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Impact of nitrogen deposition on carbon stocks in coniferous forest soils

Insights from experiments with low and high nitrogen
addition rates

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Cover: The digging of a soil test pit in one of the nitrogen addition experiments studied in this thesis.

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Abstract

Nutrient limitations constrain the capacity for northern coniferous forests to capture and store carbon (C). Nitrogen (N) deposition may enhance tree productivity in these forests, but it remains uncertain how the storage of C in soils is responding to changes in N availability, and how limitations by other elements, such as phosphorus (P), develop in response to N enrichment. In this thesis, I present results from five studies on C cycling in long-term N addition experiments in a Scots pine forest (study **I-III**) and a Norway spruce forest (study **IV-V**) in northern Sweden. In these forests, N has been added at low ($\leq 12.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) and high ($50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) rates for up to 20 years to simulate N deposition.

In the Scots pine forest, I showed that the highest N addition rate increased soil C stocks in the organic layer by $22.3 \text{ kg C kg}^{-1} \text{ N added}$ (study **I**), which supports the view that N enrichment can increase the C sink of northern coniferous forests. In study **I** and **II** I showed that N additions increased the input of C by both aboveground litter and fine-root litter, which coupled to a decrease in autotrophic soil respiration contributes to soil C accumulation. Study **II** and **III** further showed that the increase in fine-root production occurred without a concomitant increase in tree-root associated microbiota, indicating that N enabled higher retention of C in fine-root biomass production.

In the Norway spruce forest, study **IV** showed that N decreased the activity of enzymes in the soil involved in the degradation of recalcitrant organic matter, and organic N uptake, and increased the activity of enzymes involved in the degradation of simple carbohydrates, whereas enzyme activity targeting P was unaffected. Study **V** further showed that N had no effect on the mobilization of P in soil organic matter or on the fungal exploration of P rich apatite minerals in the soil. In both forests, N changed the composition of the microbial community, which is likely to impact soil respiration and decomposition. However, more studies are needed on the links between N availability and soil microbial activity. In conclusion, the result presented in this thesis support the view that N deposition is enhancing the C sink of N poor boreal forests, however at a rate that is too low to make a major contribution to the global land C sink.

Keywords: boreal forest, carbon sequestration, climate change, decomposition, ecological stoichiometry, ectomycorrhizal fungi, nitrogen deposition, *Picea abies*, *Pinus sylvestris*, soil respiration

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Effekten av kvävedeposition på fastläggning av kol i marken i barrskogar - insikter från experiment med låga och höga kvävegivor

Sammanfattning

Tillgängligheten av näringsämnen är begränsande för nordliga barrskogarnas förmåga att lagra kol. Kvävedeposition kan öka produktiviteten i dessa skogar men det är ännu oklart hur markens kolförråd påverkas av gradvisa förändringar i kvävetillgång, och hur begränsningar av andra näringsämnen, så som fosfor, påverkar denna respons. I denna avhandling presenterar jag resultat från fem studier av kolcykeln i långliggande experiment i tall- och granbestånd i norra Sverige, där kväve tillförts i låga och höga givor i upp till 20 år för att simulera olika nivåer av kvävedeposition.

I tallbeståndet visade studie **I** att kvävetillförseln ökade kolförrådet i markens organiska skikt med 22.3 kg kol kg^{-1} kväve tillfört, vilket ger stöd för att kvävedepositionen bidrar till att förstärka kolsänkan i nordliga barrskogar. Studie **I** och **II** visade vidare att denna effekt berodde på en ökad produktion av fall- och rotförna, tillsammans med minskad markandning. Studie **II** och **III** visade att ökningen i rotförnaproduktion skedde utan en motsvarande ökning i mikrober förknippade med trädens rötter, vilket tyder på att kvävetillförsel möjliggör en högre fasthållning av kolföreningar i rötterna.

I granbeståndet visade studie **IV** att kvävetillförseln minskade aktiviteten av enzymer som bryter ner komplext och svåredbrytbart organiskt material, och i upptaget av kväve i organiska former. Däremot ökade aktiviteten av enzymer involverade i nedbrytningen av enklare kolhydrater, medan den enzymatiska aktiviteten riktad mot fosfor var oförändrad. Studie **V** visade att varken frigörandet av fosfor i markens organiska material, eller marksvamparnas kolonisering av det fosforrika mineralet apatit påverkades av kvävetillförseln.

I båda bestånden förändrades sammansättningen av mikrobiella samhällen, vilket sannolikt bidragit till förändringarna i markandning och nedbrytning. Mer studier behövs dock för att förstå länkarna mellan kvävetillgång och markens mikrobiologiska aktivitet. Sammanfattningsvis visar resultaten i den här avhandlingen att kvävedepositionen bidrar till att förstärka kolsänkan i nordliga barrskogsekosystem, om än på en för låg nivå för att i sig ha avgörande inflytande på den globala landkolsänkan.

