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Effect of anthropogenic nitrogen inputs on the structure and function of ectomycorrhizal communities in spruce forests

Ph.D. Thesis

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Annotation

The effect of anthropogenic nitrogen (N) inputs on the structure of ectomycorrhizal fungal communities in Norway spruce forests was investigated. With the use of soil DNA amplicon sequencing, ectomycorrhizal communities were described in spruce forests exposed historically to different anthropogenic N deposition and experimental manipulative N treatments. This information was put into context with soil biochemical properties and rates of soil processes involved in C and N cycling in order to reveal possible consequences of ectomycorrhizal community restructuring for soil and ecosystem functioning.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. Zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Rappe-George, M.O., **Choma, M.**, Čapek, P., Börjesson, G., Kaštovská, E., Šantrůčková, H., Gärdenäs, A.I., 2017. Indications that long-term nitrogen loading limits carbon resources for soil microbes. Soil Biology and Biochemistry 115, 310–321. (IF = 5.290)
Michal Choma participated in soil sampling, biochemical analyses and PLFA extraction, was responsible for assembly of soil biochemical data and contributed to writing and revisions of the manuscript.

II. **Choma, M.**, Rappe-George, M.O., Bárta, J., Čapek, P., Kaštovská, E., Gärdenäs, A.I., Šantrůčková, H., 2017. Recovery of the ectomycorrhizal community after termination of long-term nitrogen fertilisation of a boreal Norway spruce forest. Fungal Ecology 29, 116–122. (IF = 3.990)
Michal Choma participated in soil sampling, performed the DNA extraction, processed and evaluated the sequencing data, wrote the first version of the paper and completed revisions.

III. Tahovská K., **Choma M.**, Kaštovská E., Oulehle F., Bárta J., Šantrůčková H., Moldan F., 2020. Positive response of soil microbes to long-term nitrogen input in spruce forest: results from Gårdsjön whole-catchment N-addition experiment. Soil Biology and Biochemistry. (in press, IF = 5.290)
Michal Choma processed and evaluated the sequencing data and contributed to writing and revisions of the manuscript.

IV. **Choma, M.**, Tahovská, K., Kaštovská, E., Bárta, J., Oulehle, F. Uniform rapid response of soil micorbes to acidification in spruce and beech forests. – manuscript
Michal Choma processed the sequencing data, evaluated all the data and prepared first draft of the manuscript.

Contents

Abbreviations

AM – arbuscular mycorrhiza
C – carbon
DOC – dissolved organic carbon
DON – dissolved organic nitrogen
ECM – ectomycorrhiza
EMF – ectomycorrhizal fungi
ERM – ericoid mycorrhiza
ITS – internal transcribed spacer
N – nitrogen
NH_4^+ – ammonium
NO_3^- – nitrate
S – sulphur
SOM – soil organic matter
P – phosphorus
PLFA – phospholipid fatty acid

1. General introduction

1.1. Background

The absolute majority of plants forms associations with soil microorganisms. In return for instant supply of simple organic compounds released by roots, soil microbes provide plant with multiple beneficial services. Most importantly, they enhance plant nutrient acquisition (Bais et al., 2006; Nannipieri et al., 2007). A typical form of such cooperation is symbiosis with mycorrhizal fungi. In boreal and temperate forest ecosystems (only forests thereafter), the most common type is ectomycorrhiza (ECM; Smith and Read, 2008). Soils of these ecosystems are usually rich in organic matter, but often poor in available nutrients (Högberg et al., 2017; Pan et al., 2011). Ectomycorrhizal fungi (EMF), making a substantial part of microbial biomass in forest soils (Högberg and Högberg, 2002), and aside of the saprotrophic fungi they may be effective in enzymatic decay of organic matter and a release of incorporated nutrients (Cairney, 2011; Smith and Read, 2008; Zak et al., 2019) that they further transfer to roots of their hosts (Read and Perez-Moreno, 2003). The EMF thus play key roles in C and nutrient cycling and affect the whole ecosystem functioning (Baldrian, 2017; Tedersoo and Bahram, 2019; Uroz et al., 2016; Zak et al., 2019).

In past decades, forests were exposed to increased nitrogen (N) input caused by intensive human activities (Galloway et al., 2004; Kopáček and Posch, 2011), which considerably influenced ECM associations and led to changes in EMF communities and their activity (Lilleskov et al., 2019). Commonly, EMF decrease in abundance and their community structure changes (e.g. Arnolds, 1991; Cox et al., 2010; Kjøller et al., 2012). Due to considerable differences in ability to utilize organic N sources among EMF species, the restructuring of EMF community might lead to a change in overall ecosystem functioning (Bahr et al., 2013, 2015; Högberg et al., 2014a; Kjøller et al., 2012).

1.2. Mycorrhizal symbiosis

Mycorrhiza is a symbiotic relationship between plant roots and fungi. The cooperation is based on exchange of assimilates produced by plant for nutrients and water acquired by fungus, but also other provided benefits such as a plant protection from pathogens (Smith and Read, 2008). The overwhelming majority of terrestrial plants forms mycorrhizas and the cooperation between ancient plants and first mycorrhizal fungi is hypothesised as a prerequisite for prehistoric land colonisation by higher plants (Boer et al., 2005). Mycorrhizal symbiosis is a tight relationship, in which both partners are to a large extent dependent on each other. Plants with mycorrhizal symbionts better face stressful environmental conditions and, vice versa, fungi are believed to be unable to complete their life cycle without a support of their plant partner (Courty et al., 2010; Nicholson and Jones, 2017; Taylor and Alexander, 2005). Plants may invest up to one third of photosynthetically fixed carbon (C) to fungus (Leake et al., 2004), which in turn considerably increases plant nutrient uptake. For instance, up to 86% of plant N might be acquired and transferred by fungi in nutrient poor arctic environment (Hobbie and Hobbie, 2006).

Mycorrhiza has been classically regarded as a mutually beneficial cooperation. Nevertheless, the relationship is dynamic and both partners are able to regulate the amount of compounds passed to their symbionts (Lindahl et al., 2002). While plants cannot completely cease the flow of assimilates to roots, fungi are more flexible in restricting partner's access to gathered resources. In specific conditions, such as strong N limitation, mycorrhizal fungi might completely isolate plant root tips from soil and while utilising permanent host C flow use acquired N for own purposes rather than pass it to the tree and thus even intensify its N limitation (Franklin et al., 2014; Näsholm et al., 2013). On the other hand, in specific cases such as in early spring, when deciduous trees are restricted by undeveloped foliage, mycorrhizal fungi may provide its host with C as well (Courty et al., 2007). Therefore, rather than always bilaterally beneficial, ectomycorrhizal symbiosis needs to be perceived

as a dynamic relationship, where one of partners might modify its behaviour to increase own fitness at the expense of the opposite, i.e. even parasitize (Ågren et al., 2019; Johnson et al., 1997).

Several types of mycorrhiza are distinguished according to their morphology, taxonomy and functioning. Ectomycorrhiza (ECM), ericoid (ERM) and arbuscular mycorrhiza (AM) are the most commonly found in forests. ECM is formed by trees, ERM by ericaceous shrubs and AM by both woody and non-woody plants (Smith and Read, 2008). Fungi forming ECM and ERM mostly belong to Ascomycota or Basidiomycota and often are able to mine for nutrients stored in organic matter due to production of extracellular enzymes. On the other hand AM fungi belong exclusively to Glomeromycota and lack effective enzymatic apparatus and therefore rely solely on readily available mineral or very simple organic forms of N (reviewed in Tedersoo and Bahram, 2019). In result, AM association is more abundant in nutrient rich soils, whereas ECM and ERM dominate in soils rich in organic matter but with limited availability of nutrients (Phillips et al., 2013).

1.3. Ectomycorrhiza and ectomycorrhizal fungi

Ectomycorrhizal symbiosis evolved independently multiple times in more than 80 fungal lineages (Hibbett and Matheny, 2009; Tedersoo and Smith, 2017) and so far has been described over 5,000 EMF species, mostly belonging to Basidiomycota and Ascomycota (Rinaldi et al., 2008; Taylor and Alexander, 2005; Tedersoo et al., 2009). EMF make a symbiotic relationship with over 8,000 plant, mostly woody, species (Rinaldi et al., 2008; Taylor and Alexander, 2005). Single tree species is able to cooperate with up to hundreds of EMF species, but although majority of EMF are generalists, some of them are strictly specialised to one plant family, genus or even species (Bruns et al., 2002).

The initiation of ectomycorrhizal symbiosis is preceded by bilateral chemical signalisation (Garcia et al., 2015). With help of this communication, EMF locate tree roots. EMF colonise the most subtle part of roots system –

fine roots. The root tip is covered by a compact net of hyphae that gives rise to hyphal mantle while in the root intercellular space fungal filaments create the Hartig net. The Hartig net is an interface where the communication between tree root and EMF occurs (Smith and Read, 2008). The colonisation frequency might reach almost 100 % of present root tips (Jensen et al., 2003; Taylor et al., 2000). In order to search for water and nutrients, extramatrical mycelium spreads to surrounding soil from the hyphal mantle. The reach of peripheral mycelium is species specific and might range from millimetres to tens of centimetres. Some EMF species form specific structures, for instance rhizomorphs, which allow them a transport of C and nutrients at long distances.

According to differentiation and length of extramatrical mycelia, EMF species are divided into exploration types (Fig. 1; Agerer, 2001). The EMF exploration type implies fungus' function in the soil and requirements for plant C support. The medium- and long-distance types are effective in soil proliferation, nutrient acquisition and translocation. However the production of extensive mycelia and extracellular enzymes is highly C demanding, thus requires a strong supply of plant carbohydrates (Agerer, 2001; Kohler et al., 2015; Weigt et al., 2012). Conversely, contact and short-distance EMF have relatively lower need for C, but their reach out of roots is only in the range of millimetres to centimetres. Their enzymatic potential is low, which constrains nutrient acquisition in oligotrophic soils (Agerer, 2001; Weigt et al., 2012).

The peripheral mycelia of different EMF individuals might be interconnected in the ectomycorrhizal network. This network links mycelia within one, but also among multiple EMF species including their host trees. It enables C exchange between EMFs and supposedly between different tree individuals and species (Simard and Durall, 2004). By this mean, C and nutrients might be passed from one fungus or tree to another based on their different needs or photosynthesis effectivity (Nara, 2006; Simard et al., 1997; Simard and Durall, 2004).

The area of soil under direct influence of ECM root tips and EMF extramatrical mycelium, called ectomycorrhizosphere, harbours specific

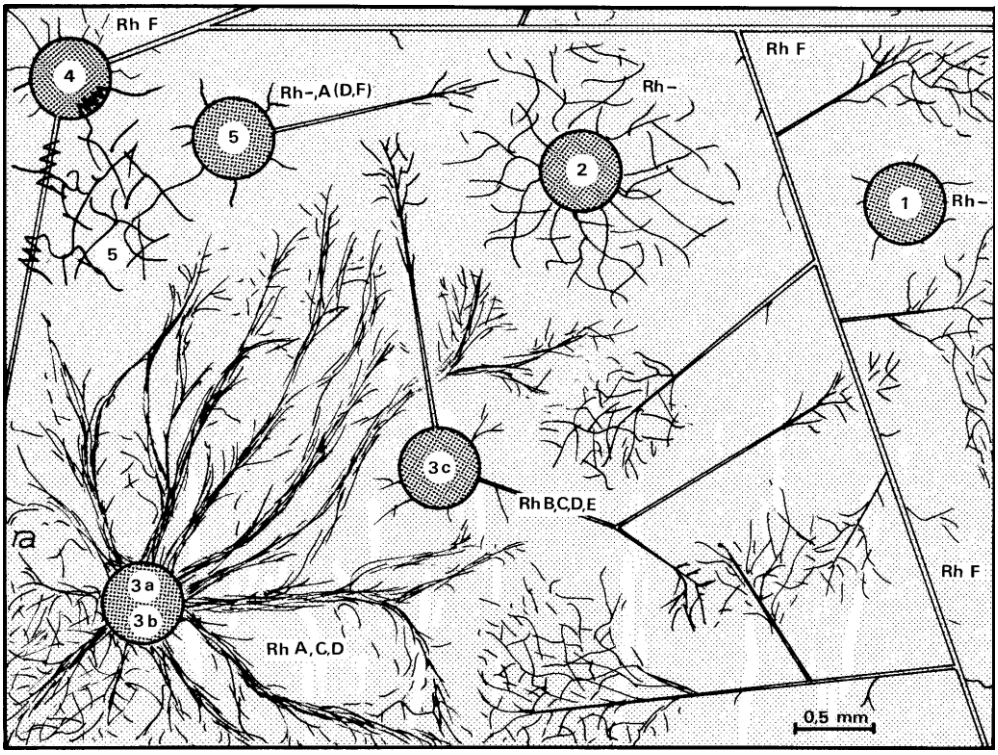

Figure 1.: *Schematic drawings of different exploration strategies, represented by cross-sections of ectomycorrhizae and the extramatrical mycelium. 1 Contact exploration, 2 short-distance exploration, 3a, b medium-distance fringe exploration and medium-distance mat exploration, 3c medium-distance smooth exploration, 4 long-distance exploration, 5 pick-a-back exploration, shown as mycorrhiza and as soil hyphae in contact and intruding into rhizomorphs and ectomycorrhizae of a longdistance exploration type ectomycorrhiza. All figures are to scale (Rh rhizomorph, – rhizomorph lacking, A–F organization types of rhizomorphs according to Agerer 1987–1998, 1991a). Reproduced with permission from Agerer (2001).*

microbial assemblage that is both taxonomically and functionally distinct from the community in the surrounding bulk soil (Calvaruso et al., 2007; Uroz et al., 2013, 2012). Due to specific adjustment of the release and nature of compounds exuded through root tips and extramatrical hyphae (Rygiewicz and Andersen, 1994; Schöll et al., 2011), the composition and functioning of microbial communities might vary between EMF species (Izumi and Finlay, 2011; Marupakula et al., 2016).

1.4. Ectomycorrhizal fungi as important players in forest soil C and N cycling

EMF are recognised as one of the key players in temperate and boreal forest ecosystems. Besides their irreplaceable support of primary production, they considerably participate in soil organic matter (SOM) and nutrient transformations, immobilisation and release (Baldrian, 2017; Tedersoo and Bahram, 2019; Uroz et al., 2016; Zak et al., 2019).

EMF are important moderators of soil C dynamics. They have exclusive direct and permanent access to plant assimilates, which is an immense competitive advantage against other soil microbes and the reason for their dominant role in forest soil microbial biomass and functioning. Via nutritional support of plant primary production, they indirectly increase C input into soils through plant litter and rhizodeposition as well as by their own biomass. The C flow from ECM root tips might be a source of more than 40 % of dissolved organic C (DOC), the form which is readily available for utilization by other soil biota (Högberg and Högberg, 2002). The fungus redistributes the tree-derived C through the soil profile to a longer distance than the tree root system alone would reach. It even influences the chemical composition of the rhizodeposition by utilizing some and releasing other compounds (Rygiewicz and Andersen, 1994; Schöll et al., 2011).

One of the crucial aspects of forest C dynamics are effects of EMFs on organic matter decomposition. EMF contribute to OM decomposition directly by production of extracellular enzymes and/or hydroxyl radicals and indirectly through interactions with saprotrophic microbes (Zak et al., 2019). EMF evolved primarily from saprotrophs across a vast number of lineages (Tedersoo and Smith, 2013). Genes for enzymes catalysing degradation of plant litter, microbial detritus and SOM were detected in many EMF species (Kohler et al., 2015). However, the potential to degrade SOM is highly variable across EMF lineages and depends on which genes the EMF species' ancestor possessed and whether they were retained in the present EMF genome (Pellitier and Zak, 2018). For example, *Amanita muscaria* evolved

within a clade of wood decomposing fungi and completely lost the ability to depolymerize SOM (Kohler et al., 2015; Wolfe et al., 2012), while multiple *Cortinarius* species are able to produce peroxidases with action comparable to enzymes produced by "true" saprotrophs (Bödeker et al., 2014). Although EMF participate in SOM decay, they probably do not utilise the released organic compounds in saprobic way, i.e. as a source of C and energy, but rather mine for incorporated nutrients (Lindahl and Tunlid, 2015; Zak et al., 2019). Experiment using stable isotope tracing showed that only ca 12 % of C in EMF biomass may originate from soil (Hobbie et al., 2014). Nevertheless, the particulate SOM is cleaved by action of EMF and soluble forms of C are released and become a part of the DOC.

EMF community does not affect soil C dynamics only directly through its active contribution to SOM decay but it is also necessary to consider their interactions with other soil biota (Lindahl et al., 2001; Olsson et al., 1996). For instance, the advantage of host C supply enables EMF to efficiently compete with saprotrophs (Fernandez and Kennedy, 2016). The antagonistic interaction, where EMF repress the activity of saprotrophs, which finally turns into weakened SOM decomposition is described as the "Gadgil effect" (Gadgil and Gadgil, 1975, 1971). Moreover, ectomycorrhizosphere bacterial community has different activity of enzymes targeting specific C compounds, compared to bulk soil (Uroz et al., 2013), which also contributes to EMF's influence on soil C dynamics.

EMFs contribute significantly to the soil C sequestration. They comprise a considerable part of microbial biomass in forest soils and, therefore, their tissues provide a large C input into SOM (Cairney, 2012; Högberg et al., 2010; Högberg and Högberg, 2002; Wallander et al., 2013). Different parts of fungal body give rise to necromass with distinct resistance to decomposition and thus different potential to contribute to a stable part of SOM (Certano et al., 2018; Fernandez et al., 2016; Fernandez and Koide, 2012). Mainly residues of fungal cell walls, specifically fungal melanins and other hydrophobic proteins are cleaved slowly, while chitin is decomposed relatively rapidly (Fernandez et al., 2016). Accordingly, the amount and

stability of the sequestered C depends upon EMF community structure, i.e. amount and composition of necromass produced by EMF (Clemmensen et al., 2015; Fernandez et al., 2016). EMF unquestionably affect C cycling through several actions, still their real contribution is hard to estimate and is under ongoing debate (Cairney, 2012; Högberg et al., 2020; Lindahl and Tunlid, 2015; Zak et al., 2019).

Besides modifying C dynamics, EMF are concerned in nutrient transformations and in special cases (especially under strong nutrient limitation), they can dictate nutrient availability to plants and other microbes (Franklin et al., 2014; Näsholm et al., 2013). Thanks to direct permanent and exclusive access to photoassimilates, they avoid the competition for available C and energy with other members of soil microbial community, which allows them to invest more effort to enzymatic production and nutrient mining from SOM (Cairney, 2011). Additionally, EMF may obtain extra P by weathering soil minerals such as apatite through secretion of solvents such as organic acids (Cairney, 2011). The acquired nutrients might then be used for their own growth and maintenance or passed to their symbionts. It seems that EMF are very flexible and adjust the portion of nutrients passed to the hosts according to their own actual needs (Franklin et al., 2014).

EMF efficiently take up and immobilize nutrients both the readily available in soils and those released by their own action (Cairney, 2011; Näsholm et al., 2013). Therefore, their biomass represents a strong sink for nutrients. Particular attention is given to the role of EMF in retention of nitrogen. Given their high contribution to soil microbial biomass and necromass (Fernandez et al., 2016; Högberg and Högberg, 2002) that are able to store immobilized N for years, they might be the principal component of soil N retention in forest ecosystems (Blaško et al., 2015).

1.5. Nitrogen cycle in forest soils

Nitrogen is a biogenic element. It is irreplaceable constituent of living bodies, for instance amino acids, proteins or nucleic acids. In the environment, it

occurs in organic forms as a part of living bodies and dead organic matter, in simple mineral forms (ammonium – NH_4^+, nitrite and nitrate – NO_2^-; NO_3^-) and gases (dinitrogen gas and N oxides). There is a large set of processes, mainly provided by microorganisms, during which N is transformed from one form to another. A simplified outline of soil N cycle in forest systems as described in Sylvia et al. (2005) follows.

Naturally, N enters the temperate and boreal forest ecosystems mainly through atmospheric deposition or biological fixation (Högberg et al., 2017). N-fixation can occur in roots of N-fixing tree species (for instance Alder), mosses, conifer needles, ectomycorrhizal root tips or in free soil (DeLuca et al., 2002; Levy-Booth et al., 2014; Moyes et al., 2016; Paul et al., 2007) and its importance in temperate and especially boreal forests is still under debate (Högberg et al., 2017; Wurzburger, 2016). In unpolluted areas and in forests without N-fixing trees, the atmospheric deposition and biological fixation are comparable at rate 1–3 kg N ha^{-1} y^{-1} (as reviewed by Sponseller et al., 2016). The incoming N is usually quickly incorporated into N-limited plant biomass and enters the soil latter through the litter and root exudates. The essential portion of N cycle thus takes part in the soil. Through the action of soil biota, particulate organic matter entering the soil is being decomposed. By combination of physical (mechanical) and biochemical (exoenzymatic) breakdown, bioavailable organic monomers are released (depolymerisation; Schimel and Bennett, 2004). These can be taken up and readily utilised by microbes. The uptaken organic N can be incorporated into biomass or converted to a mineral form, i.e. as a by-product of catabolism released to soil as ammonium (N mineralization). Ammonium might be used as a source of energy and transformed to nitrate through the process of nitrification. Nitrate might serve as an electron acceptor for denitrifying organisms and N is then emitted to atmosphere in gaseous forms or it can be converted in assimilatory (microbial assimilation) or dissimilatory nitrate reduction (back to ammonium, DNRA). Both mineral N forms present in the soil can be again immobilised into microbial or plant biomass and afterwards return to the organic matter pool.

Primarily, N is the limiting nutrient in temperate and boreal forests (Högberg et al., 2017; Vitousek and Howarth, 1991). Most of N is present in complex organic forms bound in the litter horizon or in the soil organic matter, while easily available forms of N (organic monomers and mineral forms) are scarce. The N cycle is closed. This means that the available N is being readily immobilised in plant or microbial biomass and N losses from the N limited systems (occurring predominantly in leaching of dissolved organic N) are negligible (Schimel and Bennett, 2004).

1.6. Anthropogenic N inputs, N saturation concept and acidification

Human inputs of N through increased atmospheric N deposition and fertilization considerably changed the N cycling in forest ecosystems in the past century (Galloway et al., 2004; Kopáček and Posch, 2011). N fertilization as a management practice has been applied to nutrient poor forest stands to increase their productivity (Smethurst, 2010). Atmospheric N deposition is a natural phenomenon, but it has been substantially increased by human activities (anthropogenic deposition) such as livestock production and use of fossil fuels in energetics, industry and traffic (Kopáček and Posch, 2011). In the severely polluted areas, N deposition might increase by two orders of magnitude, compared to pre-industrial era (Kopáček and Hruška, 2010). In Europe, intensity of N deposition culminated in 1980s, when the maximal doses in the most exposed regions in the Central Europe exceeded 25 kg N h^{-1} y^{-1}, and since then has been partially reduced to current level at ~10 kg N h^{-1} y^{-1} in this region (Engardt et al., 2017; Kopáček and Veselý, 2005). However, the global atmospheric N pollution increases and is expected to continue rising (Lamarque et al., 2005). Although, effects of N deposition and fertilization are not completely comparable, they might act similarly on ecosystem functioning (Gao et al., 2015). Most notably, they may lead to eutrophication and acidification of forest ecosystems.

With anthropogenic N input of external N, the balance of N transformation processes is hampered, the N cycle opens and, in longer term,

the ecosystem status might switch from N limitation to N saturation (Ågren and Bosatta, 1988). Based on hypothesis of Aber et al. (1998, 1989) and further re-visiting by Lovett and Goodale (2011) and Kopáček et al. (2013) a progression of forest ecosystem from N limitation towards N saturation might be briefly described as follows: With alleviated plant N limitation due to anthropogenic N input, plant and microbial biomass growth is no longer limited and both increase. Further, plant tissues are becoming enriched in N (i.e. the biomass C:N ratio decreases). Accordingly, N released by decomposition of enriched plant biomass increases its availability in soil. As the N limitation is relieved, a limitation of plant and microbial biomass by other resources occurs, surplus N is not completely immobilized and the N cycle opens. N mineralization and nitrification exceed the demand for N and the availability of mineral forms increases in the system. Finally, a biologic capacity of the system to immobilize incoming N is filled and exceeded, which leads to a loss of nitrates from soil. Ecosystem at this theoretical stage, i.e. in the status, when it is not able to efficiently immobilize additional N and leaches nitrates is termed as N saturated (Stoddard, 1994; Venterea et al., 2004).

Acidification, a process of soil pH lowering and/or reduction of soil acid-neutralizing capacity, usually accompanies N inputs. Atmospheric N deposition is interconnected with deposition of sulphur (S), which is a strong acidifying agent. Transformation of reactive nitrogen (ammonium and nitrate) also generates acidity (Kopáček et al., 2013). Acidified soils are depleted in base cations and DOC and nutrient availability decreases, while aluminium toxicity increases (Kopáček et al., 2013; Oulehle et al., 2011). Soil pH is one of the strongest selectors of microbial community and acid soils are hostile for plentiful of microbial groups (Lauber et al., 2009; Tedersoo et al., 2014; Zhang et al., 2017). Compared to bacteria, fungi are more resistant to direct low pH stress, still EMF communities are responsive to pH decline, probably to its indirect effects on host plants and dissolved organic C stabilization (Glassman et al., 2017; Rousk et al., 2010b; Tedersoo et al., 2014). The response of microbes to soil acidification is immediate (Oulehle et al., 2018). Acid

deposition and soil acidification strongly influence also tree health and can substantially reduce tree growth and fine root biomass (Engel et al., 2016; Schmitz et al., 2019), which has further negative impact on EMF community. Thus, both microbial and plant N immobilization potential is reduced by acidification, which might strengthen negative effects of N overloading.

1.7. Ectomycorrhizal community response to anthropogenic N inputs

Under N-limiting conditions, plants richly supply assimilates to their EMF community. The EMF community of N-limited forests is largely formed by species well adapted to conditions of low N availability. These species explore the soil with extensive mycelia, are able to enzymatically mine for nutrients stored in SOM and reallocate them via hydrophobic rhizomorphs towards places of need and partly share them with their host plants (Lilleskov et al., 2019, 2011; Weigt et al., 2012). Such ECM species form a substantial part of microbial biomass, which keeps N cycling closed with minimal losses due to the high N immobilisation capacity (Blaško et al., 2015; Franklin et al., 2014; Högberg and Högberg, 2002; Näsholm et al., 2013).

Increased N inputs strongly influence the ECM functioning and related EMF communities (Arnolds, 1991; Lilleskov et al., 2019, 2011; Treseder, 2004; Wallenda and Kottke, 1998). The pressure forced on original EMF community exposed to consequences of N inputs stems basically from changes in host C supply, increased soil N availability and acidification driven stress. When the N input relieves their limitation, trading assimilates for N becomes less profitable for trees (Brzostek et al., 2014) and the C allocation to EMF decreases (Högberg et al., 2010; Olsson et al., 2005). The first observed reaction is a decrease in sporocarp production, eventually followed by a reduction in extramatrical mycelium production, root-tip colonisation and changes in community composition and diversity (Avis et al., 2003; Cox et al., 2010; Hasselquist and Högberg, 2014; Kjøller et al., 2012; Lilleskov et al., 2002b, 2002a, 2001; Teste et al., 2012; Toljander et al., 2006; Van Der Linde et al., 2018).

EMF species respond to N inputs non-uniformly. Lilleskov et al. (2011) proposed dividing the EMF species to nitrophillic and nitrophobic. Species uniformly retreating under increased N input, for instance *Cortinarius* sp., *Suillus* sp., *Piloderma* sp, are marked as nitrophobic. These are fungi with extensive extramatrical mycelia with hydrophobic rhizomorphs (long-distance and medium-distance fringe exploration types; Fig. 1) and often substantiated oxidative or proteolytic activity (Agerer, 2001; Bending and Read, 1996; Bödeker et al., 2014; Finlay et al., 1992). These features enable them to prosper in N limited ecosystems with high host C supply. On contrary, nitrophillic taxa (e.g. some *Lactarius* or *Russula* species, *Thelephora*) lack ability to explore large volume of soil (mostly hydrophilic contact, short-distance and medium-distance smooth exploration type; Fig. 1) and often also enzymatically cleave N from complex organic matter. As they rely mostly on readily available forms of N such as dissolved simple organic and mineral N and have lower C requirements compared to nitrophobes, they thrive in forests that are not strongly N limited. Aside from species with relatively uniform reaction to N input, reaction of many EMF species might differ case-by-case assuming that other concurrent mechanisms contribute to shaping the community (Lilleskov et al., 2011).

Trees are further hypothesised to be able to actively select ECM partners. With increasing N availability, more ECM species are able to deliver desired amount of N and plants thus might choose partners with the best "trade offer", i.e. those who provide higher N transfer rate per C withdrawn (Wallander, 1995).

Changes on EMF community might be also co-driven by acidification and concurrent aluminium toxicity, which frequently accompany N inputs. With decreasing pH, richness of EMF community decreases (Suz et al., 2017, 2014), which indicates that some EMF species are less tolerant to acidification. Nevertheless, the nutritional effect of N expressed through decreased plant C supply seems to be a more influential factor than acidification in case of EMF community (Wallenda and Kottke, 1998).

The lowered tree C supply to the ECM community and consequent decrease in its biomass and a structural shift may bring substantial functional alterations. An exchange of EMF species with large proteolytic and/or oxidative capacity for species with a reduced decomposition potential may slow down the SOM decomposition (Berg and Matzner, 1997; Fog, 1988; Hagedorn et al., 2003; Knorr et al., 2005; Waldrop et al., 2004) and lead to increased C and N sequestration in forest soils (Clemmensen et al., 2015; de Vries et al., 2009; Lindahl and Tunlid, 2015; Zak et al., 2019). However, there are also opposite effects of N input on soil N dynamics through change in EMF functioning. The reduction of the EMF biomass may decrease actual N retention in forest soils (Bahr et al., 2013, 2015; Högberg et al., 2014a; Kjøller et al., 2012). A retreat of EMF within the microbial community may also reduce their negative impact on other saprotrophs (the Gadgil effect), mainly on saprotrophic fungi known by their large exoenyzmatic decomposition capacity, but bacteria as well (Maaroufi et al., 2019). These changes would have an opposite effect – the stimulation of SOM decomposition and N leaching from the forests. In summary, although EMF functions in C dynamics are recognized, their real contribution to overall soil C cycling is still under debate (Cairney, 2012; Högberg et al., 2020; Lindahl and Tunlid, 2015; Zak et al., 2019). Hence, how the EMF community response to elevated N ability contribute to determining the fate of ecosystem C and N dynamics still needs to be elucidated.

1.8. Norway spruce forests and their response to anthropogenic N input: a special case

Naturally, Norway spruce (*Picea abies*) forests (spruce forests thereafter) occur in Fennoscandia, eastern European lowlands and mountain ranges in central and south-eastern Europe (Boratyńska, 2007). Nevertheless, nowadays is Norway spruce along with European beech (*Fagus sylvatica*) the most abundant tree species also in central European temperate lowlands due to its wide use in productive forestry (Pretzsch et al., 2014).

The spruce needle litter is relatively rich in lignin and other phenolic compounds and poor in N and during its decomposition, organic acids are released (Bárta et al., 2010; Binkley, 1995; Šantrůčková et al., 2006). Due to its high leaf area index and evergreen character, spruce is more effective in interception of acid deposition, compared to deciduous trees (De Schrijver et al., 2007; Růžek et al., 2019). These features create specific properties of soils, which develop under spruce. The soils are typically acid and nutrient poor, but covered by a thick layer of accumulated slowly decomposing litter (Augusto et al., 2002; Berger and Berger, 2012; Modrzyński, 2007). Therefore, EMF species forming extensive extramatrical mycelia and/or capable of effective acquisition of N from SOM tend to dominate the EMF community in spruce forests (Asplund et al., 2019; Korkama et al., 2006; Nacke et al., 2016; Wallander et al., 2010).

Due to above discussed combination of poor litter quality and efficient interception of atmospheric deposition, spruce is acknowledged as a high potent source of soil and water acidification, when exposed to acid atmospheric deposition (Augusto et al., 2002; Rothe et al., 2002). In addition, subsoils under shallow-rooting spruce also leach more nitrates compared to beech and hence spruce stands constitute a high risk of water eutrophication (Rothe et al., 2002). The EMF communities in forests dominated by conifers are more sensitive to N inputs compared to deciduous trees (reviewed by Lilleskov et al., 2019). In result, spruce forest ecosystems exposed to anthropogenic N inputs may react more intensively compared to other tree species and thus represent a high threat for quality of themselves and consecutively connected hydrosphere. In the response of spruce forests to consequences of N input, a reaction of EMF community may play an essential role and thus desires particular attention.

1.9. Recovery of ectomycorrhizal community from N saturation and acidification

Predictions of development of N deposition differ between regions. In some areas an increased pressure of N input is expected. In some other regions with decreasing atmospheric N and S deposition, a recovery of ecosystems from acidification and N saturation proceeds or is anticipated in near future (Ferrier et al., 2001; Gilliam et al., 2019). In light of these changes, a recovery of EMF community after cessation of anthropogenic N input emerges as a hot topic. Nevertheless, studies investigating such issue are scarce. A comprehensive long-term dataset of sporocarp abundance across Netherlands showed a recovery of sporocarp presence of nitrophobic EMF species after an effective reduction of N deposition (van Strien et al., 2018). This EMF reestablishment was more evident in regions affected by lower N deposition. Hasselquist and Högberg (2014) reported a recovery of sporocarp production 23 years after cessation of N fertilization (110 kg N ha^{-1} y^{-1} for 20 years) in a boreal forest. This was also associated with an increase in N retention in the restoring EMF biomass (Hasselquist and Högberg, 2014; Högberg et al., 2011). The process of EMF recovery seems to be a long-distance run and leftover effects (legacy) of former high N inputs on EMF structure might be evident even after five decades (Strengbom et al., 2001).

1.10. Concluding remarks

In summary, the basic knowledge of effect of N inputs on amount and diversity of EMF is increasing, but how the changes in EMF community affect the ecosystem function is still an unresolved puzzle. The studied forests usually vary in species composition, age, climate and soil properties (esp. acidity, nutrients contents, original microbial community etc.) prior to N input onset. In addition, the progression of N deposition is region-specific and plot experiments vary largely in the duration, dosage (both total amount and

number of applications throughout the year) and a form of N applied. Observations and experiments attempting to understand the changes in ecosystem functioning, often focus on bulk parameters such as quantification of N input and output, impacts on soil pH and total C and N pools, on aboveground primary production and/or litter quality and the like. Although these variables might provide valuable conclusions, such coverage is insufficient to improve our understanding about change in functioning of EMF and other parts of microbial community. The feedbacks of EMF diversity and functioning to anthropogenic N inputs and their potential recovery are complex issues and more insightful and targeted research is needed to produce outcomes applicable in ecosystem management and nature conservation.

2. Aims and objectives

The overall aim of this thesis was to investigate the effect of anthropogenic N inputs and recovery therefrom on ectomycorrhizal fungal communities in Norway spruce forest ecosystems and to relate the shifts in the structure of EMF community to changes in soil functioning. Specific objectives were:

(I.) To describe and compare changes in belowground EMF community composition and abundance in spruce forest soils with different duration and dosage of N input. We expected that the N input will enhance N availability in soils, which would decrease EMF abundance and cause a shift from prevalence of nitrophobic towards higher proportion of N tolerant EMF species.

(II.) To confront the transformation of EMF community with changes in soil functioning induced by N input. We hypothesised that the decrease in EMF abundance and retreat of nitrophobic species would hamper the soil N retention capacity and lead to a considerable enhancement of N leaching from N loaded soils.

(III.) To evaluate a recovery of EMF community and related soil functioning after termination of elevated N input. We assumed that declining N availability after the cessation of N inputs would allow an increase in the abundance of EMF, nitrophobic species dominance will be restored and the N leaching will be minimized.

In order to fulfil the aims, the belowground diversity of EMF community was assessed with the use of high-throughput sequencing of amplified fungal ITS markers obtained from total soil genomic DNA. Based on taxonomic classification, exploration types were assigned to the identified EMF species and these were further divided into nitrophobic and nitrophilic groups according to their response to N input. The observed shifts in structure and

function of EMF community were always related to changes in measures of soil biochemical parameters such as microbial biomass, its respiratory and exoenzymatic activity and processes involved in N cycling including nitrate leaching. This approach enabled to identify N-invoked changes in the soil functioning and deduce the role of the EMF shifts in it.

3. Experimental sites and methods

3.1. Experimental sites

The presented research was conducted in several Norway spruce forest ecosystems. Three sites are situated in central and southern Sweden: Stråsan (ST), Skogaby (SK) and Gårdsjön (GA), two are in the Czech Republic: Načetín (NAC) and Čertovo lake (CT) (Fig. 2). First, the localities represent the natural north-south gradient of increasing total N input (and co-occurred S deposition) to the spruce forests. Additionally, some of them, ST, GA and NAC host field manipulative N addition experiments of different duration.

The northernmost site ST recently receives ~3 kg N ha^{-1} y^{-1} and the deposition haven't been previously considerably higher. The GA is located in south-western Sweden, where atmospheric N deposition is the highest in Sweden. Particularly, the N deposition in GA averaged on 9 kg N ha^{-1} y^{-1} in the last three decades with no significant trend to change (Hansen et al., 2013; Moldan et al., 2018). In addition, severe S deposition lasting until 1990s has acidified the ecosystems in south-west Sweden, which slowly recover since that time, while the acid deposition in the north and the central Sweden was lower (Moldan et al., 2018; Pihl Karlsson et al., 2011). The Central Europe has been exposed to severe N and S deposition. Both Czech localities thus received the largest total S and N inputs from the studied sites, which culminated in the 1980s, when the N deposition reached ~30 kg N ha^{-1} y^{-1}, and then largely decreased due to effective reduction of emissions (Kopáček and Hruška, 2010). Since the retreat of the S and N atmospheric deposition, forests recover from acidification and N saturation (Kopáček et al., 2001; Oulehle et al., 2011). The N bulk deposition in the NA locality currently levels at ~11 kg N ha^{-1} y^{-1} (Oulehle et al., 2016).

For the purpose of discussing the impact of elevated N input on the EMF communities, the results obtained in NA, GA and ST are selected. (We have not sufficiently detailed EMF data from other two localities). The NAC provided an insight into the fourth year of experimental N addition, the N addition experiment in GA lasted for 24 years and the plots in ST with ongoing

fertilisation receive additional N for 46 years. ST further enables to study a 23 years long recovery of the EMF from the N addition, which previously run also for 23 years. Detailed description of the selected sites follows.

Figure 2.*: Location of the study sites. Source: ESRI, created by Vojtěch Blažek.*

Stråsan (Papers I and II) is an experimental site located in central Sweden (Fig. 2). It is a Norway spruce plantation established in 1958. The stand was 55 years old at the time of soil sampling. The soils are nutrient poor, covered by very sparse understory of mosses and lichens. Both the current and historical background deposition are low (~3 kg N ha^{-1} y^{-1}). The experimental treatment designed into randomized blocks of 30 x 30 m started in 1967:

untreated control with no NH_4NO_3 addition (N0), ongoing fertilization (N1; on average 34 kg N ha^{-1} y^{-1} for 46 years) and terminated fertilization (N2; first on average 73 kg N ha^{-1} y^{-1} for 23 years, than 23 years recovery without N additions).

