e) and to total hydrolytic enzyme activity ($r = 0.32$, $P = 0.01$; Fig. 2A). The composition of bacterial communities followed the pH gradient created across both stands. This was shown by a passive projection of pH values to the NMDS ordination diagram (Fig. 1A) and confirmed by correlation with the first NMDS axis (NMDS1) score ($r = 0.73$, $P < 0.001$; Fig. S3f). The bacterial community composition was related to microbial biomass C: N ($r = -0.47$, $P < 0.001$), which was higher in communities of more acid soils (Fig. S3g). The increase in microbial biomass C: N ratio is often attributed to an increase in the F: B ratio (Strickland and Rousk 2010). However, we found no change in the F: B ratio across the pH gradient in our case. Nor did we find any relationship between C_{mic}: N_{mic} to either the F: B ratio or fungal community composition. We explain the higher C_{mic}: N_{mic} of bacterial communities in more acid soils by the selection for oligotrophic bacterial taxa (Acidobacteria, *Acidothermus*) that are expected to have higher biomass C: N (Fierer, Bradford and Jackson 2007). Bacterial community composition was further closely connected with enzymatic stoichiometric ratios, EC: EN and EN: EP ($r = -0.63$, $P < 0.001$ and $r = 0.35$, $P = 0.005$, respectively; Fig. 2B). EC: EN was higher in acidified soils with higher C_{mic}: N_{mic} and a larger share of bacterial oligotrophs. These bacteria have a high C demand (Fierer, Bradford and Jackson 2007) and additionally need to cope with decreased C availability, which was reflected by a high EC: EN related to the low pH communities (Fig. 2B). The decrease in pH was further accompanied by a reduction in PhOX activity ($r = 0.42$, $P < 0.001$, Fig. 2C). PhOX activity was related to bacterial community composition ($r = 0.64$, $P < 0.001$; Fig. 2C) and positively correlated to the relative abundance of bacterial groups, which might potentially produce enzymes involved in phenol oxidation ($r = 0.41$, $P < 0.001$; Fig. S3i). This fact is in accordance with an increasing acceptance of the considerable bacterial role in the production of enzymes involved in lignin-degrading enzymes, a role that classically has been attributed mostly to fungi (Sinsabaugh 2010; Carrara *et al.* 2018; Kang *et al.* 2018).

At the level of individual stands, the significant relationship between soil pH and bacterial community composition (NMDS1 score) was also detected ($r = 0.64$ and $r = 0.67$ for beech and spruce stands, respectively, both $P < 0.001$; Figs S4a and S5a) and represented by consistent responses of particular bacterial groups to changing pH at both sites. However, relations between bacterial abundance, community composition and enzymatic activities were significant only in the beech stand (Fig. S4b and c). In the spruce stand, bacterial community composition and abundance were disconnected from enzymatic activity. Hydrolytic activity was negatively correlated with soil solution DOC concentration ($r = -0.53$, $P = 0.003$ for total hydrolase activity; Fig. S5b; $r = -0.53$, $P = 0.003$ for EC, $r = -0.55$, $P = 0.002$ for EN and $r = -0.38$, $P = 0.034$ for EP).

In contrast to bacteria, soil fungal community composition did not show any sign of gradual distribution pattern in the NMDS ordination plot. The sample points aggregated in two clearly separated clusters representing sites of origin (spruce vs. beech; Fig. 1B). This indicates that local-specific conditions other than pH play a role in shaping soil fungal communities. This was also supported by a rather low portion of pH-explained variability in the db-RDA model (Table S3).

From hypothesis (iii), we expected an increase in production of C-mining enzymes as a response to acidity-induced reduction in DOC availability. The sole comparison of the magnitude of activities in the respective treatments failed to provide solid support for this hypothesis. However, by examining samples along the pH gradient, we discovered increasing EC: EN with increasing acidity, which agrees with expected increasing priority to enzymatical C mining in acidified soils. We found considerably higher EP in acidifying treatments in the spruce stand. This site was historically exposed to high doses of atmospheric N (Oulehle *et al.* 2011). Long-term excessive N supply might lead to an increase in relative P limitation of the soil microbial community (Forstner *et al.* 2019). The acidification-driven decrease in

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P solubility (Kaňa and Kopáček 2005) likely strengthened inherited relative soil P insufficiency, which might have overridden the effect of decreased C availability and explains the less pronounced shift in production of C-mining enzymes under acidifying treatments when the production of phosphatases to overcome P limitation was more preferred.

N addition effect on soil biochemistry, microbial communities and enzymatic activity

After 4 years of N addition, we have not observed a significant effect on soil and soil solution chemistry or in the composition of bacterial and fungal communities in either forest. The only detected change was a shift in enzymatic activity. EC: EP and EN: EP ratios decreased compared with control treatment in the spruce stand due to the enhanced activity of EP (Table 1 and Table S1). After 4 years of treatment, N addition affected the bacterial community in the spruce stand while decreasing bacterial diversity in the beech stand in comparison with individual controls (Table S8). N-induced shifts were seen only in decreased Planctomycetes and Deltaproteobacteria relative abundances (Table S5). We found no effect of N manipulation on fungi. In particular, a sensitive response to N inputs might be expected mainly from the ectomycorrhizal portion of the fungal community. A decrease in abundance accompanied by changes in taxonomic composition is a well-documented reaction of the ectomycorrhizal community to elevated N loading (Arnolds 1991; Wallenda and Kottke 1998; Lilleskov, Hobbie and Horton 2011; Lilleskov *et al.* 2019). However, the described restructuring occurs mostly in N-limited forests, where the species adapted to prosper under low N availability prevail over the ectomycorrhizal community (Hasselquist and Högberg 2014; Choma *et al.* 2017). When the N availability is enhanced by increased N input, these nitrophobic fungi retreat in reaction to reduced tree belowground C flow and are replaced by less C-demanding nitrophilic ectomycorrhizal fungi (Olsson *et al.* 2005; Högberg *et al.* 2010; Lilleskov, Hobbie and Horton 2011; Lilleskov *et al.* 2019). In both stands, the ectomycorrhizal communities were already largely contributed to by N-tolerant taxa such as *Tylospora* sp., *Russula* sp. and *Hygrophorus* sp. (Lilleskov, Hobbie and Horton 2011). These forests were in past decades exposed to enhanced N deposition (Oulehle *et al.* 2011) and thus the systems including microbial communities were likely adapted to elevated N availability prior to experimental treatment (Oulehle *et al.* 2018). Moreover, the span of beech and spruce root systems is considerably larger than the experimental plot size of 3×3 m (Göttlicher *et al.* 2008). Therefore, the N treatment may be ineffective in changing the tree C allocation, which is a crucial event in the N input effect on ectomycorrhizal fungi (Lilleskov *et al.* 2019). Therefore, hypothesis (iv) was only partly supported. We observed a weaker effect of N addition on the composition of microbial communities compared with the acidifying treatment, but we found no considerable response from the ectomycorrhizal community.