Nyckelord: boreal skog, ekologisk stökiometri, ektomykorrhiza, klimatförändringar, kolsänka, kvävedeposition, markandning, nedbrytning, *Picea abies*, *Pinus sylvestris*

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Populärvetenskaplig sammanfattning

En stor del av det globala kolet finns lagrat i marken i nordliga barrskogar. Hur effektiv skogen är på att ta upp och lagra kol i marken är därmed en viktig faktor för mängden koldioxid i atmosfären – och är därmed en viktig faktor i fråga om den globala uppvärmningen. Luftföroreningar har gjort att delar av dessa skogar under många år har fått ett betydande tillskott av kväve genom kvävenedfall. Detta kväve kan öka skogens tillväxt och kan bidra till ett större upptag av koldioxid, dock är det inte lika väl känt hur kvävet påverkar markens mikroorganismer och nedbrytningsprocesser. I denna avhandling har jag använt långliggande kväveförsök i ett område i norra Sverige med mycket lågt kvävenedfall, där kväve tillförts årligen i låga och höga givor i upp till 20 års tid, för att mäta in effekten av kväve på markens kolbalans och utveckla förståelsen för hur kvävet påverkar markkolet.

Resultaten visar att kvävenedfall ger en ökad mängd kol i marken, särskilt i humusskiktet. Detta sker genom en ökad produktion av trädbiomassa vilket leder till att mer kol kommer in i marken via både rötter och barr, och genom att omsättningen av kol i marken minskar. Gasmätningar visade att kvävet minskade koldioxidutsläppen från marken, trots att mer kol kom in i marken, vilket tyder på en mer effektiv omsättning. Även nedbrytningen av barr och humus minskade. Samtidigt förändrades också aktiviteten av enzymer som frigör specifika näringsämnen i marken, genom att enzymer som frigör kväve från markens organiska material, och enzymer som bryter ned vedämnen minskade, medan enzymer som frigör enkla kolföreningar ökade. Detta tyder på att markens mikroorganismer använder det mer lättillgängliga kvävet som tillförts utifrån istället för att bryta ner det organiska materialet i marken. Däremot påverkades inte fosforupptaget av kvävetillförseln.

Mikroorganismer spelar en central roll för markens kolbalans eftersom de bryter ner organiskt material och därmed både tillgängliggör näringsämnen för växterna och ger upphov till koldioxidutsläpp. Kvävet minskade den totala mängden mikroorganismer i marken, och förändrade artsammansättningen. Kvävet missgynnade vissa typer av bakterier och marksvampar, men vissa typer av bakterier och marksvampar gynnades istället av den ökade kvävetillgången.

Sammantaget förstärker resultaten i den här avhandlingen bilden av att kvävenedfall kan bidra till fastläggning av kol i marken, och att en ökad förnaproduktion tillsammans med minskad nedbrytning är viktiga bidragande orsaker. Den mängd kol som lagras in i marken genom kvävedepositionen är dock liten i förhållande till andra faktorer som påverkar skogsekosystemens kolbalans.

Dedication

Till mor och far.

The hardest thing of all to see is what is really there.

J. A. Baker

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List of publications

This thesis is based on the work contained in the following papers and manuscripts, referred to by Roman numerals in the text:

- I. Benjamin Forsmark*, Annika Nordin, Nadia Maaroufi, Tomas Lundmark, Michael J. Gundale. Low and high nitrogen deposition rates in northern coniferous forests have different impacts on aboveground litter production, soil respiration, and soil carbon stocks (*accepted in Ecosystems*).
- II. Benjamin Forsmark*, Annika Nordin, Nicholas Rosenstock, Håkan Wallander, Michael J. Gundale. High levels of nitrogen enrichment in boreal forests decrease soil respiration by increasing root growth and decreasing the relative transfer of carbon to ectomycorrhizal fungi (*manuscript*).
- III. Nadia Maaroufi*, Annika Nordin, Kristin Palmqvist, Niles J. Hasselquist, Benjamin Forsmark, Nicholas P. Rosenstock, Håkan Wallander, Michael J. Gundale, (2019). Anthropogenic nitrogen enrichment enhances soil carbon accumulation by impacting saprotrophs rather than ectomycorrhizal fungal activity (*Global Change Biology* 25 (9), pp 2900-2914)
- IV. Benjamin Forsmark*, Tinkara Bizjak, Nicholas Rosenstock, Håkan Wallander, Annika Nordin, Michael J. Gundale. Impact of low and high nitrogen addition on soil microbial communities and enzyme activities in a boreal forest soil (*manuscript*).
- V. Benjamin Forsmark*, Håkan Wallander, Annika Nordin, Michael J. Gundale, 2020, Does long-term nitrogen enrichment increase microbial phosphorus mobilization in soils? (*manuscript*).