Gårdsjön (Paper III) lake catchment area is situated near the west coast in southern Sweden (Fig. 2), where the N deposition averaged at ~9 kg N ha^{-1} y^{-1} in the last almost three decades. We investigated two Norway spruce dominated experimental catchments within this area. The understory was made up by blueberries (*Vaccinium myrtillus*), heather (*Calluna vulgaris*) and mosses. Both catchments had more humid parts on the bottoms of valleys and drier parts at the surrounding slopes and these parts were analysed separately. The catchment G1 (0.63 ha) served as an untreated control. The NH_4NO_3 addition to the catchment G2 (0.53 ha) started in 1991 and the dose was ~40 kg N ha^{-1} y^{-1}separeted to several small doses in each year. The stand age was 95 years and the N treatment duration was 24 years at the time of sampling.

Načetín (Paper IV) experiment launched in 2014 in the Ore Mts. in the Czech Republic (Fig. 2). The experiment simulates separated and combined deposition of N and S. It comprises a Norway spruce and an adjoining European beech (*Fagus sylvatica*) stand. The experimental treatment simulating acid deposition is designed in factorial arrangement with four repeated blocks with treatment plots 3 x 3 m. The treatments are: N (addition of NH_4NO_3 at 50 kg N $ha^{-1}y^{-1}$), S (sulphuric acid 50 kg S $ha^{-1}y^{-1}$) and S+N (above dosage combination). The spruce stand was ~90 years old and the treatment has been applied for 4 years at the time of sampling.

3.2. Brief description of methods

In all cases, organic soil horizon (corresponding to F+H layer) was sampled. From the samples, soil genomic DNA was extracted for further fungal internal transcribed spacer (ITS) amplicon sequencing (Illumina platform). In

addition, several soil biochemical and microbial parameters were measured within these sites consistently in all cases: pH, total, water extractable and microbial pools of C and N, nitrate leaching and potential activities of extracellular enzymes. For detailed method description of these (and also other here not listed) analyses, see the respective papers.

4. Discussion

4.1. Differences among the sites on the gradient of atmospheric N deposition

The results from three spruce forest experimental sites (ST, GA, NAC) are discussed within this thesis. All the sites represent field N addition manipulative experiments. Both Swedish sites have acid soils with the pH values ~4.5, which are N limited as indicated by dominance of dissolved organic N (DON) in the N pool and a high DOC:NO_3^- ratio (molar basis; 709 and 500 for ST and GA, respectively) of the soil water extracts (**I**, **III**). The soil in NAC was more acid (pH ~3.7) and with considerably higher N availability, as indicated by comparable concentrations of mineral N (predominantly NH_4^+) and DON and substantially lower DOC:NO_3^- ratio of 18 (**IV**). The lower pH and higher N availability was to a great extent attributable to more severe historical N and S deposition in the Czech Republic compared to Sweden (Kopáček and Hruška, 2010; Oulehle et al., 2011; Pihl Karlsson et al., 2011).

The composition of EMF communities largely varied among the sites in accord with their significantly different soil N availability. In the N poor Swedish sites ST and GA, EMF made up 70 % and ~44 % of all fungal ITS amplicons, respectively (**II**, **III**). Both communities were dominated by species belonging to medium-distance fringe exploration type (Agerer, 2001), namely by *Piloderma* sp. and *Cortinarius* sp. (**II**, **III**). Representatives of both genera have ability to use organic N sources (Bödeker et al., 2014; Heinonsalo et al., 2015) and sort to nitrophobic EMF species (Lilleskov et al., 2011), which are adapted to prosper in N limited soils. Considerably lower proportion, 26 % of fungal reads, were assigned to EMF in the control plots of NAC (**IV**). The species composition was largely different compared to the Swedish sites. Although *Cortinarius* sp. and *Piloderma* sp. were also present, their relative abundance was minimal and the most detected were *Tylospora*

sp., *Imleria badia* and *Hygrophorus olivaceoalbus* (**IV**). All these EMF are considered as tolerant to increased N availability (Lilleskov et al., 2011).

Despite the large difference in soil N availability and in the DOC:NO_3^- ratios, all studied ecosystems leach minimal amounts of nitrates (**I**, **III**, Oulehle et al. 2011, Růžek et al. 2019). There is no expectation of nitrate losses from the Swedish sites due to their N-poor status. However, the efficient N retention within the NAC spruce forest, where the N availability is considerably higher, is surprising. The ecosystem, which has been historically exposed to high N inputs and lost a considerable part of nitrophobic EMF species with immense extramatrical mycelial mats that are regarded to be crucial for ecosystem N retention (Bahr et al., 2015; Högberg and Högberg, 2002), is still able to retain most of the incoming N. The NAC forest systems recover from acidification, which is connected with an increase in litter decomposition and relieve of C limitation (Oulehle et al., 2011; Růžek et al., 2019). It likely supported efficient growth of other microbial components, which apparently substituted the N retention role of EMF community in NAC.

4.2. Effect of experimental N additions

4.2.1. Načetín

Four years of experimental N additions (reaching 50 kg N ha^{-1} y^{-1}) in NAC were barely reflected by fungal community. We observed no significant effect of N input on fungal community composition and biomass, as estimated by fungal marker (18S rRNA) gene abundance. Within fungal community, no significant change in proportion of EMF ITS amplicons was observed, nor any significant change in EMF species composition (**IV**). The dose of 50 kg N ha^{-1} y^{-1} is ca twice as high as it is estimated to be sufficient enough to trigger changes in EMF (Lilleskov et al., 2019; Wallenda and Kottke, 1998). Generally, the reaction of EMF community to enhanced N availability is demonstrated as a shift from prevailing nitrophobic towards nitrophilic EMF species (Lilleskov et al., 2019, 2011). That shift is commonly triggered by multiple events occurring in the forest system, in which an enhanced

concentration of mineral N forms and a reduced tree belowground C allocation are likely the most important (Lilleskov et al., 2019). However, the EMF communities in control NAC plots (without any extra N input during the experiment) are largely assembled by nitrophilic species. It indicates that the "fundamental" structural and functional shift in the EMF community already occurred before the experiment started, caused by a historical high-level N deposition in the Ore mountains. Therefore, there was not a high potential for any dramatic compositional change during the 4-year lasting N addition experiment (**IV**).

We also admit that the design of this experiment, which was not originally intended to follow a reaction of EMF, might be the cause of no apparent reaction of EMF community. The size of the experimental plots (3 x 3 m) may be insufficient to significantly influence C allocation of host trees, as spruce root system has considerably larger reach (Göttlicher et al., 2008). Thus, we can only detect an EMF response to a direct increase in soil N availability.

4.2.2. Gårdsjön

The N fertilisation of G2 catchment in GA lasted for 24 years at the time of sampling. After the onset of N addition, the nitrate concentration in runoff have been gradually rising for the first ten years, but since then no further increase was observed until the time of our sampling (Moldan et al., 2018, 2006). Currently, the soils at fertilized G2 catchment had higher total C and N contents. Fertilization also increased a contribution of mineral N forms to the available N pool (from 5–25 % in unfertilized catchment to 40–70 % in the fertilized catchment). The DOC content was also higher in the fertilized catchment and the DOC:NO_3^- ratio decreased by a factor of ~2 compared to control catchment. The increase in C and N availability was parallel in both wet and dry parts of the catchment (**III**).

In the first years of experimental treatment, the abundance and species richness of EMF sporocarps decreased in fertilized compared to unfertilized

catchment. The most negatively affected were nitrophobic *Cortinarius* species, while N tolerant species (e.g. *Lactarius* and *Craterellus*) increased their sporocarp productivity (Brandrud, 1995). After 24 years of N addition, we found that the proportion of EMF ITS amplicons within the total fungal community was reduced to half in the G2 catchment compared to control (**III**). The EMF community was transformed, the nitrophobic EMF (*Piloderma* sp. and *Cortinarius* sp.) had considerably lower relative abundance in fertilized catchment. The retreat was more pronounced in the wet parts of the catchment, located in the bottom of the valley, where the unused and leached added N can be accumulated in larger concentrations (**III**).

After an initial steep increase in nitrate leaching, the fertilized catchment is still effective in retaining most of the incoming N (Moldan et al., 2018). We attribute this to an adaptation of the whole microbial community to increasing N availability. We observed larger soil microbial biomass with lower C:N ratio in the fertilized catchment compared to control (**III**). The decrease in C:N could be indicative of decrease in fungi-to-bacteria ratio (Högberg et al., 2017) but this was not our case. We did not observe any decrease either in fungal gene marker or in its proportion to bacterial marker, which would indicate a change in fungi-to-bacteria ratio (**III**). However, both bacterial and fungal communities got enriched in copiotrophic taxa with effective performance in conditions of readily available N, while the relative abundance of oligotrophs decreased compared to controls. The EMF decreased their relative abundance within fungal community and nitrophobic EMF like *Piloderma* sp. and *Cortinarius* sp. were exchanged for N tolerant species such as *Russula* and *Lactarius*, while the proportion of saprotrophic fungi increased (**III**). Especially moulds and yeasts, which are considered to thrive in nutrient rich conditions (Botha, 2011; Brabcová et al., 2016), had higher relative abundance in N fertilized catchment. Within the bacterial community, Actinobacteria had higher relative abundance in the N fertilized catchments. These bacteria are often regarded rather as copiotrophs, some of them are able to degrade complex C sources and are favoured by higher N availability (Ho et al., 2017; Ramirez et al., 2012, 2010; Větrovský et al.,

2014). We observed also change in activity of extracellular enzymes, when the microbes within the fertilized catchment preferential mined for C and P, when the N availability increased (**III**).

In result, there was an apparent total retreat of EMF and, in particular of nitrophobic species in GA fertilization experiment, with no negative implication for the ecosystem N retention capacity. Even after a significant decline of EMF contribution to soil microbial biomass and activity, the ecosystem still loses only a small fraction of the extra N input.

4.2.3. Stråsan

The duration of ongoing fertilization treatment in ST was 46 years at the time of sampling. The almost half a century lasting N inputs tended to enhance total N content as well as concentrations of mineral N pools and caused pronounced changes in EMF communities (**I**, **II**). In the control plots EMF dominated fungal community with 70 % relative abundance, while in fertilized plots, their proportion was only 42 %. Fertilization further changed the functional composition of the EMF community, when the originally predominant nitrophobic *Cortinarius* sp. and *Piloderma* sp. retreated in favour of N tolerant species such as *Lactarius* sp. and *Tylospora* sp. (**II**). This also projected to a change in isotopic signal in spruce needles, which indicated weakened contribution of EMF in tree N acquisition (Blaško et al., 2013).

The N fertilization further decreased microbial biomass measured both as microbial biomass C and N and phospholipid fatty acid (PLFA) content, and lowered fungi-to-bacteria ratio (ratio of fungal to bacterial PLFA) (**I**). This seems to be connected with the retreat of the nitrophobic EMF, which produce extensive mycelia and contribute to the fungal PLFA pool (**II**). A shift in relative abundance of specific PLFA markers showed that the bacterial community changed towards higher proportion of Actinobacteria and other Gram-positive bacteria (**I**). The changes within fungal and bacterial communities probably occurred as a response to a decreased supply of readily available C by trees with relieved N limitation. We do not have direct evidence

for a change in the flux of photoassimilates, nevertheless the higher tree growth rate and N enrichment of spruce needles indicated weaker N limitation of trees in fertilized compared to control plots (Blaško et al., 2013). In addition, the ratio of C-to-N acquiring enzymes was higher in the fertilized plots compared to controls, which points to relatively lower availability of C in respect to N in soils of fertilized plots.

After 46 years of N fertilization, we observed only small leaching of nitrates and the ecosystem still retained vast majority of the incoming N (**I**). Similarly as in GA, the retreat of nitrophobic EMF did not dramatically decrease the soil N retention capacity.

4.3. Implication of ectomycorrhizal community restructuring for N retention

The large biomass of extensive mycelia of nitrophobic EMF is regarded as a fundamental provider of soil N immobilisation. Its reduction is thus expected to threaten soil N retention capacity (Bahr et al., 2013; Clemmensen et al., 2015; Högberg et al., 2014a). Nevertheless, we did not observe any dramatic increase of N leaching in the form of nitrate either from the soils with low relative abundance of the EMF in microbial community or from the soil with the EMF community prevailed by nitrophilic species. In NAC and fertilized ST plots, the nitrate leaching was minimal and formed negligible proportion of the incoming N (**I**, Růžek et al. 2019). In GA, the N loss from fertilized catchment was higher, but still the ecosystem retained >80 % of the total annual N input (Moldan et al., 2018). Obviously, other ecosystem constituents may substitute the immobilization role of EMF. As revealed by the studied N fertilization experiments, the N retention might be maintained by (i) the enhanced accumulation in plant biomass as indicated by both tree growth and needle N content enhanced by N loading in ST (Blaško et al., 2013), (ii) the sequestration in soil organic matter, as implied by higher soil N content and decreased C:N in ST and GA (**I**, **III**) and (iii) the effective N use by microbial community and its immobilization in microbially-transformed products. With

the increased soil N availability and reduced competitive pressure from EMF, which lost their advantage of wealthy supply of plant assimilates, saprotrophs might start to prosper and efficiently utilize the incoming N. In GA, the retreating nitrophobic EMF were partly replaced by fast growing saprotrophic moulds and yeasts (**III**) and we further observed restructuring of bacterial communities in both ST and GA. Namely relative abundance of Actinobacteria increased in fertilized plots of both ST and GA (**I**, **III**). The adapted microbial communities enhanced enzymatic C (both ST and GA) and P (GA only) mining in order to effectively utilize available N. The preservation of soil N retention capacity by other ecosystem components, is in accordance with findings of Bahr et al. (2015), who observed that initial decrease in EMF biomass considerably reduced the soil N retention capacity, but further reduction of EMF biomass didn't lead to further N leaching. They concluded that other factors might play role in ecosystem N retention.

However, the effective maintaining of soil N retention capacity by other biotic components than EMF, might be suppressed by stressful or limiting conditions. The N loading is frequently connected with acidification. Soil pH is one of the most influential factors and acidity creates direct physiological stress, causes aluminium acidity and unavailability of nutrients for both plants and microbes (Kopáček et al., 2013; Lauber et al., 2009; Rousk et al., 2010a; Tedersoo et al., 2014). Its effect on microbes, esp. bacteria, might be fast and substantial and could induce change in the community functioning (**IV**). However in our case, pH was not lowered by the N input in GA (**III**) and thus the activity of trees and microbial community leading to N retention was not hampered. In ST, there was a slight decrease in pH, which was probably the cause of decrease in microbial biomass and activity (as indicated by lowered basal respiration), but still, the ecosystem was able to immobilize >95% of the incoming N (**I**). Hence, when the dose of N is not extreme and does not induce considerable acidification, the ecosystem is able to keep up the N retention capacity and low N losses for a long-term despite a retreat of EMF.

4.4. Recovery from N additions

The N addition to the N2 treatment plots in ST was terminated 23 years prior to sampling. The total soil N content was still significantly higher compared to control treatments (**I**) and did not decrease during the recovery time (Rappe-George et al., 2013). However, the continual decrease in N content in spruce needles pointed towards reduced N availability (Blaško et al., 2013), which was also implied by minimal nitrate leaching, being at similar level as in control plots (**I**). The restoration of the EMF community was initiated. The relative abundance of EMF tended to increase (**II**) and a change in N isotopic signal in spruce needles further indicated an increasing contribution of the EMF in tree N supply (Blaško et al., 2013). However, the N tolerant species still prevailed over nitrophobic ECM (**II**). Signs of recovery were observed also within the bacterial community (**I**), which is an indication of the ongoing slow restoration of the whole microbial community.

An analogous field experiment in a Scots pine (*Pinus sylvestris*) forest documented advanced recovery of EMF sporocarp production, belowground abundance and community composition and the increasing role of EMF in tree N supply (Hasselquist and Högberg, 2014; Högberg et al., 2014b, 2007, 2011) within 16–20 years after termination of N additions. But still, the EMF community structure and its role in plant N nutrition was not fully restored to the original state (i.e. the state comparable to control). Strengbom et al. (2001) found out that even 47 years of recovery were not sufficient to re-establish sporocarp abundance and species composition in a boreal spruce forest. It can be expected, that dose and duration of N loading would be important for further fate of EMF community after N input cessation, but to date, we lack more research dedicated to such process to be able to name the factors that govern the process of recovery and how long would it take and whether is it even possible for EMF community to fully recover.

5. Conclusions and future prospects

The present research confirmed the sensitivity of nitrophobic EMF to increased N availability induced by anthropogenic N inputs. It was mainly manifested by a lowering of EMF contribution to fungal community and by shifts in their composition towards lower proportion of nitrophobic species. Contrary to our hypothesis, this systematic shifts didn't lead to a dramatic reduction in soil N retention and the N leaching remained low in all the soils affected by increased N input. We conclude that other ecosystem components, namely plant biomass and saprotrophic microbes are able to substitute the role of retreating EMF in N retention, when the dose of N is not extreme and does not induce soil acidification. Once the N loading is ceased, EMF community might start to recover, i.e. increase in proportion within the total fungal community and restore previously retreated species.

To proceed towards better understanding of EMF functioning in soils affected by anthropogenic N inputs, more targeted research is needed. The most comprehensive possible set of climatic, vegetation and soil bio-chemical parameters should be followed in ecosystem level studies to disentangle the changes in all ecosystem components. In particular, the effect of changed N availability on plant-EMF-microbes interactions and, consecutively, microbially driven processes (enzymatic activity and C, N, P transformations) should be investigated. Another task is to gain more insight into functional potential of particular EMF species. Such information could be gained by combination of cultivation tests and examination of the genetic potential. However, this is challenging as EMF are hard to cultivate and genetic potential does not necessarily imply real action. Combination of the observation of the whole ecosystem reaction with knowledge of particular EMF species behaviour and capabilities might shed more light on the role of EMF in the soil processes.

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Paper 1

Indications that long-term nitrogen loading limits carbon resources for soil microbes

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Indications that long-term nitrogen loading limits carbon resources for soil microbes

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ABSTRACT

Microbial communities in the organic horizon (O-horizon) of forest soils play key roles in terrestrial nitrogen (N) cycling, but effects on them of long-term high N loading, by N deposition or experimental addition, are not fully understood. Thus, we investigated N-loading effects on soil microbial biomass N, carbon (C) and phosphorus stoichiometry, hydrolytic and oxidative enzymes, community composition (via phospholipid fatty acids, PLFA) and soil chemistry of the O-horizon in study plots of three well-studied experimental Norway spruce (*Picea abies*) forests in Sweden and the Czech Republic. These forests span substantial gradients in current N deposition, experimental N addition and nitrate (NO_3^-) leaching. Current N deposition ranges from ~3 kg ha^{-1} $year^{-1}$ of N in central Sweden (Stråsan) to ~15 kg ha^{-1} $year^{-1}$ of N in SW Sweden (Skogaby) and Czech Republic (Čertovo). Furthermore, accumulated historical N loading during 1950–2000 (which include experimental N addition performed at Stråsan and Skogaby) ranged ~200–~2000 kg ha^{-1} of N. Across all sites and treatments, current NO_3^- leaching ranged from low (~0.1 kg ha^{-1} $year^{-1}$ of N) at Stråsan, to high (~15 kg ha^{-1} $year^{-1}$ of N) at Skogaby and Čertovo. We found significantly lower C/N ratios and greater amounts of extractable inorganic N species in the forest soils' O-horizons at the high N loading plots. Microbial biomass and basal respiration decreased under experimental N addition treatments and tended to decrease with increased N deposition. Similarly, activities of hydrolytic enzyme activity associated with N acquisition were lower, although differences in activities at specific sites with the highest and intermediate historical N deposition levels failed statistical significance. Conversely, activities of soil hydrolytic enzymes associated with C acquisition were greater in study plots exposed high N loading. PLFA profiles indicated shifts in microbial community composition induced by long-term N load, towards higher and lower relative abundance of Gram-positive and Gram-negative bacteria, respectively (but no changes in fungal relative abundance). Taken together, our results suggest that long-term N loading of N-limited Norway spruce forests aggravates limitation of other resources, likely of C, for soil microbial communities. Although microbial variables in the soil O-horizon differed between plots exposed to low and high current N loading, microbial variables in plots that leached small amounts and large amounts of NO_3^- exposed to high N load were similar.

1. Introduction

Globally, the nitrogen (N) cycle has been altered dramatically by human activities, notably releases of reactive forms of N (Vitousek et al., 1997; Fowler et al., 2013). Fossil fuel burning and animal husbandry are important sources of N released into the atmosphere, some of which is transported long distances and deposited in forest ecosystems (Galloway et al., 2004). Excessive N loading can

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saturate forest ecosystems' biotic demands for N, in the short- or long-term, and have undesirable effects such as soil acidification and N loading on other terrestrial ecosystems via N leaching (Aber et al., 1989, 1998). Nitrate (NO_3^-) leaching is pivotal in this context as it is a major pathway of N loss and connected to acidification of soils. Full characterization of the effects of enhanced N input to initially N-limited forest ecosystems requires long-term studies, since such ecosystems frequently have high capacities to retain added N (Aber et al., 1998; Binkley and Högberg, 1997; Johnson, 1992) and some of the responses may only be apparent after prolonged N loading (Aber et al., 1998; Fenn et al., 1998).

The high retention of added N often observed in temperate and boreal ecosystems is likely due to the strength of biotic sinks for N in the soil, notably immobilization by mycorrhizal fungi or free-living saprotrophs (reviewed by Aber et al., 1998). Accordingly, an important sink for added N is soil organic matter (SOM), in which 50% or more of total added N is frequently retained (Johnson, 1992; Melin et al., 1983). SOM transformations are integral components of terrestrial carbon (C) and nutrient cycles, and are driven by the energy and nutrient requirements of diverse communities of soil organisms, notably fungi and bacteria. Variations in temperature sensitivity of decomposition of SOM fractions and its interactions with soil N availability influence both heterotrophic respiration and net ecosystem productivity and hence play a pivotal role in soil-climate interactions (Gärdenäs et al., 2011). Moreover, climate change might affect responses of microbial communities to enhanced N availability.

As recently discussed (Treseder, 2008; Ramirez et al., 2012; Kopácek et al., 2013), N addition may have both direct and indirect effects on soil microbial communities, with implications for soil C and N cycling. Direct effects include, amongst others, reduction in soil microbial biomass and respiration (Janssens et al., 2010), inhibition of enzymes involved in decomposition of lignin and lignin derivatives (Fog, 1988; Gallo et al., 2004), increased abundance of electron acceptors in the form of NO_3^- (Kopácek et al., 2013), changes in outcomes of competition favoring taxa with high N demands (Fontaine et al., 2003) and/or reductions in rates of decomposition of recalcitrant organic matter due to reductions in needs to mine soil for N (Moorhead and Sinsabaugh, 2006). Reported indirect effects are, for instance, lower dissolved organic C availability as soil acidification may decrease organic C solubility (Kopácek et al., 2013). Another indirect effect is altered resource allocation of trees, whereby reduced tree belowground photosynthate C allocation in response to elevated N availability (Högberg et al., 2010) may cause reduced C availability for microbes and microbial community shift towards loss of ectomycorrhizal fungi (EMF) dominance (Wallenda and Kottke, 1997; Lilleskov et al., 2002; Nilsson and Wallander, 2003) and concomitant reductions in N immobilization (Högberg et al., 2014). Accordingly, reductions in the abundance of fungi, as indicated by a phospholipid fatty acid (PLFA) eukaryotic biomarker linoleic acid (18:2ω6,9), in the organic soil horizon (O-horizon) of boreal forests in response to experimental N addition or ambient N deposition have been linked to reductions in investment of recent photosynthate C from trees to EMF under increased N availability (Högberg et al., 2011, 2014; Bahr et al., 2013). Subsequently, Bahr et al. (2015) reported that soil water inorganic N concentration increased and EMM production decreased following N fertilization in a Norway spruce forest in southern Sweden. However, they found that N + phosphorus (NP) fertilization reduced soil water inorganic N concentration, and further decreased EMM production, suggesting that effects on not only EMF, but also the whole microbial community and N immobilization, warrants further study. van Diepen et al. (2010) reported increased ratios of cyclopropyl/precursor PLFA, interpreted as soil microbial physiological stress, after 12 years of experimental N addition and increased abundance of Gram positive bacterial PLFA biomarkers, considered to be stress tolerant (Balser, 2005), in response to long-term N addition have been reported (Blaško et al., 2013).

In the study presented here we examined effects of long-term N loading (by experimental N addition and N deposition) on soil microbial variables related to soil microbial biomass stoichiometry, enzyme activities and community composition as reflected by phospholipid fatty acid (PLFA) profiles in three Norway Spruce (*Picea abies*) forests in Sweden and the Czech Republic. The overall aim was to improve understanding of the soil microbial element (C, N and P) stoichiometry, enzyme activities, and community composition under elevated N loading of Norway spruce forests located in the temperate and boreal zones. Furthermore, we assess how these soil microbial variables vary across large ranges of ecosystem N retention and NO_3^- leaching. We hypothesized that long-term N loading to such spruce forest systems will eventually lead to limitation of C resources for the soil microbial biomass, manifested as lower soil C/N ratios, soil microbial biomass and basal respiration, altered enzyme activities reflecting a shift from N to C acquisition and an altered community with greater proportions of stress tolerant functional groups, Gram-positive bacteria, at the expense of fungi and Gram-negative bacteria.

2. Material and methods

2.1. Site description and experimental design

All studied forest sites are long-term monitoring, or experimental, sites located in Sweden and the Czech republic. The soil microbial variables were studied in three Norway spruce (*Picea abies* L. Karst.) forests (Table 1), located at and called here: Stråsan in central Sweden (60°54′N, 16°01′E), Skogaby in southern Sweden (56°33′N, 13°13′E) and Čertovo in southern Czech Republic (49°10′N, 13°11′ E). These sites have been described by Tamm et al. (1974), Bergholm et al. (1995) and Kopácek et al. (2002), respectively. All sites are dominated by mature Norway spruce forests on acid, haplic podzols. Stråsan had the lowest mean annual temperatures of 3.1 °C, Čertovo intermediate (5.4 °C), while Skogaby is slightly warmer (7.6 °C). The long-term mean annual precipitation is similar at Skogaby and Čertovo (1187 and 1413 mm $year^{-1}$, respectively), while Stråsan is considerably drier (745 mm $year^{-1}$). The sites cover a strong gradient in historic (1950–2010) N deposition, declining in the order Čertovo (1168 kg N ha^{-1}) > Skogaby (723 kg N ha^{-1}) > Stråsan (195 kg N ha^{-1}, Table 2). Each site is described in more detail below.

2.1.1. Stråsan

Stråsan is the site of a Norway spruce forest optimum nutrition experiment located in central Sweden, approximately 40 km northeast of the city Falun (Table 1). The soil is dominated by medium and fine sand, originating from glacial till (Tamm et al., 1974). In 2010 the average O-horizon depth was 10 cm and the soil O-horizon C and N contents in the control treatment (N0) were 22.6 Mg C ha^{-1} and 0.66 Mg N ha^{-1}, respectively (Rappe-George et al., 2013). The current Norway spruce forest was planted in 1958 and in 2010 it had a standing stem volume of 286 m^3 ha^{-1} on bark. Field layer vegetation is sparse, dominated by bilberry (*Vaccinium myrtillus* L.) and ground vegetation by mosses and lichens. The experiment had a randomized block design with 30 m × 30 m experimental plots replicated in two blocks and treatments started 1967. Treatments considered in this study are the control (N0), and N addition treatments (N1 and N2, Table 2). The N2 treatment was terminated in 1991 with a total load of 1760 kg ha^{-1} of N, while the N1 treatment is still on-going at a current rate of 30 kg N ha^{-1}

Table 1
General information of the sites included in the present study.

	Unit	Stråsan	Skogaby	Čertovo
Geodesic coordinates	Lat./Long.	60°54′/16°01′	56°33′/13°13′	49°09′/13°11′
MAT	°C	3.1	7.6	5.4
MAP	mm yr^{-1}	745	1187	1413
Current N deposition	Kg N ha^{-1} $year^{-1}$	3.2[a]	14.8[b]	14.6[c]
Dominant tree species	–	Norway Spruce	Norway spruce	Norway spruce
Stand age	Years	55	47	150
Bed rock	–	Granite	Granite – Gneiss	Gneiss
Ground vegetation	–	bilberry, mosses and lichens	Mosses	bilberry, mosses, lichens, grasses
Soil type	FAO class	Haplic Podzol	Haplic Podzol	Haplic Podzol
Base saturation	%	8[d]	8[e]	9[f]
Experimental treatments		N0, N1, N2	N0, NS	None

[a] Estimated from throughfall N concentrations at a monitoring station of the Swedish throughfall monitoring network (www.ivl.se) and precipitation data from a monitoring station of the Swedish Meteorological and Hydrological Institute (www.smhi.se).
[b] Estimates from Olsson et al. (2013).
[c] Estimates from Kopácek and Hruska (2010).
[d] Sampling in 1989 in control plots at 0.2–0.3 m depth (Eriksson et al., 1996).
[e] Sampling in 1989 in control plots at 0.25 m depth (Bergholm et al., 2003).
[f] Sampling in 1997 at 0.25 m depth (Kopácek et al., 2002).

Table 2
Historic nitrogen (N) load (1950–2010), recent N load (2005–2013) due to deposition and experimental N addition, and estimates of recent nitrate-nitrogen (NO_3-N) leaching (2005–2013) and proposed N retention classification of study plots at Stråsan, Skogaby and Čertovo.

Variable	Unit	Stråsan			Skogaby		Čertovo
		N0	N1	N2	N0	NS	
Historic N deposition (1950–2010)	kg ha^{-1} of N	195[a]	195[a]	195[a]	723[b]	723[b]	1168[c]
Experimental N addition	kg ha^{-1} of N	0	1570	1760	0	1400	0
Total historic N load (1950–2010)	kg ha^{-1} of N	195	1765	1955	723	2123	1168
Recent N load (2005–2012)	kg ha^{-1} $year^{-1}$ of N	3.2[a]	33.2[a]	3.2[a]	14.8[d]	14.8[d]	14.6[c]
Recent NO_3-N leaching	kg ha^{-1} $year^{-1}$ of N	0.05	1.97	0.05	1.00[d]	14.5[d]	15.0[e]
N retention class	–	Low-in, Low-out	High-in, Low-out	Low-in, Low-out	High-in, Low-out	High-in, High-out	High-in, High-out

[a] Estimated from throughfall N concentrations at a monitoring station of the Swedish throughfall monitoring network hosted by the Swedish Environmental Research Institute and precipitation data from a monitoring station hosted by the Swedish Meteorological and Hydrological Institute (www.smhi.se).
[b] Estimates from Bergholm et al. (2003), Olsson et al. (2013) and Hansen et al. (2013).
[c] Estimates from Kopácek and Hruska (2010).
[d] Estimates from Olsson et al. (2013), covering the period 2005–2010.
[e] Estimate from Kopáček, J. (pers. comm.).

$year^{-1}$ (as solid ammonium nitrate, NH_4NO_3) with a total load of 1570 kg ha^{-1} of N (year 2013).

2.1.2. Skogaby

The experimental Norway spruce forest site Skogaby is situated in southern Sweden, ca. 15 km from the city Laholm (Table 1). The soil texture is characterized as a loamy sand overlying a gneiss bedrock (Bergholm et al., 1995). The soil O-horizon C and N contents in control plots were 32.5 and 1.2 Mg ha^{-1}, respectively, in 2004 (Persson, 2004).

Before 1913 the site was a *Calluna* heathland used for grazing cattle. It was then afforested with Scots pine (*Pinus sylvestris* L.), and the current Norway spruce stand was planted with seedlings of Polish provenance in 1966 (Bergholm et al., 1995). Control plots (N0) had an average standing stem volume of 244 m^3 ha^{-1} on bark in 2010 (pers. comm., U. Johansson, Swedish University of Agricultural Sciences). There is very sparse field layer vegetation with some grasses and ground vegetation dominated by mosses. The experiment was initiated in 1988, in a randomized block design with 45 m × 45 m experimental plots replicated in two blocks (Bergholm et al., 1995). For this study, soil was sampled from experimental plots subjected to addition of ammonium sulfate (NS), terminated in 2001 with a total load of 1400 kg ha^{-1} of N, and control plots (N0).

2.1.3. Čertovo

The Čertovo study site is situated in the watershed of Čertovo lake in the Šumava Mountains, Czech Republic (Table 1). The bedrock consists of gneiss (Kopácek et al., 2002). The average O-horizon depth was 9 cm, and total estimated soil O-horizon C and N contents in 2008–2011 were 18.9 and 0.7 Mg ha^{-1}, respectively (Tahovská et al., 2013). The area is covered by a mature (~150-year-old) Norway spruce forest which has been the dominant species since the pre-boreal era (ca 10 000 years B.P. Jankovská, 2006). There are some minor contributions (~3%) of European beech (*Fagus sylvatica* L.) and silver fir (*Abies alba* Mill.). The field layer vegetation is dominated by bilberry. Samples were taken nearby the long-term investigated plot located in the lower part of the watershed. No experimental treatment is applied in this forest. Estimates of historic and current N deposition were obtained from Kopácek and Hruska (2010), according to which N loads via deposition peaked in the early 1990's at more than 20 kg N ha^{-1} $year^{-1}$ (Kopácek and Hruska, 2010).

2.2. Soil sampling

Samples of O-horizon soil were taken in spring 2013, on the 22nd of April, 2nd of May and 9th of May at Čertovo, Skogaby and Stråsan, respectively. At both Stråsan and Skogaby, two soil samples were collected from two randomly chosen perpendicular sides of

every experimental plot, at fixed distances (10 and 20 m) from their intersecting corner, resulting in eight samples per treatment. At Čertovo, four sampling points were placed with comparable spacing to those at Skogaby and Stråsan. At each sampling point, a general sample of 25 cm × 25 cm of O-horizon soil was collected. All soil samples were stored in portable cooling boxes filled with dry ice during transport to the laboratory. Roots (>1 mm diameter) were removed when the soil samples were sieved (mesh size 4 mm). Subsamples for analyses of enzymatic activities and phospholipid fatty acids (PLFAs) were stored frozen at −20 °C until further preparation. Subsamples for total C and N analyses were dried at 35 °C to constant weight and the rest of the soil (used for determinations of basal respiration, microbial biomass C and N, and extractable N species) was stored in polyethylene bags at 4 °C prior to analyses.

2.3. Laboratory analyses

2.3.1. Soil chemical characteristics

Soil pH was determined by measurements of slurries in deionized water (1:10, w/v) using a 315i pH meter (WTW, Germany). Total C (C_{tot}) and N (N_{tot}) contents were measured using a Vario MICRO cube element analyzer (Elementar GmbH, Germany). Extractable N (N_{K2SO4}) was extracted in 0.5 M K_2SO_4 (1:4, w/v) and analyzed using a LiquiTOC II TOC/TN analyzer (Elementar, Germany). Water-extractable C, N and P were extracted with deionized water (1:10, v/w), then ammonium (NH_4^+), NO_3^- and phosphate (PO_4^{3-}) were analyzed using a QC8500 Flow Injection Analyzer (Lachat Instruments, USA), while dissolved organic C (DOC) and dissolved N (DN) were analyzed using the LiquiTOC II TOC/TN analyzer. Ultraviolet (UV) absorbance of water extracts at 254 nm was measured in a 1 cm cuvette using a UV-1800 spectrophotometer (Shimadzu Corporation, Japan), and distilled water as a blank. Specific UV-absorbance (SUVA; mg C^{-1} m^{-1}) was then calculated by dividing the UV absorbance at 254 nm (m^{-1}) by the DOC concentration of each water extract (mg C L^{-1}).

2.3.2. Microbial biomass and activity

Soil microbial carbon (C_{mic}) and nitrogen (N_{mic}) contents were determined using the chloroform fumigation-extraction method according to Vance et al. (1987). Fumigated and non-fumigated soils were extracted with 0.5 M K_2SO_4 (1:4, w/v) then total C and N contents in the extracts were measured using the LiquiTOC II TOC/TN analyzer. C_{mic} and N_{mic} were subsequently calculated as the difference between C and N contents of fumigated and non-fumigated soils using correction coefficients of 0.45 for C (Vance et al., 1987) and 0.54 for N (Brookes et al., 1985). Soil microbial biomass P (P_{mic}) was analyzed according to Brookes et al. (1982). Briefly, three sets of portions of each sample — fumigated, non-fumigated and non-fumigated with added internal standard KH_2PO_4 solution — were extracted with 0.5 M $NaHCO_3$ (pH = 8.5; 1:5, w/v), their P contents were measured using a Genesys 10S UV-Vis absorption spectrophotometer (Thermo Scientific, USA), then their P_{mic} contents were calculated using a correction coefficient of 0.4 (Brookes et al., 1982). Basal soil respiration (BR) rates were measured as the increases in CO_2 concentration during 7-day incubations of soil samples at 15 °C in bottles sealed with rubber covers, detected using a gas chromatograph (6850 Series, Agilent, USA).

2.3.3. Soil enzymatic activities

Potential extracellular enzyme activities were determined by microplate fluorometric and photometric assays under standard conditions. To determine hydrolytic enzyme activities, 1 g portions of the soil samples (dry matter) were each suspended in 100 ml of distilled water and sonicated for 4 min to disrupt particles. Portions (200 μL) of the resulting suspensions were each mixed with 50 μL of methylumbelliferyl (MUF)-labeled substrates (Marx et al., 2001), placed in wells of microplates (Tecan, Germany), and incubated at 20 °C for 120 min. Fluorescence was then measured at an excitation wavelength of 365 nm and emission wavelength of 450 nm using an Infinite F200 microplate reader (Tecan, Germany). Potential activity of soil oxidative enzymes (phenoloxidases and peroxidases) were determined photometrically using l-3,4-dihydroxyphenylalanine (L-DOPA) substrate (Bárta et al., 2010). Briefly, 200 μL of filtered soil suspension was mixed with 50 μL of acetate buffer (pH = 5.0) and 50 μL of 25 mM L-DOPA solution. For peroxidase determination, 10 μL of 0.3% H_2O_2 (v/v) was added to the L-DOPA solution. The increase in absorbance at 460 nm was recorded after 18 h. The ratio of activities of the hydrolytic C-acquiring enzymes (β-glucosidases and cellobiohydrolases) to N-acquiring enzymes (leucine-aminopeptidases), here called the C/N enzyme ratio, was used as an indicator of the ratio of C/N demand of the microbial community.

2.3.4. Phospholipid fatty acids (PLFA)

PLFAs were extracted from soil samples equivalent to 0.3 g freeze-dried soil, according to Bligh and Dyer (1959), as modified by White et al. (1979). First, lipids were extracted using a mixture (1:2:0.8 v/v/v) of chloroform, methanol and citrate buffer (pH = 4). Secondly, lipids were separated on silica gel columns (Bond Elut LRC, SI 500 mg; Agilent technologies, Santa Clara, US) with chloroform, acetone and methanol in sequence (Frostegård et al., 1991) and thereafter dried under N_2-flow. Following separation on silica columns, mild alkaline methanolysis was performed on samples according to Dowling et al. (1986). The fatty acid methyl ester (FAME) methylnonadecanoate (19:0; Larodan, Malmö, Sweden) was added to the samples, as an internal standard, prior to the methanolysis. The resulting FAMEs were analyzed on a gas chromatograph (Hewlett Packard 6890) with a flame-ionization detector (GC-FID) as described by Steger et al. (2003). Individual FAMEs were determined by comparing retention times with those of FAME standards (FAME 37-47885-U; Supelco, Bellefonte, US). Chemicals used were of analytical grade and all glassware was pyrolytically cleaned (500 °C, 12 h) before use.