CONCLUSIONS

The results of our 4-year field manipulation experiment enabled us to separate the effects of soil acidification and N addition on the structure and exoenzymatic activity of the soil microbial community in two forest stands common in Europe. Sulphuric acid-induced soil acidification caused rapid distinctive changes in soil microbial community composition at both sites. Conversely, extra N addition to a previously N-affected forest without an effect on soil pH was barely reflected by soil microbial communities. The increased acidity impacted the bacterial but not the fungal part of the community. Both acid treatments (S; S+N) immediately lowered soil pH at both sites, which was accompanied by a decrease in soil DOC concentrations. Through this manipulation, we generated a wide soil acidity gradient ranging from pH 2.9 to 5.0 along which the bacterial community composition changed with common features in both forests. Regardless of the significant differences in the original soil chemistry and microbial community structure between spruce and beech stands, the same groups of bacteria retreated after acidification, which resulted in mutually decreased soil bacterial diversity, when the treatment selected for acidophilic/acidotolerant oligotrophs. The shift in the bacterial community was related to a change in enzymatic activity. The magnitude of hydrolytic activity relied on bacterial (gene marker) abundance, while the 'targeting of hydrolytic activity' was linked to the bacterial community structure. However, when the stands are taken separately, this pattern was valid in the beech stand only. In the spruce stand, the hydrolytic activity was uncoupled from the microbial community but was correlated with the concentration of soil solution DOC. Probably, energy, C and nutrient limitation due to low organic matter bioavailability in the spruce stand stimulated microbes to enhance production of all hydrolytic enzymes regardless of their taxonomic composition. Additionally, the acidification-induced changes in the bacterial community composition coincided with decreased activity of phenol oxidases. Considered together, this suggests the non-negligible role of bacteria in determining soil enzymatic activity, at least in the first years of soil acidification when fungi remained irresponsive. We cannot exclude that the fungi contributed to changed enzymatic activity (both hydrolytic and oxidative) by modification of their action. However, given the modest changes in fungal community composition in treated soils and the lack of a continuous relationship between fungal community composition and enzymatic activity, we conclude that in our study shifts in bacterial community were more likely responsible for the alteration of soil enzymatic activity.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflicts of interest. None declared.

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9.5 Publication V

Růžek, M., Tahovská, K., Guggenberger, G., Oulehle, F. (under review). Litter decomposition in European coniferous and broadleaf forests under experimentally elevated acidity and nitrogen additions. Plant and Soil. (IF_{2019} = 3.299)

Litter decomposition in European coniferous and broadleaf forests under experimentally elevated acidity and nitrogen addition

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Abstract

Background Anthropogenically induced atmospheric sulfur (S) and nitrogen (N) deposition has impacted many regions across the Northern Hemisphere inducing acidification and eutrophication of terrestrial ecosystems. However, acidification and eutrophication processes may differently impact litter decomposition and thus soil carbon (C) dynamics.

Methods In historically air polluted two mountainous temperate forest stands (*Picea abies* and *Fagus sylvatica*), there has been six years ongoing a field manipulation experiment simulating elevated acidity and N inputs. In each forest stand, four treatments were established: control, acid addition (H_2SO_4 – 50 kg $S \cdot ha^{-1} \cdot year^{-1}$), N addition ($NH_4NO_3$ – 50 kg $N \cdot ha^{-1} \cdot year^{-1}$) and their combination. We utilized this design for litter decomposition experiment, when litter of various quality and origin (green tea, rooibos tea, spruce needles and beech leaves), in total 1536 samples, was buried below the organic layer and left to decompose up to 24 months. Retrieved samples were analysed for litter mass loss, the C/N ratio, and the concentration of lignin. Measurements of decomposition dynamics were complemented by data on soil water pH and soil respiration.

Results Acid additions decreased soil water pH, soil respiration and stabilized decomposition of the high-quality litter (green tea) in both stands, whereas mass loss of low-quality litter (rooibos tea, beech leaves, spruce needles) was reduced only in the spruce stand. Nitrogen treatments started to lower soil water pH in both stands after five years since the start of the experiment. Likely, N-induced lower soil pH constrained needle decomposition in the naturally more acidic spruce stand.

Conclusion Our study demonstrates a stabilizing effect of acidity on decomposition processes and soil C dynamics. The effect of N addition was insignificant, likely because of, previous ecosystem adaptation to historical N loadings.

Keywords: Litter decomposition, Nitrogen addition, Acidity, Tea bag initiative, Norway spruce, European beech

Introduction

Plant litter decomposition is an important process in element cycling by which carbon (C) and nutrients are released to the environment (Berg and McClaugherty 2009). It largely affects the balance of C between terrestrial ecosystems and the atmosphere. Decomposition process is driven primarily by climate and litter quality (Chapin et al. 2012), the latter being considered the primary factor (Bradford et al. 2015). Slowdown of decomposition can potentially lead to C and nutrient sequestration in soils (Jandl et al. 2007). Atmospheric sulfur (S) and nitrogen (N) deposition of anthropogenic origin (Galloway and Cowling 2002) has the potential to influence the vitality of forest ecosystems (Thomas et al. 2010; Kolář et al. 2015) by affecting various processes, causing the reduction in soil organic matter (SOM) decomposition (Knorr et al. 2005; Zhang et al. 2018).

The effect of N deposition is variable depending on the amount and duration of N addition (Knorr et al. 2005; Janssens et al. 2010). Terrestrial ecosystems are naturally N-limited (Vitousek 2004; LeBauer and Treseder 2008). In such systems with low ambient N deposition, increased N input can stimulate aboveground ecosystem productivity (Liu and Greaver 2010), soil microbial biomass and activity (Tahovská et al. 2020), and SOM decomposition (Knorr et al. 2005; Vivanco and Austin 2011), which as a result enhances ecosystem C fluxes (Feng et al. 2017). Multiple N addition studies, however, have documented a slow-down of decomposition together with increased soil C storage, especially from the long-term perspective (Fog 1988; Janssens et al. 2010; Ramirez et al. 2010; Zak et al. 2017). Several hypotheses have been proposed to explain N–induced inhibition of SOM decomposition, invoking the (a) lowering of production of lignolytic extracellular enzymes (phenol oxidase, peroxidase; Carreiro et al., 2000; Frey et al., 2004), (b) abiotic formation of recalcitrant compounds (Nommik and Vahtras 1982; Fog 1988; Thorn and Mikita 1992), and (c) lowering of microbial biomass and change of microbial community structure towards less C–demanding but more N–tolerant organisms (Ågren et al. 2001; Treseder 2008; Lilleskov et al. 2019). Greater soil N availability decreases relative tree belowground C allocation (Högberg et al. 2003) and its flow to root growth because less effort is need to acquire N from soil (Johnson and Thornley 1987; Litton et al. 2007). Reduced supply of plant–derived C leads to the weakening of plant-mycorrhizal interactions, to the retreat of C-demanding mycorrhizal species with proteolytic and oxidative abilities, and thus to the slowdown of SOM decomposition (Fog 1988; Averill and Waring 2018). Long-term or excessive N input can trigger the process of soil

92 acidification (Tian and Niu 2015), which in turn reduces microbial biomass and activity
93 and alters soil C fluxes (Pregitzer et al. 2004; Janssens et al. 2010; Zhou et al. 2017).

94 Excessive input of S and N have been found to lead to soil acidification manifested
95 by pH lowering, base cation leaching, mobilization of aluminium (Breemen et al. 1984;
96 Driscoll et al. 1989), and greater ionic strength of soil solutions (Hruška et al. 2009). All
97 these processes both directly and indirectly affect litter decomposition. The direct effect
98 resides in reduced dissolved organic carbon (DOC) solubility (Evans et al., 2012) and
99 associated reduced SOM decomposition rates (Wang et al. 2010). Indirectly, acidification
100 affects SOM decomposition through changes in microbial community composition and
101 structure (Rousk and Bååth 2011), size of the microbial biomass (Oulehle et al. 2018;
102 Choma et al. 2020), and its respiration and enzyme activity, causing C accumulation in
103 acidified soils (Oulehle et al. 2011; Wu et al. 2016).