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My contribution to the papers and manuscripts included in this thesis was:

- I. Idea and hypothesis 50 %, planning and performance of work 80 %, analysis and summary of results 100 %, writing of manuscript 90 %.
- II. Idea and hypothesis 75 %, planning and performance of work 90 %, analysis and summary of results 90 %, writing of manuscript 90 %.
- III. Idea and hypothesis 20 %, planning and performance of work 30 %, analysis and summary of results 25 %, writing of manuscript 5 %.
- IV. Idea and hypothesis 50 %, planning and performance of work 80 %, analysis and summary of results 80 %, writing of manuscript 90 %.
- V. Idea and hypothesis 80 %, planning and performance of work 90 %, analysis and summary of results 90 %, writing of manuscript 90 %.

1 Introduction

1.1 Global change

The amount of reactive nitrogen (N) in the biosphere directly available for plant uptake has increased 3-5 fold during the past century (Reay et al., 2008). The productivity of ecosystems in cold areas, including the circumpolar boreal forest, are often limited by the low availability of N (Schlesinger, 2009, Tamm, 1991, Vitousek & Howarth, 1991), and the deposition of reactive N in these forests can potentially enhance their uptake and storage of carbon (C), and thereby counteract the rising atmospheric CO₂ concentration (Dentener et al., 2006, Le Quere et al., 2018). Most of the C in boreal forests are located in soils (Tarnocai, 2009), where microbes play key roles in decomposing plant litter and soil organic matter, thereby releasing nutrients in forms available for plants, and releasing C into the atmosphere (Crowther et al., 2019). While it is well established that N deposition in boreal forests can increase C inputs to soils, it is less understood how soil microbes respond to changes in N availability, and how changes in their activity influence storage of C in soils (Bradford et al., 2016, de Vries et al., 2014, Liang et al., 2017).

The concentration of atmospheric CO₂ has increased by more than 40 % since the beginning of the industrial revolution, from 278 ppm in 1750 to its current concentration at 409 ppm (December 2019) (Butler, 2019, IPCC, 2013). The combustion of fossil fuels, industrial processes, and land-use change, currently releases CO₂ equivalent to 10.7 Pg C yr⁻¹ into the atmosphere and thereby contributes to the net growth of the atmospheric C stock by 4.7 Pg C yr⁻¹, or 2 ppm yr⁻¹ (Le Quere *et al.*, 2018). The gross uptake of CO₂ in ecosystems on land is six to seven times larger than the amount humans release every year, and together with the oceans the total net sequestration of CO₂ in the biosphere is equivalent to approximately half of the human CO₂ emissions every year (IPCC,

2013, Le Quere *et al.*, 2018). A large part of the C in land ecosystems are stored in soils (Bradshaw & Warkentin, 2015, Pan *et al.*, 2011), where also a large part of the C taken up by the vegetation is decomposed and released back into the atmosphere (Falkowski *et al.*, 2000, Trivedi *et al.*, 2013). Because of the large size of the soil C stocks, even relatively small changes in their C can have a large impact on the atmospheric C balance, but it remains uncertain how various global change drivers, including changes in N availability, affect the soil C balance (Crowther *et al.*, 2019, Luo *et al.*, 2016).

Several studies have suggested that land ecosystems at the northern hemisphere constitute a large C sink (Ciais *et al.*, 2019, Myneni *et al.*, 2001, Pan *et al.*, 2011, Reay *et al.*, 2008) and that a combination of factors, including CO₂ fertilization, climate variations, land-use change, and increased N availability contributes to enhance the uptake and storage of C in the northern hemisphere, but there is considerable disagreement in the exact contribution of each of these factors (Winkler *et al.*, 2019, Zhu *et al.*, 2016). Nutrient availability frequently limits the capacity for land ecosystems to take up CO₂ (Goll *et al.*, 2017, Luo *et al.*, 2004, Terrer *et al.*, 2019), and thereby also limits the input of plant litter to soils. Moreover, soils are also the main reservoir for growth-limiting nutrients that may constrain the uptake and storage of C in land ecosystems (Fernandez-Martinez *et al.*, 2014, Luo *et al.*, 2004, Winkler *et al.*, 2019), and the uptake of nutrients, in turn, may have both positive and negative impacts on the storage of C in soil (Chen *et al.*, 2014b). To better understand the land C sink, it is therefore critical to both understand how changes in nutrient availability affect plant litter C inputs to soils, and how the decomposition processes that release nutrients for biomass production releases C back into the atmosphere.