Fatty acids were named in accordance with standard nomenclature, by sequentially stating the total number of C atoms followed by the number of double bonds and position(s) of the double bond(s) counted from the methyl end of the molecule. For example, the fatty acid palmitoleic acid (16:1ω7) has sixteen (16) C atoms in its aliphatic chain, with one (:1) double bond located at the seventh (ω7) C atom counted from the aliphatic end. *Cis* and *trans* double bond configurations were denoted by "*c*" and "*t*", respectively, the prefixes i- and a-were used to specify iso- and anteisobranching of the C chain, respectively, and the prefix "cy" when referring to cyclopropane fatty acids. In total, 30 PLFAs were identified in each sample. Generally, specific PLFAs (or combinations thereof) should be assigned to certain functional or physiological organism groups with caution (Frostegård and Bååth, 2011). We used higher-order combinations of PLFAs as indicators of relative abundances of specific groups of organisms, as follows. The PLFA 18:2ω6,9 was interpreted as indicative of eukaryotic cell membranes, which were assumed to be fungal in these forest soils (Frostegård and Bååth, 1996; Kaiser et al., 2010). Relative abundances of 18:2ω6,9 and 18:1ω9 (another potential fungal PLFA biomarker) were strongly and positively correlated across our dataset ($p < 0.01$; $R = 0.72$), corroborating the assumption (Erwin, 1972; Frostegård and Bååth, 2011). The sum of i15:0, a15:0, 15:0, i16:0, 16:1ω7, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 contents was used as a lipid signature for bacterial biomass (Frostegård and Bååth, 1996). The ratio of fungal

to bacterial biomass (F/B_{PLFA}) was calculated from the above-mentioned designations of fungal and bacterial PLFAs. The PLFAs i15:0, i16:0, a17:0 are common in Gram-positive bacteria (Zelles, 1999), so their sum was used as an indicator of these bacteria's abundance. Analogously, the sum of 16:1ω7c, 18:1ω7 and cy19:0 PLFAs was calculated as a measure of the abundance of Gram-negative bacteria (Zelles, 1999). 10Me16:0, 10Me17:0 and 10Me18:0 were used as signature lipid biomarkers for actinobacteria (Zelles, 1999). The ratio of cyclopropyl to precursor PLFA (Cy/Pre), here interpreted as indicating metabolic stress of the microbial community, was calculated as the sum of cy17:0 and cy19:0 divided by the sum of 16:1ω7 and 18:1ω7 (Bossio and Scow, 1998).

2.3.5. Soil water nitrogen fluxes

At Stråsan, soil water fluxes at 0.5 m depth during the years 2009 and 2013 were estimated by the use of a numerical hydrological model (CoupModel; Jansson and Karlberg, 2004; Jansson, 2012) and multiplied by measured soil water NO_3-N concentrations (12 measurement occasions during both 2009 and 2013, respectively; Rappe-George et al., 2013) to obtain estimates of soil water NO_3-N leaching fluxes during these years (Table 2). The major components of the water balance including evapotranspiration with the Penman-Monteith combination equation were calculated by using the CoupModel accordingly. Interception losses were calculated based on an intercepted amount of water in the forest canopy. The leaf area index (LAI) was set at 4 m^2 m^{-2} (Gårdenäs and Jansson, 1995). Water infiltrating into the soil profile was transferred between discrete, user-defined, soil layers by solving Richards equation based on soil water potentials and soil physical properties. The model's structure and parameterization were based on data obtained and relationships observed in previous studies at the Skogaby experimental site (Alavi et al., 2001; Alavi, 2002), but it was calibrated manually, varying the canopy resistance, to fit soil temperature and moisture measurements at Stråsan (see below). Brooks and Corey (1964) hydrological model parameters were based on soil samples taken at three depths (0.1, 0.2 and 0.5 m) on opposite sides of two 1 m deep pits in the control and N1 plots of both blocks. Three samples were taken at each of these depths, and the particle size distribution, bulk density, porosity, moisture content, water holding capacity at six water tensions, and saturated hydraulic conductivity of each of the resulting soil samples were determined. Soil temperature and moisture at 0.1, 0.2 and 0.5 m depth were measured at Stråsan, by CS616 Time-Domain-Reflectometry (TDR) and 105T temperature probes (both from Campbell Scientific Ltd., UK) installed in the N0 and N1 plots during the period 2012-11-30 to 2015-08-25. The TDR ($n = 16$) and temperature ($n = 16$) probes were installed in the outer 5 m of the experimental plots subjected to the N0 and N1 treatments. Volumetric water content was estimated from data acquired from the TDR-probes, calibrated following the manufacturer's instructions and accounting for variations in soil temperature. Calculated runoff with the model was compared to runoff recorded at a nearby long-term monitoring catchment operated by the Swedish Meteorological and Hydrological Institute (SMHI, 2015) to evaluate the magnitude of annual runoff and its difference between years (2009 and 2013). Leaching fluxes of N species were calculated by multiplying calculated water fluxes (at 0.5 m soil depth) with soil water concentrations measured at ~0.5 m depth on 12 occasions during both 2009 and 2013. Winter time NO_3-N concentrations were interpolated from the first and last measured soil water NO_3-N concentrations, in spring and autumn, respectively.

2.3.6. N retention classes

The experimental plots included in the study were classified according to the estimated retention of N loads based on recent (2005–2013) N loading and NO_3^- leaching estimates. Wright et al. (2001) suggested a threshold of N deposition of 10 kg ha^{-1} $year^{-1}$ of N above which elevated NO_3^- concentrations are frequently observed in streams across Europe. The experimental plots were divided into three categories based on N loading respective N leaching $\geq$10 kg ha^{-1} $year^{-1}$ of N (Table 2): *i*) "Low in, Low-out" experimental plots that currently receive low N loads (<10 kg ha^{-1} $year^{-1}$ of N) and leach <10 kg ha^{-1} $year^{-1}$ of NO_3-N, these are Stråsan N0 and N2, *ii*) "High-in, Low-out" experimental plots that currently receive relatively high N loads (>10 kg ha^{-1} $year^{-1}$ of N) but leach low amounts, these are Stråsan N1 and Skogaby N0, and *iii*) "High-in, High-out" experimental plots that currently receive relatively high N loads and leach large amounts of NO_3, these are Skogaby NS and Čertovo.

2.4. Statistical analyses

Between-sites differences in soil chemistry, microbial biomass and activity, and signature PLFAs along the N deposition gradient were evaluated using a mixed linear model including a fixed effect of site and random effect of plot (Model 1). Between-treatment differences in soil chemistry, microbial biomass and activities, and signature PLFAs at the N addition experimental sites were evaluated using a mixed linear model including a fixed effect of treatment and random effect of plot nested within block (Model 2). The parameter estimates for the fixed effects of site and treatment in models 1 and 2 were evaluated with the Wald statistic, ξ_W (Wald, 1943). Comparisons between least squares means were adjusted for multiplicity using Tukey's method and p-values <0.05 were considered significant. The assumptions underlying the analysis were checked using diagnostic plots. Variables were log-transformed where necessary to satisfy model assumptions, and back-transformed to obtain geometric means and corresponding confidence intervals in such cases.

Patterns in the PLFA data set and microbial variables for which statistically significant effects were found were explored by principal component analysis (PCA) of the covariance matrix for the PLFA data (based on mol percentages, mol %) and the correlation matrix for other microbial variables. Scores and loadings of the first two principal components were examined via scatter plots. All statistical tests and data handling were performed in R (R Core Team, 2015).

3. Results

3.1. Forest nitrogen addition experiments

3.1.1. Soil chemistry

Four soil chemical characteristics were affected by long-term N loading at the study sites (Table 3). At Stråsan, the N content of the O-horizon (N_{tot}) and water-extractable NO_3-N were significantly affected by N treatment ($p = 0.04$): both were significantly higher in the N2 plots than in the N0 controls. Conversely, the C/N ratio ($p = 0.04$) was lower in the N2 plots than in the N0 controls. These variables in the N1 plots at Stråsan were intermediary and differed non-significantly from both the N2 and the N0 plots. Extractable N contents (N_{K2SO4}, NH_4-N, NO_3-N and DON) were higher under both N treatments than under the N0 treatment, but the only significant treatment effect was found for water-extractable NO_3-N ($p = 0.04$), where N2 and N0 differed significantly. At Skogaby, extractable N contents did not differ between treatments, but the C/N ratio of the O-horizon was affected by N treatment ($p = 0.02$) and was lower in the NS than N0 plots. No treatment effects on pH were detected at either site. SUVA was affected by N treatment at both Stråsan and

Table 3
Chemical variables of O-horizon sampled from plots in the Stråsan and Skogaby nitrogen addition experiments. Values are means, with 95% confidence intervals in brackets (n = 8). Wald test scores (ξ_W) for treatment effects are shown; multiple comparisons were adjusted with the Tukey method. Within row and experimental site, variable means with different superscripts (^{a,b}, and ^{c}) differ at p < 0.05. Significant treatment effects are marked in bold. d.w. – dry weight.

Variable	Unit	Stråsan					Skogaby			
		ξ_W	p-value	N0	N1	N2	ξ_W	P	N0	NS
pH	–	1.60	0.45	4.54 (4.36–4.73)	4.44 (4.25–4.63)	4.46 (4.27–4.65)	0.02	0.90	4.45 (4.36–4.54)	4.44 (4.35–4.53)
C_{tot}	mg g^{-1}	5.17	0.08	277 (228–325)	346 (297–394)	321 (273–369)	2.73	0.10	436 (406–467)	404 (373–434)
N_{tot}	**mg g^{-1}**	**6.67**	**0.04**	**8.09^{a} (5.78–10.4)**	**10.8^{ab} (8.53–13.2)**	**11.4^{b} (9.04–13.7)**	0.11	0.74	15.4 (13.7–17.0)	15.8 (14.1–17.4)
C/N	**g C g N^{-1}**	**6.38**	**0.04**	**35.0^{a} (31.7–38.6)**	**31.9^{ab} (28.4–35.3)**	**28.9^{b} (25.4–32.3)**	**5.61**	**0.02**	**28.4^{a} (26.9–29.9)**	**25.9^{b} (24.5–27.4)**
N_{K2SO4}	μg g^{-1} d.w.	5.29	0.07	69.8 (0–167)	143 (46.5–240)	128 (30.7–225)	0.79	0.38	155 (107–203)	125 (76.9–172)
DN	μg g^{-1} d.w.	0.94	0.62	61.8 (27.5–96.1)	73.4 (39.1–108)	72.4 (38.1–107)	2.01	0.16	51.9 (37.2–66.6)	36.9 (22.2–51.6)
NH_4-N	μg g^{-1} d.w.	2.99	0.22	9.22 (0–29.5)	14.7 (0–34.9)	17.6 (0–37.9)	1.79	0.18	11.6 (4.74–18.4)	4.97 (0–11.8)
NO_3-N	**μg g^{-1} d.w.**	**6.50**	**0.04**	**1.33^{a} (0.85–1.81)**	**1.44^{ab} (0.96–1.93)**	**2.15^{b} (1.67–2.64)**	0.05	0.83	1.90 (1.46–2.34)	1.83 (1.39–2.27)
DON	μg g^{-1} d.w.	0.435	0.81	51.2 (35.5–66.9)	57.2 (41.6–72.9)	52.6 (36.9–68.3)	2.15	0.14	38.5 (30.5–46.4)	30.1 (22.2–38.0)
DOC	μg g^{-1} d.w.	0.003	0.99	1100 (830–1400)	1120 (834–1400)	1120 (824–1390)	1.61	0.20	927 (716–1140)	775 (564–986)
$SUVA_{254}$	**l mg C^{-1} m^{-1}**	**17.0**	**<0.01**	**2.45^{a} (1.82–3.08)**	**3.12^{ab} (2.49–3.75)**	**4.20^{b} (3.57–4.83)**	**4.16**	**0.04**	**2.74^{a} (2.01–3.47)**	**3.51^{b} (2.78–4.24)**

Skogaby ($p < 0.01$ and $p = 0.04$, respectively) and was higher in the terminated N-treated plots N2 at Stråsan and NS (Skogaby) than their respective control (Stråsan N0 and Skogaby N0).

3.1.2. Soil microbial variables

Significant between-treatment differences in soil microbial variables were found at Stråsan and Skogaby (Table 4). At Stråsan, both C_{mic} and N_{mic} were lower in the ongoing N treatment (N1 plots) than in the N0 plots (with p-values for treatment effects on C_{mic} and N_{mic} of <0.01 and 0.01, respectively). The reductions in C_{mic} and N_{mic} were accompanied by lower basal respiration ($p = 0.02$). Similar N treatment tendencies were observed at Skogaby. Both N_{mic} and P_{mic} were significantly lower in the NS than in N0 plots ($p = 0.02$ and $p < 0.01$, respectively), while C_{mic} was slightly (but non-significantly) lower. Basal respiration was also significantly lower in NS plots than in N0 plots ($p = 0.01$).

The activity of several C-acquiring hydrolases was affected by the ongoing N treatment N1 at Stråsan. β-glucosidase and cellobiohydrolase activities differed between N treatments at this site ($p < 0.01$ and $p = 0.01$, respectively). The β-glucosidase activity was higher in the N1 than in both N0 and N2 plots, and the activity of C-acquiring cellobiohydrolase was significantly higher in N1 than in N2, but not N0, plots. The activity of leucine-aminopeptidases was also affected by the N treatments at Stråsan ($p < 0.01$), and was lower in the fertilized N1 and N2 plots than in the N0 plots. The C/N enzyme ratio was much higher in the N1 plots than N0 and N2 plots at Stråsan. Similarly, leucine-aminopeptidase activity was significantly lower in Skogaby NS plots than in N0 plots ($p = 0.02$), while the C/N enzyme ratio was higher ($p = 0.04$) in NS than N0 plots at Skogaby. Soil peroxidase activity differed between N treatments at Skogaby ($p = 0.01$), but did not differ between N treatments at Stråsan. Potential soil chitinase and phenoloxidase activities did not

Table 4
Microbiological variables of O-horizon sampled from plots in the Stråsan and Skogaby nitrogen addition experiments. Values are means, with 95% confidence intervals in brackets (n = 8). Wald test scores (ξ_W) for treatment effects are shown; multiple comparisons were adjusted with the Tukey method. Within row and experimental site, variable means with different superscripts (^{a,b}, and ^{c}) differ at p < 0.05. Significant treatment effects are marked in bold.

Variable	Unit	Stråsan					Skogaby			
		ξ_W	p-value	N0	N1	N2	ξ_W	p-value	N0	NS
C_{mic}	**μg g^{-1}**	**23.9**	**<0.01**	**2790^{a} (2280–3300)**	**1430^{b} (921–1950)**	**1690^{b} (1180–2200)**	2.64	0.10	2380 (1620–3140)	1710 (952–2480)
N_{mic}	**μg g^{-1}**	**9.80**	**0.01**	**532^{a} (410–654)**	**258^{b} (136–380)**	**372^{ab} (250–494)**	**5.39**	**0.02**	**429^{a} (348–510)**	**294^{b} (213–375)**
P_{mic}	**μg g^{-1}**	4.53	0.10	328 (233–423)	221 (125–316)	189 (93.5–284)	**14.4**	**<0.01**	**363^{a} (299–427)**	**192^{b} (128–256)**
C_{mic}/N_{mic}	mol:mol	0.63	0.73	5.15 (3.88–6.42)	5.45 (4.17–6.72)	4.95 (3.68–6.23)	1.08*	0.41	5.25 (4.24–6.51)	5.78 (4.67–7.16)
C_{mic}/P_{mic}	mol:mol	1.14	0.57	19.3 (6.94–31.6)	17.1 (4.71–29.4)	25.1 (12.7–37.4)	3.69	0.06	13.6 (8.89–20.9)	19.7 (12.9–30.3)
N_{mic}/P_{mic}	mol:mol	3.63	0.16	3.75 (2.46–5.04)	3.11 (1.82–4.40)	4.83 (3.54–6.12)	2.43	0.12	2.81 (2.26–3.37)	3.44 (2.88–3.99)
Basal respiration*	**μmol C g^{-1} d^{-1}**	**7.74**	**0.02**	**9.26^{a} (7.12–12.0)**	**5.85^{b} (4.50–7.61)**	**5.88^{ab} (4.53–7.64)**	**6.43**	**0.01**	**5.99^{a} (5.19–6.79)**	**4.58^{b} (3.78–5.38)**
β-glucosidase	**nmol g^{-1} h^{-1}**	**19.7**	**<0.01**	**245^{a} (86.5–404)**	**646^{b} (501–790)**	**226^{a} (66.8–385)**	0.30	0.58	471 (328–614)	528 (384–671)
Phosphatase	nmol g^{-1} h^{-1}	1.78	0.41	597 (228–967)	564 (195–933)	887 (518–1260)	0.24	0.62	536 (435–637)	500 (399–601)
Cellobiohydrolase	**nmol g^{-1} h^{-1}**	**13.6**	**<0.01**	**37.3^{ab} (17.4–57.2)**	**66.3^{a} (48.2–84.4)**	**16.9^{b} (0–36.8)**	1.03	0.31	37.8 (20.3–55.3)	50.6 (33.1–68.1)
Chitinase*	nmol g^{-1} h^{-1}	3.46	0.18	160 (114–226)	132 (94.0–187)	102 (72.1–143)	0.02	0.89	99.5 (69.6–142)	95.9 (67.1–137)
Phenoloxidase	nmol g^{-1} h^{-1}	3.34	0.19	773 (453–1090)	779 (459–1100)	1130 (806–1450)	0.66	0.41	1085 (610–1561)	830 (355–1306)
Peroxidase	**nmol g^{-1} h^{-1}**	0.34	0.85	1450 (0–2930)	1150 (0–2630)	1770 (292–3250)	**6.18**	**0.01**	**694^{a} (416–973)**	**1160^{b} (880–1440)**
Leu-aminopeptidase	**nmol g^{-1} h^{-1}**	**39.9**	**<0.01**	**47.6^{a} (42.6–52.6)**	**30.6^{b} (25.6–35.6)**	**26.7^{b} (21.7–31.7)**	**5.94**	**0.02**	**39.2^{a} (31.5–46.9)**	**26.7^{b} (19.0–34.4)**
C/N enzyme ratio	**-**	**18.9**	**<0.01**	**5.97^{a} (0.00–12.60)**	**24.7^{b} (18.5–30.9)**	**9.69^{a} (3.06–16.3)**	**4.17**	**0.04**	**13.1^{a} (4.65–16.1)**	**24.6^{b} (16.1–33.0)**
Total PLFA	**nmol g $soil^{-1}$**	**12.9**	**<0.01**	**602^{a} (528–676)**	**443^{ab} (369–517)**	**426^{b} (345–507)**	**8.42**	**0.04**	**558^{a} (515–601)**	**468^{b} (425–511)**
F/B_{plfa}	–	1.35	0.51	0.225 (0.154–0.296)	0.169 (0.099–0.241)	0.178 (0.107–0.249)	1.05	0.34	0.166 (0.135–0.197)	0.161 (0.131–0.192)
Cy/Pre	**-**	**8.36**	**0.02**	**0.70^{a} (0.55–0.84)**	**0.96^{b} (0.81–1.10)**	**0.95^{ab} (0.81–1.09)**	1.02	0.31	1.49 (1.22–1.78)	1.70 (1.42–1.98)
Actino	**Mol %**	**19.9**	**<0.01**	**7.73^{a} (0.812–1.10)**	**11.2^{b} (9.92–12.4)**	**9.08^{ab} (7.83–10.3)**	1.52	0.22	10.2 (8.22–12.2)	11.9 (9.98–13.9)
Gram +	**Mol %**	**22.0**	**<0.01**	**18.4^{a} (17.3–19.5)**	**21.9^{b} (20.8–23.0)**	**19.4^{a} (18.4–20.5)**	0.36	0.99	23.9 (22.5–25.3)	23.9 (22.5–25.3)
Gram -	**Mol %**	**7.29**	**0.03**	**18.1^{a} (15.6–20.6)**	**13.3^{b} (10.7–15.8)**	**14.8^{ab} (12.3–17.3)**	0.84	0.36	12.3 (10.4–14.1)	11.5 (9.65–13.4)

*Statistical tests performed on logged variables, so presented values are geometric means and corresponding confidence intervals.

differ between N treatments at neither Stråsan nor Skogaby.

Analyses of total extracted PLFAs (nmol g dry soil^{-1}, Table 4) confirmed that there were significant treatment effects on microbial biomass at both Stråsan and Skogaby ($p < 0.01$ for both) and less PLFAs indicative of both fungal and bacterial biomass were extracted in soil samples from the N loaded plots. No significant treatment effect on the fungi/bacteria biomarker ratio (F/B_{PLFA}) was detected at either site. However, there were significant treatment effects on various other PLFA biomarkers at Stråsan, including the cyclopropyl/precursor PLFA ratio (Cy/Pre; $p = 0.02$), which was higher in the ongoing N1 than N0. In addition, the relative abundance of PLFAs indicative of actinobacteria was affected by treatment ($p < 0.01$) with significantly higher abundance in N1 than N0 plots at Stråsan. The relative abundances of PLFAs associated with Gram-positive and Gram-negative bacteria were affected by the N treatment at Stråsan as well ($p < 0.01$ and $p = 0.03$, respectively). In plots receiving the ongoing N treatment (N1), but not N2 plots, Gram-positive and Gram-negative PLFA biomarkers were higher and lower, respectively, than in the control plots. At Skogaby, no significant treatment effects on PLFA biomarkers were found, although there were similar tendencies of N treatments to those observed at Stråsan.

3.2. Nitrogen deposition gradient

3.2.1. Soil chemistry

The non-fertilized plots Stråsan N0, Skogaby N0 and Čertovo displayed a soil chemistry gradient (Table 5) which was in several variables correlated to the gradient in long-term N deposition spanned by the sites (Tables 1 and 2). The Czech Republic site, Čertovo, had lower soil pH and O-horizon C/N ratios, while higher N_{K2SO4}, NO_3-N and DON contents than one or both of the Swedish sites. At the intermediate N deposition site Skogaby, the C_{tot} was higher than at the two other sites, while the C/N ratio and NK_2SO_4 were intermediate, but the latter two variables at Skogaby did not significantly differ from either those at Stråsan or Čertovo. Water-extractable NO_3-N contents were much higher at Čertovo than at both Stråsan and Skogaby; however, water-extractable NH_4^+ contents did not differ between the sites. Water-extractable NH_4^+-N was the dominant fraction of extractable N (accounting for 86% of the total on a molar basis) at both Stråsan and Skogaby, whereas NO_3-N was the dominant extractable N fraction at Čertovo. SUVA values tended to increase across the N deposition gradient (being lowest at Stråsan and highest at Čertovo) but the differences between sites were not statistically significant.

3.2.2. Soil microbial variables

The microbial variables of Skogaby N0 differed most frequently from one or both other sites (Table 6); basal respiration differed between sites ($p = 0.04$) and was lower at Skogaby N0 than at Stråsan N0. Similarly, leucine-aminopeptidase activity differed between sites ($p = 0.03$) and was much lower at Skogaby N0 than at Stråsan N0, while the C/N enzyme ratio was higher. Peroxidase activity differed between the sites ($p = 0.01$) and was lower at Skogaby N0 than Čertovo but intermediary at Stråsan N0. None of the microbial biomass pools or their ratios (C_{mic}, N_{mic}, P_{mic}, C/N_{mic}, C/P_{mic} or N/P_{mic}) differed significantly between sites, but C_{mic} and N_{mic} tended to be lower at Čertovo and Skogaby than at Stråsan.

Similarly as for the other microbial variables, the PLFA biomarkers of Skogaby N0 differed more frequently from one or both sites of Stråsan N0 and Čertovo (Table 6). The F/B_{PLFA} tended to be lower at Skogaby N0 and Čertovo, but differences between sites of this variable were not significant. Neither did total extracted PLFA contents (per mg soil) differ significantly between the three sites. The Cy/Pre ratio was higher at Skogaby N0 than Stråsan ($p < 0.01$). Gram-negative bacteria PLFA biomarkers were lower in Skogaby N0 than both Čertovo and Stråsan ($p < 0.01$), while the Gram-positive bacteria in Skogaby were higher than in Stråsan ($p < 0.01$) and intermediary at Čertovo. Gram-positive bacteria PLFA biomarkers accounted for 18.4, 23.9 and 22 percent of total PLFA at Stråsan, Skogaby and Čertovo, respectively. Corresponding proportions of Gram-negative bacteria PLFA biomarkers were 18.1, 12.3 and 19.1 percent, respectively. The relative abundance of actinobacteria PLFA biomarkers also differed between sites ($p = 0.03$), being higher at Čertovo than at Stråsan while Skogaby N0 was intermediary and differed non-significantly from the other two sites.

3.3. Relationships between soil microbial variables and ecosystem N retention

The data set included measurements and estimates of diverse chemical and microbial variables associated with sites and treatments spanning a wide range of historic N loads from 195 to 2123 kg ha^{-1} of N since 1950 (Table 2). Estimates of current N loading also spanned a large range: 3.1–33 kg ha^{-1} year^{-1} of N during the period 2005–2013. PCA of the microbial variables sensitive to N loading, across all study plots, separated soil samples collected from plots with low and high current N loads along PC1 (Fig. 1). Samples from "Low-in, Low-out" (Group 1) were predominantly of lower, while the "High-in, Low-out" and "High-in, High-out" (Groups 2 and 3) were predominantly of higher, scores of principal component 1. PC1 was most strongly correlated with

Table 5
Chemical variables of O-horizon sampled across the nitrogen deposition gradient (at Stråsan, Skogaby and Čertovo sites). Values are means, with 95% confidence intervals in brackets ($n = 8$ for Stråsan and Skogaby, $n = 4$ for Čertovo). Wald test scores (ξ_W) for site effects are shown, multiple comparisons were adjusted with the Tukey method. Within row, variable means with different superscripts (^{a,b}, and ^{c}) differ at $p < 0.05$. Significant treatment effects are marked in bold. d.w. – dry weight.

Variable	Unit	ξ_W	p-value	Stråsan	Skogaby	Čertovo
pH	-	**18.0**	<**0.01**	**4.54^{a} (4.38–4.71)**	**4.45^{a} (4.28–4.61)**	**3.94^{b} (3.71–4.17)**
C_{tot}	**mg g^{-1} d.w.**	**47.6**	<**0.01**	**277^{a} (243–310)**	**436^{b} (403–470)**	**304^{a} (256–351)**
N_{tot}	**mg g^{-1} d.w.**	**44.1**	<**0.01**	**8.10^{a} (6.54–9.66)**	**15.4^{b} (13.8–16.9)**	**13.7^{ab} (11.5–15.9)**
C/N	**g C g N^{-1}**	**22.3**	<**0.01**	**35.1^{a} (31.9–38.3)**	**28.4^{ab} (25.3–31.6)**	**22.2^{b} (17.7–26.7)**
N_{K2SO4}	**μg g^{-1} d.w.**	**14.2**	<**0.01**	**69.7^{a} (0.00–142)**	**155^{ab} (82.5–228)**	**223^{b} (121–326)**
NH_4-N	μg g^{-1} d.w.	0.11	0.95	9.21 (0.00–20.3)	11.6 (0.50–22.6)	10.9 (0.00–22.6)
NO_3-N *	**μg g^{-1} d.w.**	**170**	<**0.01**	**1.27^{a} (0.917–1.766)**	**1.87^{a} (1.35–2.59)**	**24.4^{b} (17.1–34.8)**
DON	**μg g^{-1} d.w.**	**9.42**	**0.01**	**51.2^{ab} (36.7–65.8)**	**38.5^{a} (23.9–53.0)**	**71.7^{b} (55.9–87.4)**
DN	**μg g^{-1} d.w.**	**10.8**	<**0.01**	**61.77^{ab} (21.4–102)**	**51.9^{a} (11.5–92.4)**	**115^{b} (74.8–155)**
DOC	μg g^{-1} d.w.	5.17	0.08	1110 (856–1370)	927 (670–1180)	1360 (1080–1640)
$SUVA_{254}$	L mg C^{-1} m^{-1}	4.27	0.12	2.45 (1.80–3.09)	2.74 (2.09–3,38)	3.62 (2.71–4.53)

*Statistical tests performed on logged variables, so presented values are geometric means and corresponding confidence intervals.

Table 6
Microbiological variables of O-horizon sampled across the nitrogen deposition gradient (at Stråsan, Skogaby and Čertovo sites). Values are means, with 95% confidence intervals in brackets (n = 8 for Stråsan and Skogaby, n = 4 for Čertovo). Wald test scores (ξW) for site effects are shown, multiple comparisons were adjusted with the Tukey method. Within row, variable means with different superscripts (a,b, and c) differ at $p < 0.05$. Significant treatment effects are marked in bold.

Variable	Unit	ξ_W	p-value	Stråsan N0	Skogaby N0	Čertovo
C_{mic}	μg g^{-1}	0.81	0.67	2790 (2000–3580)	2380 (1590–3170)	2300 (1480–3110)
N_{mic}	μg g-1	3.22	0.20	532 (434–631)	429 (331–528)	419 (316–522)
P_{mic}	μg g^{-1}	0.34	0.84	328 (242–414)	363 (277–448)	357 (236–479)
C_{mic}/N_{mic}	mol:mol	0.55	0.76	5.15 (1.97–8.32)	5.45 (2.23–8.58)	6.72 (3.54–9.91)
C_{mic}/P_{mic}	mol:mol	2.19	0.34	19.3 (13.7–24.9)	15.2 (9.51–20.8)	12.3 (4.34–20.3)
N_{mic}/P_{mic}	mol:mol	4.93	0.09	3.75 (3.15–4.35)	2.81 (2.22–3.41)	3.03 (2.19–3.88)
Basal respiration	**μmol C g^{-1} d^{-1}**	**9.67**	**0.01**	**9.26^{a} (7.53–11.4)**	**5.96^{b} (4.85–7.33)**	**8.71^{ab} (6.50–11.7)**
β-glucosidase	nmol g^{-1} h^{-1}	5.84	0.05	245 (109–382)	471 (346–596)	368 (191–545)
Cellobiohydrolase	nmol g^{-1} h^{-1}	0.64	0.73	37.3 (19.7–54.8)	37.8 (21.7–53.9)	48.0 (25.3–70.8)
Chitinase	nmol g^{-1} h^{-1}	4.99	0.08	185 (117–253)	119 (50.7–187)	53.5 (0.00–150)
Phenol Oxidase	nmol g^{-1} h^{-1}	1.15	0.56	773 (295–1250)	1090 (607–1564)	1150 (476–1830)
Peroxidase	nmol g^{-1} h^{-1}	**9.44**	**0.01**	**1450^{ab} (400–2490)**	**694^{a} (0.00–1740)**	**3520^{b} (2040–5000)**
Leu-aminopeptidase	nmol g^{-1} h^{-1}	**7.28**	**0.03**	**47.6^{a} (36.9–58.3)**	**26.7^{b} (15.9–37.5)**	**37.8^{ab} (22.7–53.0)**
Phosphatases	nmol g^{-1} h^{-1}	0.47	0.79	597 (411–784)	536 (349–723)	644 (380–908)
C/N enzyme ratio	**-**	**13.5**	**<0.01**	**5.97^{a} (3.09–8.85)**	**13.1^{b} (10.4–15.8)**	**11.3^{ab} (7.58–15.1)**
Total PLFA	nmol g $soil^{-1}$	0.98	0.61	602 (532–673)	558 (488–629)	553 (453–652)
F/B_{plfa}	–	3.04	0.22	0.225 (0.152–0.298)	0.166 (0.093–0.239)	0.116 (0.013–0.219)
Cy/Pre	**-**	**33.4**	**<0.01**	**0.70^{a} (0.50–0.90)**	**1.50^{b} (1.31–1.69)**	**1.05^{ab} (0.78–1.33)**
Actinobacteria	**Mol %**	**6.87**	**0.03**	**7.73^{a} (6.08–9.38)**	**10.2^{ab} (8.55–11.8)**	**11.1^{b} (8.76–13.4)**
Gram +	**Mol %**	**35.2**	**<0.01**	**18.4^{a} (17.1–19.7)**	**23.9^{b} (22.6–25.2)**	**22.0^{ab} (20.2–23.8)**
Gram -	**Mol %**	**11.5**	**<0.03**	**18.1^{a} (15.3–20.9)**	**12.3^{b} (9.50–15.1)**	**19.1^{a} (15.1–23.0)**

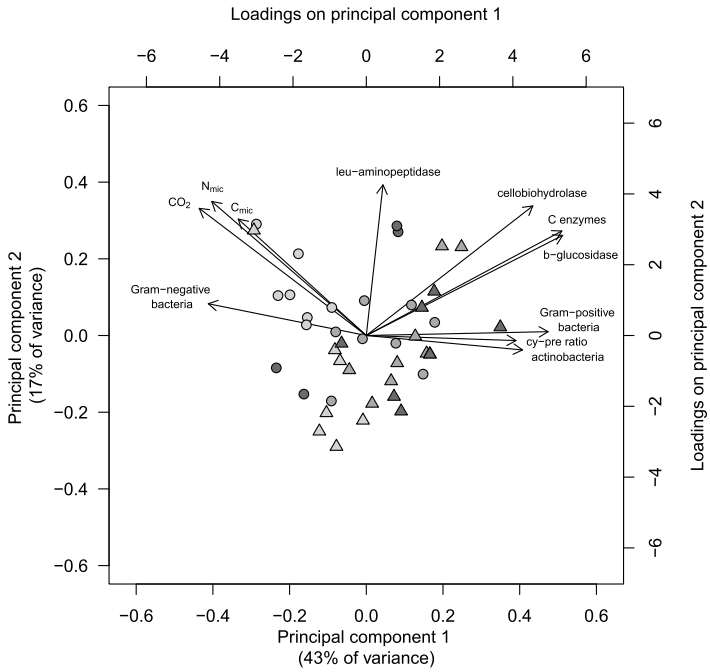

Fig. 1. Biplot of principal component scores (scaled to unit variance) and loadings of selected microbial variables determined in soil samples collected from all experimental plots (at Stråsan, Skogaby and Čertovo). Principal component scores are grouped by N retention class: green indicates plots with low current N load and low NO_3-N leaching ("Low-in, Low-out", Stråsan control and N2 plots), yellow indicates plots with high current N load but low NO_3-N leaching ("High-in, Low-out", Stråsan N1 and Skogaby control plots), and red indicates high current N load and high current NO_3-N leaching ("High-in, High-out", Skogaby NS and Čertovo plots). Dots indicate natural sites that have not received experimental N addition and triangles indicate experimental plots where either NH_4NO_3 or NH_3SO_4 have been added. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

basal respiration (CO_2), C-acquiring enzyme activities, and Gram-positive/Gram-negative bacteria (as reflected by their loadings). PC2 was most strongly correlated with activities of leucine-aminopeptidase, cellobiohydrolase and N_{mic} (as reflected by their

loadings).

Similarly, PCA of the PLFA profiles separated between samples from plots exposed to low and high current N loads along PC1 (Fig. 2). PC1 was most strongly correlated with 18:1ω7, i16:0 and 10Me16:0 while PC2 was most strongly correlated with 18:2ω6,9, 18:1ω7, 16:0 and 16:1ω9 (as reflected by their loadings). As shown in Fig. 2, the PLFA profiles corroborated the differences in soil samples from study plots in Group 1 (Low-in, Low-out) and plots in groups 2 and 3 (High-in, Low-out and High-in, High-out). However, the principal component scores of soil samples from plots in Groups 2 and 3 obtained from the PCA of both summary microbial variables (Fig. 1) and PLFA profiles (Fig. 2) were more similar, despite the large differences in estimated ecosystem N retention.

4. Discussion

Overall, the results presented here document that both long-term experimental N addition and site differences in N deposition resulted in reductions in O-horizon C/N ratios, increases in soil inorganic N availability (reflected in water-extractable inorganic N contents), and varying levels of NO_3^- leaching. Accompanying reductions in microbial biomass C and basal respiration rates were also detected at both of the N addition experimental sites, along with increases in cyclopropyl/precursor PLFAs and the soil C/N enzyme ratio. Thus, most criteria for C limitation in our hypotheses were confirmed except that fungi/bacteria ratio was not altered. Interestingly, fewer responses to N loading were observed in the N deposition gradient compared to in the N addition experiments. The N deposition loads were lower than the experimental N addition. In addition N deposition occurs in small doses all-year around, mainly connected to precipitation, while the experimental N addition occurred in a yearly single dose. Moreover, there may be differences in environmental conditions between sites in the N deposition gradient which were unaccounted for. These include, but are not limited to, differences in geology, pedogenesis and historical land-use – all of which can influence the states and functioning of the soil microbial community. Further, the statistical evaluation of the N deposition gradient was somewhat weakened by a lack of complete block replication at the Czech site, Čertovo. However, although fewer variables differed with statistical significance (at $p < 0.05$) at the N deposition gradient compared to the N addition experiments, the tendency in mean values were the same. Therefore, we argue the data reported here provide indications of overall effects of N loading, across relevant temporal and spatial scales, on soil microbial variables in Norway spruce forests. Some indications were also given here to suggest that microbial variables responded stronger to recent N loading than soil chemical variables (compare N1 and N2 at Stråsan in Tables 3 and 4) which suggests that the microbial community is more sensitive to the current rate of N loading on an ecosystem rather than the historically

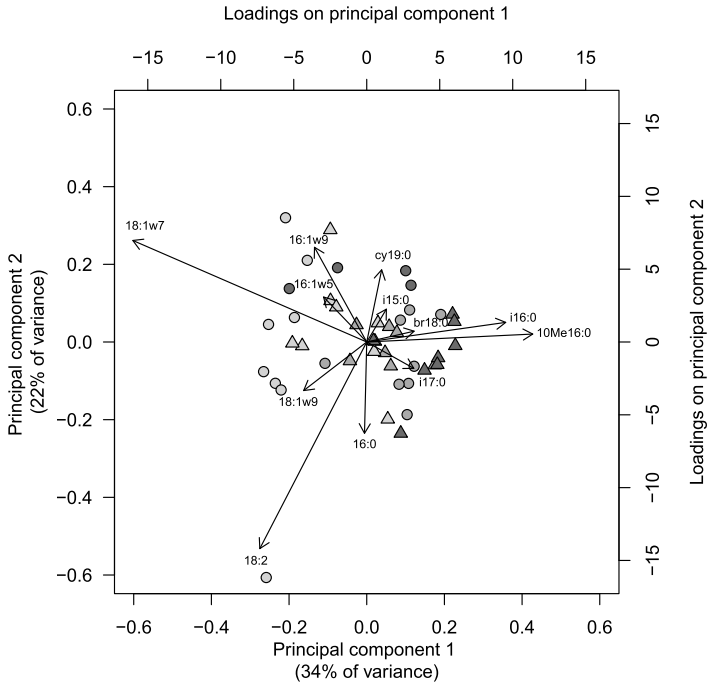

Fig. 2. Biplot of principal component scores (scaled to unit variance) and loadings of PLFA profiles determined in soil samples collected from all experimental plots (at Stråsan, Skogaby and Čertovo). Only PLFA's with the twelve highest loadings on the first two principal components are shown to improve readability. Principal component scores are grouped by N retention class: green indicates plots with low current N load and low NO_3-N leaching ("Low-in, Low-out", Stråsan control and N2 plots), yellow indicates plots with high current N load but low NO_3-N leaching ("High-in, Low-out", Stråsan N1 and Skogaby control plots), and red indicates high current N load and high current NO_3-N leaching ("High-in, High-out", Skogaby NS and Čertovo plots). Dots indicate natural sites that have not received experimental N addition and triangles indicate experimental plots where either NH_4NO_3 or NH_3SO_4 have been added. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

accumulated N load.