104 Decomposition is a sequential process in which, simple C compounds are
105 decomposed first, followed by more complex C compounds. Therefore, litter mass loss
106 decreases with increasing time. Berg and Staaf (1980) proposed a two-phase model
107 describing the fate of plant litter during decomposition. The initial phase is controlled by
108 decomposition of water-soluble compounds and non-protected cellulose. The late phase is
109 represented mainly by degradation of lignified structures. Lignin is a major component of
110 plant litter with a polymeric structure of aromatic rings (Kögel-Knabner 2002), whose
111 decay is energy–demanding and needs to be broken down by extracellular enzymes such
112 as phenol oxidase or peroxidase (Sinsabaugh 2010).

113 Litter decomposition might be also explained through litter quality. Litter quality is
114 a factor affecting the activity of decomposers and their ability to degrade plant litter. Two
115 aspects – litter recalcitrance and stoichiometric microbial demands – drive, how fast
116 decomposition will run. Decomposition rates were correlated positively with lower plant
117 litter C/N and/or lignin/N ratios (Melillo et al. 1982; Taylor et al. 1989). However,
118 additional N input might affect the strategy of decomposers at sustaining their
119 stoichiometric balance. There are two opposite mechanisms explaining the effect of N
120 addition. In the first mechanism, decomposition is determined by stoichiometric litter
121 composition and microbial demands, decomposition being faster when the C/N litter ratio
122 is lower (Sterner and Elser 2002). The second mechanism is outlined by the microbial
123 nutrient mining hypothesis (Moorhead and Sinsabaugh 2006), according to which microbes

use simple C fractions to decompose more stable compounds and gain limiting nutrients. In this case, N addition would rather reduce decomposition through alleviation of N limitation. Thus, external atmospheric (i.e. nitrogen) input might distinctly affect decomposition rates depending on litter quality. Meta-analysis of Knorr et al., (2005) has shown that external N input has both negative and positive effects on the decomposition of low-quality and high-quality litter, respectively.

Natural forest composition in the Central European region would be mix of coniferous and broadleaved species (Szabó et al. 2017). However, for silvicultural reasons in the past, most forests have been converted into Norway spruce monocultures. Despite certain economic benefits for foresters in timber production, spruce monocultures are generally known for their low biodiversity or slowly decomposable litter (Augusto et al. 2015). Spruce litter accumulates on surface, creating relatively thick organic layer with the higher C/N ratio compared to beech litter (Vesterdal et al. 2008). Moreover, spruce needles are more efficient at scavenging atmospheric compounds, enhancing acidic deposition through greater dry deposition (Augusto et al. 2002; Schrijver et al. 2007). Soils under spruce are therefore usually more acidic, less fertile and more sensitive to further acidification, so the composition of soil microbial communities differs from those under beech (Oulehle and Hruška 2005; Bárta et al. 2017). Because Central Europe has been exposed to high S and N deposition over the last century, extensive spruce-dominated stands more lowered the quality of forest soil by acidification and eutrophication than beech-dominated stands would (Oulehle and Hruška 2005).

In the present study we conducted a field decomposition experiment to disentangle the effects of changing soil acidity and N availability on litter decomposition in stands dominated by different tree species. The experiment took place in two adjacent forest stands, one broadleaved (dominated by European beech, *Fagus sylvatica L.*) and one coniferous (a Norway spruce, *Picea abies (L.) Karst.*, monoculture), in a temperate mountainous region of Central Europe that has been greatly polluted by S and N deposition. Such comparison can further reveal the effects of tree species identity on decomposition under changing soil chemistry. The vicinity of forest stands filters out the effect of macroclimate as important driver of decomposition enabling the manifestation of stand-induced effects, including microclimate. The experiment was designed to identify direct effects of acidity and N addition on litter mass loss and lignin concentration of various litter types. We selected four types of material: two native lignin-rich litter types (needles and

leaves) and two standardized types of tea litter (green tea and rooibos), representing labile and more stable C forms, respectively (Djukic et al. 2018). Utilizing the infrastructure of a long-term field manipulation experiment (Oulehle et al. 2018), we supplemented our decomposition data by field measurement of soil respiration and soil water pH. Our hypotheses were that: (1) elevated soil acidity reduces litter decomposition and that (2) N addition stimulates the decomposition of high-quality litter (low C/N) and reduces the decomposition of low-quality litter (high C/N and lignin N).

Methods

Sites and experimental design

An acidity and N availability soil manipulation experiment has been running since 2014 (Oulehle et al. 2018). The experiment was established in two adjacent forests representing main types of Central European forest – 120 years old mature beech (*Fagus sylvatica* L.) and 80 years old spruce stands (*Picea abies* (L.) Karst.). The area, located on the border of Czechia and Germany (50.59 N, 13.26 E), has been greatly affected by acidic atmospheric deposition, which peaked in the 1980s (Dambrine et al. 1993; Oulehle et al. 2006). Because of the long-term acidic deposition, the soils in the area are characterized by low base saturation (7.5% and 6.4% in the spruce and the beech stand, respectively) and by low soil water pH (3.8 and 4.2 in spruce and beech stands, respectively; Růžek et al., 2019).

The experimental forest stands are situated close to each other (1 km), so they are exposed to the same climatic (6.3°C of mean annual temperature, mean annual precipitation ≈1,000 mm) and geological (gneiss bedrock) conditions. Soils at the stands are classified as Dystric Cambisol according to the WRB FAO classification (FAO 2014). Ground cover vegetation in the spruce stand is formed mainly by *Avenella flexuosa* L. Drejer with an admixture of *Vaccinium myrtillus* L., *Dryopteris dilatata* (Hoffm.) A. Gray, *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Galium saxatile* L. The ground cover vegetation in the beech stand (1.5%) consists primarily of *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Avenella flexuosa* L. Drejer. The annual ambient bulk deposition of S and N is 4.3 kg S $ha^{-1}yr^{-1}$ and 9.7 kg N ha^{-1} yr^{-1}, respectively (Růžek et al. 2019).

The soil manipulation experiment consisted of sixteen plots (3 × 3 m) subjected to four types of treatment with four replications in a randomized block design. The treatments were denoted as ‘control’ (Ctrl), ‘acid’ (S, 50 kg S ha^{-1} yr^{-1} as H_2SO_4), ‘nitrogen’ (N, 50 kg N $ha^{-1}\cdot yr^{-1}$ as NH_4NO_3) and ‘acid + nitrogen’ (S+N, 50 kg S ha^{-1} yr^{-1} as H_2SO_4 + 50 kg N

189 $ha^{-1} \cdot yr^{-1}$ as NH_4NO_3). Treatments were randomly distributed within each block. Between
190 years 2014 and 2019, the treatment solutions were added at monthly intervals during the
191 snow-free period between April and November, mixed with rainwater collected at the sites
192 and spread using 15-l watering cans evenly across the plots, including control plots. After
193 the application of solutes, an additional 15 l of rainwater were added to all the plots,
194 including the control ones, to ensure proper seepage of chemicals into the soil. The total
195 amount of water added was 27 $mm \cdot yr^{-1}$, which is equivalent to 2.5% of average rainfall.

196 After three years of our addition experiment (2016), both soils similarly responded
197 to the acid inputs by reductions in pH of 0.4–0.5 pH values and DOC concentrations by
198 35% and 48% in the spruce and the beech stand, respectively, in soil solutions draining the
199 forest floor (Oulehle et al. 2018). In the spruce stand, these changes were followed by a
200 decrease in soil respiration by 6–10% and changes in microbial structure composition such
201 as an increased fungi-to-bacteria ratio and increased ratios of enzymes involved in C
202 acquisition to those involved in N acquisition (Oulehle et al. 2018). By contrast, only
203 limited effects of acid input were observed in the beech stand. After three years, the N
204 addition alone did not significantly affect any of the response variables in either forest stand
205 (Oulehle et al. 2018).