1.2 The nitrogen and phosphorus cycles

Nitrogen (N) and phosphorus (P) are the two main elements that limit primary production (Harpole *et al.*, 2011). Nitrogen is the central building block of proteins and nucleic acids, whereas P is a central component of the phospholipids in cell membranes and the nucleotides that play a central role in cellular energy transfer and nucleic acids (Elser *et al.*, 2003). The reactive N that can be used in these molecules are made available from the large pool of inert dinitrogen (N₂) in the atmosphere by the highly energy-demanding process of N₂ fixation (Atkins & Jones, 2010), including processes such as lightning strikes and wildfires that generate oxidized forms of N that is deposited into ecosystems (Noxon, 1976, Xuexi *et al.*, 2002). In addition, N is also fixed within the ecosystem by microbes such as cyanobacteria, actinobacteria, and rhizobium (Davidson, 2008, Houlton *et al.*, 2008), often in symbiotic relationships with

vascular plants (Binkley *et al.*, 1992) or mosses (DeLuca *et al.*, 2002). Phosphorus, on the other hand, is primarily entering the ecosystem by weathering of minerals in the soil, such as apatite (Vitousek *et al.*, 2010). In low pH soils, P is often immobilized by occlusion with iron oxides, which can impair its rate of internal recycling.

The availability of these elements can become limiting if they are lost more rapidly than they are replenished by fixation and weathering, a situation that may occur by leaching and wildfire, or harvest of plant biomass (Akselsson *et al.*, 2008, Tamm, 1991). The internal uptake of these elements is typically large compared to the inputs (Högberg *et al.*, 2017, Knicker, 2004), and the internal recycling by litter decomposition and release may, therefore, be the rate-limiting step in the supply chain (Orwin *et al.*, 2011, Stevenson & Cole, 1999). As a consequence, the incorporation of these elements into inaccessible forms in plant biomass and soil organic matter, or immobilization by microbes in the soil, may also lead to limitations despite that the elements are abundantly present in the environment (Clarholm *et al.*, 2015, Kögel-Knabner & Amelung, 2014, Vitousek & Howarth, 1991). Young soils in cold ecosystems are typically rich in mineral P, whereas the fixation and deposition of N are relatively low, and in combination with a slow turnover of soil N pools contributes to the widespread N limitations of land ecosystems in the northern hemisphere (LeBauer & Treseder, 2008). However, as ecosystems accumulate organic matter over time, N stocks are frequently increasing, whereas P is redistributed from the mineral soil into organic pools, where it is prone to loss by e.g. leaching, which may lead to P limitations either by depletion of the primary source or in relation to the availability of N (Wardle *et al.*, 2004). The relative availability of these elements is, therefore, key to the uptake of C in forests, and the release of limitations from these elements, by e.g. N deposition, enables a higher C capture until other elements such as P, or other factors becomes limiting.

1.3 Changes in the global nutrient cycles

The access to cheap energy in fossil fuels is a major driver of global change (IPCC, 2013), and in addition to the direct emissions of CO₂, it has also had profound impacts on the global N cycle (Galloway *et al.*, 2008). The method to fix atmospheric N under high pressure that was developed by Fritz Haber and Carl Bosch is one of the most significant innovations in human history, as it enables us to circumvent the slow biotic N₂ fixation process (Erisman *et al.*, 2008, Galloway *et al.*, 2003, Steffen *et al.*, 2015). The Haber-Bosch method converts N₂ under high pressure and temperature to forms that are available to plants and can be applied as fertilizers to enhance crop-yields, and be used for

many industrial applications (Galloway *et al.*, 2013, Smil, 2001). In addition to the fixation of N₂ by the Haber-Bosch process, the use of fossil fuels in a wide range of combustion engines and power plants is also an important source of fixation of atmospheric N₂ and release of reactive N into the atmosphere and has together with management of biotic N₂ fixation contributed to a doubling of N₂ fixation at a global scale (Fowler *et al.*, 2013). In contrast to the availability of N that has increased in many ecosystems, and the higher concentration of C in the atmosphere, P availability has not increased to the same degree, and may become increasingly limiting for CO₂ uptake in future (Peñuelas *et al.*, 2013).

On a local scale, N compounds and associated particles contribute to air quality issues in densely populated areas, and the deposition of N, including nitrous and sulfuric acids, and other pollutants can decrease biodiversity (Bobbink *et al.*, 2010), and have other undesirable impacts on ecosystem functions (Aber *et al.*, 1989, Goulding *et al.*, 1998, Nihlgård, 1985). Moreover, reactive N released into the atmosphere have negative impacts on the ozone layer and are potent greenhouse gases (IPCC, 2013). To mitigate these effects, several countries in Europe and North America has agreed to decrease the emissions of N, which successively decreased reactive N emissions from its peak emissions during the 1980ies (Erisman *et al.*, 2008, Fowler *et al.*, 2013, Holland *et al.*, 2005). Nonetheless, N deposition is still high in some parts of the world, particularly in parts of Asia (Liu *et al.*, 2013). In areas where N has decreased during the latest decades, such in southern Sweden, N deposition is still high compared to in the north, farther from major pollution sources (Fig. 1) (Binkley & Högberg, 2016, Pihl-Karlsson *et al.*, 2017). Current upper-level N deposition rates in the boreal region add c. 12 kg N ha⁻¹ yr⁻¹, however, the majority of the boreal region receive considerably lower N deposition rates (Gundale *et al.*, 2011), which can be compared to the natural input of N by fixation and background deposition that is approximately 2-6 kg N ha⁻¹ yr⁻¹ in this region (Högberg *et al.*, 2017).