Reductions in soil microbial biomass in response to N addition and N deposition have been reported in several previous investigations (Nilsson and Wallander, 2003; Wallenstein et al., 2006; Ramirez et al., 2012), and reduction in heterotrophic soil respiration rates appears to be a general response to N deposition across boreal and temperate forest ecosystems (Janssens et al., 2010). The lower microbial biomass C found in N addition experiments and in the N deposition gradient might reflect a change in the microbial community composition, including a lower abundance of EMF under high N availability. Belowground C allocation of recent photosynthate is a large and rapid flux of C in boreal forest ecosystems and varies with changes in nutrient availability for trees, notably of N (Högberg et al., 2008, 2010). Thus, elevated N availability may lead to reductions in belowground allocation of recent tree photosynthate C by trees and (*inter alia*) a concomitant reduction in EMF (Lilleskov et al., 2002; Högberg et al., 2010, 2014). However, although we found a tendency for F/B_{PLFA} to decline with increases in N loading (under N treatments and across the N deposition gradient) - and lower relative abundance of DNA sequences attributable to EMF have been found in N1 plots than in N0 plots at Stråsan (Choma et al., 2017) - differences in F/B_{PLFA} were not statistically significant in the present investigation.

We found lower abundance of soil PLFA biomarkers indicative of Gram-negative than Gram-positive bacteria under high long-term N loading. Low availability of C sources such as root exudates may adversely affect the abundance not only of fungal, but also Gram-negative PLFA biomarkers (Griffiths et al., 1998) because these bacteria tend to be rapidly growing, rhizosphere specialists (Schlegel, 1992), while Gram-positive bacteria are slow-growing but stress tolerant (Balser, 2005). The distinct changes in C and N hydrolytic enzyme activities due to long-term N loading suggest that the soil microbes oriented their resources towards acquisition of C substrates under elevated N availability. Moreover, the increase in aromaticity of the water-extractable DOC (as indicated by increases in SUVA) may indicate increased utilization of non-aromatic dissolved organic C by the soil microbial community under elevated N availability. Sjöberg et al. (2004) did not find differences in the chemical structure of SOM (i.e. abundance of aromatic C compounds in O-horizon) between NS and N0 plots at Skogaby. The response of C and N hydrolytic enzyme activities to N loading reported here are similar to those reported for a N fertilized boreal forest in Alaska (Allison et al., 2008) and in response to N additions over three years in North American deciduous forests (Waldrop et al., 2004). Andersson et al. (2004) also found that soil enzyme (cellulase and chitinase) activities correlated negatively with N deposition across European forests. We found a similar tendency at our study sites, although differences between N treatments and sites were not significant. Rather, we observed more frequently increases in activities of hydrolytic C-acquiring enzymes, and in C-acquiring to N-acquiring enzyme activity ratios, in response to long-term N loading. Such changes are consistent with expectations and likely reflect adaptations of the soil microbial community's metabolic requirements as enzyme synthesis is costly and thus strictly regulated (Schimel and Weintraub, 2003; Allison et al., 2011).

High N availability may also induce increases in microbial carbon use efficiency (CUE), linked to reductions in respiration rates, according to theoretical modeling by Schimel and Weintraub (2003). From a meta-analysis of 20 studies Janssens et al. (2010) reported insignificant reductions in leaf litter decomposition mass losses but significant reductions in heterotrophic respiration in response to N addition. Furthermore, Spohn (2015) found positive correlations between the soil microbial metabolic quotient (basal respiration per unit soil microbial biomass, qCO_2) and litter C/N ratios in a meta-analysis of 14 experimental studies covering tropical, boreal and temperate forests. However, we observed similar responses of soil microbial biomass and basal respiration (both decreased under high N loading), resulting in no indications of increased microbial CUE under long-term N loading in the focal Norway spruce forests. Although ratios of cyclopropyl PLFA to precursor PLFAs are confounded by changes in community composition (Frostegård and Bååth, 2011), the increase in this ratio in response to N addition, together with increased soil C/N enzyme ratios, points to substrate limitation of the growth of the microbial community under long-term N loading. The lack of an increase in Gram-negative bacteria abundance to increased N availability (as indicated by extractable N forms in the O-horizon) found in Stråsan N1 and N2, also suggests limitation of resources other than N of the soil microbial community in response to long-term N loading. Over timescales of several decades, indirect effects of N loading, e.g. changes in trees' C allocation patterns, nutrient attainment strategies, litter quantity and quality, are likely to be quantitatively important. Thus, taken together, our results are not consistent with increased substrate availability for soil microbial biomass under long-term N loading of Norway spruce forests, as proposed by Schimel and Weintraub (2003). Rather, our results suggest that long-term N loading aggravated limitations of resources other than N for the soil microbial community, possibly of C due to reduced belowground C allocation of recent photosynthate (Högberg et al., 2010).

In the here presented study we examined relationships between historical (1950–2010) N loads, N leaching and microbial variables. However, we detected only minor differences in the considered microbial variables between plots with high current N loads (>10 kg ha^{-1} $year^{-1}$ of N) that currently leach large amounts of NO_3^- (High in, High-out; Čertovo and Skogaby NS plots) and those that leach only small amounts of NO_3^- (<2 kg ha^{-1} $year^{-1}$ of NO_3-N). It is possible that the high leaching rates of NO_3^- from the High-in, High-out plots is partly due to limitations or saturation of EMF immobilization capacities for N (Högberg et al., 2014), but our investigation could not show such differences statistically. Recoveries of EMF after termination of N loading in boreal Scots pine (Högberg et al., 2011; Hasselquist and Högberg, 2014) and Norway spruce (Choma et al., 2017) forests in Sweden have been reported. However, in all of the cited investigations, the ambient N deposition was low (ca. 4 kg ha^{-1} $year^{-1}$ of N), while at Skogaby and Čertovo ambient N deposition has remained high (ca. 15 kg ha^{-1} $year^{-1}$ of N). N mineralization and NH_4^+ consumption remained elevated 14 years after termination of experimental N addition to a boreal Scots pine forest in northern Sweden (Chen and Högberg, 2006), and 19 years after termination of N applications in the N2 treatment at Stråsan (Blaško et al., 2013). Soil NH_4-N turnover probably remained high in the Skogaby NS and Čertovo plots, as O-horizon C/N ratios were low (<26 g C g N^{-1}). Due to the low annual mean precipitation and runoff at Stråsan, relatively high soil water NO_3^- concentrations will be required to generate NO_3^- leaching losses that approach current N loading (33 kg ha^{-1} $year^{-1}$ of N). Our observations, thus, suggest that in addition to adaptations of the soil microbial community, changes to substrate properties and turnover and current N loading likely affect ecosystem N retention. This is consistent with findings that NO_3^- leaching across European forests correlates negatively with soil O-horizon C/N ratios (Gundersen et al., 1998) and positively with N deposition rates (Dise et al., 1998).

We detected only small between-treatment and between-site differences in pH of the soil O-horizons. O horizon pH was lower at Čertovo than at the other two sites, likely due to high historical sulfate deposition reported for this part of Europe (Kopácek and Hruska, 2010). The total proton load under the NS treatment at the Skogaby experimental site during 1988–1998 was mainly

driven by soil N cycling according to Bergholm et al. (2003). It is likely that NO_3^- leaching will cause further soil acidification in the Skogaby NS and Čertovo plots if it continues at current rates.

5. Conclusions

Long-term N addition and N deposition have altered soil O-horizon chemical properties, most notably it decreased the C/N ratio, in the studied forests. The O-horizon pH was not affected by the N treatments in the addition experiments, but it was lower at the site with the highest historical N deposition, likely due to acid sulfate deposition and current high NO_3^- leaching rates. Microbial biomass and basal respiration were reduced by the N addition treatments, especially the on-going N treatment, and tended to decline with increasing N deposition. Similarly, N-acquiring hydrolytic enzyme activity tended to decline while hydrolytic enzyme activities relating to C acquisition generally increased in response to long-term N loading. PLFA profiles indicate that long-term N loading induced shifts in microbial community composition, towards higher and lower relative abundance of Gram-positive and Gram-negative bacteria, respectively, without changes in fungal abundance. Overall, our results indicate that long-term N loading to N-limited Norway spruce forests aggravates limitations of other resources than N, likely that of easily available C, for soil microbial communities. Although microbial variables in the soil O horizon differed between plots with low and high current N loads, microbial variables in plots that leached small amounts and large amounts of NO_3^- exposed to high N load were similar.

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Paper 2

Recovery of the ectomycorrhizal community after termination of long-term nitrogen fertilisation of a boreal Norway spruce forest

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Recovery of the ectomycorrhizal community after termination of long-term nitrogen fertilisation of a boreal Norway spruce forest

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ABSTRACT

Ectomycorrhizal fungi (ECM) are a fundamental component of boreal forests promoting tree growth and participating in soil nutrient cycling. Increased nitrogen (N) input is known to largely influence ECM communities but their potential recovery is not well understood. Therefore, we studied the effects of long-term N-fertilisation on ECM communities, and their recovery after termination of N treatment. Fungal ITS sequencing data indicated that N-fertilisation (34 kg N ha^{-1} y^{-1}) for 46 y decreased the relative abundance of ECM species in the fungal community and suppressed originally dominating medium-distance fringe exploration types adapted to N-limited conditions, while the ECM diversity remained unaffected. In other plots, 23 y after termination of fertilisation at 73 kg N ha^{-1} y^{-1} for 23 y, the relative abundance of ECM species shifted closer to, but did not reach, control levels. These observations indicate only slow recovery of ECM community, likely due to a high soil N retention capacity.

1. Introduction

Ectomycorrhizal fungi (ECM) are fundamental components of boreal forest ecosystems. As tree root symbionts, they improve capacities of their hosts to acquire water and nutrients by increasing their effective uptake surface area and soil exploration capacity. They also play important roles in soil organic matter decomposition (Lindahl and Tunlid, 2015), and both carbon (C) and nitrogen (N) sequestration (Högberg et al., 2014a; Clemmensen et al., 2015). However, different ECM taxa have different abilities to explore soil in search for nutrients and different capacities to utilize available substrates (Lilleskov et al., 2011). Therefore, the ECM community's composition is an important factor that strongly affects not only tree growth, but also soil organic matter and nutrient cycling.

Soil N availability is one of the most important factors affecting ECM community composition and functioning. Numerous investigators, using various measures of ECM abundance and community composition, have found that ECM abundance decreases in response to increases in N availability, either through N fertilisation or atmospheric N deposition (e.g. Arnolds, 1991; Lilleskov et al., 2002b; Treseder, 2004; Kjøller et al., 2012; Högberg et al., 2014a). However, there are a few exceptions (Wallenda and Kottke, 1997; Kalliokoski et al., 2009). Besides the local soil conditions, the magnitude of the ECM community response to elevated N probably depends on the N dose and duration of elevated N inputs (Hasselquist and Högberg, 2014).

The generally observed decline in abundance of ECM fungi after increase in soil N availability is likely driven by a reduction in the allocation of photosynthate C to the below-ground parts of trees and thus the ECM (Högberg et al., 2003, 2011; Hobbie, 2006; Janssens et al., 2010). However, plants allocate less photosynthate belowground only if no other nutrient (e.g. P) becomes limiting (Johnson and Gehring, 2007). When plant C allocation below-ground decreases, ECM lose their main advantage in competition

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with other soil microorganisms (Högberg et al., 2003). These changes can be rapid: reductions in ECM sporocarp and extramatrical mycelium production have been observed within 5 y after commencement of N addition (Lilleskov et al., 2011).

Numerous studies have also reported negative effects of elevated N supply on ECM diversity (Lilleskov et al., 2001, 2002a, 2002b; Avis et al., 2003; Toljander et al., 2006; Cox et al., 2010; Kjøller et al., 2012; Hasselquist and Högberg, 2014). However, sensitivity of the ECM community to soil N enrichment is dependent on its taxonomic composition (Lilleskov et al., 2011). Some genera are generally sensitive to N additions and decrease in abundance, such as *Cortinarius* (Brandrud, 1995; Lilleskov et al., 2002a; Carfrae et al., 2006) and *Piloderma* (Lilleskov et al., 2002a; Cox et al., 2010), while others, such as *Lactarius* (Brandrud, 1995; Wiklund et al., 1995; Lilleskov et al., 2002a) and *Laccaria* (Termorshuizen, 1993), are generally regarded as N tolerant. The responses of many taxa to N additions also seem to depend on site characteristics, N enrichment parameters and temporal aspects of N addition (Lilleskov et al., 2011). The sensitivity of particular ECM taxa to soil N enrichment seems to be connected to their capacity to utilize complex N sources (e.g. proteins). Taxa growing effectively on protein-derived N sources are often reduced in abundance when soil N status is increased (Taylor et al., 2000; Lilleskov et al., 2002b, 2011).

Agerer (2001) classified ECM into distinct 'exploration types' based on their ability to forage resources in the soil environment, and properties such as presence/absence of rhizomorphs, hydrophobicity and extramatrical mycelium branching patterns. The extramatrical mycelial growth of contact and short-distance ('short' hereafter) exploration types is limited to a few millimetres from the root tip, while ECM of medium- and long-distance exploration types capable of producing rhizomorphs may spread tens of centimetres from the root tip. Exploration type and susceptibility to N loading seem to be correlated, and long-distance, medium-distance fringe (medium fringe hereafter) and medium-distance mat exploration types seem to be the most sensitive (Lilleskov et al., 2011). These fungi are capable of producing long, hydrophobic rhizomorphs, which allow them to explore remote parts of the soil environment in searches for nutrients, and transport acquired resources over long distances. They also often have high capacity to utilize complex organic N sources (Finlay et al., 1992; Lilleskov et al., 2002b). Such attributes provide these ECM with potent advantages under N-limited conditions. However, production of rhizomorphs and the enzymes required to break down complex N sources is C- and energy-demanding, and thus disadvantageous in N-rich environments with reduced C supplies from trees. Under such conditions, less C- and energy-demanding contact and short ECM types may thrive.

Although N deposition rates have likely declined in Europe as a whole since 1990, there have been large temporal and spatial variations, and projections of future global N deposition do not indicate that levels will be decreasing in the near future (Galloway et al., 2004; Tørseth et al., 2012). Reductions in N deposition might result in recovery of N-saturated ecosystems, which could be accompanied by functional and structural changes of ECM communities. There have been few studies of such responses. Hasselquist and Högberg (2014) recently assessed the recovery of ECM communities from long-term high N loads in a boreal forest ecosystem (the Norrliden experimental site, Sweden). Three years after the treatment had ceased they found lower ECM sporocarp production and species richness in plots subjected to annual N additions (70 kg N ha^{-1} y^{-1}) for 38 y compared to control plots. However, in plots that initially received a higher load (110 kg N ha^{-1} y^{-1} for 20 y) and subsequently have been left to recover for 23 y, sporocarp abundance and ECM species richness had returned to those of control plots. Moreover, the role of ECM fungi in ecosystem N retention had been restored (Högberg et al., 2011, 2014b). To improve our understanding of the ability of ECM communities to recover structurally and functionally after termination of high N loading, additional similarly focused studies are needed.

The Stråsan experimental site in Sweden is ideal for such studies, as it allows comparison between experimental plots subjected to both ongoing and terminated N treatments and comparable, unfertilised control plots. Thus, we assessed changes in composition and diversity of ECM communities in these plots. For this purpose, we sequenced fungal internal transcribed spacer (ITS) amplicons obtained from soil DNA and assigned the detected ECM taxa to exploration types, in order to evaluate the functional potential of ECM communities in plots with different N loading histories. The primary aims of the study were to characterise responses of the ECM community in the N-limited Norway spruce boreal forest at Stråsan to N addition in detail, and evaluate its recovery 23 y after termination of annual N additions that had been ongoing for 23 y.

2. Materials & methods

2.1. Study site

E26A Stråsan (Tamm et al., 1974) is the site of a long-term fertilisation experiment, located in central Sweden (60°55′ N, 16°01′ E), in which Norway spruce (*Picea abies*) forest stands have been subjected to various fertilisation treatments (some ongoing and some now terminated). The stands were 55 y old when samples of soil, classified as a haplic podzol (Berggren Kleja et al., 1998), were collected from the site for this study (in 2013). The experiment has a randomized block design with two blocks of 30 m × 30 m experimental plots assigned to treatments, which are represented once within each block. The peripheral 5 m borders of the plots are treated as buffer zones, thus the inner 25 × 25 m areas are used for sampling and measurements. The treatments have included three levels of N addition (as NH_4NO_3 annually in May) since 1967: N0 – control with no N addition, N1 – ongoing fertilisation with 34 kg N ha^{-1} y^{-1} on average (hereafter called 'fertilisation'), and N2 – fertilisation with 73 kg N ha^{-1} y^{-1} on average until cessation in 1990 (hereafter called 'recovery'). The current rate of N deposition at the site is low at 3.2 kg N ha^{-1} y^{-1} (Rappe-George et al., 2013). For detailed information on N additions and soil chemistry see Table 1.

2.2. Soil sampling

The sampling was performed during spring (9th May) and autumn (11th October) of 2013. In total, 4 samples of the soil organic layer (25 cm × 25 cm) were collected for each plot per each sampling event. Thus in total, we collected 16 soil samples per each of the three treatments.

The samples were transported on dry ice to the laboratory, sieved through a 4 mm mesh and stored at −80 °C until preparation for analysis.

DNA was isolated from the samples using a FastDNA Spin Kit for Soil (MP Biomedicals, USA), including additional treatment with 5.5 M guanidinium thiocyanate (molecular grade; Sigma-Aldrich, USA) to remove polymerase chain reaction (PCR) inhibitors according to the manufacturer's instructions. DNA was stored in 2 ml microtubes at −20 °C until analysed.

2.3. Illumina sequencing of ITS

LGC Genomics GmbH (Berlin, Germany) sequenced the ITS2 region in the DNA samples, including all of the preparation steps.

Table 1
Details of treatments and chemical variables of the soils subjected to them. For the methods details, see Supplementary information. n.a. = not applicable.

		Control	Fertilisation	Recovery
Treatment code		N0	N1	N2
Total N fertilisation (1967–2013)	kg ha^{-1}	0	1570	1760
Average dose of N during treatment	kg ha^{-1} y^{-1}	n.a.	34	73
Fertilisation duration	years	n.a.	46	23
Recovery duration	years	n.a.	0	23
pH		4.33	4.32	4.16
Soil total C	mg g^{-1} dry soil	277	346	321
Soil total N	mg g^{-1} dry soil	8.4	10.8	11.4
Dissolved organic C	μg g^{-1} dry soil	1144	1130	1083
Water-extractable N-NH_4^+	μg g^{-1} dry soil	7.7	15.6	15.9
Water-extractable N-NO3-	μg g^{-1} dry soil	1.53	1.86	1.78
Number of soil samples analysed		14	16	16

PCR mixtures each included about 5 ng of extracted DNA, 15 pmol of forward primer; fITS7 (Ihrmark et al., 2012) and reverse primer; ITS4 (White et al., 1990) in 20 μl of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline, USA) and 2 μl of BioStabII PCR Enhancer (Sigma Aldrich, USA). For each sample, the forward and reverse primers were fitted with the same 8-nt barcode sequence. The PCR amplification program consisted of 2 min denaturation at 96 °C, followed by 30 cycles of 96 °C for 15 s, 50 °C for 30 s and 72 °C for 60 s. About 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. DNA samples for which PCR failed were diluted 10-fold and the PCR reaction was repeated. The amplicon pools were purified with one volume Agencourt AMPure XP beads (Beckman Coulter, USA) to remove primer dimers and other small mispriming products, then further purified using MinElute columns following the manufacturer's instructions (QIAGEN, Germany). About 100 ng of each purified amplicon-pool was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1–96 (NuGEN, UK). Illumina libraries were pooled and size-selected by preparative gel electrophoresis, then sequenced on an Illumina MiSeq platform using V3 Chemistry (Illumina, USA).

2.4. Data analysis

Paired-end reads were joined using Mothur v.1.25.0 (Schloss et al., 2009). The ITS2 region was extracted using ITSx v. 1.0.11 (Bengtsson-Palme et al., 2013) and gathered sequences were quality filtered (min phred score 19, no ambigous bases, maxhomopolymer 6). Sequences shorter than 130 bp were discarded. OTUs were assembled and taxonomy assigned in the frame of the QIIME v. 1.9.0 script "pick_open_reference_otus.py" (Caporaso et al., 2010), in which OTUs were constructed using USEARCH v. 6.1 (Edgar, 2010) by clustering with 98.5% similarity and taxonomy was assigned using the UNITE database, v. 7.1 (Koljalg et al., 2013). A subset of 7000 sequences was randomly chosen from each sample for taxonomic summary at the species level. One sample from a control plot in spring failed to provide sufficient quality data and was thus omitted (together with the corresponding replicate from the autumn sampling) from further analyses.

Only OTUs assigned to ECM taxa were included in further analyses. Exploration types were assigned to ECM OTUs according to Agerer (2001, 2006) and DEEMY (Information System for Characterization and Determination of Ectomycorrhizae; www.deemy.de) (Supplementary table 1).

Species richness and Shannon-Wiener and Pielou's indices (Magurran, 2004) based on species relative abundance in the ECM community were calculated in R v. 3.3.1 (R Core Development Team, 2016) using package vegan 2.4–0 (Oksanen et al., 2016).

The effect of treatment on biodiversity measures, relative abundance of ECM in total fungal community, proportions of exploration types and relative abundance of OTUs assigned to ECM genera and species was analysed using linear mixed-effect models with treatment and time as fixed effects and plots nested within blocks as random effects. Statistical analyses were conducted in R v. 3.3.1 (R Core Development Team, 2016) using the package lme4 (Bates et al., 2015). Differences were considered significant at $p < 0.05$. Tukey's post-hoc pairwise comparison test was calculated using package multcomp (Hothorn et al., 2008).

Treatment effects on overall ECM community composition were tested by PERMANOVA analysis of Bray-Curtis dissimilarities at both genus and species level with 999 permutations using package vegan 2.4–0 (Oksanen et al., 2016). A model accounting for the effects of treatment, time and plot nested in block was used.

2.5. Data deposition

Data were deposited in the European nucleotide archive (ENA) under accession number ERS1294511.

3. Results

The resulting dataset originally contained 1 396 383 sequences but was randomly subsampled to 322 000 sequences (7000 per sample). Across all samples, sequences were assigned to 124 ectomycorrhizal species of 26 genera. 3 genera belonged to Ascomycota and the other 23 genera belonged to Basidiomycota (Supplementary table 1).

According to PERMANOVA analysis, the ECM community was affected by the treatments at both the genus (Df = 2, pseudo-F = 4.27, $p < 0.001$) and species level (Df = 2, pseudo-F = 4.36, $p < 0.001$).

The relative abundance of ECM showed a clear negative response to both N treatments. The proportion of ECM sequences in total fungal sequences (hereafter 'proportion of ECM') was significantly lower in both fertilisation and recovery plots than in controls, and non-significantly higher in recovery than in fertilisation plots (Table 2).

The ECM diversity did not significantly change in response to N fertilisation. Still, species richness, Shannon-Wiener index and Pielou index showed a tendency to approach higher values on fertilised plots compared to controls. All diversity measures were intermediate in recovery plots (Table 2).

Proportions of reads assigned to taxa within specific soil exploration types were also influenced by treatments. The ECM community in control plots was dominated by the medium fringe type (Table 2), mostly of the genera *Piloderma* (*Piloderma fallax* and *Piloderma bicolor*) and *Cortinarius* (*Cortinarius testaceofolius*, *Cortinarius lux-nymphae* and *Cortinarius humboldtensis*) (Table 3).

Table 2
Biodiversity indices, relative abundance (in %) of sequences assigned to ectomycorrhizal fungi in the total fungal sequences and relative abundance (in %) of sequences assigned to exploration types in total ectomycorrhizal sequences in indicated treatment plots. Values are means with standard errors in brackets. Different lower-case letters indicate significant differences at the $p < 0.05$ level.

	χ^2	p	Control	Fertilisation	Recovery
Species richness	2.72	0.256	16.9 (0.5)	19.0 (1.1)	17.3 (1.2)
Shannon-Wiener index	2.79	0.247	1.25 (0.12)	1.47 (0.07)	1.28 (0.13)
Pielou's index	2.02	0.364	0.44 (0.04)	0.51 (0.03)	0.45 (0.04)
ECM in total fungal community (%)	19.62	<0.001	69.5 (3.6)a	42.5 (5.8)b	49.2 (5.8)b
Medium-distance fringe (%)	8.32	0.016	46.0 (9.0)a	18.3 (5.4)b	19.4 (5.3)b
Medium-distance mat (%)	2.05	0.359	0.0	<0.1	0.0
Medium-distance smooth (%)	1.85	0.396	1.0 (0.5)	2.0 (1.6)	0.1 (0.1)
Long-distance (%)	2.04	0.360	<0.1	<0.1	5.1 (5.0)
Short-distance (%)	11.14	0.004	9.2 (2.3)a	30.0 (4.7)b	30.2 (7.1)b
Contact (%)	3.6	0.165	29.0 (10.0)	33.1 (6.4)	8.2 (3.9)
Unassigned exploration type (%)	9.15	0.010	14.8 (5.2)a	16.5 (3.6)a	37.0 (8.2)b

Table 3
Relative abundance (in %) of sequences assigned to most abundant ectomycorrhizal species in the total ectomycorrhizal sequences in the indicated treatment plots, and their exploration types. Values are means with standard errors in brackets. Different lower-case letters indicate significant differences at the $p < 0.05$ level. Exploration types according to Agerer (2001, 2006) and DEEMY (Information System for Characterization and Determination of Ectomycorrhizae; www.deemy.de).

Species	Exploration type	χ^2	p	Relative abundance (%)		
				Control	Fertilisation	Recovery
Amanita fulva	unassigned	3.12	0.210	<0.1	0	1.9 (1.5)
Amphinema byssoides	medium-distance fringe	1.37	0.504	0.3 (0.2)	1.8 (1.1)	1.5 (1.2)
Byssocorticium pulchrum	long-distance	2.05	0.359	<0.1	0 (0)	5.0 (5.0)
Cenococcum geophilum	short-distance	0.60	0.739	1.5 (1.2)	0.6 (0.3)	1.4 (1.1)
Clavulina coralloides	short-distance	2.19	0.335	0.1 (0.1)	<0.1	1.6 (1.5)
Cortinarius anomalus	medium-distance fringe	2.46	0.314	0	4.4 (4.0)	<0.1
Cortinarius camphoratus	medium-distance fringe	2.17	0.337	0	<0.1	1.4 (1.3)
Cortinarius humboldtensis	medium-distance fringe	5.69	0.058	6.5 (4.3)	<0.1	<0.1
Cortinarius lux-nymphae	medium-distance fringe	2.53	0.282	1.5 (1.5)	0	0
Cortinarius obtusus	medium-distance fringe	5.97	0.051	0	0	3.0 (1.8)
Cortinarius privignipallens	medium-distance fringe	3.20	0.202	<0.1	<0.1	2.0 (1.6)
Cortinarius testaceofolius	medium-distance fringe	2.56	0.278	5.6 (5.5)	<0.1	<0.1
Cortinarius traganus	medium-distance fringe	2.06	0.357	<0.1	<0.1	5.2 (5.2)
Hygrophorus olivaceoalbus	unassigned	1.29	0.525	4.9 (2.4)	4.2 (2.6)	1.8 (0.9)
Lactarius necator	contact	14.66	<0.001	1.1 (1.1)a	8.1 (2.4)b	1.6 (0.7)a
Lactarius rufus	contact	4.67	0.097	15.7 (7.5)	24.7 (5.9)	6.5 (3.5)
Piloderma bicolor	medium-distance fringe	4.92	0.085	15.5 (4.6)	3.6 (2.0)	2.6 (1.6)
Piloderma byssinum	medium-distance fringe	1.44	0.486	3.9 (3.6)	2.1 (2.1)	<0.1
Piloderma fallax	medium-distance fringe	4.49	0.106	23.9 (8.1)	4.5 (2.6)	3.1 (2.0)
Piloderma olivaceum	medium-distance fringe	2.96	0.226	0.1 (0.1)	3.8 (3.1)	<0.1
Piloderma sphaerosporum	unassigned	12.23	0.002	9.6 (3.3)ab	1.9 (1.2)a	23.8 (7.3)b
Pseudotomentella humicola	medium-distance smooth	2.17	0.339	1.0 (0.5)	1.9 (1.6)	
Pseudotomentella mucidula	unassigned	8.76	0.013	<0.1a	8.0 (3.1)b	3.3 (1.6)ab
Russula consobrina	unassigned	6.42	0.040	<0.1	<0.1	4.8 (2.7)
Russula decolorans	contact	5.24	0.073	12.1 (8.3)	<0.1	0
Tomentella stuposa	unassigned	5.71	0.058	<0.1	1.6 (0.8)	0.6 (0.4)
Tylospora asterophora	short-distance	4.90	0.086	0.5 (0.3)	2.1 (0.8)	0.5 (0.3)
Tylospora fibrillosa	short-distance	7.54	0.023	6.9 (1.5)a	27.2 (4.4)b	26.0 (6.5)ab

The next most common exploration type in control plots was the contact type (Table 2) with *Lactarius rufus* and *Russula decolorans* being the most abundant representatives (Table 3).

In fertilisation plots, representatives of *Piloderma* and *Cortinarius* decreased markedly (yet both not significantly) compared to control plots (Tables 3 and 4). This resulted in a statistically significant decrease of the medium fringe type (Table 2). In contrast, the proportion of fungi of the short type was three-fold higher (Table 2), primarily due to significant increase of *Tylospora fibrillosa* (Table 3). In general, the total proportion of contact type ECM remained unaffected (Table 2), but in more detail, the contact type representatives *L. rufus* and *Lactarius necator* increased, while *R. decolorans* practically disappeared (Table 3). Almost 17% of the ECM in fertilised plots were composed of species with uncertain exploration type (Table 2). The most abundant members of this group were *Pseudotomentella mucidula* and *Hygrophorus olivaceoalbus* (Table 3).

In recovery plots, *Piloderma* and *Cortinarius* representatives had higher relative abundance than in fertilisation plots (Table 4). The relative abundance of *Piloderma sphaerosporum* was significantly higher compared to the fertilisation treatment, but lower relative abundances of sequences of other *Piloderma* species were observed (Table 3). An analogous shift was observed within the genus *Cortinarius* (Table 3). We found neglectable abundance of *Cortinarius* species which were present on control and fertilised plots, while *Cortinarius camphoratus*, *Cortinarius privignipallens*, *Cortinarius traganus* and *Cortinarius obtusus* occurred on recovery plots (Table 3).

The relative proportion of short type fungi remained at the same level as in fertilised plots and *T. fibrillosa* remained a strong dominant of this group. Contact exploration type decreased (all representatives decreased their relative abundance), while medium

Table 4
Relative abundance (in %) of sequences assigned to most abundant ectomycorrhizal genera in the total ectomycorrhizal sequences in the indicated treatment plots. Values are means with standard errors in brackets. Different lower-case letters indicate significant differences at the $p < 0.05$ level.

Genus	χ^2	p	Relative abundance (%)		
			Control	Fertilisation	Recovery
Amanita	3.17	0.205	<0.1	<0.1	1.9 (1.5)
Amphinema	1.37	0.504	0.3 (0.2)	1.8 (1.1)	1.5 (1.2)
Byssocorticium	2.05	0.359	<0.1	0	5.0 (5.0)
Cenococcum	0.61	0.738	1.5 (1.2)	0.6 (0.3)	1.4 (1.1)
Clavulina	2.09	0.351	0.3 (0.2)	0.2 (0.1)	1.7 (1.5)
Cortinarius	2.45	0.294	17.1 (7.1)	6.0 (3.9)	14.8 (5.4)
Hygrophorus	1.20	0.549	4.9 (2.4)	4.3 (2.6)	1.9 (0.9)
Lactarius	8.70	0.013	16.8 (7.9)ab	32.9 (6.4)a	8.2 (3.9)b
Piloderma	3.50	0.174	38.2 (8.8)	12.4 (4.0)	27.0 (7.4)
Pseudotomentella	12.18	0.002	1.0 (5.3)a	10.3 (3.3)b	3.4 (1.6)ab
Russula	3.41	0.182	12.2 (8.3)	0.3 (0.1)	5.0 (2.7)
Tomentella	5.63	0.060	<0.1	1.6 (0.8)	0.6 (0.4)
Tylospora	7.96	0.019	7.5 (1.5)a	29.4 (4.7)b	26.5 (6.6)ab

fringe slightly increased, both non-significantly. The greatest proportion of ECM sequences (37%) in recovery plots was covered by species with unassigned exploration type (Table 2).

4. Discussion

The ecosystem at the Stråsan experimental site is characterized as N-limited with closed N cycling, mediated by a high soil N retention capacity (Blaško et al., 2013), resulting in minimal N leaching (Rappe-George et al., 2013). This N-limited state was reflected in the fungal community composition of control plots, where ECM dominated over saprotrophs, accounting for 70% of the analysed fungal sequences. The ECM community was dominated by genera assigned to the medium fringe exploration type, e.g. *Piloderma* and *Cortinarius*. Representatives of this exploration type have densely ramified interconnected rhizomorphs capable of nutrient foraging in large volumes of soil (Agerer, 2001) and high capacity to use proteins as sources of N (Finlay et al., 1992; Lilleskov et al., 2002b). Both of these capabilities enhance competitiveness in N-limited soils (Lilleskov et al., 2011). Moreover, the dominance of the medium fringe exploration type may explain the high N retention capacity of the soil. We base this possible explanation on the facts that medium exploration type ECM form more extramatrical mycelia biomass than contact and short exploration types (Weigt et al., 2012), and N retention was shown to correlate with ECM biomass (Högberg et al., 2014a).

The ongoing fertilisation treatment (N1), involving long-term N addition at 34 kg N ha^{-1} y^{-1} on average for 46 y, has increased soil N turnover (gross N mineralization and immobilization as determined used the ^{15}N pool dilution technique) and tree N uptake (Blaško et al., 2013) and slightly increased dissolved organic nitrogen contents in the mineral B horizon (Rappe-George et al., 2013). Tree responses include significantly enhanced growth and increases in needle N concentration (Blaško et al., 2013). Our results show that these responses are associated with a substantial decrease in the relative abundance of ECM in the fungal community, from 70% to 43%, in accordance with the generally observed negative response of ECM to N enrichment (e.g. Arnolds, 1991; Wallenda and Kottke, 1997; Treseder, 2004; Kjøller et al., 2012; Högberg et al., 2014a). The most strongly repressed genera were two of the dominants in the originally N-limited system, *Piloderma* and *Cortinarius*, which both decreased their relative abundance approximately to a third.

The decline of the dominant *Piloderma* and *Cortinarius* contributed to the decline of the representation of the medium fringe type of ECM in fertilisation plots, which more than halved compared to controls (from 46% to 18%), while contact type slightly increased and was the dominant exploration type with more than 33%. Short distance ECM had three-fold their relative abundance (30%) in fertilised plots, which is consistent with a hypothesis of Lilleskov et al. (2011), who designated the medium fringe exploration type "nitrophobic", as it is prone to decline after N enrichment. Thus, the fertilisation treatment has induced a functional shift in the ECM community, involving replacement of taxa with supposed high C and energy demands by taxa that may have lower requirements of photosynthate C supply. These findings are indicative of higher N availability and potentially a result of reductions in trees' allocation of C resources to belowground parts.

Detection of DNA from fungal necromass, spores or dormant structures can provide a biased picture of the active fungal community. Indeed, redundant structures might persist in soil for many years (McCormack et al., 2010). However, taking the long duration of the Stråsan experiment (46 y) into account, the treatment effect bias due to inclusion of fungal necromass DNA should be negligible. We also do not expect spore contamination as detrimental for comparison of treatments, since we expect a uniform enrichment of external spores among the closely located plots.

A majority of studies investigating effects of N availability on ECM community are in agreement that increases in N availability decreases ECM community diversity (Lilleskov et al., 2001, 2002a, 2002b; Avis et al., 2003; Cox et al., 2010; Kjøller et al., 2012; Hasselquist and Högberg, 2014), even though exceptions occur (Kranabetter et al., 2009; Hay et al., 2015). These studies discuss N deposition gradients (Lilleskov et al., 2002a) as well as fertilisation experiments (Hasselquist and Högberg, 2014), covering forest ecosystems of North American temperate (Avis et al., 2003), European temperate (Suz et al., 2014), North American boreal (Kranabetter et al., 2009) as well as Scandinavian (Hasselquist and Högberg, 2014) regions. ECM diversity has usually been estimated based on sporocarp observation (e.g. Hasselquist and Högberg, 2014) or root tip colonisation (e.g. Suz et al., 2014).

Unfortunately, studies comparable to our originally N poor Norway spruce forest subjected to N fertilisation (34 kg N. ha^{-1} y^{-1} for 46 y) are scarce. Hasselquist and Högberg (2014) postponed a fertilisation experiment in an analogous Scots pine (*Pinus sylvestris*) forest. However, they based the estimate of species richness on sporocarp production, which makes it difficult to compare with our soil (incl. some smallest fine roots) oriented survey, because sporocarp diversity does not necessarily follow the belowground state (Douhan et al., 2011). In other work, Hay et al. (2015) presented data from interior spruce (*Picea glauca* x *engelmannii*) forest fertilisation experiments located in British Columbia and estimated the species richness and diversity based on a root tips survey. Still, this study differs to ours due to younger stand age and shorter length of fertilisation treatment.

Hasselquist and Högberg (2014) showed a clear decrease in ECM species richness in response to long-term fertilisation, while Hay et al. (2015) did not find any effect on ECM in their three sites. In our study, all diversity indicators used – species richness, Shannon-Wiener index and Pielou evenness index – remained unaffected. We only observed a non-significant tendency of slight increase of diversity measures in fertilised plots. Such conflicting outcomes from studies of comparable experiments are in agreement with the expectation of Hasselquist and Högberg (2014), who suggested that responses of ECM community to increasing N availability are more complex than simply negative.

Beside other factors, the manner of N input (amount, length, dosage) might influence the ECM community response. Wallenda and Kottke (1997) tried to estimate a critical N load sufficient to

negatively affect ECM. Based on their review, they expect a dose of 35 kg N ha^{-1} y^{-1} to be sufficient to trigger drastic changes in ECM sporocarp formation and abundance, while their estimation of a dose critical for belowground ECM diversity (20–60 kg N ha^{-1} y^{-1}) was more uncertain due to discrepancy between pot and field experiments. The results of Hasselquist and Högberg (2014) are in agreement with a critical load threshold of 35 kg N ha^{-1} y^{-1} for sporocarp production, as they observed no effect on sporocarp abundance in 20 kg N ha^{-1} y^{-1}, while a 100 kg N ha^{-1} y^{-1} dose led to a drastic reduction. In the Stråsan fertilisation treatment, 34 kg N ha^{-1} y^{-1} did not reduce the ECM diversity similar to another even higher dose (50–70 kg N ha^{-1} y^{-1}) in an experiment in British Columbia (Hay et al., 2015). However, other studies have reported significant reductions in ECM diversity at lower N doses (e.g. Lilleskov et al., 2002a). Apparently, the dose of N cannot by itself be a used as a predictor of the ECM diversity response.

In the recovery plots, 23 y after cessation of the N addition at an annual average dose of 73 kg N ha^{-1} y^{-1} over 23 y, N contents remained similar to fertilised plots (Table 1), but mineral N concentrations in soil leachate (Rappe-George et al., 2013) and N concentration in Norway spruce needles reduced (Blaško et al., 2013) indicating that N availability is gradually declining. However, soil N turnover (gross N mineralization and immobilization, as determined using the ^{15}N pool dilution technique) is still high, according to (Blaško et al., 2013). Moreover, the total soil N content in recovery plots did not significantly change between 1995 and 2010 (Rappe-George et al., 2013), supporting tree growth rates similar to those observed in fertilized plots, indicating high soil N retention capacity. The results presented here indicate that recovery of the ECM community under these conditions has been slow. We detected a higher proportion of ECM in recovery plots (49%) compared to fertilised (43%) plots, but it was still much lower than the proportion (70%) in control plots.