206 **Litter decomposition experiment**

207 Between 2017 and 2019, litter bag decomposition experiments with native and standardized
208 litter were conducted in the experimental plots. As native litter we used naturally shed
209 leaves of *Fagus sylvatica* (L.) and needles of *Picea abies* (L.) Karst. growing at the study
210 sites. The litter was collected during 2016 using five litter baskets distributed evenly across
211 the forest stands. The litter was air dried at room temperature and 1.5-g portions of it were
212 packaged in polyethylene bags (mesh size: 0.1 mm) tied closed with plastic string and
213 marked with a tag.

214 Two kinds of standardized tea material were used for the experiment – green tea
215 (*Camellia sinensis*; EAN no.: 8 722700 055525) and rooibos tea (*Aspalanthus linearis*;
216 EAN no.: 8 722700 188438). Each tea bag consists of a string with a tag and a synthetic
217 bag (mesh size: 0.25 mm) filled with 2 g of tea material. Both materials represent extremes
218 in degradability rates, with expected high and low mass losses for green tea and rooibos
219 tea, respectively. In recent years, these materials were used as standard by several

international decomposition research initiatives (Keuskamp et al. 2013; Djukic et al. 2018; Mueller et al. 2018) as well as in local studies (Didion et al. 2016).

Tea and native litter differ in their quality. Green tea is rich in N and poor in lignin whereas rooibos tea has the opposite properties. Spruce needles are higher in both lignin and N compared to beech leaves; however, the lignin/N ratio is identical for both litter types. The native litter studied here is compositionally closer to rooibos tea, especially beech leaves with the same C/N ratio (Table 1).

Before being placed into the soil, all litter bags were air-dried and their initial weight was measured. The incubation time for all the materials was 3, 6, 12 and 24 months. For the whole experiment 1,536 bags were prepared (2 forest stands × 4 samplings times × 4 types of material × 4 treatments × 4 treatment replications × 3 replicates per plot). The experiment started in April 2017 and all litter bags were inserted beneath the O_h horizon of the forest floor and on top of the mineral soil (A horizon). After a given time, the tea bags were retrieved, oven-dried at 70°C for 48 hours, cleansed of soil particles and roots, and the remaining material was weighed to calculate mass loss. For identification of initial values, sort of material was analysed prior to decomposition. Three tea bags of individual tea type were mixed to get composite sample. For native litter, composite sample was obtained by litter homogenisation with collection of 5 samples.

Analytical methods

Collected samples were homogenized using an analytical mill IKA A11 (IKA®- Werke GmbH & Co. KG., Staufen, Germany). Prior to the analyses of C and N content, three replications of each litter type were mixed per plot, yielding composite samples for each plot. The contents of C and N in decomposed material were determined simultaneously by a Carlo-Erba Fusion 1108 analyser (Thermo, Torino, Italy).

Initial lignin content was analysed in all types of litter before the start of decomposition, furthermore in collected tea samples, which were incubated 3, 12 and 24 months. We mixed samples from four blocks together, creating one composite sample per treatment per site. Lignin-derived phenols were determined using alkaline CuO oxidation at 170°C for two hours (Hedges and Ertel 1982), followed by solid-phase extraction with C-18 columns (Mallinckrodt Baker Corp., Phillipsburg, New Jersey, USA). Phenols were eluted with ethyl acetate, dried under N_2, and derivatized with a 1:1 mixture of pyridine

and N,O-bis(trimethysilyl) trifluoroacetamide. The trimethylsilyl derivatives were separated and quantified using a gas chromatography column equipped with a mass-sensitive detector (GC/QP-2100, Shimadzu Corp., Kyoto, Japan) and an SPB-5 fused silica capillary column (30 m length, 0.25 mm inner diameter, 0.25-mm film; Supelco, Bellefonte, Pennsylvania, USA). Lignin phenols were identified by target ions and quantified according to response factors of external phenol standards. Ethylvanillin was added as an internal recovery standard prior to the CuO oxidation and phenylacetic acid was added before derivatization. CuO oxidation yields phenolic products (vanillyl, syringyl and cinnamyl units) that derive from lignin. Vanillyl (V) and syringyl (S) units may be present as aldehydes, carboxylic acids, and ketones, whereas cinnamyl (C) units include p-coumaric acid and ferulic acid. The sum of these phenolic compounds (V + S + C = VSC) is indicative of lignin content (Thevenot et al. 2010).

Soil respiration and soil solution pH

Soil CO_2 efflux (soil respiration) measurements were performed every month during the vegetation season (April – November) between 2017 and 2019 using a LiCor Survey Chamber (8100A-103 20 cm survey chamber; LiCor Biosciences, NE, USA) attached to an LiCor LI-8100A gas analyser. At treatment each plot, four soil collars were permanently placed 5 cm deep into the forest floor. Efflux of CO_2 was calculated based on the linear increase in chamber CO_2 concentration over the measurement period of 75 seconds.

Soil solution samples were taken during the vegetation season (April – November) when moisture conditions allowed it. To avoid any direct effect of treatment application on soil solution pH, samples were collected after rainfall events and before the application of treatments. Soil solution samples were collected by Rhizon® suction samplers (Rhizosphere Research Products, Wageningen, Netherlands) consisting of a 10 cm long 2.5-mm diameter porous membrane attached to 50 ml syringes. Five to seven Rhizon samplers were randomly installed into the forest floor of each plot to get one composite sample per plot. Samples were stored at 4°C and analysed for pH using a TTT-85 radiometer (Copenhagen, Denmark) with a combination electrode.

Data analysis

Data were tested for normality (Shapiro-Wilk), homogeneity of variance and skewness using Levene's test. Treatment effects on the measured variables (mass loss, C/N, lignin

concentration) were analysed using linear mixed-effects models with three fixed effects – Material, Stand and Treatments, and one random effect – Block (referring to block design). The analysis was conducted using the *lme4* package (Bates et al. 2015). Because the samples for analysis of lignin concentration were composites over blocks, we omitted Block as a factor. Post-hoc comparison of treatments within respective stands was done using estimated marginal means – *emmeans* v.1.5.2-1 (Lenth et al. 2020). One-way ANOVA was also used to assess differences in soil solution pH for each year (2017, 2018 and 2019). Soil respiration rates were tested for each year using repeated measures ANOVA (treatment as a fixed factor) for each stand and measurement period. Significant differences were further analysed by Fisher's LSD multiple comparison test for detecting differences between treatments. All statistical tests were performed with a significance level of 5% and were conducted using R 4.0.2 (R Development Core Team 2020).

Results

Mass loss

All types of material lost weight over the course of the study depending on the type of litter material, forest stand, time and treatment (Table 2). Averaged across both stands, the greatest total mass loss after 24 months was recorded for green tea and further decreased in the following order: green tea < rooibos < leaves ≈ needles (Table 3). The impact of tree species identity on mass loss depended on the type of material. While no effect of stand was observed during the decomposition of green tea, the beech stand increasingly profiled itself as more effective at the decomposition of all the complex materials (rooibos, leaves, needles) over time (Table 3). The acid treatments (S, S+N) reduced the decomposition of the green tea material in the spruce stand, with significant differences at months 12 and 24, when mass loss was lower by 8–11% compared to the control. In the beech stand, acid addition significantly affected decomposition only once, when S+N treatment reduced mass loss of green tea material by 7–8% relative to the control. For the other materials, the S-containing treatments reduced decomposition of other materials significantly in the spruce stand only. The S treatment had a retarding effect on mass loss at months 6 and 24 and at months 3 and 6 on rooibos tea and leaves, respectively (Table 3). Both S-containing treatments (S; S+N) reduced needle decomposition at month 24. Nitrogen addition only reduced needle decomposition in the spruce stand after 24 months of the experiment.