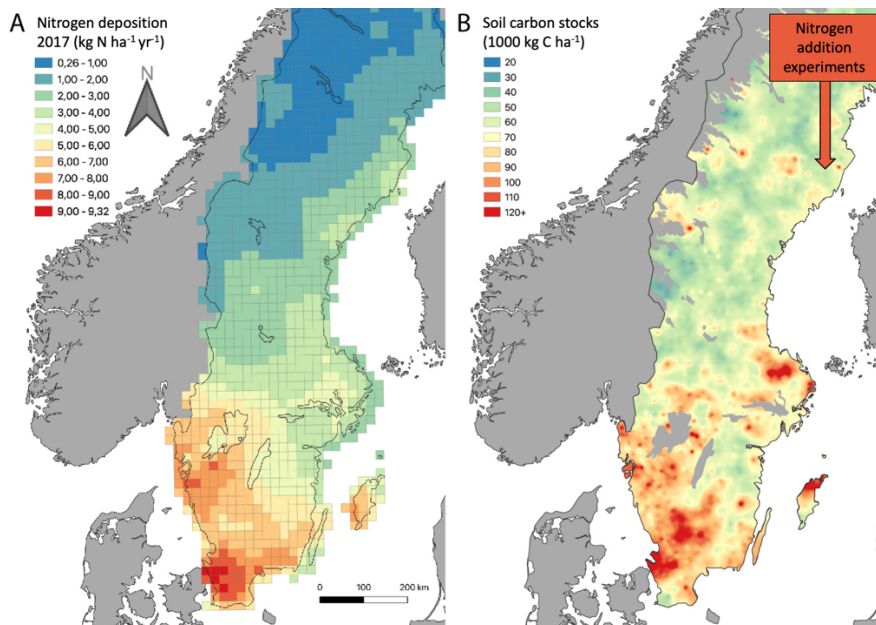


Figure 1: Current nitrogen deposition rates (A) and soil carbon stocks (B) in Sweden. Nitrogen deposition in panel A is the sum of NH_4^+ and NO_x , data from the Swedish Meteorological and Hydrological Institute. Soil carbon stocks in panel B includes the organic layer and mineral soil down to 50 cm at productive forest land, excluding peatlands and areas set aside from forestry, data from the Swedish Forest Soil Inventory. Note that soil C stocks are plotted on top of land-use types other than productive forest land. The arrow in panel B show the location of the N addition experiments used in this thesis. Maps produced by Carl Vigren.

1.4 Nitrogen deposition and the soil carbon balance

The storage of C in soils depend on several factors covarying across broad geographical scales, including precipitation and temperature, as well as with historical and current N deposition rates (Olsson *et al.*, 2009), which makes it difficult to disentangle the impact of N deposition as such (e.g. Fig. 1) (Sutton *et al.*, 2008). Studies across such gradients have shown that the tree productivity in areas with high N deposition is higher than expected from local site factors alone (Solberg *et al.*, 2009), and that the storage of C in soils at fertile sites, including sites in areas with high N deposition, are systematically underestimated by several soil C models (Tůpek *et al.*, 2016), which indicates that changes in N availability may play a key role in regulating the C sink.

Experiments in boreal forests adding high levels of N frequently report on increased aboveground growth (Nohrstedt, 2001), decreased decomposition and respiration, and increased soil C storage, which indicate that N deposition can potentially increase soil C storage (Berg, 2014, Hyvönen *et al.*, 2008, Janssens

et al., 2010). However, most of these experiments have added one order of magnitude more N than current upper-level N deposition rates (Hasselquist *et al.*, 2012), and may not be representative of N deposition for the majority of the northern coniferous forests (Gundale *et al.*, 2011). Indeed, some experiments adding N to initially N poor soils, or at rates more representative for N deposition, have reported on increased decomposition (Knorr *et al.*, 2005), and sometimes increased soil respiration (Bowden *et al.*, 2004, Hasselquist *et al.*, 2012, Janssens *et al.*, 2010), suggesting that different rates of N input may have different impact on soil C. However, these experiments are few, and key information on how low rates of N deposited into N poor forest soils affect the ecosystem C balance is missing.