No change was observed in proportions of medium fringe and short type fungi compared to fertilisation plots, while contact type tends to decrease. This might assume a low recovery potential of nitrophobic medium fringe type. A possible explanation can be that despite observed signs of decrease the N availability still remains too high to allow a full recovery of both original total ECM relative abundance and nitrophobic exploration types' dominance. However, it should be noted that our exploration type composition estimates in recovery plots are biased by a high proportion (37%) of sequences belonging to ECM with uncertain exploration type, thus comparability to the other treatments with ~15% sequences of unassigned exploration type is weak.

Hasselquist and Högberg (2014) reported ECM sporocarp production to recover to the levels of control plots during 23 y recovery from an even higher dose of N (110 kg N ha^{-1} y^{-1}). Despite the dissimilarities of the two studies (sporocarps vs. ITS reads and negative vs. not observed effect on diversity), both studies indicate a potential of ECM (relative) abundance to recover from the long term N fertilisation towards the original state. Unfortunately, we are not able to investigate the recovery of exploration types with sufficient certainty due to inability to assign an exploration type to a satisfying proportion of sequences.

In conclusion, persistently high soil N content and turnover rates on recovery plots (Blaško et al., 2013) constrain the full recovery of the ECM community. Still, the lower needle N concentrations (Blaško et al., 2013), lower N leaching from the soil (Rappe-George et al., 2013) and increasing relative abundance of ECM are indicative of a reversion tendency to a N-limited ecosystem. The process is slow, however, possibly due to the high N retention capacity of the soil in the studied ecosystem. We conclude that more time (>23 y) is needed for the full recovery of ECM community structure and functioning of the Stråsan forest from prolonged experimental N addition.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.10.002.

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Paper 3

Positive response of soil microbes to long-term nitrogen input in spruce forest: results from Gårdsjön whole-catchment N-addition experiment

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Positive response of soil microbes to long-term nitrogen input in spruce forest: Results from Gårdsjön whole-catchment N-addition experiment

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ABSTRACT

Chronic nitrogen (N) deposition from anthropogenic emissions alter N cycling of forests in Europe and in other impacted areas. It disrupts plant/microbe interactions in originally N-poor systems, based on a symbiosis of plants with ectomycorrhizal fungi (ECM). ECM fungi that are capable of efficient nutrient mining from complex organics and their long-distance transport play a key role in controlling soil N mineralization and immobilization, and eventual nitrate (NO_3^-) leaching. Current meta-analyses highlight the importance of ECM biomass in securing the large soil N pool. At the same time, they point to the adverse effect of long-term N input on ECM fungi. The functioning of N-poor and N-overloaded forests is well understood, while the transient stages are much less explored. Therefore, we focused on the spruce-forest dominated catchment at Gårdsjön (Sweden) that received N addition of 40 kg N $ha^{-1}yr^{-1}$ over 24 years (a cumulative N input of >1200 kg N ha^{-1}) but still loses via runoff only <20% of annual N input (deposition + addition) as NO_3^-. We found that, compared to the control, the N-addition catchment had a much larger soil microbial biomass. The N addition did not change the fungi/bacteria ratio, but a larger share of the bacterial community was made up of copiotrophs. Furthermore, fungal community composition shifted to more nitrophilic ECM fungi (contact and short exploration type ECM species) and saprotrophs. Such a restructured community has been more active, possessed a higher specific respiration rate, enhanced organic P and C mining through enzymatic production and provided faster net N mineralization and nitrification. These may be early indications of alleviation of N limitation of the system. We observed no signs of soil acidification related to N additions. The larger, structurally and functionally adapted soil microbial community still provides an efficient sink for the added N in the soil and is likely to be one of the explanations for low NO_3^- leaching that have stabilized in the last decade. Our results suggest that a microbial community can contribute to effective soil N retention in spite of the partial relative retreat (20–30%) of nitrophobic ECM fungi with large external mycelia, provided the fungal biomass remains high because of replacement by other ECM and saprotrophic fungi. Furthermore, we assume that N retention of similar C-rich boreal forests (organic soil molar C/N ~35) is not necessarily threatened by a large cumulative N dose provided N enters at a moderate rate, does not cause acidification and the soil microbial community has time to adapt through structural and functional changes.

1. Introduction

A low nitrogen (N) supply limits plant productivity in most temperate and boreal forests (LeBauer and Treseder, 2008). To ensure their nutrition, plants provide C-assimilates to the root-associated ectomycorrhizal fungi (ECM) possessing the proteolytic and oxidative enzymatic apparatus necessary to mine nutrients from organic sources and, in turn, get the N in available forms. Thanks to C supplied microbial activity, gross soil N transformations are generally considerable (Hart et al., 1994; Högberg et al., 2017) but the N produced through mineralization is efficiently immobilized by N-limited plants and microbes (Schimel and Bennett, 2004). This keeps the ecosystem N cycling close,

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with small mineral N pools and no or negligible mineral N losses. However, the N constraints on plant growth have been historically, to some extent, relieved because of human activities that elevated N input in ecosystems globally over the long term (Galloway et al., 2004; Vitousek et al., 2010; Kopáček and Posch, 2011). Such elevated depositions of reactive N and forest fertilization practices changed forest productivity, biodiversity and function (Bobbink et al., 2010; Erisman et al., 2013; Kopáček et al., 2013; Tian and Niu, 2015; Du and de Vries, 2018; Schulte-Uebbing and de Vries, 2018; Cheng et al., 2019). One of the major adverse consequences of elevated anthropogenic N input is the overloading of forest N retention capacity evident in NO_3^- leaching to surface waters (N saturation concept; Agren and Bosatta, 1988; Aber et al., 1998).

In the context of increased N loads, a number of studies from the last thirty years have been focused on the understanding of mechanisms of forest transition from N limitation to N saturation. Nitrogen addition experiments carried out in forests produced mixed results. In some systems, N fertilization over time caused faster net N mineralization and significant NO_3^- losses, while other forests efficiently retained the large quantities of added N over the long term (Emmett et al., 1998; Aber et al., 2003; Curtis et al., 2011; Rowe et al., 2012; Zhou et al., 2017; Fuss et al., 2019). The heterogeneous ecosystem responses result from both the differences in experimental designs (such as duration, dose and the form of N addition) and various initial conditions of N limitation at individual forest sites. While N depositions steadily decline in Europe (Kopáček and Posch, 2011), the importance of atmospheric N emissions and related depositions increases globally because of more intensive agricultural and industrial activities worldwide (Simpson et al., 2014; Xian et al., 2019). Therefore, disentangling the causation of forest transition from N limitation to N saturation remains of current significance.

Traditionally, considerable attention was given to soil chemical parameters such as C availability (Goodale et al., 2005; Evans et al., 2006a; Kopáček et al., 2013) and the soil C/N ratio (Dise et al., 1998, 2009; Gundersen et al., 1998; Evans et al., 2006b), both of which have been closely correlated to N leaching from forest soils. In fact, these parameters reflect biological interactions between plants and soil organisms in the system because they are derived from the composition of above- and belowground plant inputs, their following microbial processing (Nadelhoffer and Fry, 1988; Kögel-Knabner, 2002) and final plant and microbial utilization. Plant and microbial biomass, and microbially transformed and stabilized soil organic matter are important pools for the N immobilization. In N-poor coniferous forests, the extensive mycelia of ECM fungi could reach hundreds to thousands mg^{-1} of soil (reviewed in Tedersoo and Bahram, 2019) and immobilize N for long periods of months or even years (Clemmensen et al., 2015), thus constituting the main sink for N in the soils (Högberg et al., 2007; Blaško et al., 2015). Similarly, Bahr et al. (2013) found that soil N leaching correlated negatively with mycorrhizal production across 29 Norway spruce stands in Sweden.

The formation and maintenance of the ECM mycelia with its exoenzymatic activity is highly C demanding, taking 7–30% of plant C fixation (Leake et al., 2004), so it is completely dependent on plant C supply. Under elevated N input, coniferous trees substantially reduce the relative allocation of photosynthates to their roots (Högberg et al., 2010), which weakens the C-demanding ECM symbiosis (Lilleskov et al., 2019). Consequently, a significant decline in fungal biomass is often reported from N-fertilized forests (Treseder, 2004; Janssens et al., 2010; Zhou et al., 2014). Lilleskov et al. (2011) further suggested a restructuring of the ECM community: ECM taxa vary in their sensitivity to increasing N input depending on the nature of their mycelia and the ability to obtain and transport resources from the soil. The ECM fungi forming extensive mycelia and rhizomorphs (medium- and long-distance exploration types) and having proteolytic and oxidative abilities show a consistent negative response to N fertilization (N sensitive, nitrophobic taxa). Some other taxa, commonly belonging to contact and short-distance types with a higher affinity for inorganic N forms but mostly lacking a strong degradation machinery (reviewed in Tedersoo and Bahram, 2019), could survive and even prosper in an N-rich environment (N tolerant, nitrophilic). The restructuring of the ECM community after N fertilization of the spruce forest from originally dominant medium-distance fringe and long-distance exploration types towards contact and short-distance types was further observed, for example, by Kjøller et al. (2012) and Choma et al. (2017). This shift can lead to a decline in proteolytic and oxidative capacities of the community and potentially result in slower organic matter decomposition (Lilleskov et al., 2019; Zak et al., 2019). Additionally, the C shortage in the ECM community may benefit bacteria and saprotrophic fungi, allow nitrifying bacteria to compete for the ammonia released by C-limited organisms and cause the opening of N cycle (Högberg et al., 2017). Thus, plant and soil microbial physiologies may vary across a gradient of increasing N supply, with an important impact on the balance of the ecosystem N cycle. The structure and functioning of N-poor and N-overloaded forest systems are well understood, while transient stages are much less explored.

Nitrogen addition experiments consistently show that the higher the dose and longer the duration of N input, the greater the negative effect on soil microbial biomass is (Wallenstein et al., 2006; Liu and Greaver, 2010; Boot et al., 2016; Zhou et al., 2017). Yet, there are also studies showing the positive effect of N addition on soil microbial biomass (Janssens et al., 2010; Zhou et al., 2017). These studies are commonly conducted in seriously N-limited organic-rich forests or use low N-fertilization rates (<50 kg ha^{-1} $year^{-1}$). These better represent ambient N-deposition inputs than experiments using large unrealistic N loading rates but are, with only few exceptions (6 studies from 134 lasted over 20 years), conducted for a relatively short period of <15 years (Zhou et al., 2017). Our study was conducted in the coniferous forest experimental catchment in the vicinity of Gårdsjön Lake, Sweden (a part of NITREX project from 1991) that receives moderate N deposition in comparison with the rest of Europe (Pihl-Karlsson et al., 2011). Despite more than 24 years of N addition (NH_4NO_3, ~40 kg N $ha^{-1}y^{-1}$ on average), representing a cumulative amount of >1.2 t N ha^{-1}, the NO_3^- outputs through the runoff stabilized between 4 and 16% of the annual catchment N input (N deposition + addition) after the first decade and do not rise thereafter (Moldan et al., 2018). The NH_4^+ runoff increased to a much lesser extent than that of NO_3^-, and the dissolved organic N (DON) leaching remained unchanged (Moldan et al., 2018). Kjønaas and Stuanes (2008) and Moldan et al. (2006) estimated that 30–43% of the added N was stored in the aboveground plant biomass. Moldan et al. (2006) further calculated that ca 65% of added N was stored in the soil, based on differences in soil C and N pools measured prior to the N addition and 12 years after. Although the 119 soil cores used in the study were not sufficient to obtain the statistical significance of the detected increase in the soil N pool, it demonstrates that soil represented a large sink for added N (5540 kg N ha^{-1} in 1990 before the start of N additions). Except for early retreat of some nitrophobic ECM fungi (Boxman et al., 1998; Brandrud and Timmermann, 1998), there is no further information about the soil microbial community size, composition and activity related with soil N transformation processes and no assessment of its status on the gradient from N to C limitation connected with the on-going N addition experiment at Gårdsjön.

Our research aims were to assess the size of soil microbial biomass, its composition and rates of N mineralization and nitrification processes in the N-addition catchment (24 years since the onset of the fertilization additions). Comparing those to the same characteristics measured in the adjacent control catchment (receiving N only from atmospheric deposition), we wanted to know the N-related changes in communities and their C/N limitation status. Possible changes in the balance between N immobilization/mineralization processes related to long-term N addition could then indicate whether the soil system approaches a transition to C limitation/N saturation and whether there is an increasing risk of significant NO_3^- leaching to surface waters. Because there has been high

soil N retention observed in the N-addition catchment, we hypothesize that soil microbial biomass has not been reduced in response to N addition and that its growth and activity have not been constrained by adverse environmental conditions. For this purpose, we measured the microbial components of soil C and N in relation to soil chemistry. We further hypothesize that N addition has induced structural changes in the soil microbial community, especially in its fungal part, connected with shifts in respiratory and exoenzymatic degradation activity, which, however, did not result in a significant increase in NO_3^- availability and leaching risk. For this purpose, we characterized the microbial communities using amplicon sequencing and taxonomy assignment of fungi and prokaryotes, measured hydrolytic enzyme activities and rates of gross and net N mineralization and nitrification.

2. Methods

2.1. Study catchment description

The experimental area at Gårdsjön Lake (58°04′ N, 12°01' E) is situated near the west coast of Sweden, approximately 15 km inland at an elevation of 130 m a.s.l. The whole area has been recovering from acidification of soils and waters driven by declining deposition of sulphur (S) since the 1990s [from ~17 kg S ha^{-1} yr^{-1} in 1989–1990 to 1.5 kg S ha^{-1} yr^{-1} in 2015–2016 (sea-salt corrected throughfall)]. The N deposition decreased much less, reaching on average 9 kg N $ha^{-1}yr^{-1}$ between 1989 and 2016 (sum of NO_3^- and NH_4^+, measured in open field precipitation; Moldan et al., 2018). Research was conducted at two study catchments, the control catchment (G1; 0.63 ha) and the N-addition catchment (G2; 0.525 ha).

The N-addition catchment G2 is covered by a 95-year-old Norway spruce forest [Picea abies (L.) Karst] with an admixture of Scots pine (*Pinus sylvestris*). The bedrock is composed of gneiss. Soils are predominantly silty and sandy podzols of a mean depth of 38 cm. The organic LFH horizon is 10–15 cm thick (Kjønaas and Wright, 1998). The total capacity of soil N before the onset of N addition was 5,540 kg N ha^{-1} of which ~40% was in the LFH horizon (Moldan et al., 2006). The G2 forest floor is mainly covered with *Vaccinium myrtillus*. *Calluna vulgaris* is found higher up at the hillslopes. Dominant moss species are *Dicranum majus*, *Leucobryum glaucum* and *Plagiothecium undulatum*, with *Sphagnum girgensohnii* being present in the more humid parts in the valley, which comprise ~25% of the catchment area. The annual average precipitation (1989–2016) was 1146 mm; annual discharge was 537 mm, and the mean annual air temperature was 7.4 °C. Seepage to groundwaters was at both sites considered negligible due to the relatively good balance between measured chloride input and runoff output. Long-term throughfall deposition of chloride was 16% lower than runoff output at G2 (27 years average; Moldan et al., 2018) and 2% higher at G1 (24 years average; Moldan et al., 1998 and unpublished data). Both values are well within the observed imbalances between chloride inputs and outputs in boreal catchments without a significant groundwater component (Svensson et al., 2012).

The G1 is a part of the same forest and situated next to G2. G1 is marginally larger than G2, and has slightly steeper slopes and narrower valley bottom, with a less *Sphagnum girgensohnii* dominated area in the valley (~10% of the catchment area). G1 catchment has slightly less bedrock outcrops, which is reflected in its average higher soil thickness of 43 cm (Andersson et al., 1998). Soil types, forest structure and vegetation cover are similar at the two catchments (Andersson et al., 1998). At G1, the input of N was reduced when the whole catchment was for 10 years (1991–2000) covered by a plastic roof. During that experiment, atmospheric acid deposition was replaced by the addition of KNO_3, thus resulting in N input of 3.5 kg N ha^{-1} yr^{-1} (Moldan et al., 2004).

2.2. Experimental design of long-term N addition at G2

The NITREX experiment at G2 started in 1991. NH_4NO_3 dissolved in deionised water was sprayed over the forest floor in annual amounts ranging between 28.9 and 51.6 kg N $ha^{-1}yr^{-1}$ (averaging 39.5), divided into 12–24 doses each year. The stepwise increase in the N input resulted in a gradual increase in NO_3^- leaching from largely below the detection limit values (NO_3–N <0.002 mg l^{-1} a NH_4–N <0.02 mg l^{-1}) before the onset of the N application (1989–1990) to annual concentrations around 60 μeq l^{-1} after the first 10 years. In the following 15 years, no marked increase in NO_3^- in the runoff was detected. NH_4^+ has increased to a much lesser extent than NO_3^- while concentrations of DON did not change (Moldan et al., 2018). The concentrations of NO_3^- in the runoff from the control catchment G1 were only rarely above the detection limit over the whole time period 1989–2015 (Moldan et al., 2004 and unpublished results).

2.3. Soil sampling

A whole-catchment sampling assay was run in late April 2015. From each catchment, 16 soil cores were collected using a soil corer (8 cm in diameter), of which 8 were from wet (valley) and 8 were from dry (sloped) areas. Soil from cores was divided into the organic O horizon [composed of partially decomposed Oe (fermentation F layer) + well-decomposed Oa (humification H layer)] and 5–15 cm of topsoil mineral A horizon. In G2, only 6 samples of A horizon were obtained from the dry part. In total, there were 62 samples (G1: 8 organic-wet, 8 mineral-wet, 8 organic-dry, 8 mineral-dry; G2: 8 organic-wet, 8 mineral-wet, 8 organic-dry, 6 mineral-dry). After the sampling, soil was sieved (4 mm) and kept at 4 °C during transport to the laboratory (3 days). Immediately after soil arrival, samples for molecular analyses were frozen at −80 °C and for enzyme analyses at −21 °C. All other chemical and microbial analyses started within one week (soil kept under 4 °C) after soil arrival.

2.4. Soil analyses

2.4.1. Soil physico-chemical characteristics

Soil pH was determined by a pH meter in deionised water (water: fresh soil, 25 : 10, v/w) by agitating for 5 min and letting stand for 0.5 h. Total soil C and N contents were measured in dried (60 °C, 48 h) and milled soil samples (true replicated) using an NC analyser (Vario Micro Cube, Elementar, Germany). Amounts of water-soluble C and N, representing microbially available forms, were assessed as follows. Fresh soil was extracted with cold distilled water (true replicated = 62 samples, water: soil, 10 : 1, v/w, shaken 60 min/150 opm, centrifuged 4000 rpm/10 min, filtered through acid washed 0.45 μm glass-fiber filter), total C and N concentrations in water extracts were measured using a LiquiTOC II (Elementar, Germany) and used to assess available substrate stoichiometry (C/N water).

2.4.2. Soil microbial biomass and its growth limitation parameters

Soil microbial biomass was assessed using the fumigation extraction method. Fresh soil samples (10 g) were placed into glass flasks in two sets (true-replicated samples $2 \times 62 = 124$ flasks in total). One set of the samples was directly extracted with 0.5 M K_2SO_4 (extractant: soil, 4 : 1, v/w, shaken 45 min/150 opm, centrifuged 4000 rpm/10 min, filtered through 0.45 μm glass-fiber filter) while another set was extracted after a 24-h fumigation with amylene-stabilized chloroform. Organic C and total N amounts in the K_2SO_4 extracts were measured on a LiquiTOC II (Elementar, Germany). Microbial biomass C (Cmic) and N (Nmic) were calculated as the difference in organic C and total N, respectively, between fumigated and non-fumigated soils and corrected by the factors of 0.38 for Cmic (Vance et al., 1987) and 0.54 for Nmic (Brookes et al., 1985). Extracts from non-fumigated soils were further analysed for extractable ammonium (NH_4–N) and nitrates (NO_3–N) by a flow injection analyser (QuickChem 8500, Lachat Instruments, USA). By

subtracting the sum of both inorganic N forms (DIN) from the total N in the non-fumigated extracts, DON was calculated. Organic C in the same extracts is equal to dissolved organic carbon (DOC).

We determined whether the microbial growth is C or N limited. Microbial biomass critical C/N ratio (C/N critical) was calculated to assess microbial requirements for balanced growth and compared to the C/N of available substrate represented by C/N water (Manzoni et al., 2008). First, carbon use efficiency (CUE) was calculated as CUE = 0.43* ((C/N water)/(Nmic/Cmic))^0.6, then C/N critical = 0.5*((Cmic/Nmic)/CUE). When the C/N water is lower than the C/N critical, microbial growth is limited by C availability. When it is higher, the N limitation prevails.

2.4.2.1. Basal and specific microbial respiration rates. Microbial activity was characterized by basal and specific respiration rates. The basal microbial respiration rate was determined as a CO_2 production rate from the soil without any substrate addition. Fresh soils (10g) were moistened to 60–70% water holding capacity and pre-incubated in the dark at 15 °C for a week. Then the flasks (250 ml) were tightly closed, and an increase in CO_2 concentration in the headspace during 24 h was measured on a gas chromatograph (Agilent GC HP 6850, USA). Analyses were performed in three laboratory replications for each soil sample (3 × 62 = 186 flasks in total). Specific respiration equals the basal respiration rate per unit of Cmic.

2.4.2.2. Net and gross N mineralization and nitrification. To assess net N mineralization and nitrification rates, fresh soil samples (5 g) were placed into glass flasks (250 ml, 2 sets of 62 true-replicated samples, 124 flasks in total), adjusted to 60–70% of water holding capacity, sealed with perforated Parafilm and incubated at 15 °C in the dark. One set was extracted using 0.5 M K_2SO_4 after one week and the other after three weeks (extractant: soil, 4 : 1, v/w, shaken 45 min/150 opm, centrifuged 4000 rpm/10 min, filtered through 0.45 μm glass-fiber filter) (Ste-Marie and Pare, 1999). Net N mineralization and nitrification rates were calculated as the difference between the final (21 days) and initial (7 days) concentration of NH_4–N and NO_3–N in extracts, respectively, divided by the incubation time.

Gross N mineralization and nitrification were run in four composite samples representative of a specific horizon (O/A) from the dry or wet area of each catchment (three for A from dry part of G2). Each composite sample was prepared by mixing two samples randomly selected from the 8 original samples (6 for A from dry part of G2) representing the horizon and dry/wet part of the respective catchment. A pool dilution assay was used. Fresh mixed soil samples (25 g) moistened to 60–70% of WHC were incubated in glass flasks (250 ml, covered with perforated Parafilm) at 15 °C for three weeks to reach a stable temperature response. Then 5 g from each sample was put into 50 ml centrifuged tubes and labelled either with $^{15}NH_4$–N or with $^{15}NO_3$–N to achieve N addition of 2 μg N (99% ^{15}N) per 1 g of soil dry weight. Soils were stirred manually and incubated for 4 or 24 h (15 °C). Samples were then extracted through agitation (45 min/150 opm) with 0.5 M K_2SO_4 solution (extractant: soil, 4 : 1, v/w), centrifuged (15 min/4000 rpm) and the supernatant was filtered through a 0.45 μm glass fiber filter. The concentrations of NH_4–N and NO_3–N were measured using the flow injection analyser (FIA; QuickChem 8500, Lachat Instruments, USA). A diffusion method adapted from Goerges and Dittert (1998) was used to separate NH_4–N and NO_3–N. Extracts (containing at least 20 μg N) were put into glass flasks (250 ml). In the case of NH_4–N separation, 10 ml of 10 M NaOH was added, and the flasks were made airtight with a double lid containing acidified traps (⌀ = 0.5 cm, ashless filter paper, Whatman, UK, treated with 5 μl of 2.5 M $KHSO_4$ solution) embedded in two PTFE tape strips. Diffusion was carried out on a horizontal agitator for 6 days (140 opm, 24 °C). In the case of NO_3–N separation, extracts with NaOH were first left opened for 6 days to remove NH_4–N and then sealed with similarly prepared traps after the addition of 400 mg of Devarda's alloy and diffused for another 6 days. After diffusion, filters were dried overnight in a desiccator with H_2SO_4, packed in tin capsules and measured for the $^{15}N/^{14}N$ ratio and total N on IR-MS (Delta X Plus, Finnigan, Germany) connected to the NC analyser (Elementar analyser FLASH 2000, Thermo Fisher Sci., Germany). The correction of actual atm% ^{15}N enrichment was done using a calculated blank (Stark and Hart, 1996). The gross mineralization and nitrification rates were calculated based on the theoretical equation of Kirkham and Bartholomew (1954) by Hart et al. (1994).

2.4.2.3. Extracellular enzyme activity. A microplate fluorometric assay was used to determine extracellular enzyme activities. One gram of defrosted soil was suspended in 100 ml of distilled water and sonicated (4 min). 200 μl of soil suspension was added to 50 μl methylumbelliferyl (MUF) 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 substrate solution for leucine-aminopeptidase (LEU) determination (Bárta et al., 2014). Three concentrations of each substrate were tested (50, 100 and 300 μM). The concentration with the highest enzymatic activity (substrate saturated enzyme) was selected. Plates were incubated at 20 °C for 120 min. Fluorescence was quantified at an excitation wavelength of 365 nm and emission wavelength 450 nm (Infinite F200 Microplate Reader, TECAN, Germany). The ratio of C-enzymatic activity (BG + CEL) to N-enzymatic activity (LEU + NAG) was calculated (Sinsabaugh et al., 2009) and is referred to as the C/N enzyme ratio (E_C/E_N).

2.4.3. Total DNA extraction, purification and quantification

Nucleic acid extractions were made from all soil samples according to a modified bead-beating protocol (Urich et al., 2008) and further purified using the CleanAll DNA/RNA Clean-up and Concentration Micro Kit (Norgen Biotek Corp., Ontario, Canada). Total DNA was quantified using SybrGreen (Leininger et al., 2006). To quantify the bacterial and fungal SSU rRNA gene, the FastStart SybrGREEN Roche® Supermix and Step One system (Life Technologies, USA) were used. Each reaction mixture (20 μl) contained 2 μl DNA template (~1–2 ng of DNA), 1 μl of each primer (0.5 pmol μl^{-1}, final concentration), 6 μl PCR H_2O, 10 μl FastStart SybrGREEN Roche® Supermix (Roche, France) and 1 μl BSA (Fermentas, 20 mg μl^{-1}). In the case of bacteria, the primers were 341F and 534R (Muyzer et al., 1993), and PCR conditions were: initial denaturation (120s, 95 °C), followed by 30 cycles of 30 s at 95 °C, 30 s at 62 °C, 15 s at 72 °C and fluorescence signal measurement at 80 °C. A serial 10x dilution of the known amount of purified PCR product obtained from genomic *Escherichia coli* ATCC 9637 was used for standard curve generation. The qPCR conditions for fungal quantification were as follows: primers nu-SSU-0817-5′ and nu-SSU1196-3′ (Borneman and Hartin, 2000), initial denaturation (10 min, 95 °C), 40 cycles of 60 s at 95 °C, 60 s at 56 °C, 60 s at 72 °C, fluorescence signal measurement at 72 °C. Standard curve was derived from dilution series PCR product obtained from genomic *Aspergillus niger* DNA. In both cases, product specificity was confirmed by melting point analysis (52 °C–95 °C, read in 0.5 °C step) and agarose gel electrophoresis.

2.4.4. Microbial community composition

Microbial community composition was determined by barcoded amplicon sequencing of the prokaryotic SSU rRNA gene and ITS fungal region. The PCR primers (515F/806R) that targeted the V4 region of the gene of the small ribosomal subunit (SSU) rRNA, previously shown to yield accurate phylogenetic information and to have only a few biases against any bacterial taxa were used (Liu et al., 2007; Bates et al., 2011; Bergmann et al., 2011). The PCR primers ITS1f and ITS2 (White et al., 1990), targeting the fungal hypervariable internal transcribed region (ITS1), were used. PCR reactions contained 13 μl MO BIO PCR-grade water, 10 μl 5 PRIME HotMasterMix, 0.5 μl each of the forward and reverse primers (0.2 μM final concentration), and 1 μl genomic DNA.

Reactions were sustained at 94 °C for 3 min to denature the DNA, with amplification proceeding for 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. The reverse primer contained a 12-base error-correcting Golay barcode to facilitate multiplexing. Each sample was amplified in triplicate, combined and quantified using Invitrogen PicoGreen and a plate reader, and equal amounts of DNA from each amplicon were pooled into a single 1.5 ml microcentrifuge tube. Cleaned amplicons were quantified using Picogreen® dsDNA reagent in 10 mM Tris buffer (pH 8.0). A composite sample for sequencing was created by combining equimolar ratios of amplicons from the individual samples and cleaned using the Ultra Clean® htp 96-well PCR clean up kit (MO BIO Laboratories). Purity and concentration of the samples were estimated by spectrophotometry and verified using agarose gel. Amplicons of 250 bp were sequenced pair-end (150x150 cycles) on the Illumina-MiSeq platform (ARGONE Lab, Illinois, USA).

Prokaryotic (bacterial and archaeal) paired-end reads were joined using ea-utils (Aronesty, 2013). In the case of fungi, only forward reads were analysed due to poor quality of reverse reads. Raw sequences were quality filtered (min. quality mean 25, no ambiguous bases) using PRINSEQ-lite 0.20.4 (Schmieder and Edwards, 2011). The ITS1 region was extracted from fungal reads using the ITSx 1.0.11 algorithm (Bengtsson-Palme et al., 2013). Furthermore, fungal ITS1 sequences were length-filtered to a min. length of 150. Prokaryotic sequences were filtered and trimmed to a length of 250 using PRINSEQ-lite. USEARCH 8.1 (Edgar, 2010) was used for OTU table construction (singletons discarded, similarity level cut-off 97% and 98.5% for prokaryotes and fungi, respectively). Taxonomy assignment was done using the BLAST algorithm (Altschul et al., 1990) via parallel_assign_blast.py script within QIIME v 1.9.1 pipeline (Caporaso et al., 2010) against the Silva 132 (Quast et al., 2013) and UNITE 7.2 (Koljalg et al., 2013) databases for prokaryotes and fungi, respectively. The detailed workflow of sequence data processing is available at https://github.com/chomic-kbe/gardsjon. OTU tables were further processed with the phyloseq package 1.22.3 (McMurdie and Holmes, 2013) for statistical program R (R Core Team, 2017). At least 2000 reads per sample are regarded as sufficient to align with the main pattern in the microbial community (Caporaso et al., 2011). Samples with lower sampling depth were discarded (one in prokaryotic dataset, four in fungal dataset). Following recommendations of McMurdie and Holmes (2014), sequences were rarefied to a minimal sequencing depth in each dataset (11,319 and 2989 for prokaryotes and fungi, respectively) prior to alpha diversity analyses, while other calculations (beta diversity based on the Bray-Curtis sample dissimilarity matrix, taxa relative abundances etc.) were performed on nonrarefied data. Alpha diversity measures, i.e. OTU richness and the Shannon index (Magurran, 2004), were calculated at the OTU level. Prokaryotic genera with members possessing genes for any enzyme involved in denitrification pathway (NarGHI, NapAB, NirK, NirS, NorBC and NosZ) as revealed by search in FunGene v 9.8 (Fish et al., 2013) and KEGG pathway databases (Kanehisa and Goto, 2000) were considered as potential denitrifiers. Lifestyle was manually assigned to fungal OTUs [OTUs with assigned genus or species level with the help of FUNGuild database v 1.1 (Nguyen et al., 2016) and literature research; reference sequences of the other OTUs were BLAST compared to the NCBI database, and metadata of ≥99% of hits were searched for information leading to lifestyle assignment] until at least 90% of sequences in each sample were covered. Furthermore, exploration types were assigned to ECM species OTUs according to Agerer, 2001, 2006 and DEEMY (Information System for Characterization and Determination of Ectomycorrhizae; www.deemy.de). Complete list of fungal OTUs with assigned lifestyles and exploration types is available at https://chomic-kbe.github.io/gardsjon/. Sequence data are stored at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the study accession number PRJEB33059.

2.5. Calculations and statistics

All biochemical and microbial data parameters were expressed per gram of soil dry weight (105 °C; 5 h) and their normality was checked prior to the analyses by histogram plots and a Kolmogorov-Smirnov test. Means and standard deviations were calculated (n = 8 for soil biochemical and microbial parameters except mineral soil samples from dry part of G2 catchment where n = 6; n = 4 for ^{15}N pool dilution except mineral soil samples from dry part of G2 catchment where n = 3). All C/N stoichiometric ratios were calculated on a molar basis. Linear and exponential regressions were used to test the interrelations among microbial biomass, basal respiration, enzymatic activity and Ntot. We used Factorial ANOVA with interactions [treatment (N-addition catchment; control catchment), moisture (dry part; wet part), horizon (organic horizon; mineral horizon) as factors with fixed effects, Statistica 64, version 13.0 (Dell, USA)] followed by a multiple comparison of means (Tukey HSD tests) for identifying their significant effects on soil parameters and microbial activities including relative abundances of individual microbial groups. Homogeneity of variances was verified by Bartlett's test. Data were log transformed, when needed, to satisfy ANOVA assumptions. All proportional data were square root transformed prior to ANOVA. The community composition difference was tested by a permutational multivariate analysis of variance (PERMANOVA, function *adonis*; 9999 permutations) of Bray-Curtis dissimilarity matrices derived from the OTU's square-root transformed relative abundance (function *metaMDS*) with the use of the vegan package for R v.2.5–4 (Oksanen et al., 2019).

3. Results

3.1. Comparison of soil chemistry between N-addition and control catchments

Soils at the N-addition catchment (G2) had significantly higher concentrations of total C and N than soils from the control catchment (G1) both in the organic and in the mineral horizons (Table 1). Furthermore, the N-addition catchment soils had higher pH by 0.1–0.4 pH units, higher concentrations of DOC and of all forms of extractable N and especially of extractable NH_4–N. Therefore, DON comprised 75–95% of total extractable N at the control catchment, while it was only 32–60% at the N-addition catchment (Table 1). The ratio of organic-to-mineral N forms (DON/DIN) in the soil extracts was thus 5–25 times lower and the DOC/NO_3–N ratio 1.3–2 times lower in the N-addition catchment compared to the control site. The above-described differences between catchments occurred consistently in both the organic and the mineral horizon (N x horizon interaction mostly insignificant, Table 1) and usually were more pronounced in the wet parts compared to dry parts of the catchments (significant N x moisture, Table 1). All the chemical parameters indicated a larger N accumulation and higher N availability in the soils of the N-addition catchment compared to the control site, with more pronounced differences in their wet parts located in valleys.

3.2. Comparison of soil microbial biomass and its growth limitation between the catchments

Consistently with the higher total soil C and N content, the soil microbial biomass C and N were significantly higher at the N-addition catchment compared to the control catchment (on average by 5–68% and 14–106% for Cmic and Nmic, respectively; Table 2), and always larger in their wet than dry parts (Table 2). The microbial C/N ratio was lower in the N-addition than control site but only in the organic horizon. As a result, both the C/N critical as a measure of microbial CN requirements for balanced growth and the C/N water as a measure of available substrate stoichiometry were lower and closer to each other in the organic horizon at the N-addition catchment than the control one

Table 1
Soil chemical characteristics in organic and mineral soils of the wet and dry parts of control and N-addition catchments. Mean ± SD are given (n = 8 except dry-mineral horizon of N addition catchment where n = 6). Instead of SD a range is given for pH. Average pH equals -log (average H^+ concentration). Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + p < 0.05, ++ p < 0.01, +++ p < 0.001, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*p < 0.05, **p < 0.01, ***p < 0.001).

	F values (ANOVA)					organic horizon				mineral horizon			
	N	moisture	horizon	N x moisture	N x horizon	wet		dry		wet		dry	
						control	N	control	N	control	N	control	N
pH_{H2O}	30^{+++}	6.8^{+}	32^{+++}	ns	ns	4.6 (4.5–4.8)	5.0*** (4.8–5.2)	4.6 (4.4–4.7)	4.7 (4.5–4.8)	4.4 (4.3–4.9)	4.5 (4.3–4.7)	4.4 (4.3–4.6)	4.6 (4.4–4.9)
Ctot (mmol g^{-1})	21^{+++}	114^{+++}	354^{+++}	9.7^{++}	11^{++}	39 (1.2)	40 (0.9)	37 (1.7)	38 (2.0)	27 (5.3)	36 (6.7)***	6.0 (1.4)	7.8 (1.9)
Ntot (mmol g^{-1})	45^{+++}	104^{+++}	286^{+++}	11^{++}	ns	1.2 (0.12)	1.3 (0.15)*	1.0 (0.058)	1.2 (0.13)*	0.57 (0.24)	1.2 (0.28)***	0.14 (0.03)	0.16 (0.04)
Ctot/Ntot (mol/mol)	10^{++}	31^{+++}	55^{+++}	5.1^{+}	ns	34 (3.6)	31 (3.4)	38 (2.5)	31 (2.8)	40 (3.5)	32 (4.4)**	45 (7.1)	49 (6.7)
DOC (μmol g^{-1})	16^{+++}	20^{+++}	86^{+++}	6.3^{+}	ns	55 (12)	68 (20)	49 (6.4)	60 (10)	24 (10)	53 (19)***	19 (6.1)	17 (3.8)
DON (μmol g^{-1})	13^{+++}	21^{+++}	15^{+++}	ns	ns	4.7 (1.1)	6.8 (2.8)	3.3 (1.7)	5.2 (1.3)	2.7 (3.3)	6.6 (4.0)*	0.57 (0.36)	0.95 (0.40)
NH_4-N (μmol g^{-1})	61^{+++}	ns	21^{+++}	ns	20^{+++}	0.62 (0.62)	10 (5.8)***	0.12 (0.068)	11 (6.0)***	0.37 (0.45)	4.1 (2.9)	0.16 (0.11)	1.9 (1.1)
NO_3-N (μmol g^{-1})	24^{+++}	ns	6.5^{+}	ns	ns	0.11 (0.024)	0.26 (0.17)	0.090 (0.013)	0.31 (0.14)*	0.062 (0.027)	0.30 (0.27)*	0.033 (0.017)	0.051 (0.025)
DON/DIN	37^{+++}	ns	9.1^{++}	ns	15^{+++}	14 (11)	0.82 (0.56)***	17 (8.8)	0.64 (0.39)***	6.9 (3.1)	1.5 (0.93)	3.2 (1.6)	0.53 (0.12)
DOC/NO_3-N	21^{+++}	ns	ns	ns	ns	535 (159)	348 (176)	551 (103)	252 (180)**	404 (160)	302 (185)	577 (151)	375 (146)

(Fig. 1). It indicated that the microbial communities in the organic horizon of the N-addition catchment approached the situation where they may switch from N to C limitation (N saturation). Such a situation did not occur in the mineral horizon.