Litter C/N ratio

Litter C/N ratios were significantly affected by the stand, time and the type of material, but not by the treatment (Table 2). After its initial decrease, the C/N ratio of green tea changed significantly depending on the forest stand (Table 4). In the beech stand, the C/N ratio of decomposing green tea remained stable over time (≈ 9.8) whereas in the spruce stand it increased to 11.3. The C/N ratios of the other types of litter decreased over the course of the decomposition. Between-stand differences increased with the progression of decomposition, with lower C/N values recorded in the beech stand than in the spruce stand (Table 4).

Lignin concentration

The concentration of lignin-derived phenols (VSC) in both analysed materials (green tea and rooibos tea) increased in the initial months of decomposition. For green tea, no significant differences in lignin concentrations between the treatments were detected in either of the stands (Table 5). In the beech stand, lignin concentrations in rooibos tea were significantly different from the control plots under the S+N treatment only (Table 5). Rooibos lignin concentrations in the spruce stand differed significantly at month 3, when samples subjected to the S and S+N treatments contained less lignin compared to the control treatments.

Soil water pH

In the control plots, the pH of soil water in the spruce stand (3.79 ± 0.04) was significantly lower by 0.48 pH units ($p < 0.001$) than in the beech stand (4.24 ± 0.04; Figure 2). The acid treatments significantly decreased the pH of soil water in both stands. The pH of soil water from the beech plots under acid treatments declined more (by 0.44–0.55 pH units) than in the spruce stand (by 0.34–0.37 pH units). Nevertheless, the increase of H^+ concentration in acid treatments was greater in the spruce stand because of the overall lower soil solution pH compared to the beech forest. Nitrogen addition significantly lowered soil water pH in the spruce stand in 2019. In the beech stand, N enrichment decreased the average soil water pH over the average period of 2017–2019.

Soil respiration

The mean soil respiration rate was significantly greater in the spruce stand compared to the beech stand (3.27 ± 0.09 μmol CO_2 m^{-2} s^{-1}; 2.93 ± 0.10 μmol CO_2 m^{-2} s^{-1}, for spruce and beech, respectively; $p = 0.013$). Acid additions (S, S+N) reduced the annual CO_2 efflux in

both stands. Averaging over the observed period, the acid treatments reduced soil respiration by 13% and 15%, in the spruce and the beech stand, respectively. N addition had no effect on soil respiration in either stand (Figure 3).

Discussion

Our results show that induced soil acidity affects C-related soil processes, namely litter decomposition and soil respiration efflux in both the beech stand and the spruce stand. Soil respiration and decomposition of high-quality litter, represented by green tea, were reduced by acid treatments similarly in the two stands whereas the decomposition of low-quality litter (rooibos, leaves, needles) was suppressed only in the spruce stand. Nitrogen addition lowered soil water pH in the end of our observation in both stands, retarded needle decomposition after 24 months in the spruce stand only, otherwise had no other effect on decomposition nor soil respiration effluxes. These responses are consistent with previous results and extend observations of long-term a manipulative experiment of acid and N addition commenced in 2014 (Oulehle et al. 2018; Choma et al. 2020).

Effect of litter material and forest stand on decomposition

In line with previous studies (Melillo et al. 1982; Taylor et al. 1989), high-quality litter (green tea) with high initial N content, low C/N and/or lignin/N ratios got decomposed more than materials with the opposite properties after two years of *in-situ* decomposition (rooibos tea, beech leaves, spruce needles; Table 3). High-quality green tea contains large amounts of easily removable water-soluble fractions (49%), which most probably caused rapid mass loss in first months of incubation (Keuskamp et al. 2013). The limited mass loss of the native litter in both stands could be related to (a) the more complex structure of leaves and needles and/or to (b) the smaller mesh size of litter bags for needles and leaves samples compared to rooibos tea bags. Rooibos tea had less favourable initial litter characteristics than the native litter materials, but it was decomposed to a greater extent at the end of the study period (Table 1). On the other hand, native litter contains large amounts of other less degradable compounds (i.e. phenols), which were not part of our analyses (Johansson 1995). Despite this, we suppose that the mesh size of litter bags likely had a certain effect on the reduced decomposition of leaves and needles. Smaller mesh size can exclude access to litter by members of the larger soil fauna (Bradford et al. 2002). However, similar decomposition studies or meta-analyses (Knorr et al. 2005; Zhang et al. 2016) have shown

that N addition regulates litter decomposition equally regardless of the presence of the soil fauna.

The type of forest stand affected decomposition depending on litter quality. Whereas high-quality green tea was decomposed similarly in both stands, low-quality litter with higher lignin content was decomposed less under the spruce stand over time. It seems that litter decomposition is slower with increasing complexity of C in the spruce stand than in the beech stand. An inhibition effect of litter decomposition in the spruce stand has also been observed in other experiments (Albers et al. 2004; Berger and Berger 2014). It is frequently ascribed to unfavourable conditions of the spruce organic layer (Albers et al. 2004), which is made up of polyphenol rich needles. Polyphenols inhibit decomposition processes (Gallet and Lebreton 1995). Both forest stands share the same abiotic conditions (climate, bedrock, soil type) and an identical acidification history (Oulehle et al. 2016; Růžek et al. 2019). However, they differ in physiological stand parameters such as leaf area, leaf longevity, litter quality, root depth or stand age (Schrijver et al. 2007; Augusto et al. 2015). In our study, such parameters led towards (a) greater soil N availability in the beech stand (Oulehle et al. 2016), causing lower C/N in decomposing litter (Table 4); (b) increased acid inputs into the spruce stand, making soils under spruce naturally more acidic (pH ≈ 3.8) under ambient conditions than the beech stand (pH ≈ 4.2). Thus, another factor affecting litter decomposition was initial soil acidity.

Increased acidity as a decomposition driving factor

In the two distinct forest stands, manipulated acid addition created a pH gradient spanning in the ranges of 3.7–4.2 pH and 3.3–3.8 pH units in the beech and the spruce stand, respectively (Figure 1). The presented decomposition experiment showed a clear suppressing effect of acidity on litter decomposition, as was hypothesized. Nevertheless, the effects of experimental acid addition differed depending on litter quality.