Based on a large number of N addition experiment, de Vries *et al.* (2014) hypothesized that the efficiency of N deposition to enhance C sequestration in forest ecosystems depends on three critical thresholds, where different rates of N input will affect net primary production (NPP) and heterotrophic respiration differently. At the lowest rates of N input ($< 15 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), N is expected to be well retained in soils due to strong competition between tree roots and other sinks for N on the forest floor and in the soil that may limit tree access to N (de Vries *et al.*, 2014, Gundale *et al.*, 2011, Kaye & Hart, 1997, Melin *et al.*, 1983, Templer *et al.*, 2012, Tietema *et al.*, 1998), which may lead to very low C accumulation, or even negative impact on the C balance by stimulating heterotrophic respiration (Bowden *et al.*, 2004, Hasselquist *et al.*, 2012). At slightly higher N input rates ($20\text{-}50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), such competition will decrease and more N will be available for plant uptake, which is expected to increase NPP and decrease the supply of labile C to soil heterotrophs, and thus a maximum positive effect on the ecosystem C balance. At even higher N addition rates ($> 100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), N limitations on NPP is expected to decrease as other limitations come into effect, and leaching of N and cations contribute to acidification (de Vries *et al.*, 2014). Thus, to more accurately predict the impact of N deposition on the ecosystem C balance, it is critical to develop a more mechanistic understanding of how N affects key ecosystem processes changing the input and output of C to soils.

1.5 Nitrogen effects on soil carbon - specific mechanisms

1.5.1 Changes in carbon use and allocation

Carbon is taken up in the canopy and transported through a network of transport highways to sinks above- and belowground. Along the way, C is lost from the

system by respiration, and the fraction that becomes incorporated in plant biomass forms the C and energy base of the ecosystem. The ecosystem C sink is determined by the difference between C taken up, and the total amount of C respired. Carbon enters soils by above- and belowground litter, mainly by needle and fine-root biomass, the most dynamic components of the plant biomass. Forest trees allocate 25-63 % of their photo-assimilates belowground (Litton *et al.*, 2007). This C is used to produce and maintain roots, part of which eventually ends up in root litter. However, a large and potentially dynamic flux of C is exuded into the root-zone (Canarini *et al.*, 2019), and transferred to root-associated mycorrhizal fungi (Simard *et al.*, 2002), which then contributes to enhance nutrient uptake (Orwin *et al.*, 2011). This C is a central source of energy and C for microbes in the root-zone, which contributes to litter inputs by microbial biomass turnover (Clemmensen *et al.*, 2013, Godbold *et al.*, 2006, Vicca *et al.*, 2012), but is also a driver of decomposition processes (Craine *et al.*, 2007, Kuzyakov, 2010). From a plant allocation perspective, the supply of C to these processes competes with biomass production in other parts of the plant, and can, therefore, be expected to be down-regulated as nutrients become more available (Bloom *et al.*, 1985, Hermans *et al.*, 2006).

The perhaps most obvious way N deposition could increase soil C storage is by increasing plant production and thereby litter inputs to soils. Increased aboveground litter production is expected from the higher aboveground growth often observed after N additions (Nohrstedt, 2001). Biomass production belowground is more difficult to assess, yet litter produced in the root-zone is a potentially important source of soil C, as it is more likely to become stabilized in the soil (Schmidt *et al.*, 2011), and thereby also likely to remain longer than aboveground litter inputs (Clemmensen *et al.*, 2013, Rasse *et al.*, 2005, vandenEnden *et al.*, 2018).

1.5.2 Changes in microbial community composition and function

Soil microbes are the main agents transforming organic matter in soils (Paul, 2015), and different groups of soil microbes may respond differently to changes in N availability, and in turn affect soil C in different ways (Crowther *et al.*, 2019). Nitrogen concentrations in coniferous plant litter and soils are typically low compared to the N concentrations in microbial biomass (Cleveland & Liptzin, 2007, McGroddy *et al.*, 2004, Wallander *et al.*, 2003), and a higher N concentration in plant litter is therefore likely to stimulate microbial activity (Fierer *et al.*, 2009, Högberg *et al.*, 2003). Nitrogen can, on the other hand, also influence soil microbes indirectly, for example by changing plant C allocation patterns, determining the availability of C in the root-zone, as outlined above.

Different groups of soil microbes are likely to respond differently to these changes, and depending on functional traits of these microbes affect soil C in different ways (Koide *et al.*, 2014, Lilleskov *et al.*, 2019).

Ectomycorrhizal fungi (EMF) colonize the root-tips of most common boreal forest tree species such as Scots pine and Norway spruce, where they receive C directly from photosynthesis. Some of this C is used for the production of biomass including recalcitrant cell wall components, generating litter presumably with long turnover time in soils (Clemmensen *et al.*, 2013, Fernandez & Koide, 2014). In boreal forests, EMF may constitute a large part of the total biomass production (Ekblad *et al.*, 2013), yet considerable uncertainty remains regarding the regulation of C supply to roots at different rates of N deposition, where N could decrease belowground litter inputs by decreasing C allocation belowground, or by decreasing C allocation to root microbes such as EMF (Liang *et al.*, 2017, Vicca *et al.*, 2012).