3.3. Comparison of microbial activities between N-addition and control catchments

Basal and specific respiration rates as well as total enzymatic activity followed microbial biomass results and were significantly higher at the N-addition catchment compared to the control catchment, and always larger in their wet than dry parts (Tables 2 and 3). All the measured enzymes, except the N acquiring NAG, were higher at the N-addition catchment. The differences in activities of both C-acquiring (BG + CEL) and P-acquiring (PME) hydrolases between the N-addition and control catchment were more pronounced in their wet than dry parts (significant N x moisture, Table 3). Gross rates of N-transformation processes [N mineralization (ammonification) and nitrification] were comparable between catchments, while net N mineralization and nitrification were faster in the soils from the N-addition catchment. The catchment difference in the net N mineralization rate was more pronounced in the organic horizon than mineral horizon (significant N x horizon interaction, Table 2). In case of net nitrification (and also basal respiration), the positive effect of N addition was larger in wet than dry parts of the catchment (significant N x moisture interaction, Table 2).

3.4. Linkages between microbial biomass and its activity across a span of soil N

Microbial biomass was a driver of soil activities such as soil basal respiration (Fig. 2a) and total enzymatic activity and for the enzymes the relation was closer in the mineral horizon ($R^2 = 0.68$ and 0.69 for Cmic and Nmic respectively, $p < 0.001$) than in the organic horizon ($R^2 = 0.21$ and 0.31 for Cmic and Nmic, $p < 0.01$). Specifically, in the mineral horizon, the microbial biomass C was closely correlated related to N-mining enzymes ($R^2 = 0.82$, $p < 0.001$). All the characteristics significantly increased with the soil N content. In particular, there was a significant positive relation between soil N content and basal respiration (Fig. 2b) and microbial biomass C and N (Fig. 3a and b). The ratio of Nmic/Ntot was much higher in the organic horizon at N-addition than in the control catchment. It was 50 and 46 at the N-addition catchment while it was the 40 and 37 at control catchment, in the wet and dry parts, respectively. The activities of all enzymes also increased with soil N content. The PME increased the most, followed by C and N acquisition enzymes, though the differences in the rates of increases were not statistically significant (Fig. 4). Consequently, the Ec/En ratio was markedly higher in the N-addition than the control catchment in both wet and dry parts: by 2.6 times in the organic horizon and by 1.7 times in the mineral one (Table 3).

3.5. Inter-catchment comparison of soil microbial community

The abundance of the fungal 18S rRNA marker genes were higher in the N-addition than the control catchment in both horizons, though the stronger positive effect of N addition was observed in the mineral horizon (Table 4). Abundances of prokaryotic 16S rRNA marker genes were also higher in the N-addition compared to the control site, but only in the mineral horizon (N x horizon, Table 4). The fungi/bacteria ratio (Table 4) and overall prokaryotic and fungal community diversity measures – OTU richness and the Shannon index – did not differ between the two catchments, while composition of both communities did (Fig. 5). PERMANOVA revealed significant difference in fungal and prokaryotic community composition between the catchments (Table 5) being influenced by both position in the catchment (wet and dry parts) and in the soil profile (horizon). Fungal community was more impacted by moisture, while the effect of horizon was greater in the case of

Table 2
Soil microbial biomass and activity parameters in organic and mineral soils in wet and dry parts of control and N addition catchments. Mean ± SD are given (n = 8 except dry-mineral horizon of N addition catchment where n = 6). Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

	F values (ANOVA)					organic horizon				mineral horizon			
	N	moisture	horizon	N x moisture	N x horizon	wet		dry		wet		dry	
						control	N	control	N	control	N	control	N
Cmic (μmol g^{-1})	6.2^{+}	35^{+++}	244^{+++}	ns	ns	657 (102)	695 (175)	555 (88)	612 (131)	237 (72)	399 (144)*	60 (23)	66 (23)
Nmic (μmol g^{-1})	15^{+++}	17^{+++}	121^{+++}	ns	ns	48 (7.6)	65 (24)	37 (5.2)	55 (18)	15 (6.1)	31 (18)	4.8 (1.4)	5.5 (2.3)
Cmic/Nmic (mol/mol)	6.3^{+}	ns	ns	ns	5.5^{+}	14 (1.6)	11 (1.5) **	15 (1.4)	12 (1.6) ***	15 (4.0)	15 (3.6)	12 (2.9)	12 (1.9)
basal respiration (μmol $g^{-1}d^{-1}$)	16^{+++}	25^{+++}	163^{+++}	4.04^{+}	ns	5.1 (1.4)	6.6 (1.6)	5.0 (0.69)	5.7 (0.73)	2.2 (1.4)	4.1 (1.4)*	0.60 (0.20)	0.97 (0.22)
specific respiration (mmol C mol $Cmic^{-1}h^{-1}$)	5.6^{+}	5.2^{+}	9.8^{++}	ns	ns	7.8 (2.2)	9.5 (1.0)	9.0 (1.2)	9.6 (1.5)	9.5 (3.7)	11 (3.7)	11 (5.0)	16 (6.6)
net N mineralization (nmol $g^{-1}d^{-1}$)	21^{+++}	ns	37^{+++}	ns	17^{+++}	59 (102)	310 (163)	47 (45)	205 (158)*	5.0 (13)	14 (28)	14 (8)	23 (17)
net nitrification (nmol $g^{-1}d^{-1}$)	5.9^{+}	4.8^{+}	ns	6.8^{+}	ns	2.7 (2.1)	6.9 (12)	4.9 (2.3)	3.9 (3.5)	0.60 (0.59)	17 (17)**	0.17 (0.073)	0.30 (0.44)
gross N mineralization (nmol $g^{-1}d^{-1}$)	ns	ns	ns	ns	ns	1468 (670)	4254 (1378)	929 (234)	1639 (862)	553 (592)	148 (43)	138 (107)	98 (63)
gross nitrification (nmol $g^{-1}d^{-1}$)	ns	ns	23^{+++}	ns	ns	160 (106)	428 (151)	183 (108)	206 (167)	74 (68)	97 (72)	42 (34)	27 (5)

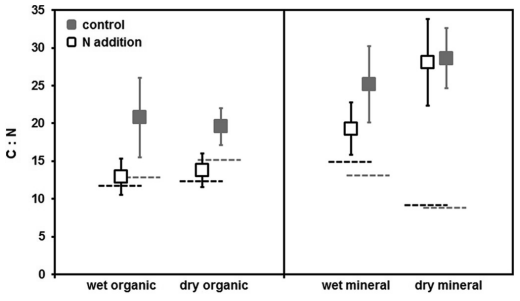

Fig. 1. The C/N ratio of soil water extract (C/N water; box and whiskers; mean ± sd, n = 6–8) as a measure of substrate stoichiometry and the C/N critical (displayed as dashed lines) as a measure of microbial CN requirements for balanced growth in the organic and mineral horizons in wet and dry parts of control (grey coloured) and N addition (black coloured) catchments.

prokaryotes. Furthermore, the N-addition effect on both prokaryotic and fungal communities differed between wet and dry parts as showed by significant N x moisture interaction (Table 5).

In both horizons at the N-addition catchment, prokaryotic communities were enriched in Actinobacteria while being poorer in Planctomycetes and Chlamydiae compared to the control catchment (Fig. 5, Table S1). Furthermore, the communities in the organic horizon at the N-addition catchment had lower relative abundances of Acidobacteria and Deltaproteobacteria, while those of Alphaproteobacteria, Gammaproteobaceria and Chloroflexi were higher in comparison to control soils. The only identified phylum representing Archaea (Thaumarchaeota) had higher relative abundance at the N-addition catchment namely in its mineral-wet soils. The N effect on community composition was stronger in wet than dry soils in case of increasing contributions of Armatimonadetes, Chlamydiae, Alphaproteobacteria and Thaumarcheota (N x moisture, Fig. 5, Table S1).

The fungal community in the N-addition catchment was relatively enriched in saprotrophs such as *Penicillium* and *Solicoccozyma,* which replaced some receding ECM genera (Fig. 6, Table S1). Specifically, the largest and consistent reduction compared to the control site was observed for two nitrophobic ECM genera of *Cortinarius* and *Piloderma*, and also for *Hygrophorus* in both soil horizons. The N effects on other ECM genera *Clavulina* and *Russula* were dependent on the position in the catchment. In both horizons of the N-addition catchment, relative abundance of *Clavulina* decreased in wet parts, while it thrived in dry parts. The opposite effect was observed for *Russula* that negatively responded to N in dry sloped parts while it thrived in wet valleys (Fig. 6, Table S1). As a result, the ECM communities established in the two catchments significantly differed in the proportions of ECM exploration type groups (Table 6). The proportion of nitrophobic medium-distance fringe ectomycorrhizal fungi was much lower at the N-addition than the control catchment, forming <32% and >43% of ECM ITS amplicons, respectively, in the organic horizons (Table 6), and 6–34% and 18–48% of the ECM community, respectively, in the mineral horizon (insignificant difference). In contrast, the ECM communities in both soil horizons of the N-addition catchment were enriched in nitrophilic species compared to the control site. The difference for nitrophilic contact exploration type species was as great as ~5% and ~12% of all ECM sequences in organic and mineral wet horizons, respectively, compared to <0.1% at the control catchment. Nevertheless, their relative proportion in dry conditions was minimal regardless of N treatment. The dry sloped parts of the N-addition catchment were enriched in nitrophilic short-distance type ECM species and organic horizons of dry and wet parts in medium-distance smooth exploration type species (Table 6). Additionally, the wet part of the N-addition catchment was richer in the genera of *Russula* and partly also *Lactarius* than the control catchment (https://chomic-kbe.github.io/gardsjon/). For several species within these genera, we were not able to assign their exploration types exactly and thus labelled a substantial part as a group of an unknown-type.

Table 3
Soil enzymatic activity (C acquiring β-glucosidase and Cellobiohydrolase; N acquiring N-Acetyl-β-D Glucosaminidase and Leu-aminopeptidase; P acquiring Phosphatase) and C-to-N acquiring enzyme activity ratio (E_C/E_N) in organic and mineral soils in dry and wet parts of the control and N addition catchments. Mean ± SD are given (n = 8 except dry-mineral horizon of N addition catchment where n = 6). Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (dry; wet) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + p < 0.05, ++ p < 0.01, +++ p < 0.001, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N additions within dry/wet parts of catchments and for organic/mineral horizons separately (*p < 0.05, **p < 0.01, ***p < 0.001).

	F values (ANOVA)					organic horizon				mineral horizon			
	N	moisture	horizon	N x moisture	N x horizon	wet		dry		wet		dry	
						control	N	control	N	control	N	control	N
β-glucosidase (nmol $g^{-1}h^{-1}$)	36^{+++}	16^{+++}	4.8^{+}	12^{++}	ns	414 (317)	2074 (1258) ***	604 (483)	1134 (686)	485 (273)	1820 (716)**	121 (75)	400 (228)*
Cellobiohydrolase (nmol $g^{-1}h^{-1}$)	23^{+++}	12^{++}	ns	8.6^{++}	ns	34 (41)	414 (369) ***	49 (44)	192 (148)	74 (65)	320 (163) ***	14 (5.4)	26 (19)
N-Acetyl-β-D Glucosaminidase (nmol $g^{-1}h^{-1}$)	ns	12^{++}	8.3^{++}	ns	ns	136 (71)	294 (109)	122 (113)	94 (59)	81 (31)	190 (90)	20 (11)	20 (6.8)
Leu-aminopeptidase (nmol $g^{-1}h^{-1}$)	4.8^{+}	9.4^{++}	ns	ns	ns	16 (11)	39 (34)	17 (10)	22 (16)	26 (17)	45 (33)	10 (5.0)	10 (2.6)
Phosphatase (nmol $g^{-1}h^{-1}$)	33^{+++}	50^{+++}	ns	11^{++}	ns	3645 (1946)	7024 (2152) **	1149 (570)	2838 (1698)	1825 (1080)	8022 (4402) ***	430 (195)	1288 (855)*
E_C/E_N (mol/mol)	17^{+++}	6.4^{+}	ns	ns	ns	2.4 (1.5)	6.3 (2.6)	4.9 (2.7)	13 (8.0)**	5.7 (3.2)	9.8 (3.8)	6.3 (5.7)	11 (6.0)

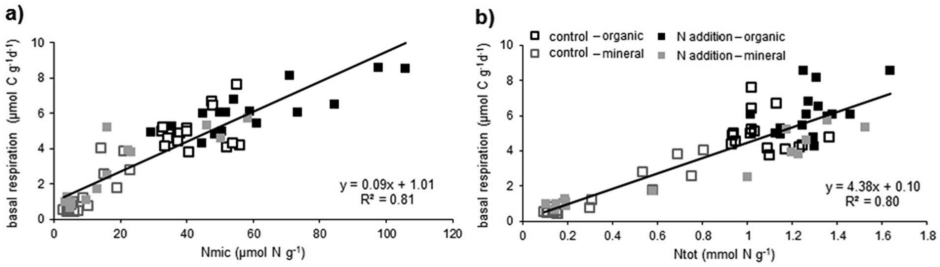

Fig. 2. The linear regression between microbial biomass N and respiration rate (a) and total soil N and basal respiration rate (b) for both N addition and control catchments together (n = 62). R^2 is coefficient of determination (p < 0.001).

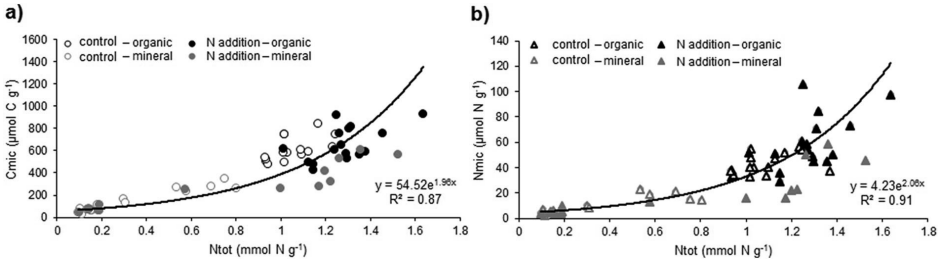

Fig. 3. The exponential regression of total soil N and microbial biomass C (a) and N (b) for both N addition and control catchments together, n = 62. R^2 is coefficient of determination (p < 0.001).

4. Discussion

4.1. Long-term N loading enhanced soil microbial biomass

The N application at the moderate rate of ~40 kg N $ha^{-1}y^{-1}$ since 1991 significantly enhanced soil microbial biomass evidenced by the larger content of microbial C and N and numbers of bacterial and fungal marker genes per gram of soil at the Gårdsjön coniferous forest catchment. This result coincides with a previous finding of Moldan et al. (2006) that the soil at the Gårdsjön N-addition catchment represented a significant sink of >60% of added N and conforms well to our expectation that the long-term enhanced N input to this particular catchment

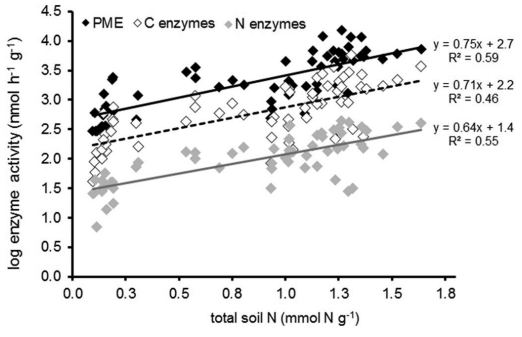

Fig. 4. The relationship between C (BG + CEL), N (LEU + NAG) and P (PME) acquiring enzyme activities and total soil N content for both the N addition and control catchments together, n = 62. R^2 is coefficient of determination ($p < 0.001$). Notice that Y axis is in log-transformed scale.

did not cause a decline in soil microbial biomass. Correspondingly, Janssens et al. (2010) pointed out that in seriously N-limited forests, i.e. rich in organics with a high C/N ratio of 30–50 (0–30 cm of soil profile, Mulder et al., 2015), N addition could support soil microbial biomass and its activity. Interestingly, in the case of the Gårdsjön catchment, the stimulating N effect persists for 24 years of an on-going enhanced N supply despite the cumulative N input already reaching ~1200 kg N ha^{-1} in these days. This contradicts most of the prevailing observations, which content that the negative effects of long-term enhanced N input on soil microbial biomass, specifically its fungal communities in temperate and boreal regions (e.g. Soderstrom et al., 1983; Treseder, 2008; Liu and Greaver, 2010; Zhou et al., 2017; Zhang et al., 2018). This raises the question: why did the Gårdsjön catchment respond differently to the long-term N treatment than many others in the above-cited studies? We address possible explanations and mechanisms in the following discussion.

4.2. Absence of acidifying effect of N addition

We suggest that a very important prerequisite why we did not find negative N-related effects on microbial biomass and its activity is the absence of an acidifying effect that is often connected with enhanced N addition (Högberg et al., 2006; Evans et al., 2008; Horswill et al., 2008; Tian and Niu, 2015). Soil acidification is coupled with aluminium or ammonium toxicity, base cation depletion, and chemical stabilization of available C (Evans et al., 2012; reviewed in Kopáček et al., 2013). These adverse effects of soil acidification largely exceed the potential nutritional effect of N addition and bring about physiological constraints that reduce microbial growth (Geisseler and Scow, 2014; Tian and Niu, 2015; Chen et al., 2016; Averill and Waring, 2018; Ye et al., 2018) and soil respiration including its heterotrophic part (Chen et al., 2016; Li et al., 2018; Oulehle et al., 2018). However, in our case, the soil pH was comparable between the two Gårdsjön catchments (even the N-addition catchment had a soil pH up to 0.4 units higher that the control one in organic horizon). The N-addition effect on soil pH depends on the form of N input and on its subsequent transformations. Processes like plant/microbial ammonium uptake and nitrification generate acidifying H^+, while NO_3^- reduction and assimilation processes alkalize the environment by consuming H^+. In our case, the added ammonium nitrate contained both forms equally. However, the actual NH_4^+ availability largely exceeded that of NO_3^- in the N-addition catchment (on average 20–50 times), while acidifying processes of gross and net nitrification remained slow. The relatively low NO_3^- availability in the soils of the N-addition catchment points to its efficient uptake and/or transformation to other N forms in processes, which do not generate acidifying H^+. The potential sinks for the added NO_3^- could be both plants and the large soil microbial biomass, if they are not severely C-constrained (Hart et al., 1994; Tahovská et al., 2013). In summary, both acidifying and alkalizing processes are still in balance at the N-addition catchment, which keep the soil pH level unchanged or even higher. As such, the soil biochemical and microbial responses, which we point out and discuss below, can be ascribed solely to the nutritional effect of N.

4.3. Shifts in composition and functioning of the microbial community in response to long-term N addition

We suggest that moderate annual N input into the system that was, moreover, divided into several small doses applied during the year enabled gradual adaptation of the microbial community to the slowly increase N availability. As a result, the N-supplied soil microbial community had a larger biomass with a lower C/N ratio (significant only in the organic horizon) compared to those in the N-poorer control soils. Many studies point out that the N-related lowering of biomass C/N in forests is connected with the decreasing fungi/bacteria ratio (reviewed in Högberg et al., 2017; Zhou et al., 2017). In this study, soil

Table 4
Total prokaryotic (bacteria and archaea) and fungal community gene abundances in organic and mineral soils in dry and wet parts of control and N addition catchments. Relative abundance of sequences assigned to ectomycorrhizal taxa (% ITS amplicons) among all fungal sequences and fungi-to-bacteria ratio is shown. Mean ± SD (n = 6–8). Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

	F values (ANOVA)					organic horizon				mineral horizon			
	N	moisture	horizon	N x moisture	N x horizon	wet		dry		wet		dry	
						control	N	control	N	control	N	control	N
fungal abundance (18S rDNA copies g^{-1})	4.2^{+}	40^{+++}	ns	ns	5.9^{+}	8.1×10^8 (2.7 $\times 10^8$)	8.1×10^8 (2.6 $\times 10^8$)	4.4×10^8 (1.7 $\times 10^8$)	4.7×10^8 (1.8 $\times 10^8$)	5.1×10^8 (3.7 $\times 10^8$)	$1.1 \times 10^{9**}$ (6.0 $\times 10^8$)	1.5×10^8 (1.1 $\times 10^8$)	2.6×10^8 (1.4 $\times 10^8$)
ECM in total fungal community	ns	ns	ns	ns	ns	43 (22)	26 (31)	45 (12)	21 (16)	72 (63)	42 (65)	20 (18)	40 (19)
prokaryotic abundance (16S rDNA copies g^{-1})	ns	80^{+++}	31^{+++}	4.8^{+}	6.1^{+}	9.2×10^{10} (1.4×10^{10})	9.8×10^{10} (3.6×10^{10})	5.4×10^{10} (1.5×10^{10})	5.2×10^{10} (6.5×10^9)	4.6×10^{10} (2.0×10^{10})	9.5×10^{10} (4.1×10^{10})	8.4×10^9 (2.7 $\times 10^9$)	1.0×10^{10} (4.5×10^9)
fungi/bacteria ratio	ns	7.2^{++}	18^{+++}	ns	ns	0.009 (0.004)	0.009 (0.005)	0.008 (0.002)	0.009 (0.003)	0.011 (0.006)	0.013 (0.009)	0.018 (0.013)	0.026 (0.009)

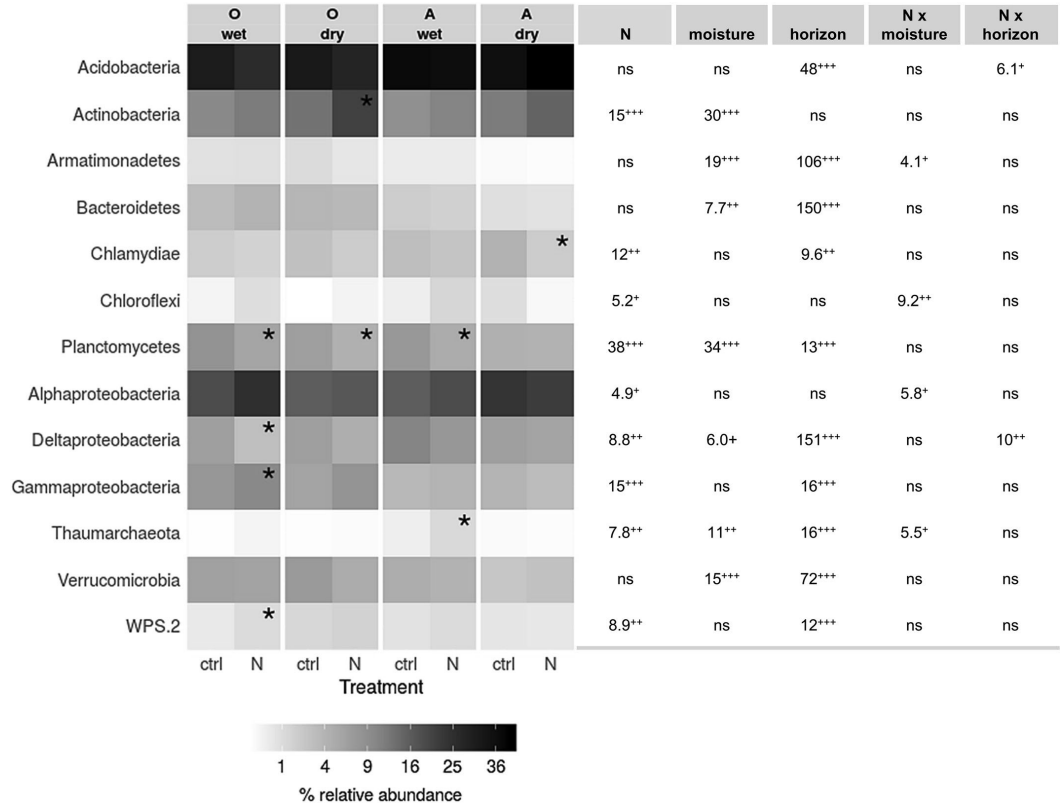

Fig. 5. Prokaryotic relative abundances within the soil microbial community (O; organic horizon, A; mineral horizon) in wet and dry parts of control and N addition catchments. Only the most abundant prokaryotic phyla (at least 5% in one of the soil sample categories) are shown. Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 5
Nitrogen addition, moisture and horizon effects on prokaryotic and fungal community composition including effect's interactions. Results of permutational multivariate analysis (PERMANOVA) based on Bray-Curtis sample dissimilarities are shown.

	Prokaryotes			Fungi		
	F	R^2	p	F	R^2	p
N addition	4.8	0.054	<0.001	4.0	0.055	<0.001
moisture	6.0	0.068	<0.001	8.7	0.120	<0.001
horizon	16	0.176	<0.001	10	0.139	<0.001
N x moisture	2.9	0.032	0.002	2.9	0.040	<0.001
N x horizon	ns			ns		

communities of differently N-treated catchments had a comparable fungi/bacteria ratio and were of similar taxonomic diversities. Nevertheless, the lower biomass C/N and higher specific respiration rate of the community adapted to N supply showed that it contained more resource-acquisitive taxa with larger energetic demands, intensive hydrolytic enzyme production and faster growth and turnover (Vanveen et al., 1984). Correspondingly, the N-supplied prokaryotic community was poorer in N-sensitive phyla of Deltaproteobacteria and Acidobacteria (and by Chlamydiae and Planctomycetes) but enriched in Actinobacteria, Alphaproteobacteria, Gammaproteobacteria and Chloroflexi, considered to include many copiotrophic taxa (Fierer et al., 2012; Mannisto et al., 2016).

The first changes in the fungal community have already been observed a few years after the onset of N addition at Gårdsjön in 1991. Boxman et al. (1998) found 30–40% decrease of ECM fruit-body richness and Brandrud et al. (1998) observed a retreat of N-sensitive species such as *Cortinarius*, while stress tolerant species from *Lactarius* or *Craterellus* genera prevailed (data from sporocarp production). Such N-driven early retreat of the sensitive ECM fungal taxa is consistent with results of many other N addition studies (Arnolds, 1991; Treseder, 2004; Högberg et al., 2014; Morrison et al., 2016). The estimated threshold level causing the regress of ECM (10–20 kg $ha^{-1}yr^{-1}$, van der Linde et al., 2018), was greatly exceeded in the case of Gårdsjön N-addition catchment. Consequently, our current data (based on molecular techniques) gained at the same site after 24 years show that *Cortinarius* and also *Hygrophorus* are almost absent. The other N-sensitive species *Piloderma* is less abundant, while *Craterellus* taxa predominate ECM fungal communities in the wet parts of the N-addition catchment, especially in the

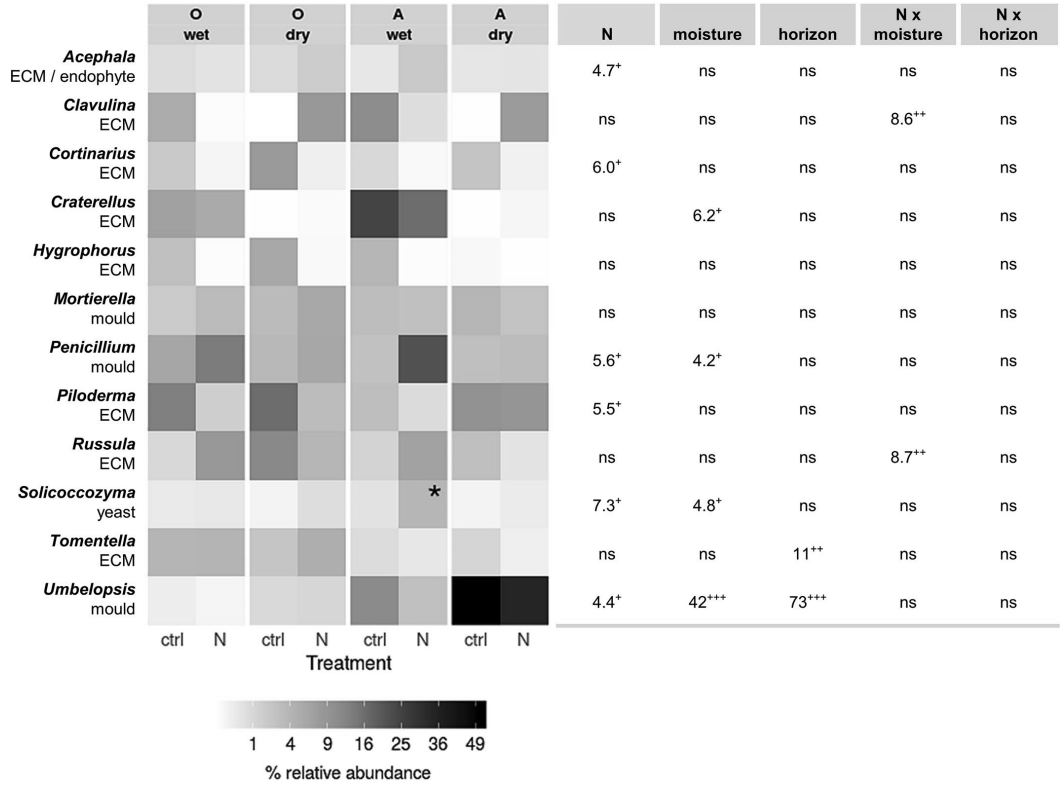

Fig. 6. Fungal relative abundances within the soil microbial community (O; organic horizon, A; mineral horizon) in wet and dry parts of control and N addition catchments. Only the most abundant fungal genera (at least 2% in one of the soil sample categories) are shown. Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 6
Relative abundance of sequences (% ecm ITS amplicons) assigned to nitrophilic (contact, short and medium smooth), nitrophobic (medium-fringe and long) exploration types and unknown types in all ectomycorrhizal sequences. Mean ± SD (n = 6–8). Results of factorial ANOVAs (F values) of the effects of N addition (N), horizon (organic; mineral), moisture (wet; dry) and selected interactions (N x moisture; N x horizon) are presented. Statistical significances of F values are marked as follows: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ns – not significant. Asterisks mark statistical significances of post-hoc comparison (Tukey HSD test) between controls and N-additions within dry/wet parts of catchments and for organic/mineral horizons separately (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

		F values (ANOVA)					organic horizon				mineral horizon			
		N	moisture	horizon	N x moisture	N x horizon	wet		dry		wet		dry	
							control	N	control	N	control	N	control	N
Nitrophilic	contact	11^{++}	10^{++}	ns	7.9^{++}	ns	<0.1	5.2 (5.6) **	<0.1	0.16 (0.27)	0.10 (0.15)	12 (22)*	<0.1	<0.1
	short	ns	9.3^{++}	ns	5.6^{+}	ns	28 (20)	13 (23)	0.12 (0.26)	23 (35)	51 (44)	32 (42)	4.8 (7.4)	18 (23)
	medium-smooth	ns	ns	9.1^{++}	ns	5.0^{+}	12 (11)	15 (14)	4.6 (3.7)	28 (26)	5.0 (9.8)	6.5 (15)	6.8 (5.4)	0.45 (0.62)
Nitrophobic	medium-fringe	14^{+++}	14^{+++}	ns	ns	ns	43 (20)	4.8 (5.7) **	54 (31)	32 (28)	18 (21)	5.9 (13)	48 (32)	34 (37)
	long	ns	ns	ns	ns	ns	<0.1	<0.1	<0.1	0.10 (0.11)	10 (26)	<0.1	<0.1	0.14 (0.21)
Unknown type		ns	ns	ns	7.1^{+}	ns	18 (26)	63 (37)	41 (30)	17 (18)	16 (23)	44 (33)	40 (29)	47 (39)

mineral horizon. The retreating species *Piloderma* and *Cortinarius* are typical nitrophobic ECM fungi with hydrophobic rhizomorphs (Agerer, 2001; Lilleskov et al., 2011) and great proteolytic capabilities (Bodeker et al., 2014; Phillips et al., 2014; Kohler et al., 2015). These are, however, redundant for the associated trees under increasing availability of mineral N forms in the soil. Therefore, a decline of such highly C-demanding ECM fungal species (Clemmensen et al., 2015) is likely connected with reduced relative allocation of plant assimilates belowground and their preferential flow to the root growth (Litton et al., 2007; Lilleskov et al., 2019), leading to a reduced fraction of mycorrhizal root tips (Majdi et al., 2001). Accordingly, actual data from Gårdsjön showed higher total root biomass at the N-addition compared to the control catchment by ca 300 g m^{-2} (Moldan and Oulehle, *unpublished data*).

The soil/root niche that was relaxed by retreating nitrophobic ECM species was partly occupied by the nitrophilic ECM species belonging mainly to contact and short exploration types in wet and dry conditions, respectively. Both exploration types likely involve host generalists that grow their extramatrical mycelia only at small mm-cm scales close to the roots thus having relatively lower requirements for plant C supply (Agerer, 2001; van der Linde et al., 2018). Others were *Russula* and *Lactarius* of uncertain exploration types that are both also considered rather nitrophilic (Avis, 2012). The nitrophilic fungal taxa commonly lack proteolytic abilities and rely on mineral N (Lilleskov et al., 2019). Their low C-requirements and high affinity for mineral N (Lilleskov et al., 2019) preferred them over nitrophobic ECM fungi under high soil N supply. The fungal communities of N-addition catchment soils were further enriched in fungi with a saprotrophic life strategy as observed also, for example, by Morrison et al. (2016). Especially certain moulds (*Mortierella, Penicillium*) and yeasts (*Solicoccozyma*) almost doubled their relative abundances compared to control conditions. They are acknowledged mainly as efficient carbohydrate and fungal biomass decomposers (Brabcova et al., 2016). Thus, they may take advantage of the faster turnover of the microbial community in the N-supplied soils. We are aware that the growth of the moulds, known as disturbance-adapted fungi (Brabcova et al., 2016), could be also supported by soil processing – specifically by soil sieving before its storage and analysis. Their greater representation in soils of N addition catchment could also indicate their larger potential to proliferate in N-rich conditions than in N-poor soils of the control catchment.

4.4. The current status of the microbial community in relation to the opening of soil N cycle

The microbial community adapted by taxonomical restructuring towards a prevalence of copiotrophic, faster growing, more resource-acquisitive groups of prokaryotes, ECM and saprotrophic fungi, while N-sensitive taxa retreated. Its large microbial biomass secured itself a larger N-retaining capacity per g of soil than the smaller community in the N-poorer control soils. This was obvious not only from a higher biomass N concentration per gram of soil but also from a higher Nmic-to-soil N ratio in N-addition compared to control soils. These parameters, however, also indicate that the soil N in the N-addition catchment is more labile, because it is replenished more by N pools with a fast turnover than in the original N-poor conditions. The larger microbial biomass provided faster degradation of organic matter through higher activity of extracellular hydrolytic enzymes. Compared to control soils, the enzymatic investments were, with N addition, shifted to a preferential gain of C and P over N. This reflects microbial adaptation to higher soil N availability in order to compensate enhanced C and P demands to build the biomass, synthesize enzymes and obtain energy (Allison and Vitousek, 2005; Moorhead et al., 2012; Wutzler et al., 2017; Averill and Waring, 2018). An increasing N excess in the soil system was indicated by an enhanced rate of net N mineralization and, to a much lesser extent, also of net nitrification, which led to detectable NO_3^- leaching from the N-addition catchment. The NO_3^- leaching, however, has remained stable for more than a decade despite the ongoing N additions to the soils (Moldan et al., 2018).

We cannot calculate the whole N budget of the Gårdsjön N-addition catchment including soil and vegetation N pools from currently available data. However, based on all known input-output data and the current physiology of the soil microbial community given in this study, we suggest that a critical point of ecosystem N saturation has probably not been reached. The deposition + addition input of ~40 kg N kg $ha^{-1}yr^{-1}$ is not rivalled by outputs. Nitrate runoff losses form up to 20% of the total annual N input and the DON plus NH_4^+ leaching is commonly lower or exceptionally equal to nitrate N losses (Moldan et al., 2018). Considering other pathways of potential ecosystem N losses, there is no evidence for a significant N leaching to groundwater in the studied catchment (Moldan et al., 2018). We do not have data about denitrification rates in Gårdsjön but according to other published values, N losses through denitrification usually reach 3–4 kg N $ha^{-1}yr^{-1}$ in northern hardwood forests (Kulkarni et al., 2015) and also in coniferous forests (Gundersen et al., 1998; Galloway et al., 2004). The functional potential to denitrification (mainly *Acidothermus*) ranged from 7% to 18% of relative abundances in mineral and organic horizons, respectively, but with no effect of N addition. Even if we, contradict of found functional potential, consider the 100% increase of the denitrification N losses in N-supplied forest soils (Barnard et al., 2005), they could not outbalance the remaining >50% of the annual N input (i.e. >20–25 kg N kg $ha^{-1}yr^{-1}$). Denitrification and its response to N addition further depend on C availability and moisture (Mason et al., 2017). Because microbial communities in the N-addition catchment at Gårdsjön show signs of forthcoming C limitation, we do not expect enormous increase in N losses through denitrification. Therefore, the studied N-supplied forest system is still able to retain a part of added N in the plant-microbe-soil system.

4.5. Implications for the N retention in Gårdsjön catchment under ongoing N addition

The current state of the plant-microbe-soil system can be characterized as follows. The 95-year-old coniferous forest without the potential for a significant growth (and additional N immobilization) in future is associated with a large resource-acquisitive soil microbial community, dominated by nitrophilic ECM and saprotrophic fungi and copiotrophic groups of bacteria. Although they intensively decompose and mineralize organic matter and are able to immobilize N in their large biomass, their metabolism shows signs of C limitation/N excess. Nevertheless, the C-supply is still sufficient to sustain the large microbial biomass, the activity of which outcompetes autotrophic nitrifiers, so the excess N accumulates in the soil mainly in the form of NH_4^+. This situation corresponds well to the third out of the four stages of N saturation concept formulated by Schimel and Bennett (2004). The third stage, before NO_3^- is prevalent in soil, is characterized by an increased soil N mineralization rate and domination of NH_4^+ in the available soil N pool. In such conditions, plants still have the capacity to immobilize the NH_4^+ surplus and promote production, and nitrification is thus limited only to NH_4^+-rich but C-limited microsites. Relatively low concentration of NO_3^- in the Gårdsjön G2 runoff are likely not a sign of system N saturation but rather indicated that nitrification occurred at microsites with exhausted available C. Evidently, the system is shifting from N to C limitation but without an acidification effect connected with N-input and with a current plant C supply to the soil microbial community. We would hardly expect risk of a significant increase of nitrate leaching to surface waters in the following years. However, an occurrence of dry seasons in the context of climate change and a risk of pest infections in the relatively old-aged forest could markedly change the situation.

5. Conclusions

We conclude that the forest soil microbial community at Gårdsjön has adapted through structural and functional changes to long-term N

addition to maintain sufficient growth, microbial biomass and N immobilization. The microbial biomass, in spite of the relative retreat of fungi with large external mycelia, retains N, being relatively enriched by certain copiotrophic bacteria, nitrophilic fungi and saprotrophs. We observed, that an alleviation of microbial N limitation is continuing in the organic soil horizon which, however, took 24 years in systems with 70 tonnes of soil C per hectare and molar C/N ~35 in the organic horizon. We assume that microbial biomass and its metabolic activity is not necessarily threatened by a large cumulative N dose, provided N is added at a moderate rate, and the persistent and active microbial biomass thus substantially contribute to soil N retention.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.107732.