Degradation of high-quality green tea was identically suppressed under acid addition in both stands. Based on the high proportion of an easily decomposed fraction in green tea, we assume that the reduced mass loss was a result of lower decomposition of labile C fraction. The mechanisms responsible for this pattern might be (a) reduced availability of litter C to decomposers, which is evidenced by lower DOC concentrations under S treatments (Oulehle et al. 2018); (b) changes in bacterial community composition and microbial biomass, especially in spruce stand (Choma et al. 2020), making

409 decomposers ineffective at the standard degradation of labile C. Limited accessibility of
410 labile C may be relevant for the degradation of less degradable C fractions, because easily
411 degradable organic compounds serve as energy sources for microorganisms supporting the
412 decomposition of more complex C such as lignin (Klotzbücher et al. 2011). As a result,
413 unavailability of labile C might cause reduced decomposition of recalcitrant organic matter.
414 Mass loss of rooibos tea, leaves and needles was suppressed in the spruce stand but
415 remained unresponsive in the beech stand. A similar manipulation experiment (64 kg $S \cdot ha^{-1} \cdot yr^{-1}$)
416 carried out in a boreal spruce forest (Berg, 1986) also showed retarded
417 decomposition of spruce needles. Theoretically, low-quality litter should be composed of a
418 relatively smaller proportion of rapidly decomposing soluble compounds but a greater
419 amount of recalcitrant lignified fractions than high-quality litter (Berg and McClaugherty
420 2009). However, the composition of each litter type is specific. Depending on the type of
421 litter, mass loss was suppressed in both the first months (rooibos, beech leaves) and the
422 later months of the experiment (rooibos, spruce needles). With respect to the two-phase
423 decomposition model (Berg and Staaf 1980), we assume that acidity addition manifested
424 itself in the stabilization of low-quality litter in two ways. The first mechanism is related
425 with reduced decomposition of labile C in the first months as well as green tea (see above).
426 Evidence supporting this explanation is provided by a lower rooibos VSC yield under the
427 acid treatments at month 3 in the spruce stand, which might correspond with a greater
428 content of the stabilized labile C fraction (Table 5). The second mechanism was limited
429 decomposition of lignified tissues in later months. Because of its complex structure, lignin
430 is decomposed using extracellular enzymes. These enzymes are often synthesized by fungi,
431 but bacteria may also be relevant to their production (de Gonzalo et al. 2016). In a parallel
432 study, Choma et al. (2020) found acidity-driven shifts in bacterial community composition,
433 accompanied by decreased phenol oxidase activity. Likely, acidity-induced changes in
434 bacterial communities resulted in reduced lignin degradation and therefore slower litter
435 decomposition. However, our analysis of lignin content in decomposing rooibos material
436 did not reveal any differences between treatments in the spruce stand in later months,
437 probably because lignin concentration, and not lignin mass, was measured. In comparison,
438 too low VSC yields in the S+N treatment in the beech stand were most probably a result of
439 more extensive presence of fungal hyphae (visually observable) in decomposed samples.
440 Litter decomposition and soil respiration thought to be tightly linked with microbial
441 decomposers that transform litter C to CO_2. Soil respiration only partly reflects soil
442 microbial activity because, beside a heterotrophic fraction, it also contains an autotrophic

component (Högberg et al. 2001). It is therefore difficult to detect changes in soil CO_2 efflux that are caused by heterotrophic decomposition of litter and SOM. In the spruce stand, the acidity-induced decrease in decomposition coincided with lower overall *in situ* measured soil respiration efflux by 13% compared to the control plots. Similar responses to acidity were reported elsewhere (Feng et al. 2017). Moreover, on our locality the acid treatments reduced soil microbial biomass in the spruce stand (Choma et al. 2020). Taken altogether, the effect of acidity was so significant that lower soil microbial biomass reduced litter decomposition and, in turn, soil respiration. The response of beech soil respiration was similar (15%) but without any connection to litter decomposition. The non-uniform response in beech soil C dynamics might be related to lower soil acidity, which would not affect all soil C processes. Furthermore, changes in CO_2 effluxes produced by autotrophic organisms, for example by root respiration, might affect soil respiration unpredictably (Hanson et al. 2000).

Soil system responses to elevated nitrogen availability

As nitrogen is a nutrient, N and C dynamics are tightly interlinked in SOM transformations. Since the start of the experiment in 2014, no responses induced by N treatment, such as changes in microbial community composition (Choma et al. 2020) or DOC production (Oulehle et al. 2018), were observed in any of the stands. In the period of 2014–2018, N inputs did not affect soil water pH. After 2018, decreases in soil water pH were observed in both stands but did not reach the levels seen in acid-treated plots (Figure 1). Similarly, no response in soil respiration (Figure 2) and a limited response of litter decomposition (Table 3) were observed under the N treatment. Only spruce needle decomposition was reduced in the spruce stand.

Numerous studies have observed that N addition often increases mass loss of materials with low lignin content but decrease that of high-lignin litter (Berg and Matzner 1997; Carreiro et al. 2000; Knorr et al. 2005). This pattern was explained by N stimulation of cellulose decomposition in high-quality litter, and inhibition of lignin decomposition was explained by suppressed activity of lignin-degrading enzymes (Carreiro et al. 2000; Magill and Aber 2000; DeForest et al. 2004). However, other studies found only a suppressing effect on litter decomposition irrespective of litter quality (Zhang et al. 2016). None of the theories on decomposer strategy turned out to be valid, because N addition did not alter mass loss in most of the cases. The observed less advanced spruce needle decay

476 in the spruce stand compared to the control plots could be a result of (a) reduced soil pH,
477 or (b) abiotic interaction of external N with phenolic compounds (Fog 1988; Janssens et al.
478 2010). Although newly produced stable compounds were observed in the soil after N
479 fertilization (Nommik and Vahtras 1982; Thorn and Mikita 1992), more recent studies did
480 not ascribe them major importance for the recalcitrance of SOM (Sjöberg et al. 2004).
481 Because the reduction in needle mass loss under the N treatment was comparable with the
482 analogous reductions under the S and S+N treatments, we suggest that N addition affected
483 decomposition through a decrease in pH, with the same consequences as in the acid
484 treatments. Likely, the lack of responses in the decomposition of the other types of litter
485 under elevated N was a result of high historical ambient N inputs. Forest soils have
486 probably adapted to high N availability, so artificially elevated N inputs have no effect on
487 C dynamics after six years of N addition of a cumulative dose of 300 kg $N \cdot ha^{-1}$.
488

489 ## Conclusion

490 Our decomposition experiment clearly demonstrates how acidity constrains litter
491 decomposition in the spruce and the beech forest stands. Whereas high-quality litter (green
492 tea) was degraded equally in both stands, the naturally less acidic beech stand was able to
493 faster decompose low-quality litter (rooibos, leaves, needles). The acid treatment, which
494 decreased soil water pH by ~0.4 pH units, uniformly resulted in limited decomposition of
495 high-quality litter in both forest stands, probably as a result of inaccessibility of labile litter
496 C. Initially more acidic soil in the spruce stand was more sensitive to acid inputs than soil
497 under beech, as the acid treatments reduced both decomposition of low-quality litter and
498 soil respiration. By contrast, in the beech stand, only lowered soil respiration was observed.
499 After six years of experimental addition, elevated N inputs started to decrease soil water
500 pH in both stands, which retarded needle mass loss in the spruce stand, but not soil
501 respiration. The naturally more acidic spruce stand was sensitive to decomposition changes
502 caused by acid treatment additions compared to the beech stand. We suggest that spruce
503 forests are more sensitive to elevated deposition because decomposition of their litter slows
504 down more than in beech forests. Spruce forests would thus accumulate more C under acid
505 input into the soil than beech forests.

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Figure 1: Mean annual and average (2017–2019) soil water pH in beech and spruce forest stand plots subjected to control, nitrogen (N), sulfur (S) and sulfur + nitrogen (S+N) treatments (n=4 per treatment and forest stand). Different lowercase letters indicate significant differences found by one-way ANOVA between treatments at $p < 0.05$. In the spruce stand, a lack of observations from control plots did not permit statistical evaluation in 2018.