1.5.3 Changes in soil respiration

Respiration is the main source of CO₂ emission from forest soils where carbohydrates are used as an energy source in roots and microbial biomass (Berg *et al.*, 2012). Depending on its main source of C, the emissions of C by respiration can be ascribed to either a heterotrophic food web, respiring C supplied by plant litter, or an autotrophic food web respiring C supplied by photosynthates in the root-zone including both the tree root and the organisms that depend directly on that C (Högberg *et al.*, 2001, Moore & William Hunt, 1988). Depending on their inherent differences in C source, these two components of respiration can be expected to respond in different ways to N additions. The response of these components can provide valuable information about soil C dynamics (Olsson *et al.*, 2005, Pumpanen *et al.*, 2015). If N increases the total plant production, it would provide more C for the heterotrophic community to respire, by increasing both above- and below-ground litter C production (Chen *et al.*, 2014a), whereas a decreased allocation of C to the root system in general, or to root-associated microbes with a high respiration rate, would decrease the autotrophic component (Janssens *et al.*, 2010). Similarly, a larger soil C stock and a larger microbial biomass C pool would also lead to higher soil respiration. On the other hand, N could also enable both autotrophic and heterotrophic organisms to incorporate more C into biomass, and thereby reduce the C used for respiration (Manzoni *et al.*, 2012, Pregitzer *et al.*, 2008, Schimel & Weintraub, 2003).

1.5.4 Changes in decomposition

Decomposition is a broad term that refers to the changes in the composition of plant litter during the humification process and is central for the release of C and nutrients (Berg & McClaugherty, 2014). Carbon is rapidly lost from plant litter during early stages of decomposition by the action of hydrolytic enzymes targeting carbohydrates such as cellulose that releases C for assimilation, C that can be used to build biomass, produce new enzymes, or be respired to generate energy (Allison *et al.*, 2011, Moorhead & Sinsabaugh, 2006). As decomposition progresses, an increasing fraction of the remaining C occurs in recalcitrant forms such as lignin, and depolymerization of these forms becomes increasingly limiting for further decomposition (Schimel & Weintraub, 2003) and is frequently the rate-limiting step for late stages of decomposition (Berg, 2014, Melillo *et al.*, 1982). In addition to such selective preservation (Knicker, 2004) of recalcitrant cell wall components from plant biomass and other sources, the synthesis of microbial products deposited into the substrate during decomposition also contributes to the formation of a stable residue that may be resistant to further degradation (Liang *et al.*, 2017). During the transition to late stages of decomposition, the microbial communities shift towards types with abilities to degrade these recalcitrant forms of C, using oxidative enzymes that breaks down the matrix into smaller pieces, such as Laccases, or mineralize organic matter completely using e.g. Manganese peroxidase (Carreiro *et al.*, 2000, Sinsabaugh & Shah, 2011). The activities of these enzymes may, therefore, play a key role in controlling soil C storage (Stendahl *et al.*, 2017).

In parallel with the loss and transformations of C during the early stages of decomposition, the nutrient concentrations of the litter typically increase due to the colonization of microbes with high nutrient concentrations, and the rapid loss of labile C from the litter (Berg & McClaugherty, 2014). These nutrients are tied up in various molecular structures that are both synthesized during the decomposition process, to form recalcitrant molecular structures with proteins and phenols (Knicker, 2004), or forming loosely held together supramolecular aggregates where nutrients are inaccessible due to physical barriers preventing access by enzymes (Clarholm *et al.*, 2015, Piccolo, 2001, Schmidt *et al.*, 2011). Nutrients are taken up from this material by hydrolytic enzymes targeting specific bonds, proteins are released by peptidases, and aminosugars in microbial necromass are released by enzymes such as chitinases, and P is released from organic matter by phosphatases (Sinsabaugh *et al.*, 2008). However, to access these resources, barriers in soil organic matter may need to be degraded by the action of oxidative enzymes, and nutrient acquisition thus lead to loss of soil C (Sinsabaugh & Shah, 2011, Talbot *et al.*, 2008).

2 Objectives

The main aim of this thesis was to clarify how different rates of N addition affect soil C storage in strongly N limited coniferous forests and to evaluate how changes in C inputs by aboveground litter and roots, and changes in microbial community composition and function contribute to these effects (Fig. 2). I have used two long-term N addition experiments (Fig. 3) and a combination of measurements to address the impact of N on the input and output of C to the soil.

My overarching hypothesis was that a reduction in tree C allocation to roots in response to N enrichment would enable a higher production of aboveground biomass, and thus increase aboveground litter C inputs, while simultaneously decreasing the belowground supply of C to roots and associated soil organisms that would decrease the decomposition of soil organic matter.