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Paper 4

Indications that long-term nitrogen loading limits carbon resources for soil microbes

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Uniform rapid response of soil microbes to acidification in spruce and beech forests

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Abstract

In past decades, anthropogenically enhanced atmospheric deposition of sulfur (S) and nitrogen (N) influenced forest ecosystems in Central Europe. The soils were simultaneously acidified and enriched in N. However, both mechanisms of S and N impact on microbial communitites and consequences to microbially driven soil functioning differ. We run a two-forest stand (mature Norway spruce and European beech) replicated field experiment involving acidity (H_2SO_4 addition) and N (NH_4NO_3) manipulation and their combination. During four years, we traced a separate response of soil microbial communities to acidification and N loading and searched for the relations with changes in activity of extracellular enzymes. Acidification selected for acidotolerant and oligotrophic taxa of Acidobacteria and Actinobacteria and decreased bacterial community richness and diversity. This shift was fast and parallel in both stands disregarding their original dissimilarities in soil chemistry and microbial communities. The acidity-driven shifts in bacterial community composition influenced the stoichiometry, while the bacterial abundance a magnitude of enzymatic activity. Differently, bacterial response to experimental N addition occurred only after four years and was weak probably due to historically enhanced N availability in these systems. Fungi

were not influenced by any treatment during 4-year manipulation. We suggest that in the first years of acidification when fungi remain irresponsive, bacterial reaction might drive the changes in soil enzymatic activity

Introduction

Enhanced atmospheric deposition of sulfur (S) and nitrogen (N) during the second half of the 20th century caused acidification and eutrophication of forest ecosystems across Europe and North America. In Central Europe, atmospheric deposition was significantly reduced during 1990s (Kopáček and Veselý, 2005). Yet, forests still face its consequences and their full recovery is not expected in near future (Alewell et al., 2000).

As a result of acidification, soils are depleted in base cations, an availability of dissolved organic carbon (DOC) is reduced, while toxic Al^{3+} is mobilized (Evans et al., 2012; Kopáček et al., 2013; Oulehle et al., 2011). Besides these indirect acidification effects, soil microorganisms are under immediate physiological pH-induced stress (Prescott et al., 2005). Soil pH is widely accepted as one of the most influential factors shaping microbial communities. Low soil pH reduces cell growth rate and thus soil microbial biomass, enhances cell maintenance requirements and selects for acidotolerant and acidophilic microbial species. (Fierer, 2017; Glassman et al., 2017; Lauber et al., 2009; Rousk et al., 2010a; Rousk and Bååth, 2011; Tedersoo et al., 2014). The metabolic requirements and limitations of present microbial community together with an actual substrate availability drive secretion of extracellular enzymes, which cleave complex organics to release utilizable forms of C, nutrients and energy (Burns et al., 2013; Mooshammer et al., 2014). Accordingly, through a modification of substrate quality, availability and restructuring microbial community and its activity, acidification alters essential soil processes (Kopáček et al., 2013).

N input via atmospheric deposition triggers changes in processes of C and N transformation (Aber et al., 1998; Gao et al., 2015; Janssens et al., 2010; Kopáček et al., 2013). One of the important mechanisms is the N-induced

change in soil microbial community. It commonly involves a retreat accompanied by changes in taxonomic composition of ectomycorrhizal fungi and a decrease of overall fungi-to-bacteria ratio (F:B), which results in a lower N retention in the fungal biomass (Bahr et al., 2013; Choma et al., 2017; Frey et al., 2004; Högberg et al., 2007; Janssens et al., 2010; Treseder, 2004) and a lower activity of exoenzymes involved in organic matter decomposition (Gallo et al., 2004). In result, the processes of microbial SOM formation connected with an efficient N immobilization can be slowed down and a risk of excess N leaching increases. Nevertheless, the progression of N-induced changes in ecosystem functioning does not accurately follow this widely accepted scenario in all cases. The ecosystem response depends on the nature of N input (dose, duration, regularity and form of N) and on its initial conditions, esp. the stage of N limitation (Tahovská et al., 2020).

The commonly observed negative consequences of the N input may likely be connected to accompanying acidification, which results from reactive N transformations (Chen et al., 2016; Gao et al., 2015; Kopáček et al., 2013) and coupled S deposition, rather than the nutritional N effect itself. Additionally, studies questioning a superiority of fungal role in soil enzymatic activity and N retention capacity begin to emerge (Carrara et al., 2018). This highlights a need for more targeted effort in understanding of separated impact of acidification and nutritional N effects on soil microbial communities and their functioning.

The ecosystem response to acidification and elevated N input further depends on the stand tree species composition (Rothe et al., 2002; Růžek et al., 2019). Individual tree species create specific soil conditions through differences in litter and rhizodeposition inputs, in forming associations with various soil microorganisms and in varying potential to intercept water and atmospheric deposition (Augusto et al., 2015; Calvaruso et al., 2011; De Schrijver et al., 2007; Prescott and Grayston, 2013). In Central Europe, Norway spruce (*Picea abies* (L.) Karst.) and European beech (*Fagus sylvatica* L.) are the most common tree species. Spruce litter is more acid and of higher C:N compared to beech (Bauer et al., 1997; Calvaruso et al., 2011).

Additionally, due to higher leaf area and year-round foliage, spruce is more efficient in atmospheric compounds interception (De Schrijver et al., 2007). In result, soils under spruce are usually more acid, less fertile and more susceptible to further acidification (Augusto et al., 2002). Consequently, soil microbial communities under spruce and beech differ in their taxonomic composition and metabolic potential (Asplund et al., 2018; Bahnmann et al., 2018; Bárta et al., 2017; Nacke et al., 2016).

In this study, we attempt to disentangle the separated effects of acidity and increased N availability on soil microbial communities under beech and spruce. The Načetín experimental site (Ore Mts., Czech Republic) consists of intensively studied adjacent beech and forest stands similar in abiotic conditions (climate, deposition, bedrock, original soil type). Parallel acidity (sulfuric acid addition) and N input manipulation (NH_4NO_3) experiment in full factorial design was established in both forest stands to examine their separated and combined effects on microbial community and linked soil processes. In this study, we aimed to: (1) differentiate between the effect of acidification and N input on composition of soil bacterial and fungal communities using amplicon sequencing (2) compare the changes between beech and spruce stands and (3) relate the shifts in community composition to changes in exoenzymatic activity. We hypothesized that (i) the acidification will markedly change the structure of soil microbial community and decrease its diversity, with a stronger impact on bacteria than fungi. The nutritional effect of N will be less pronounced, as the systems are not primarily N limited. We expect that the ectomycorrhizal fungi will be the most affected by N addition. (ii) The acidification driven changes in microbial communities will be more pronounced in the spruce than in the beech stand due to its lower original pH. (iii) An acidification-induced decrease in C availability will enhance activity of C mining enzymes and shift the enzymatic stoichiometry in behalf of C acquisition. We further analyzed soil physico-chemical properties to seek for driving factors of observed structural and functional changes.

Material and methods

Sites and experimental design

At each forest stand, a full factorial manipulation experiment with four treatment variants was created: control (Ctrl), nitrogen (N), acid (S) and acid + nitrogen (S+N) treatments in a randomized blocked design. In October 2012, four complete blocks of 3 x 3 m plots were established resulting in four treatments replicates available, followed by a period of pre-treatment measurements (April 2013 – March 2014). The manipulative experiment itself 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 using 15 l watering cans evenly across the plots, followed by addition of extra 15 l rainwater to properly seep all added chemicals into the soil. 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 average annual amount of rainfall. The NH_4NO_3 addition did not significantly alter pH of added solution (pH of applied solution was ~ 5.4).

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 conditions permitted the collection. Importantly, soil water was always collected before treatment application. Soil solution samples were collected using Rhizon® suction samplers (Rhizosphere Research Products, Wageningen, 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. We measured 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), dissolved organic carbon (DOC), dissolved nitrogen (DN), PO_4^{-} and total phosphorus. However, detailed methodology is listed only for variables that significantly affected the microbial community composition (acc. to step-wise selected models, see below): Solution pH (pH) was determined using Radiometer TTT-85 with a combination electrode. Concentrations of dissolved organic carbon (DOC) and dissolved nitrogen (DN) were measured in the filtered solution samples (glass fiber Macherey-Nagel) using Tekmar-Dohrmann Apollo 9000 analyzer. Dissolved organic nitrogen (DON) in the solution was calculated as a 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 (F+H horizon layer) was performed in years 2014 – 2017, always in May and September, for each treatment plot two samples were combined. However, the soil chemical parameters, microbial biomass, marker gene abundances and enzymatic activities presented in this study come from samples taken in 2017, the other years' soil and soil solution data was published previously (Oulehle et al., 2018). Microbial community composition was analyzed for all sampling occasions, nevertheless, only the 2017 year data is presented in detail, while only partial results from years 2014–2016 are used as supporting information.

Total soil carbon (C_{tot}) and N (N_{tot}) were determined in dried (60°C, 48h), milled soil samples using elemental analyzer (Vario Micro Cube, Elementar, Germany). Soil extractable NH_4^{+} and NO_3^{-} were measured after the extraction with 0.5M K_2SO_4 (extractant: soil, 4 : 1, v/w; shaken 45 min/150 opm; centrifuged 4,000 rpm/15 min; supernatant filtered through 0.45 μm glass-fiber filter) by flow injection analysis (QuickChem 8500, Lachat Instruments, USA). Soil microbial biomass carbon (C_{mic}) and N (N_{mic}) were measured by fumigation extraction method. Fresh soil samples (10 g) were

put into glass flasks in two sets. One set was directly extracted with 0.5 M K_2SO_4 in the same manner as for mineral nitrogen determination while another set was extracted after a 24-h fumigation (amylene-stabilized chloroform). Organic carbon and total N in the K_2SO_4 extracts were measured on a LiquiTOC II (Elementar, Germany). C_{mic} and N_{mic} were calculated as the difference in organic carbon and total N, respectively, between fumigated and non-fumigated samples and corrected by the factors of 0.38 for C_{mic} (Vance et al., 1987) and 0.54 for N_{mic} (Brookes et al., 1985). All data were then expressed per gram of soil dry weight (5h, 105°C). We also determined soil water extractable DOC, DN, dissolved organic N (DON), basal respiration, specific respiration, net ammonification and net nitrification, but these variables were not shown to explain differences in microbial communities and are not presented in detail.

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 is saturated was picked. Plates were incubated at 20 °C for 120 min. Fluorescence was measured at an excitation wavelength 365 nm and emission wavelength 450 nm using Infinite F200 microplate reader (TECAN, Germany). Activity of carbon (C), N and phosphorus (P) mining enzymes was calculated as EC=BG+CEL, EN=NAG+LEU and EP=PME, respectively (Sinsabaugh et al., 2009). Therefrom, ratios EC:EN, EC:EP and EN:EP were derived. Total hydrolytic activity was calculated as E_{tot}=EC+EN+EP.

DNA was obtained from 0.5 g of soil with the use of extraction buffer containing 5% hexadecyltrymethilammonium bromide (in 0.7M NaCl, 120 mM potassium phosphate, pH 8.0) and 0.5 ml of phenol-chloroform-isoamylalcohol (25:24:1). The first step was performed in FastPrep™ Lysis Matrix E tube and FastPrep Instrument (MP Biomedicals, Solon, OH, USA) for 45s. Then, the samples were extracted with chloroform and precipitated in a PEG 6000/1.6M NaCl solution. Pellets were washed with 70% ethanol and re-suspended in molecular biology grade water. Quantus™ (Promega, USA) was used for DNA concentration measurement.

Quantitative PCR (qPCR) of markers specific for bacteria and fungi – genes for ribosomal small subunit: 16S rRNA and 18S rRNA genes, respectively, was performed with use of FastStart SybrGREEN Roche® Supermix (Roche, France) and StepOne system (Life technologies, USA) as described previously (Bárta et al., 2017; Gittel et al., 2014). Fungi-to-bacteria ratio (F:B) was calculated as count of 18S copies divided by 16S copies.

Barcoded amplicon sequencing was performed in order to assess the microbial community composition. The PCR primers (515F/806R) targeting the V4 region of the small ribosomal subunit (SSU) rRNA gene were used in case of bacteria (Caporaso et al., 2011) while primers ITS1f and ITS2 were used for fungi (White et al., 1990). PCR reactions for fungal ITS amplification consisted of 0.8 µl PCR-grade water, 1.5 µl 10x PCR buffer w/o $MgCl_2$, 1.5 µl $MgCl_2$ (25mM), 0.3 µl dNTP's (10mM), 0.75 µl DMSO, 0.15 µl FastStart polymerase (Roche), 2 µl of each primer (1.6 µM) and 6 µl of genomic DNA (1 ng. μl^{-1}). PCR setup was as follows: 95°C for 10 min to denature the DNA, with amplification proceeding for 10 cycles at 95°C for 30 s, 55°C for 40 s, and 72°C for 120 s; 8 cycles at 95°C for 30 s, 80°C for 30 s, 60°C for 40 s and 72°C for 120 s; 22 cycles at 95°C for 30 s, 55°C for 40 s, and 72°C for 120 s; with a final extension of 5 min at 72°C to ensure complete amplification. Bacterial V4 region PCR reaction contained 8 µl of GoTaq Master Mix (Promega), 2 µl of each primer (1.6 µM) and 4 µl of genomic DNA (1 ng. μl^{-}

[1]). PCR: 95°C for 10 min to denature the DNA, with amplification proceeding for 10 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 60 s; 8 cycles at 95°C for 15 s, 80°C for 30 s, 60°C for 30 s and 72°C for 60 s; 14 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 120 s; final extension 5 min at 72°C. The primers contained a 10-base MID barcode to facilitate multiplexing. The sequencing library was prepared with use of NEBNext Ultra II DNA Library Prep Kit for Illumina kit and NEBNext Multiplex Oligos for Illumina adaptors. Quantification wass carried out with help of Agilent Bioanalyzer 2100, High Sensitivity DNA Kit and KAPA library Quantification Kit. Amplicons of were sequenced on the Illumina HiSeq2500 platform. The amplicon library preparation and sequencing were contracted (SEQme s.r.o., Czech Republic).

Bacterial paired-end reads were joined using -fastq_mergepairs command within USEARCH 8.1 (Edgar, 2010), while only forward reads were further processed in 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). Then, bacterial sequences were filtered and trimmed to length of 250 using PRINSEQ-lite. 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 help of BLAST (Altschul et al., 1990) implemented within QIIME v1.9.1 pipeline (Caporaso et al., 2010). SILVA 132 (Quast et al., 2013) and UNITE 7.2 (UNITE Community, 2017) databases were used for annotation in case 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). 2,000 reads per sample are accepted as sufficient to cover main pattern in the microbial community (Caporaso et al., 2011). Only single bacterial sample did not reach this limit and was discarded. The fungal samples had

overall lower read counts, thus more relaxed limit 1,300 reads per sample was chosen as a compromise to preserve reliable part of samples within dataset (2 samples were discarded). Alpha diversity was estimated from datasets rarefied to minimal sequencing depth (6,065 and 1,310 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 as recommended by 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 genus or species level was searched in FUNGuild database v1 (Nguyen et al., 2016) and available literature. Reference sequences of other OTUs were BLAST compared to GenBank NCBI database and for hits with ≥ 99% identity, metadata was searched for information implying lifestyle. The effort was taken to cover at least 90% of sequences in each sample. Sequence data are stored at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the study accession number PRJEB36421.

Statistical analysis

All steps of statistical evaluation were performed in R 3.6.1 (R Core Development Team, 2019), the considered significance level was α=0.05. The treatment effect on soil and soil water physico-chemical variables, activity of hydrolytic enzymes, marker gene abundances, diversity measures and taxa relative abundance was tested using linear mixed-effects models with stand, treatment and sampling time (May and September) as fixed effects and block as a random effect using package *lme4* ver. 1.1-20 (Bates et al., 2015). Relative abundance data was square-root transformed prior to testing. Post-hoc comparison of treatments within respective stands was done using estimated marginal means – *emmeans* package v.1.4.1 (Lenth et al., 2019). The stand effect was evaluated by a linear model (stand ad sampling time as factors) that included only control treatment plots.

Treatment effect on overall bacterial and fungal communities was examined with help of the package *vegan* v.2.5-4 (Oksanen et al., 2019). PERMANOVA analysis (9,999 permutations) of Bray-Curtis dissimilarities at OTU level was based on a model considering stand, treatment and sampling time as explanatory variables (permutations restricted to treatment blocks). To reveal stand effect only control treatments data was evaluated by 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 differences between microbial communities, distance-based redundancy analysis (db-RDA) model forward selection was performed for complete dataset as well as only for 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

Primary stand differences in soil biochemistry and microbial activity

Adjacent beech and spruce stands share the same bedrock, soil type, climate, as well as acidification history. However, long-term presence of different tree dominants projected to significantly different soil biochemical properties. The spruce stand originally had significantly lower soil solution pH (~3.9 and ~4.3, in spruce and beech, respectively), more C_{tot} with higher C_{tot}:N_{tot} ratio and higher soil solution DOC concentration than the beech stand (Tab.1 & S1, Fig.S1). DON and NH_4^+ dominated dissolved N pool in both stands, but NO_3^- concentration was seven-fold higher in the beech than in the spruce stand (Tab.1 & S1). Microbial biomass C and activities of C acquiring enzymes were comparable at both sites, while microbial biomass N and activities of N and P mining enzymes were significantly higher in the beech stand (Tab.1 & S1).

Treatment effects on soil biochemistry and microbial activity

At both stands, S and/or combined S+N treatments strongly and consistently affected soil and soil solution biochemistry and hydrolytic activity, while the effect of N treatment was mostly negligible. Both S and S+N treatments significantly acidified the soil solutions in both stands by ca 0.4 pH unit compared to control treatments (Tab.1 & S1). The acidification was accompanied by 40% reduction of soil solution DOC concentrations on average in both stands and also DON decrease (by 30%) in the beech stand only. In the spruce stand, C_{mic} was reduced by 20% (Tab.1). Acidifying treatments further induced changes in activities of individual hydrolases. Specifically, both S and S+N treatments increased E_{tot} in the spruce stand, mainly due to a stimulation of PME, and decreased C/P and N/P enzyme activity ratios compared to control (Tab.1 & S1). In the beech stand, the S treatment but not the S+N treatment decreased both LEU and PME activities, which significantly enhanced C/P enzyme activity ratio compared to control (Tab.1 & S1). Despite no observable change in soil chemistry, a shift in enzymatic activity was detected in N treatment. Thus, the only effect of N addition was decreased C/P and N/P enzyme activity ratios compared to control treatment in the spruce stand due to enhanced activity of PME (Tab.1 & S1).

Primary stand differences in microbial communities' composition

Sequencing of year 2017 samples yielded 2,044,725 bacterial and 222,477 fungal high-quality sequences with a range 6,065–55,933 and 1310–8342 sequences per sample for bacteria and fungi, respectively. Clustering identified 2,154 bacterial and 1,168 fungal unique OTUs across all samples.

Beech stand soil bacterial community with average of 572 OTUs and mean Shannon diversity at 5.03 was more diverse compared to the spruce stand with 464 OTUs and Shannon index 4.78 (Tab.1). Fungal OTU richness was higher in spruce than in beech (163 and 141, respectively) as well as

Table 1: Soil and soil solution physico-chemical parameters, activity of hydrolytic enzymes, bacterial and fungal diversity measures and marker gene abundances in respective treatments in both stands – their mean±sd. Within each stand, significant difference ($p<0.05$) to control treatment denoted by asterisks (*). The last column shows stand differences (i.e. 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 diff. (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 water 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)	++
BG	nmol g^{-1} h^{-1}	1473(347)	1566(428)	1320(356)	1958(1000)	1539(434)	1715(489)	1968(752)	1851(723)	n.s.
CEL	nmol g^{-1} h^{-1}	330(90)	381(95)	324(84)	455(315)	315(123)	403(136)	427(160)	416(160)	n.s.
LEU	nmol g^{-1} h^{-1}	40.3(18.5)	25.8(16.1)	18.2(9.4) *	18.6(15.4) *	10.6(8.8)	1.75(2.25)	7.38(8.88)	7.50(6.57)	+++
NAG	nmol g^{-1} h^{-1}	783(260)	770(424)	532(272)	1028(978)	309(99)	260(81)	337(90)	382(159)	+++
PME	nmol g^{-1} h^{-1}	1860(412)	1628(391)	931(604) *	1723(793)	828(582)	1387(316)	1489(537) *	1476(662) *	++
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.
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.

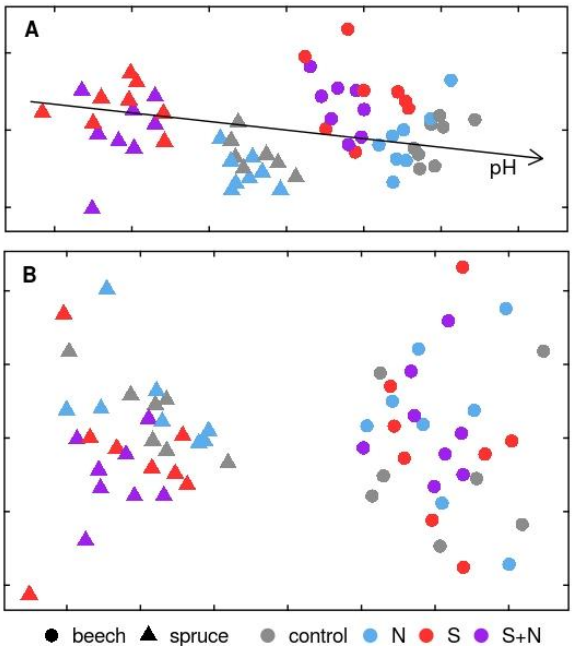

Figure 1: Visualization of a nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity of bacterial (A) and (B) fungal communities composition (stress = 0.06 and 0.13, respectively). Soil solution pH in panel (A) was passively projected.

Shannon diversity (though insignificantly; 3.80 and 3.47, respectively; Tab.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 (Tab.1).

The original soil microbial communities (control treatments only) differed considerably between beech and spruce stands as shown by significant stand effect in PERMANOVA analysis explaining 46.1 % and 26.2 % of variability for bacterial and fungal community, respectively (both $p<0.001$; Tab.S2). NMDS visualization of all the samples clearly separated beech and spruce stand bacterial communities (Fig.1A). 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 (Tab.S3). The other significant factors were DOC, C_{tot} and NO_3^- with 4.5 %, 2.5 % and 2.3 %, respectively (Tab.S3).

Generally, Acidobacteria contributed to more than 50 % of bacterial sequences, which made them dominant in both stands (Tab.2). The following high abundant phyla were Proteobacteria, Actinobacteria, Verrucomicrobia, and Planctomycetes (Tab.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: several Alphaproteobacterial orders (Caulobacterales, Micropepsales, Reyranellales and Rhodospirillales), Verrucomicrobia, Planctomycetes, and Deltaproteobacteria (Tab.2 & S4).

Fungal community dissimilarity between stands is also clearly presented by NMDS projection (Fig.1B). Step-wise built model found that C_{tot}:N_{tot} was the strongest predictor among the soil chemical variables with 16.4 % explained variability. The other significant variables DON, pH and C_{tot} accounted for lower portion of explained variability (5.1 %, 2.4 % and 2.4 %, respectively; Tab.S3). 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 higher abundance in the spruce stand compared to the beech stand (Tab.3). Regarding Basidiomycota, spruce soils contained more sequences assigned to ectomycorrhizal genera *Tylospora* and *Hygrophorus* (Tab.3). In the beech stand, genera *Amanita*, *Inocybe, Russula* and *Piloderma* (marginally insignificant) were more abundant compared to the spruce stand, where they accounted to only fractions of percent in relative abundance (Tab.3). Mortierellomycota reached higher abundance in the beech stand. Other fungal lineages such as Rozellomycota, Chytridiomycota or Glomeromycota were detected as well, but in negligible counts (Tab.3). Examining lifestyle groups, moulds were more abundant under beech, while spruce soils were richer in ericoid mycorrhizal or root associated fungi (Tab.S5).

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 stand. Asterisks denote stand differences significance (* - p<0.05, **-p<0.01, ***-p<0.001, n.s.-not significant). Higher (+) or lower (-) relative abundance in respective treatments compared to control marked for differences at p<0.05 for each stand spearately.

			% mean relative abundance in control			Treatment significant change to control					
			beech	spruce	stand diff.	beech			spruce		
	Phylum	Lower nested taxon				N	S	S+N	N	S	S+N
Phyla with 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	n.s.		+	+			
Phyla with 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	***		-	-	-	-	-

Treatment effect on microbial communities' composition

S and S+N treatments decreased bacterial diversity (OTU richness and Shannon index) in both stands (Tab.1). Fungal diversity was not affected by any treatment in any stand. We found no effect of treatment on the abundances of 16S and 18S rRNA genes and their ratios in both stands (Tab.1). Bacterial community composition changed shortly after both acidifying treatments onset in both stands (Tab.S6 & S7). 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 (Tab.S6). In 2017, additionally, N treatment affected bacterial community in the spruce stand while decreased bacterial diversity in the beech stand in comparison to individual controls (Tab.S7). N induced shifts projected only in decrease of Planctomycetes and Deltaproteobacteria relative abundances (Tab.S4).

A detailed view on compositional changes showed that the bacterial communities of acidified (S and S+N) and control treatments were remarkably distinct and clustered separately in NMDS ordination plot (Fig.1A). Although starting from different original communities, the acidification-induced changes were analogous in both stands. Majority of responding taxa showed the same positive or negative reactions in both stands (Tab.2). The relative abundance of dominant whole phylum Acidobacteria was unaffected, but at deeper taxonomical resolution, acidification enhanced relative abundance of Acidobacteriales at the expense of Solibacteralales in both stands and also at the expense of Subgroup 2 in the spruce stand (Tab.2). Numerous bacterial groups significantly decreased their relative abundances in response to acid additions mutually in both stands. Namely Armatimonadetes, Bacteroidetes, Elusimicrobia, Fibrobacteres, Gemmatiomonadetes, Planctomycetes and Verrucomicrobia as well as proteobacterial Azospirillales, Reyranellales and Rhodospirillales (all Alphaproteobacteria) and Deltaproteobacteria. Betaproteobacteriales decreased significantly only in S+N treatment in both stands. Further, in the spruce stand, Caulobacterales, Micropepsales,

Table 3: The selected fungal taxonomic groups – their mean relative abundance in the control treatment plots of the beech and the spruce stand. Asterisks denote stand differences significance (* - p<0.05, **-p<0.01, ***-p<0.001, n.s.-not significant, n.t.-not tested). Taxonomic level: p-phylum, c-class, o-order, f-family, g-genus.

	Taxonomic level	% mean relative abundance in control		stand diff.
		beech	spruce	
Ascomycota	p	31.3	38.0	n.t.
Dothideomycetes	c			
Venturiales	o	2.4	2.1	n.s.
Eurotiomycetes	c			
Capnodiales	o	0.3	0.7	**
Chaetothyriales	o	2.6	4.1	n.s.
Eurotiales	o	0.5	1.1	*
Leotiomycetes	c			
Helotiales	o	18.0	25.0	n.s.
Hyaloscyphaceae	f	3.5	7.2	**
Helotiaceae	f	2	7.9	***
Thelebolales	o	0.4	<0.1	***
Pezizomycetes	c			
Byssonectria	g	2.7	1.3	n.s.
Basidiomycota	p	35.3	39.6	n.t.
Microbotryomycetes	c			
Leucosporidiales	o	0.6	0.2	**
Agaricomycetes	c			
Amanita	g	6	0.5	*
Cortinarius	g	0.4	0.7	n.s.
Hygrophorus	g	0.2	1.7	*
Inocybe	g	0.5	<0.1	**
Atheliaceae	f	9.7	13.8	n.s.
Tylospora	g	1.1	10.2	**
Piloderma	g	6.2	0.4	n.s.
Boletales	o	7.8	7.8	n.s.
Sistotrema	g	0.6	0.2	n.s.
Phallus	g	0.6	<0.1	n.s.
Russula	g	5.1	<0.1	*
Tremellomycetes	c			
Cystofilobasidiales	o	0.8	0.5	n.s.
Solicoccozyma	g	2.8	2.8	n.s.
Mortierellomycota	p	30.5	18.2	n.t.
Mortierellales	o	30.5	18.2	*
Other lineages		0.2	0.2	n.t.
Unassigned fungal sequences		2.7	4	n.t.

Rhizobiales and Rickettsiales (all Alphaproteobacteria) along with Gammaproteobacteria decreased in relative abundance as well (Tab.2). Positive reactions to acid additions were less numerous. Actinobacteria were the only phylum consistently increasing its relative abundance across all acid treated soils, and, it was especially pronounced in case of genus *Acidothermus* (Frankiales; Tab.2). Moreover, in the beech stand, Elsterales increased in both acidified treatments while Acetobacterales increased in both stands, but under S+N treatment only (Tab.2).

In contrast to a fast and complex bacterial reaction, the response of fungal community composition to treatment additions was far weaker. The first evidence of systematic treatment effect appeared in 2017 in the spruce stand, when fungal communities in acid treatments differed from control (Tab.S6 & S7) due to relative abundance increase of *Cladophialophora*, *Inocybe* and Capnodiales (in S+N only) and declining Filobasidiales and *Cortinarius* (Tab.S8). The response of fungal functional groups was limited only to a decrease of yeasts in S treatment in the spruce stand (Tab.S5).

Relations of microbial characteristics along generated soil pH gradient

The sulphuric acid addition created a robust continuous pH gradient, on which plots in beech and spruce stands overlapped. The soil solution pH gradient ranged from ~2.9 in acidified plots in the spruce stand to ~5.0 in control and N treated plots in the beech stand (Fig.S1). None of chemical soil and soil water 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.S2A,B,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.S2D,E) and to total hydrolytic enzyme activity ($R=0.32$, $p=0.01$; Fig.2A).

Bacterial community composition consistently shifted along whole pH gradient. This was shown by a passive projection of pH values to NMDS

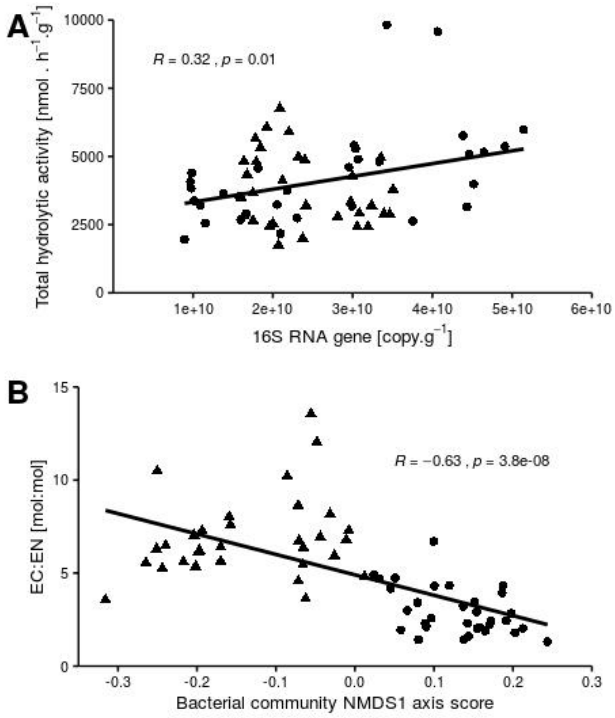

Figure 2: The relationship between (A) bacterial marker (16S rRNA) gene abundance and total hydrolytic activity and (B) bacterial community composition (NMDS1 axis score) and ratio of C-to-N mining enzymes among both stands (beech – circles, spruce – triangles). Correlation coefficients and corresponding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

ordination diagram (Fig.1A) and confirmed by correlation with the first NMDS axis (NMDS1) score (R=0.73, p<0.001; Fig.S2F). Bacterial NMDS1 score correlated with microbial biomass C:N ratio (R=-0.47, p<0.001; Fig.S2G) and N acquiring enzymes activity (R=0.57, p=<0.001; Fig.S2H). In result, shifts in bacterial community composition were also 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, Fig.S2I).

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 stand, respectively, both p<0.001; Fig.S3A & S4A), 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.S3B & C). In the spruce stand, bacterial community composition and abundance were disconnected from enzymatic activity. The hydrolytic activity was negatively correlated with soil solution DOC concentration (R=-0.53, p=0.003 for total hydrolase activity; Fig.S4B; R=-0.53, p=0.003 for EC hydrolyses, R=-0.55, p=0.002 for EN hydrolases and R=-0.38, p=0.034 for PME).

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

Discussion

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 soil microbial community in two forest stands common in Central Europe. Sulphuric acid-induced soil acidification caused rapid distinctive changes in soil microbial community composition at both sites. Bacterial but not fungal part of the community was impacted by increased acidity. Regardless 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. These systematic shifts of bacterial community were connected with alteration of soil exoenzymatic stoichiometry that, however, was not uniform and depended on the stand type. Differently, the N addition (as ammonium nitrate) didn't acidify the soil and an impact on soil microbes did not appear until the fourth year of the treatment. Then we detected the incipient shifts in the composition of bacterial community in the spruce stand and a decreased bacterial diversity in the beech stand. Given that the impacts of S and N addition on microbial communities

differed, but the effects were similar for both forest stands, below we discuss the S and N effects in detail separately.

The effect of acidification

By their nature, soils of both stands differed in soil pH before the start of experiment. The soil at spruce stand had soil pH of ~3.9, while pH of soils at beech stand was by 0.4 unit higher. Both acid treatments (S; S+N) further lowered soil pH at both sites, which was accompanied by a decrease in soil DOC concentrations (Oulehle et al., 2018). By this manipulation, we generated wider soil acidity gradient ranging from pH 2.9 to 5.0 (Fig. S1) along which bacterial community composition changed with common features in both forests. The acidity-induced shift in bacterial communities already occurred in the first year of the experiment, regardless of supplementary N addition (no difference between the response in S and S+N). The patterns of the shifts within bacterial community, leading to a decrease in bacterial OTU richness and Shannon diversity index were comparable in both forests, however, the magnitude was larger and changes started earlier in the spruce stand. This was likely connected to its lower original soil pH and thus to stronger treatment-induced physiological stress of bacteria.

After four years, the soils of both acid treatments were still dominated by Acidobacteria, which are common in acid forest soils (Baldrian et al., 2012; Nacke et al., 2016). Their relative abundance was not affected by acid treatment. However, within the group, Acidobacteriales known by low pH optima in range 3.8–6.0 (Campbell, 2014) partly replaced other Acidobacteria, mainly the Subgroup 2 and Solibacterales. 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., 2014, 2010; Thrash and Coates, 2015). We further observed 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). Large part of Actinobacterial reads belonged to *Acidothermus* sp.

tolerant to low pH (Barabote et al., 2009), which is probably the cause of their success in these acidified soils. Majority of other present bacterial lineages declined in acidified soils. These were e.g. Bacteroidetes, Gemmatimonadetes and Fibrobacteres, which were previously shown to depress in acid soils, but also Armatiomonadetes, Elusimicrobia, Verrucomicrobia or Planctomycetes, for which no obvious pH preference was observed since they prosper uniformly on wide pH ranges (Lauber et al., 2009; Rousk et al., 2010a; 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 probably induced stress also to these bacterial generalists. Alongside pH stress, bacteria with rather copiotrophic lifestyle such as Bacteroidetes and Gammatimonadetes (reviewed by Ho et al., 2017) were probably mutually disadvantaged by low C availability in acidified soils. On contrary, many acidotolerant Acidobacteria and Actinobacteria are capable to degrade polysaccharides (Barabote et al., 2009; Lladó et al., 2016; Větrovský et al., 2014), which probably contributed to their higher relative abundance in the acid-treated soils of both stands together.

In contrast to bacteria, the reaction of fungal community to acidification was weak. Modest compositional changes occurred in the spruce stand only. It is in accordance with generally observed lower susceptibility of fungi to direct pH stress compared to bacteria, because fungal communities are rather shaped by indirect pH effects through changed resource availability and competition with bacteria (Glassman et al., 2017; Rousk et al., 2010b, 2010a). In the spruce stand, yeasts (namely *Solicoccozyma terricola*) decreased under acid treatment. Yeasts usually do not produce enzymes involved in degradation of complex C compounds and rely on products of degradation by other soil microbes (Botha, 2011), thus their lower relative abundance in the acidified spruce soils likely stems from decreased C availability.

The composition of bacterial communities followed the treatment pH gradient created across both stands (Fig. S2F). The bacterial community composition was positively related to microbial biomass C:N, which was

higher in communities of more acid soils (Fig. S2G). The increase in microbial biomass C:N ratio is often attributed to increase in F:B ratio (Strickland and Rousk, 2010). However, F:B ratio did not change across the pH gradient in our case and we did not found any relationship of C_{mic}:N_{mic} either with F:B or fungal community composition. We suggest that the reason of higher C_{mic}:N_{mic} of bacterial communities in more acid soils is that low pH selected for oligotrophic bacterial taxa (Acidobacteria, *Acidothermus*) that are expected to have higher biomass C:N (Fierer et al., 2007). These bacteria have high C demand 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). In previous years, Oulehle et al. (2018) found higher EC:EN in the acid treatments than in untreated soils in both stands. They attributed it to organic matter stabilization and reduced C availability to microbes in acidified soils and hence relatively higher activity of EC compared to EN. Here we provide a direct link between the stoichiometric ratio of enzymatic activity and bacterial community composition. Moreover, total hydrolase activity positively correlated with bacterial biomass (16S rRNA gene abundance), while was uncoupled from C_{mic}, N_{mic} and fungal 18S rRNA gene abundance. It indicates that both the magnitude and stoichiometry of hydrolytic activity was mainly driven by bacteria. While the total hydrolase activity is related to their abundance, the "targeting of hydrolytic activity" is linked to bacterial community structure. However, taken the stands separately, this pattern was valid in the beech stand only. In the spruce stand, the hydrolytic activity was uncoupled from microbial community but was correlated with 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 a production of all hydrolytic enzymes regardless its taxonomic composition. We cannot exclude that the fungi contributed to changed enzymatic activity by modification of their action. However, given the modest changes in fungal community composition in treated soils and a lack of continuous relationship between fungal community composition and

enzymatic activity, we conclude that in our study rather shifts in bacterial community drove the alteration of enzymatic activity.

Effect of N addition

Four years of N addition have not yet been sufficient to considerably alter soil and soil solution chemistry and composition of bacterial and fungal communities in both forests. Particularly, sensitive response to N inputs might be expected mainly from ectomycorrhizal part of fungal community. A decrease in abundance accompanied by changes in taxonomic composition is well-documented reaction of ectomycorrhizal community to elevated N loading (Arnolds, 1991; Lilleskov et al., 2019, 2011; Wallenda and Kottke, 1998). However, described restructuring occurs mostly in N limited forests, where the species adapted to prosper under low N availability prevail the ectomycorrhizal community (Choma et al., 2017; Hasselquist and Högberg, 2014). When the N availability is enhanced by increased N input, these so called nitrophobic fungi retreat in reaction to reduced tree belowground C flow and are replaced by less C demanding nitrophilic ectomycorrhizal fungi (Högberg et al., 2010; Lilleskov et al., 2019, 2011; Olsson et al., 2005). In both stands, the ectomycorrhizal communities were already largely contributed by N tolerant taxa such as *Tylospora* sp., *Russula* sp. and *Hygrophorus* sp. (Lilleskov et al., 2011). These forests were in the 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 x 3 m (Göttlicher et al., 2008). Hence, the N treatment may be ineffective in changing the tree C allocation, which is a crucial event in N input effect on ectomycorrhizal fungi (Lilleskov et al., 2019).

N treatment itself didn't change the acid-base chemistry and thus did not decrease soil pH and DOC availability in contrast to the acidified

treatments. Together with high pre-treatment N availability, these are the most important reasons for no apparent shifts in bacterial and fungal communities. Nevertheless, the decrease in bacterial OTU richness and Shannon diversity in the beech stand and a modest change in bacterial community structure and a decrease in EC:EP and EN:EP in the spruce stand foreshadow a commencing microbial community reaction. It probably needs longer treatment duration before the ecosystems approach closer to N saturation and response of soil bacteria and fungi is launched out.

Conclusions

The reaction of bacterial community to simulated acidification was rapid and parallel in both stands, despite their originally different forest floor chemistry and microbial communities. Decrease in pH and DOC availability favored acidotolerant oligotrophs and lowered bacterial diversity. The shift in bacterial community was related to a change in hydrolytic activity, which highlighted non-negligible role of bacteria in determining soil enzymatic activity, at least in the first years of soil acidification when fungi remained irresponsive. In contrast, extra N addition to previously N loaded forest without an effect on soil pH was barely reflected by soil microbial communities.