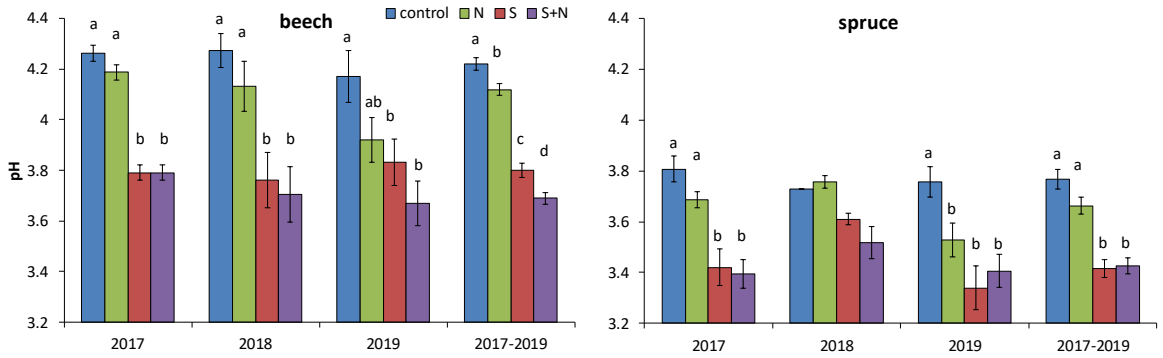
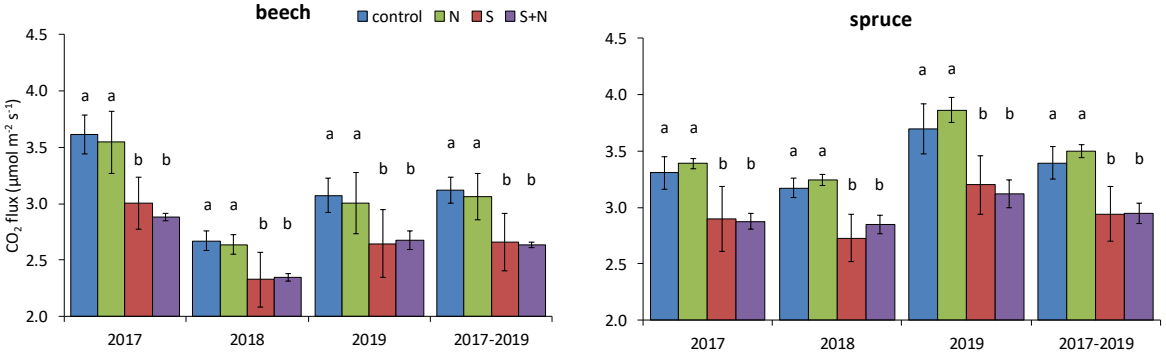


Figure 2: Mean annual and average (2017–2019) soil CO_2 efflux in beech and spruce forest stand plots subjected to control, nitrogen (N), sulfur (S) and sulfur + nitrogen (S+N) treatments (n=4 per treatment and forest stand). Different lowercase letters indicate significant differences identified by repeated measures ANOVA between treatments at $p < 0.05$.



768 Table 1: Initial characteristics of decomposed litter materials.

	Lignin (mg/g)	N (mg/g)	Lignin/N	C/N
Green tea	5.5	35.2	0.2	13.4
Spruce needles	29.7	10.6	2.8	45.3
Beech leaves	24.0	8.9	2.7	53.2
Rooibos tea	34.7	8.8	3.9	53.2

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770 Table 2: Results of factorial ANOVAs (F- and p-values) of the effects of forest stand, treatment, time and material on mass remaining and the C/N ratio.

	Mass remaining		C/N ratio	
	F-value	p-value	F-value	p-value
Stand	36.1	<0.0001	11.8	0.0006
Treatment	9.7	<0.0001	2.3	0.07
Time	576.9	<0.0001	40.8	<0.0001
Material	2160.3	<0.0001	1641.9	<0.0001

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Table 3: Mass remaining of decomposed litter samples after 3, 6, 12 and 24 month. Values are presented as percentages (%) of initial litter weight. Means ± SE (n=4) are presented. Capital letters indicate significant differences ($p<0.05$) between materials within each stand, month and treatment. Small letters denote significant differences ($p<0.05$) between treatments within each stand, material and month. Missing letters signify non-significant differences. The last column indicates stand differences at $p<0.05$ (+), $p<0.01$ (++) and $p<0.001$ (+++); n.s. – not significant.

		Beech				Spruce				Stand diff.
	Month	Control	N	S	S+N	Control	N	S	S+N	
Green tea	3	43(2.8)^{A,a}	47(1.5)^{A,ab}	46(1.9)^{A,ab}	49(0.6)^{A,b}	47(1.6)^{A,ab}	45(2.6)^{A,a}	50(0.7)^{A,b}	50(1.0)^{A,b}	n.s.
	6	36(2.4)^{A,a}	39(1.4)^{A,ab}	40(1.9)^{A,ab}	43(1.0)^{A,b}	38(2.2)^{A}	37(2.0)^{A}	42(1.5)^{A}	43(1.7)^{A}	n.s.
	12	33(1.0)^{A}	31(2.2)^{A}	36(2.5)^{A}	38(1.0)^{A}	28(1.5)^{A,a}	29(1.0)^{A,a}	39(2.4)^{A,b}	36(0.8)^{A,b}	n.s.
	24	30(2.2)^{A,a}	26(1.1)^{A,a}	30(3.4)^{A,a}	38(0.8)^{A,b}	27(2.3)^{A,a}	29(0.7)^{A,ab}	37(1.3)^{A,b}	35(1.9)^{A,b}	n.s.
Rooibos	3	80(0.8)^{B}	79(0.7)^{B}	80(1.2)^{B}	78(0.4)^{B}	79(1.0)^{B,ab}	78(0.9)^{B,a}	81(0.2)^{B,b}	81(0.3)^{B,b}	n.s.
	6	67(1.8)^{B}	69(1.0)^{B}	69(0.7)^{B}	68(1.4)^{B}	68(0.6)^{B,a}	69(1.5)^{B,a}	73(0.6)^{B,b}	70(0.9)^{B,a}	+
	12	57(2.6)^{B}	59(1.1)^{B}	61(2.5)^{B}	61(2.7)^{B}	62(1.7)^{B}	63(0.6)^{B}	66(1.1)^{B}	64(1.3)^{B}	+++
	24	45(4.4)^{B}	47(3.4)^{B}	47(4.2)^{B}	38(5.9)^{A}	51(1.7)^{B,a}	53(1.7)^{B,a}	57(1.5)^{B,b}	54(0.9)^{B,a}	+++
Leaves	3	91(0.8)^{C}	91(1.1)^{C}	90(0.9)^{C}	89(0.3)^{C}	88(0.2)^{C,a}	89(0.6)^{C,ab}	91(1.1)^{C,b}	90(1.3)^{C,ab}	n.s.
	6	80(1.8)^{C}	79(1.4)^{C}	79(1.3)^{C}	78(0.8)^{C}	80(1.1)^{C,a}	81(0.4)^{C,ab}	84(1.2)^{C,b}	83(1.1)^{C,ab}	++
	12	73(2.1)^{C}	72(2.9)^{C}	74(1.1)^{C}	74(0.6)^{C}	74(0.7)^{C}	76(0.6)^{C}	78(2.5)^{C}	77(1.5)^{C}	++
	24	60(4.4)^{C}	65(2.1)^{C}	62(4.2)^{C}	64(1.3)^{B}	69(1.3)^{C}	72(1.4)^{C}	71(0.9)^{C}	69(1.3)^{C}	+++
Needles	3	85(0.4)^{D}	86(0.6)^{D}	86(0.3)^{D}	85(0.5)^{D}	85(0.6)^{C,ab}	85(0.7)^{C,ab}	86(0.7)^{D,a}	84(0.7)^{B,b}	n.s
	6	79(0.4)^{C}	80(0.8)^{C}	79(0.6)^{C}	79(0.6)^{C}	79(0.6)^{C}	80(0.6)^{C}	80(1.0)^{C}	79(1.1)^{C}	++
	12	74(1.5)^{C}	74(0.4)^{C}	75(1.6)^{C}	75(1.2)^{C}	75(1.0)^{C}	75(0.6)^{C}	77(0.6)^{C}	76(0.5)^{C}	++
	24	61(1.5)^{C}	60(4.1)^{C}	59(5.2)^{BC}	63(0.5)^{B}	65(1.2)^{D,a}	70(1.2)^{C,b}	70(0.5)^{C,b}	69(1.4)^{C,b}	+++

Table 4: Carbon-to-nitrogen ratios in decomposed litter samples after 3, 6, 12 and 24 month. Means ± SE (n=4) are presented. Capital letters indicate significant differences ($p<0.05$) between materials within each stand, month and treatment. Small letters denote significant differences ($p<0.05$) between treatments within each stand, material and month. Missing letters signify non-significant differences. The last column indicates stand differences at $p<0.05$ (+), $p<0.01$ (++) and $p<0.001$ (+++); n.s. – not significant.