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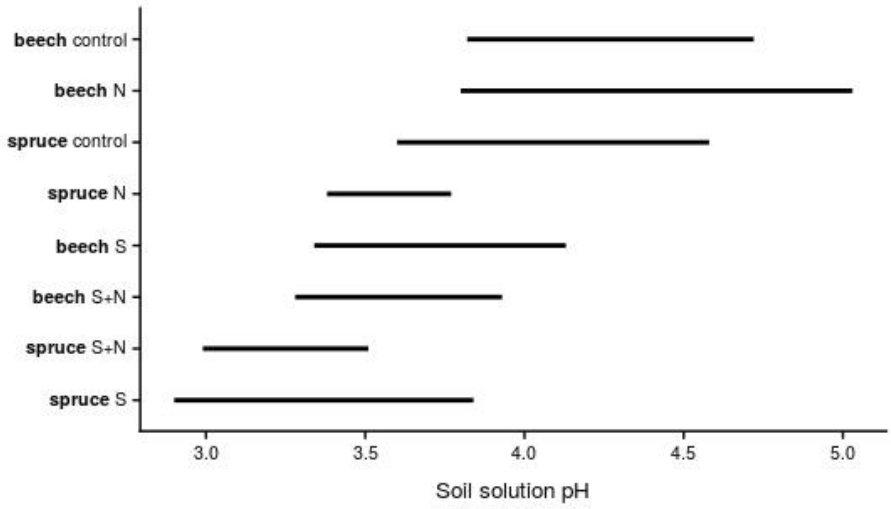

Figure S1: The range of soil solution pH in respective treatments in the beech and the spruce stand.

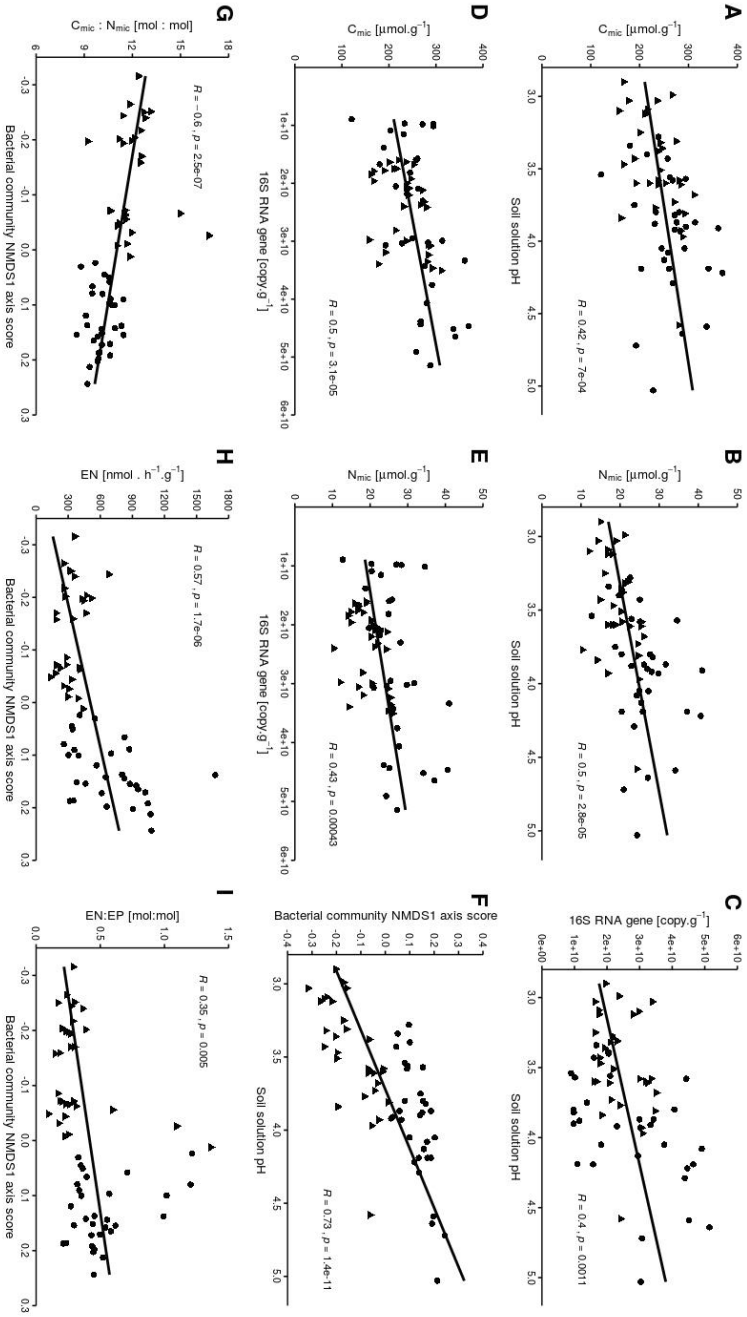

Figure S2: Soil solution pH, bacterial marker (16S rRNA) gene abundance and bacterial community composition (NMDS1 axis score), their mutual relationships and connection to microbial biomass and enzymatic activity among both stands (beech – circles, spruce – triangles). Correlation coefficents and corresponding p-values indicated

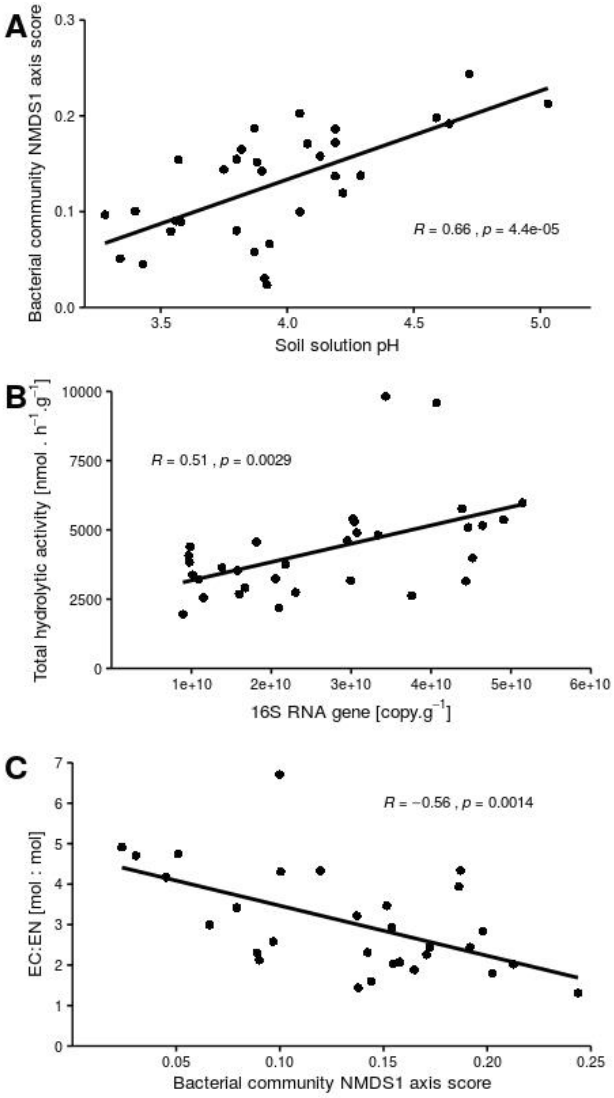

Figure S3: The relationship between (A) soil solution pH and bacterial community composition (NMDS1 axis score), (B) bacterial marker (16S rRNA) gene abundance and total hydrolytic activity and (C) bacterial community composition (NMDS1 axis score) and ratio of C-to-N mining enzymes in the beech stand only. Correlation coefficents and corresponding p-values indicated.

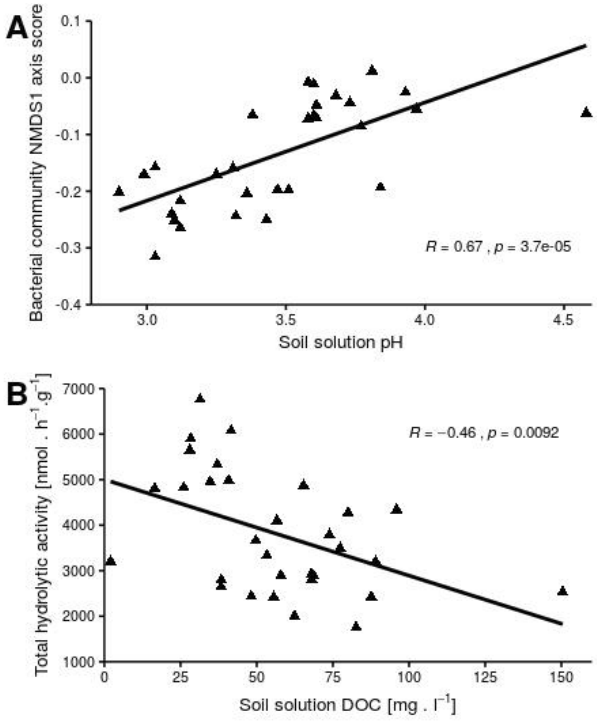

Figure S4: The relationship between (A) soil solution pH and bacterial community composition (NMDS1 axis score) and (B) soil solution DOC concentration and total hydrolytic activity in the spruce stand only. Correlation coefficents and corresponding p-values indicated.

Table S1: Stand effect (only control treatments compared) and the treatment effect on the soil and soil solution physico-chemical parameters, activity of hydrolytic enzymes, bacterial and fungal diversity measures and marker gene abundances.

	Stand difference (controls)		Treatment effect beech		Treatment effect spruce	
	F	p	c^2	p	c^2	p
Soil solution pH	10.7	0.007	81.9	<0.001	68.6	<0.001
Soil solution DOC	13.3	0.003	1.15	0.765	3.15	0.369
Soil solution DON	0.18	0.677	1.44	0.695	4.62	0.202
Soil solution DOC:DON	26.4	<0.001	2.08	0.556	11.8	0.008
Soil C_{tot}	6.62	0.024	23.9	<0.001	32.6	<0.001
Soil N_{tot}	4.18	0.064	17.3	<0.001	8.92	0.03
Soil C_{tot}:N_{tot}	2.37	0.149	23.0	<0.001	39.7	<0.001
Soil NH_4^+	1.51	0.242	1.19	0.755	4.16	0.245
Soil NO_3^-	36.0	<0.001	3.84	0.280	10.4	0.016
C_{mic}	0.15	0.705	4.54	0.209	19.1	<0.001
N_{mic}	7.93	0.016	2.40	0.494	8.89	0.031
C_{mic}:N_{mic}	10.3	0.007	1.42	0.700	0.69	0.877
BG	0.05	0.831	14.9	0.002	7.93	0.048
CEL	0.07	0.800	5.38	0.146	5.95	0.114
LEU	26.6	<0.001	22.8	<0.001	9.02	0.029
NAG	22.5	<0.001	5.40	0.144	8.62	0.035
PME	14.7	0.002	17.9	<0.001	12.4	0.006
Etot	11.1	0.006	12.6	0.006	11.1	0.011
EC	0.01	0.918	12.5	0.006	7.58	0.055
EN	24.4	<0.001	5.32	0.150	9.12	0.028
EC:EN	9.75	0.009	4.50	0.212	7.31	0.063
EC:EP	7.26	0.020	16.1	0.001	14.6	0.002
EN:EP	0.77	0.397	6.56	0.087	17.5	<0.001
Bac. Shannon	36.2	<0.001	36.5	<0.001	60.4	<0.001
Bac. OTU richness	92.0	<0.001	41.2	<0.001	132	<0.001
Fun. Shannon	3.88	0.074	3.63	0.304	7.47	0.058
Fun. OTU richness	6.11	0.031	3.41	0.333	4.45	0.271
Bac. 16S rRNA gene	3.85	0.074	6.97	0.072	9.27	0.026
Fun. 18S rRNA gene	0.26	0.617	2.30	0.513	3.04	0.386
F:B	0.02	0.904	10.4	0.016	3.42	0.332

Table S2: Stand effect on soil bacterial and fungal community composition - results of PERMANOVA. Only control treatment data were included in analysis.

		Df	SS	F	R^2	p
Bacteria	Stand	1	0.277	11.2	0.461	<0.001
	Season	1	0.027	1.08	0.044	0.295
	Residuals	12	0.296		0.494	
Fungi	Stand	1	0.604	4.80	0.282	<0.001
	Season	1	0.153	1.22	0.071	0.212
	Residuals	11	0.296		0.646	

Table S3: Results of permutation tests of db-RDA models based on forward-selected soil and soil solution variables that best explained differences in bacteria and fungal communities.

		Df	SS	F	R^2	p
Bacteria	C_{tot}:N_{tot}	1	0.889	28.88	0.262	<0.001
	pH	1	0.438	14.21	0.129	<0.001
	DOC	1	0.152	4.95	0.045	0
	C_{tot}	1	0.086	2.79	0.025	0.024
	NO_3^-	1	0.079	2.57	0.023	0.029
	Residual	57	1.755		0.516	
Fungi	C_{tot}:N_{tot}	1	1.668	12.73	0.164	<0.001
	DON	1	0.515	3.93	0.051	<0.001
	pH	1	0.246	1.88	0.024	0.03
	C_{tot}	1	0.245	1.87	0.024	0.030
	Residual	57	7.466		0.736	

Table S4: Relative abundance (mean±sd) of bacterial phyla and selected nested groups (treatment difference to control at p<0.05 denoted by asterisks) and stand (i.e. difference between control treatments) and treatment effects.

		% relative abundance						Stand diff. (controls)		Treatment effect			
		beech			spruce					beech		spruce	
Phylum	Lower nested taxon	ctrl	N	S	S+N	ctrl	N	S	S+N	F	p	e^2	p	e^2	p
Phyla with positive or no reaction to acidifing treatments															
Acidobacteria		51.2(4.7)	52.5(5.7)	51.8(4.5)	50.4(3.7)	52(3.9)	55(5.7)	50.4(4.7)	49.3(5.9)	0.579	0.461	1.23	0.746	11.2	0.011
	Acidobacteriales	24.6(2.1)	26.4(4.1)	27.7(1.2) *	28.9(2) *	33.3(3)	36.1(5.1)	37.8(3.2) *	37(5.3)	101	<0.001	15.3	0.002	8.1	0.044
	Subgroup2	14(2.5)	15(3.9)	14.5(3.3)	13.5(2.3)	8.2(1.5)	9.1(4.2)	3.9(1.6) *	4.5(1.3) *	99.9	<0.001	1.13	0.769	43.6	<0.001
	Solibacterales	12.2(1.1)	10.8(1.8)	9.2(1.6) *	7.7(1.6) *	10.2(0.8)	9.5(1.3)	8.6(2.4)	7.8(1.7) *	3.32	0.073	56.1	<0.001	12.9	0.005
Actinobacteria		9.2(1.4)	10.5(1.8)	13.5(3.9) *	14.1(2.4) *	17.4(2.8)	14.4(3.4)	26.1(5.2) *	24.7(3.5) *	71.3	<0.001	33.5	<0.001	87.9	<0.001
	Acidimicrobiia	0.7(0)	0.9(0.2)	1.2(0.4) *	1.4(0.3) *	1.5(0.2)	1.3(0.2)	2.6(0.4) *	2.6(0.4) *	50.2	<0.001	43.7	<0.001	136	<0.001
	Catenulisporales	<0.1	0.1(0)	0.1(0.1) *	0.1(0) *	<0.1	0.1(0)	0.1(0.1)	0.1(0)	0.066	0.799	24	<0.001	4.04	0.257
	Frankiales	5.4(1)	6.1(1.1)	8.1(2.4) *	7.9(1.5) *	11.4(2.4)	9.4(2.2)	17.2(4.1) *	16.2(2.5) *	73.3	<0.001	24.9	<0.001	78.2	<0.001
	IMCC26256	0.7(0)	0.9(0.2)	1.2(0.4) *	1.4(0.3) *	1.5(0.2)	1.3(0.3)	2.6(0.4) *	2.6(0.4) *	50.6	<0.001	47.3	<0.001	132	<0.001
	Solirubrobacterales	<0.1	<0.1	<0.1 *	<0.1 *	<0.1	<0.1	<0.1 *	<0.1	2.54	0.117	19.3	<0.001	26	<0.001
Chloroflexi		0.2(0.1)	0.2(0.1)	0.3(0.2)	0.3(0.1)	0.4(0.2)	0.4(0.1)	0.5(0.4)	1.1(0.8) *	4.4	0.057	8.62	0.035	15.9	0.001
Patescibacteria		1(0.2)	0.7(0.3)	1.3(0.5)	1.6(0.2) *	0.6(0.2)	0.7(0.2)	1.4(0.7) *	1.2(0.6)	18.0	0.001	47.3	<0.001	20.3	<0.001
Thaumarchaeota		<0.1	<0.1	0.2(0.2)	0.1(0.1)	<0.1	<0.1	<0.1	0.2(0.1) *	0.534	0.479	2.58	0.461	51.8	<0.001
WPS2		1.8(0.5)	1.4(0.5)	2.5(1.1) *	2.4(0.7) *	2(0.5)	2.7(0.6)	2.4(1.2)	3.2(1.4)	0.448	0.516	37.4	<0.001	7.24	0.065
Phyla with negative reaction to acidifying treatments															
Armatimonadetes		0.2(0)	0.2(0)	<0.1 *	<0.1 *	0.1(0)	0.1(0.1)	<0.1 *	<0.1 *	4.34	0.059	175	<0.001	130	<0.001
Bacteroidetes		3.2(0.7)	2.5(1.1)	1.7(0.5) *	1.6(0.4) *	2.8(0.6)	3.1(0.6)	0.9(0.4) *	1(0.4) *	3.45	0.088	42.2	<0.001	156	<0.001
Elusimicrobia		0.2(0.1)	0.2(0.1)	<0.1 *	<0.1 *	<0.1	<0.1	<0.1 *	<0.1 *	102	<0.001	60.3	<0.001	25.9	<0.001
Fibrobacteres		<0.1	<0.1	<0.1 *	<0.1 *	<0.1	<0.1	<0.1 *	<0.1 *	17.2	0.001	26.5	<0.001	23.5	<0.001
Gemmatimonadetes		1(0.2)	0.8(0.2)	0.5(0.1) *	0.4(0.1) *	0.4(0.1)	0.5(0.1)	0.2(0) *	0.2(0.1) *	61.3	<0.001	75.1	<0.001	172	<0.001
Verrucomicrobia		6.5(1.3)	4.9(1.7)	3.7(1.4) *	3.6(0.8) *	2.4(0.8)	2.2(0.5)	0.9(0.5) *	1.2(0.3) *	61.5	<0.001	28.8	<0.001	50	<0.001
Planctomycetes		3.3(0.5)	2.3(0.6) *	1.9(0.4) *	2(0.4) *	2.3(0.5)	1.4(0.4) *	1(0.3) *	1(0.3) *	14.8	0.002	43.9	<0.001	55.3	<0.001
Proteobacteria		21.8(1.7)	23.4(2)	22(2.8)	22.8(1.5)	19.2(2)	19.2(2.4)	15.9(1) *	16.5(2.2) *	11.0	0.006	3.99	0.263	34.5	<0.001
	Alphaproteobacteria	19.3(1.5)	21.3(2)	20.7(2.7)	21.8(1.5)	17.7(1.9)	18.3(2.4)	15.6(0.9)	16.1(2.2)	4.2	0.063	9.19	0.027	19.9	<0.001
	Acetobacterales	4.1(0.4)	4.1(0.8)	4.5(0.5)	5.3(0.6) *	4(0.9)	4(0.3)	4.8(0.7)	5.4(1.3) *	0.031	0.864	21.8	<0.001	20.9	<0.001
	Azospirillales	0.1(0)	0.2(0)	<0.1 *	<0.1 *	0.1(0)	0.1(0)	<0.1 *	<0.1 *	0.255	0.623	63.1	<0.001	125.6	<0.001
	Caulobacterales	1.7(0.4)	1.7(0.4)	1.5(0.4)	1.6(0.4)	1.1(0.2)	1.4(0.2)	0.5(0.2) *	0.6(0.2) *	9.21	0.010	2.82	0.421	84.7	<0.001
	Elsterales	2.6(0.3)	3.2(0.6)	4(0.9) *	3.9(0.8) *	2.5(0.8)	2.9(1.1)	3.2(1)	3.3(0.9)	0.313	0.586	29.6	<0.001	6.4	0.094
	Micropepsales	2.4(0.3)	2.6(0.3)	2.2(0.5)	2.1(0.3)	2.1(0.3)	2.4(0.2)	0.7(0.4) *	0.9(0.2) *	4.88	0.047	15.1	0.002	330	<0.001
	Reyranellales	0.1(0.1)	0.1(0)	0.1(0)	<0.1 *	<0.1	<0.1	<0.1 *	<0.1 *	17.3	0.001	109	<0.001	52.1	<0.001
	Rhizobiales	7(0.8)	8.1(1) *	7.5(0.8)	7.9(0.8)	6.9(1.2)	6.6(0.5)	5.9(0.7)	5.5(0.8) *	0.194	0.667	8.67	0.034	18.8	<0.001
	Rhodospirillales	0.7(0.1)	0.7(0.1)	0.4(0.2) *	0.4(0.1) *	0.4(0.2)	0.3(0.1)	0.1(0.1) *	<0.1 *	42.6	<0.001	48.7	<0.001	97.5	<0.001
	Rickettsiales	0.3(0)	0.3(0.1)	0.3(0.1)	0.3(0.1)	0.3(0.1)	0.3(0)	<0.1 *	<0.1 *	0.071	0.795	5.84	0.12	141	<0.001
	Sphingomonadales	0.1(0)	0.1(0)	<0.1	0.1(0.1)	0.1(0)	0.2(0.1)	0.2(0)	0.2(0.2)	2.4	0.164	3.88	0.275	4.39	0.222
Gammaproteobacteria		0.4(0.1)	0.5(0.1)	0.4(0)	0.4(0.1)	0.4(0.1)	0.4(0.1)	0.2(0.1) *	0.3(0.1) *	0.365	0.557	15.9	0.001	37.7	<0.001
	Betaproteobacteriales	<0.1	<0.1	<0.1	<0.1 *	<0.1	<0.1	<0.1	<0.1 *	0.579	0.462	13	0.005	11.3	0.01
	WD_260	0.1(0)	0.1(0)	0.1(0.1)	<0.1 *	0.1(0)	0.1(0)	<0.1 *	<0.1 *	0.272	0.611	32	<0.001	72.8	<0.001
Deltaproteobacteria		2.1(0.2)	1.7(0.2)	1(0.4) *	0.6(0.3) *	1(0.4)	0.5(0.2) *	0.1(0.1) *	0.1(0.1) *	47.7	<0.001	160	<0.001	150	<0.001
Bdellovibrionales		<0.1	<0.1	<0.1 *	<0.1 *	<0.1	<0.1 *	<0.1 *	<0.1 *	10.0	0.006	68.9	<0.001	107	<0.001
Myxococcales		1.9(0.1)	1.6(0.4)	0.9(0.1) *	0.6(0.2) *	1(0.4)	0.5(0.1) *	0.1(0.3) *	0.1(0) *	42.4	<0.001	152	<0.001	162	<0.001

Table S5: Relative abundance (mean±sd) of fungal lifestyle groups (treatment difference to control at p<0.05 denoted by asterisks) and stand (i.e. difference between control treatments

	% relative abundance								Stand diff. (controls)		Treatment effect			
	beech				spruce						beech		spruce	
	control	N	S	S+N	control	N	S	S+N	F	p	χ^2	p	χ^2	p
Moulds	31.4(9.5)	28.2(11.3)	29.7(9.4)	33.6(11.9)	20.3(6.6)	20.2(8.1)	24.3(13.4)	18.7(7.4)	6.1	0.031	n.t.		2.2	0.527
Saprotrophs	12.3(11.2)	1.9(0.7)	5.9(4.9)	7.8(7.5)	7.0(12.0)	2.9(3.2)	1.7(1.2)	1.6(1.)	1.0	0.34	n.t.		8.0	0.045
Yeasts	4.4(1.6)	2.9(1.5)	6.4(2.0)	5.9(3.1)	3.9(2.1)	2.1(0.8)	1.9(1.7) *	2.8(1.3)	0.1	0.758	n.t.		10.3	0.016
Ectomycorrhizal	28.6(11.6)	51.4(14.7)	37.3(14.8)	31.5(20.6)	25.5(6.7)	37.2(12.9)	27.6(13.7)	26.4(14.4)	0.2	0.641	n.t.		5.3	0.151
Ericoid mycorrhizal	0.7(0.4)	0.8(0.6)	1.0(0.7)	0.8(0.3)	5.9(2.9)	5.6(2.1)	7.5(3.1)	8.0(2.9)	40.7	<0.001	n.t.		6.9	0.075
Root associated	5.3(2.6)	4.4(1.9)	5.2(2)	7.9(3.4)	12.8(4.8)	8.6(3.9)	12.5(8.2)	12.3(4.4)	15.2	0.002	n.t.		6.5	0.089
Uncertain	10.8(12.7)	5.5(2.3)	7.9(3.5)	6.5(2.4)	17.0(8.6)	17.5(6.2)	18.0(8.5)	21.9(8.9)	1.8	0.206	n.t.		2.6	0.464
Lichenised	0	<0.1	0	<0.1	<0.1	<0.1	<0.1	0.1(0.2)	5.1	0.046	n.t.		18.1	<0.001
Not attempted	6.5(2.3)	4.9(1.9)	6.5(1.8)	6.0(1.5)	7.7(1.4)	5.8(1.9)	6.4(2.4)	8.2(1.0)		n.t.	n.t.			n.t.

Table S6: Treatment offect on microbial communities - results of PERMANOVA of bacterial and fungal commuinty composition in the beech and the spruce stand in respective years 2014 -2017.

			beech					spruce				
	Year		Df	SS	F	R^2	p	Df	SS	F	R^2	p
Bacteria	2014	Treatment	3	0.273	1.208	0.106	0.058	3	0.271	1.116	0.102	0.124
		Season	1	0.276	3.665	0.107	<0.001	1	0.206	2.538	0.077	<0.001
		Residuals	27	2.036		0.787		27	2.190		0.821	
	2015	Treatment	3	0.313	1.457	0.132	<0.001	3	0.499	2.509	0.196	<0.001
		Season	1	0.128	1.786	0.054	0.002	1	0.258	3.883	0.101	<0.001
		Residuals	27	1.931		0.814		27	1.791		0.703	
	2016	Treatment	3	0.372	2.018	0.195	<0.001	3	0.410	2.530	0.208	<0.001
		Season	1	0.248	4.047	0.130	<0.001	1	0.423	7.838	0.215	<0.001
		Residuals	21	1.289		0.675		21	1.134		0.576	
	2017	Treatment	3	0.246	3.007	0.239	<0.001	3	0.460	5.899	0.387	<0.001
		Season	1	0.048	1.747	0.046	0.033	1	0.054	2.058	0.045	0.056
		Residuals	27	0.736		0.715		26	0.677		0.568	
Fungi	2014	Treatment	3	0.701	1.557	0.194	0.007	3	0.565	1.027	0.139	0.384
		Season	1	0.365	2.434	0.101	0.002	1	0.388	2.118	0.095	0.003
		Residuals	17	2.550		0.705		17	3.117		0.766	
	2015	Treatment	3	0.547	1.083	0.122	0.124	3	0.502	1.012	0.112	0.294
		Season	1	0.580	3.446	0.129	<0.001	1	0.346	2.090	0.077	<0.001
		Residuals	20	3.367		0.749		22	3.639		0.811	
	2016	Treatment	3	0.427	1.052	0.105	0.328	3	0.400	1.058	0.109	0.364
		Season	1	0.816	6.029	0.200	<0.001	1	0.885	7.019	0.240	<0.001
		Residuals	21	2.842		0.696		19	2.394		0.651	
	2017	Treatment	3	0.474	1.225	0.117	0.001	3	0.506	1.491	0.137	<0.001
		Season	1	0.226	1.755	0.056	<0.001	1	0.241	2.128	0.065	<0.001
		Residuals	26	3.353		0.827		26	2.943		0.798	

Table S7: Microbail community pairwise treatment comparison - results of pairwise PERMANOVA of bacterial and fungal commuinty composition in the beech and the spruce stand in respective years 2014 -2017.

	Year	beech				spruce			
Bacteria			ctrl	N	S		ctrl	N	S
	2014	PERMANOVA not significant				PERMANOVA not significant			
	2015	N	0.692			N	0.488		
		S	0.043	0.097		S	0.003	0.001	
		S+N	0.016	0.043	0.692	S+N	0.001	<0.001	0.486
	2016	N	0.104			N	0.767		
		S	0.025	0.038		S	0.010	0.014	
		S+N	0.025	0.038	0.178	S+N	0.010	0.010	0.444
	2017	N	0.181			N	0.013		
		S	<0.001	<0.001		S	<0.001	<0.001	
		S+N	<0.001	<0.001	0.216	S+N	<0.001	<0.001	0.034
Fungi	2014	N	0.173			N			
		S	0.045	0.173		S	PERMANOVA not significant		
		S+N	0.173	0.516	0.270	S+N			
	2015	N	PERMANOVA not significant			N	PERMANOVA not significant		
		S				S			
		S+N				S+N			
	2016	N	PERMANOVA not significant			N	PERMANOVA not significant		
		S				S			
		S+N				S+N			
	2017	N	0.248			N	0.384		
		S	0.361	0.119		S	0.021	0.004	
		S+N	0.361	0.119	0.603	S+N	0.005	0.007	0.364

Table S8: Relative abundance (mean±sd) of selected fungal groups and teratment effect in the beech and spruce stand (treatment difference to control at p<0.05 denoted by asterisks). Taxonomic level: p-phylum, c-class, o-order, f-family, g-genus.

	Taxonomic level	% relative abundance							Treatment effect		treatment effect	
		beech				spruce			beech		spruce	
		control	N	S	S+N	control	N	S	S+N	χ^2	p	χ^2	p
Ascomycota	p												
Dothideomycetes	c												
Venturiales	o	2.4(3.2)	1.0(0.6)	1.1(0.7)	0.8(0.8)	2.1(3.1)	1.2(0.9)	0.8(0.4)	0.8(0.9)	not tested		4.3	0.23
Eurotiomycetes	c												
Capnodiales	o	0.3(0.2)	0.2(0.2)	0.4(0.2)	0.3(0.2)	0.7(0.2)	0.6(0.3)	1.3(0.8)	1.5(1.1) *	not tested		13.8	0.003
Chaetothyriales	o	2.6(1.5)	2.9(1.5)	3.1(1.3)	3.4(1.3)	4.1(2.3)	3.0(2.4)	4.3(1.6)	4.9(1.1)	not tested		11.6	0.009
Eurotiales	o	0.5(0.2)	0.4(0.3)	0.7(0.5)	0.4(0.1)	1.1(0.5)	0.7(0.6)	1.3(0.8)	0.9(0.9)	not tested		6.4	0.095
Leotiomycetes	c												
Helotiales	o	18.0(10.7)	10.1(3.9)	12.5(6.9)	12.8(3.9)	25.0(8.4)	21.7(10.1)	26.7(11.0)	31.5(10.5)	not tested		9.5	0.023
Hyaloscyphaceae	f	3.5(1.6)	2.3(0.8)	3.6(1.9)	3.4(1.6)	7.2(4.8)	3.6(2.6)	5.7(5.1)	5.2(1.2)	not tested		6.4	0.096
Helotiaceae	f	2.5(1.3)	3.3(1.9)	4.3(4.7)	3.2(1.0)	8.2(2.4)	8.3(5.0)	9.3(5.3)	11.3(5.8)	not tested		0.9	0.825
Thelebolales		0.4(0.2)	0.2(0.1)	0.5(0.3)	0.5(0.5)	<0.1	<0.1	0.2(0.1)	0.1(0.1)	not tested		5.6	0.133
Pezizomycetes	o												
Byssonectria	c	2.7(6.5)	0.4(0.3)	0.5(0.5)	2.7(6.6)	1.3(1.6)	1.7(3.3)	1.4(1.1)	0.9(0.9)	not tested		0.6	0.89
Basidiomycota	p												
Microbotryomycetes	c												
Leucosporidiales	o	0.6(0.4)	0.5(0.5)	0.5(0.2)	0.6(0.2)	0.2(0.1)	0.2(0.1)	0.2(0.2)	0.2(0.1)	not tested		0.8	0.846
Agaricomycetes	c												
Amanita	g	6.0(6.0)	16.1(8.0)	16.4(23.1)	14.0(17.0)	0.5(0.2)	0.8(0.8)	0.5(0.4)	1.7(2.8)	not tested		4.5	0.209
Cortinarius	g	0.4(0.2)	0.2(0.1)	0.6(0.4)	1.4(2.2)	0.7(0.7)	0.2(0.2) *	<0.1 *	0.1(0.1) *	not tested		22.8	<0.001
Hygrophorus	g	0.2(0.2)	0.1(0.1)	0.4(0.3)	0.5(0.3)	1.7(1.9)	7.9(9.4)	3.7(8.6)	3.8(6.0)	not tested		4.6	0.203
Inocybe	g	0.5(0.6)	8.8(14.4)	2.4(3.2)	6.5(16.9)	<0.1	<0.1	0.1(0.1) *	0.2(0.2) *	not tested		17.1	<0.001
Atheliaceae	f	9.7(9.1)	12.6(16.2)	10.4(14.3)	5.8(4.7)	13.8(7.6)	23.9(10.8)	12.3(11.9)	12.7(12.1)	not tested		8.0	0.046
Tylospora	g	1.1(0.7)	0.5(0.2)	0.9(0.7)	0.8(0.3)	10.2(7.4)	12.6(4.5)	8.7(12.2)	7.0(9.7)	not tested		6.1	0.109
Piloderma	g	6.2(8.9)	7.5(16.6)	0.6(0.6)	2.2(3.4)	0.4(0.2)	0.4(0.3)	0.7(0.5)	0.4(0.2)	not tested		3.5	0.319
Boletales	o	7.8(9.3)	14.4(22.2)	7.1(7.2)	3.8(2.5)	7.8(6.8)	6.8(5.5)	12.1(10.5)	8.6(3.9)	not tested		2.0	0.564
Sistotrema	g	0.6(1.5)	0.1(0.2)	0.4(0.4)	0.1(0.2)	0.2(0.3)	0.1(0.2)	0.2(0.3)	<0.1	not tested		2.2	0.543
Phallus	g	0.6(0.8)	0.2(0.1)	3.0(5.0)	2.6(4.7)	<0.1	0.1(0.1)	<0.1	<0.1	not tested		15.5	0.001
Russula	g	5.1(8.3)	0.6(1.2)	1.8(2.9)	1.1(2.2)	<0.1	<0.1	0.1(0.1)	0.1(0.1)	not tested		1.4	0.716
Tremellomycetes	c												
Cystofilobasidiales	o	0.8(0.5)	0.5(1)	1.1(1.5)	1.7(2.3)	0.5(0.6)	0.6(0.5)	0.6(0.9)	1(0.8)	not tested		3.2	0.363
Solicoccozyma	o	2.8(1.2)	1.8(1)	4.6(1.3)	3.5(2.2)	2.8(1.4)	1(0.3) *	1(0.8) *	1.4(1) *	not tested		21.3	<0.001
Mortierellomycota	p												
Mortierellales	o	30.5(9.6)	27.5(11.3)	28.5(9.0)	32.7(11.8)	18.2(6.9)	18.8(8)	21.5(12.9)	16(7.6)	not tested		2.1	0.548

Curriculum vitae

Curriculum vitae

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Education

2012–present day	Faculty of Science, University of South Bohemia - Ph.D. program in Ecosystem biology (supervisor doc. Mgr. Eva Kaštovská, Ph.D.)
2009–2012	Faculty of Science, University of South Bohemia - M.Sc in Applied ecology (supervisor prof. Ing. Hana Šantrůčková, CSc.)
2006–2009	Faculty of Science, University of South Bohemia - B.Sc in Environmental Management (supervisor doc. Mgr. Eva Kaštovská, Ph.D.)

Scientific focus

Ecology of soil microorganisms in disturbed forest ecosystems

Work and science experience

Participation at Czech Science Foundation projects:

GA20-19471S – *GeoMicLink: Microbial imprint in catchment scale nutrient retention* (team member)

GA20-19284S – *Phosphorus leaching from undeveloped alpine soils: Biotic or abiotic control?* (team member)

GA20-14704Y – *Integrating experimental and modelling approach to understand the effect of redox potential on microbial carbon use efficiency* (team member)

GA19-16605S – *An interdisciplinary study on element cycling in mountain catchment-lake systems regenerating from tree dieback* (team member)

GA18-19561S – *Decay resistance of* Sphagnum – *biochemical causes and consequences* (team member)

P504-17-15229S – *Phosphorus dynamics in unmanaged terrestrial ecosystems: Links with nitrogen and carbon cycling.* (team member)

P503-14-09231S – *An integrated study on effects of changing environmental chemistry and climate on biogeochemical processes in soils (weathering, mineralization, P and Al mobility) and lakes (nutrient availability, toxicity) and their biodiversity (soil and water invertebrates).*

P504/12/1218 – *The effect of natural dieback of mountain spruce forest on microclimate, chemistry, and biodiversity of terrestrial and aquatic ecosystems.*

Courses and certificates

Summer school NGS data analysis (2016), Practical course on high-throughput sequencing data analysis (2016), Stable Isotope Course, Freising, Germany: an introduction to uses in ecology and plant physiology (2014), Basic Real-Time PCR Training (2013), TOEFL ITP (2011)

Teaching

Biology of microorganisms (tutorial lecturer, since 2013)
Soil ecology (tutorial lecturer, since 2014)
Environmental DNA (lecturer, since 2019)
Ecology of soil organisms for Environmental Management (lecturer, since 2019)

Publications in peer-reviewed scientific journals

Tahovská, K., **Choma, M**., Kaštovská, E., Oulehle, F., Bárta, J., Moldan, F., 2020. Positive response of soil microbes to long-term nitrogen input in spruce forest: Results from Gårdsjön whole-catchment N-addition experiment, Soil Biology and Biochemistry. Elsevier Ltd. doi:10.1016/j.soilbio.2020.107732

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Choma M., Bárta J., Šantrůčková H., Urich T. 2016. Low abundance of Archaeorhizomycetes among fungi in soil metatranscriptomes. Scientific Reports 6: 38455.

Participation in international conferences

Choma M., Tahovská K., Kaštovská E., Bárta J., Oulehle F. Effect of simulated nitrogen and sulphur deposition on soil microbial community and enzymatic activity in mountain forests. 4th Thünen Symposium on Soil Metagenomics 2019, Braunschweig, Germany. (poster)

Choma M., Bárta J., Kaštovská, E., Valtera M., Šantrůčková H., Šamonil P.: Soil microbial community composition in natural *Picea abies* mountain forest with different time since the last disturbance. Biogeomon 2017. Litomyšl, Czech Republic. (poster)

Choma M., Bárta J., Čapek P., Kopáček J., Šantrůčková H., Kaštovská E., 2016. Effect of simulated warming on soil N and P cycling and microbial community composition and functioning in a mountain grassland ecosystem. 3rd Thünen Symposium on Soil Metagenomics 2016, Braunschweig, Germany (poster).

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Choma M., Čapek P., Gärdenäs A., Kaštovská E., Rappe-George M., Šantrůčková H.: Minor answer of soil microbial biomass and activity of a Norway spruce forest ecosystem to changes in soil chemical properties caused by nitrogen deposition. Biogeomon 2014. Bayreuth, Germany. (poster)

Other professional activities

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Other skills

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