		Beech				Spruce				Stand diff.
	Month	Control	N	S	S+N	Control	N	S	S+N	
Green tea	3	8.5(0.2)^{A}	9.0(0.3)^{A}	8.8(0.3)^{A}	9.2(0.2)^{A}	9.3(0.3)^{A}	9.5(0.2)^{A}	10.1(0.3)^{A}	9.6(0.2)^{A}	++
	6	8.8(0.4)^{A}	8.5(0.3)^{A}	8.6(0.2)^{A}	8.9(0.1)^{A}	10.2(0.4)^{A}	9.7(0.2)^{A}	10.2(0.3)^{A}	9.9(0.4)^{A}	+++
	12	10.1(0.7)^{A}	10.0(0.4)^{A}	9.9(0.4)^{A}	9.3(0.5)^{A}	13.2(0.2)^{A,a}	11.4(0.5)^{A,ab}	11.0(0.6)^{A,b}	10.6(0.4)^{A,b}	+++
	24	10.3(0.5)^{A}	9.8(0.4)^{A}	9.8(0.4)^{A}	9.1(0.3)^{A}	12.3(0.5)^{A}	11.2(0.4)^{A}	10.8(0.2)^{A}	10.9(0.2)^{A}	+++
Rooibos	3	36.5(2.1)^{B}	36.1(1.9)^{B}	39.8(1.9)^{B}	38.7(2.4)^{B}	38.9(2.5)^{BC}	40.0(2.2)^{BC}	41.5(1.3)^{B}	41.0(2.5)^{B}	n.s.
	6	33.4(2.0)^{B}	29.7(2.1)^{B}	29.0(1.4)^{B}	32.2(1.5)^{B}	40.7(1.1)^{B}	37.1(2.1)^{BC}	38.2(2.1)^{B}	34.8(2.6)^{B}	+++
	12	34.6(1.9)^{B}	33.6(1.4)^{B}	37.4(3.3)^{B}	35.8(3.5)^{B}	43.0(1.4)^{B}	38.4(1.5)^{B}	38.8(1.1)^{B}	37.6(1.0)^{B}	+
	24	30.9(1.2)^{B}	31.7(0.8)^{B}	31.0(0.3)^{B}	26.1(2.1)^{B}	36.5(0.7)^{B}	36.0(1.2)^{B}	35.6(1.7)^{B}	34.4(1.8)^{B}	+++
Leaves	3	43.8(1.6)^{BC}	43.4(1.9)^{BC}	39.0(1.9)^{B}	40.4(2.3)^{B}	36.7(1.5)^{B}	37.6(2.7)^{B}	40.6(3.6)^{B}	40.3(2.6)^{B}	n.s.
	6	38.2(2.6)^{B}	38.3(0.4)^{C}	36.8(1.8)^{C}	35.0(1.2)^{B}	33.6(1.2)^{C,ab}	32.1(3.2)^{B,a}	39.0(2.7)^{C,b}	35.7(1.9)^{BC,ab}	n.s.
	12	35.7(0.7)^{B}	33.3(0.5)^{B}	34.6(1.1)^{B}	35.2(1.5)^{B}	35.2(1.4)^{C}	35.2(2.2)^{B}	39.4(2.6)^{B}	35.5(1.7)^{B}	n.s.
	24	31.3(1.2)^{B}	29.1(0.7)^{B}	29.1(0.6)^{B}	28.6(0.5)^{B}	33.7(1.2)^{B}	32.1(1.2)^{B}	34.7(1.1)^{B}	34.3(0.5)^{B}	+++
Needles	3	44.5(1.4)^{C}	46.7(2.0)^{C}	43.5(1.4)^{C}	43.8(0.4)^{C}	51.7(3.4)^{C}	51.8(2.7)^{C}	49.8(2.8)^{B}	49.8(3.5)^{B}	++
	6	45.5(1.6)^{C,ab}	42.3(1.9)^{C,a}	43.0(1.8)^{D,b}	41.7(2.7)^{C,ab}	45.4(1.9)^{B}	41.4(1.2)^{C}	49.4(1.1)^{B}	44.5(2.7)^{C}	n.s.
	12	48.8(2.6)^{C}	48.2(2.0)^{C}	50.3(1.6)^{C}	49.5(2.5)^{C}	50.1(1.1)^{B}	47.3(1.4)^{C}	48.7(0.7)^{C}	48.7(1.4)^{C}	n.s.
	24	38.1(0.9)^{C}	42.5(1.4)^{C}	43.8(1.8)^{C}	41.1(2.9)^{C}	38.2(1.5)^{B}	35.7(0.8)^{C}	41.3(1.6)^{C}	38.2(1.2)^{C}	+

790 Table 5: Lignin concentrations in decomposed green tea and rooibos tea samples in respective treatments in both stands. Values are sums of concentrations
791 ($mg \cdot g^{-1}$ C_{org}) of lignin-derived phenols (vanillyl, syringyl, cinnamyl). Data are presented as a mean ± SE (n=2). Small letters indicate significant differences
792 between treatments within each stand, month and litter material.

		Beech				Spruce			
	Month	Control	N	S	S+N	Control	N	S	S+N
Green tea	0	11.6(0.4)				11.6(0.4)			
	3	14.9 (1.9)	14.3(1.9)	14.9(1.9)	14.3(2.7)	13.6(1.8)	12.4 (1.8)	12.2(1.8)	11.1(2.5)
	12	17(1.9)	18.1(1.9)	16.3(1.9)	19.2(1.9)	23 (1.8)	19.5(1.5)	18.0(1.8)	17.5(1.8)
	24	15.3(1.9)	16.1(1.9)	14.3(1.9)	14.1(1.6)	18.6(1.5)	15.4(1.8)	13.9(1.8)	16.3(1.8)
Rooibos	0	74.2(1.1)				74.2(1.1)			
	3	96.2(1.6)^{a}	92.4(1.9)^{a}	94.9(1.9)^{a}	82.9(1.9)^{b}	104.4(1.5)^{a}	98.4(1.8)^{ab}	91.8(1.8)^{bc}	89.3(1.8)^{c}
	12	94.1(1.4)^{a}	88.8(1.6)^{ab}	91.2(1.6)^{a}	83.7(1.9)^{b}	103.4(1.5)	105.3(1.8)	102.4(1.8)	100.5(1.8)
	24	101.1(1.9)^{a}	95.6(1.9)^{a}	95.3(1.9)^{a}	74.2(1.9)^{b}	81.3(1.5)^{ab}	83(1.8)^{ab}	87.4(1.8)^{a}	79.8(1.8)^{b}

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