The specific objective of each study was to:

- I. Quantify the rate of soil C accumulation, aboveground litter C input, and C outputs by soil respiration per unit of N added.
- II. Quantify the effect of N on needle, fine-root, and EMF biomass production, and the respiration of tree roots and their associated microbiota.
- III. Clarify the role of saprotrophic versus tree-root associated microbiota on decomposition at different N addition rates.
- IV. Identify shifts in the activities of soil enzymes and shifts in functional groups and species of soil microbes in response to N addition.
- V. Evaluate the impact of long-term N enrichment on soil P mobilization and its impact on soil C stocks.

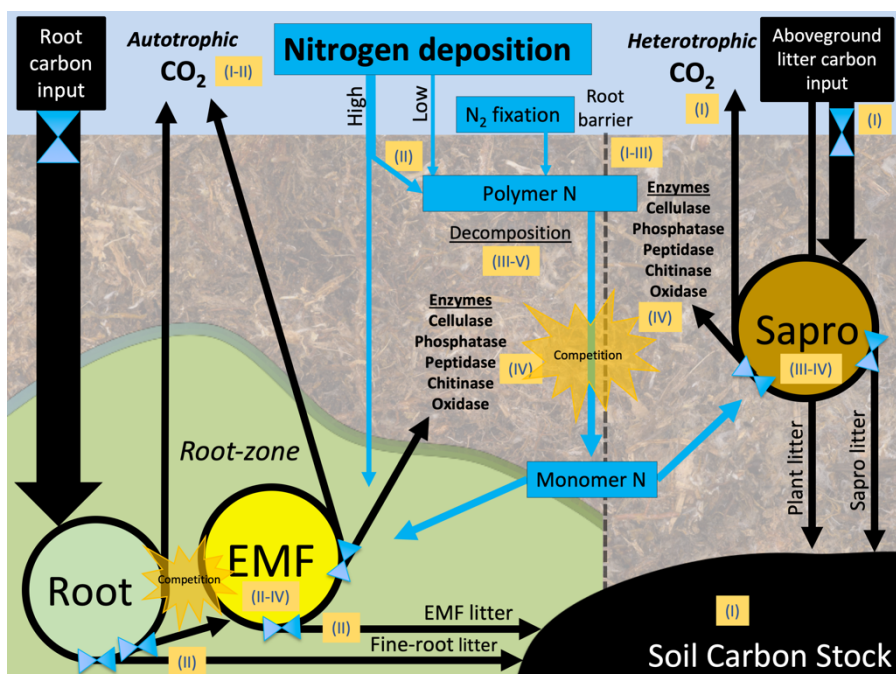


Figure 2. Overview of carbon (black) and nitrogen (blue) pools and fluxes in the top soil of boreal forest and the two main functional groups of soil decomposers studied in this thesis, ectomycorrhizal fungi (EMF) and saprotrophic microbes (sapro). Carbon enters the soil by aboveground litter (upper right), which feeds into a saprotrophic food-web, or via roots (upper left), which feeds into a community of root associate microbes dominated by EMF. Nitrogen enters the soil via N₂ fixation, above ground litter, and nitrogen deposition, and can be incorporated in soil organic matter, and subsequently released by decomposition, or leached further down into the soil e.g. to the main rooting zone of the trees. Blue valves on top of carbon flux illustrate hypothetical regulation points where the availability of nitrogen is likely to control the flux of carbon to subsequent processes. Roman numerals in yellow boxes refer to the papers and manuscripts in this thesis where the impact of nitrogen was studied. The dashed line indicates the trenching treatment used in study I-III to study the interactions between tree-roots and saprotrophs.

3 Major methods

3.1 Nitrogen addition experiments

All studies in this thesis are based on two long-term N addition experiments in the Svartberget experimental forest in Vindeln in north Sweden. The experiments are designed to simulate different rates of N deposition, and is located in an area with low background N deposition ($<2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) (Fig. 1), in the middle boreal zone ($64^{\circ} 13' \text{N}$, $19^{\circ} 48' \text{E}$, 200 above sea level) with a mean annual temperature of $+1^{\circ}\text{C}$ and mean annual precipitation of 600 mm. The two experiments are located within 3 km along a hill-slope, with Scots pine (*Pinus sylvestris* L.) dominated forest on an alluvial sand-silt plain (“Åheden” (Persson, 2000)) established after a forest fire approximately 140 years ago, and an approximately 120 year old Norway spruce (*Picea abies* Karst.) forest on glacial till uphill (“Svartberget”) (From *et al.*, 2016). Scots pine and Norway spruce are the dominant tree species in Eurasian boreal forests and together constitute nearly 80 % of the forest tree volume in Sweden, with nearly equal contribution of each species (Fransson, 2017).

Both experiments were established in randomized complete block designs with a combination of control, low and high N applied in the form of solid ammonium-nitrate applied manually every year after snowmelt, which usually occurs by the end of May. Both experiments include control treatments that only receive the ambient N deposition, and a high N treatment of $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ is added, that represents an extremely high N deposition rate, and serves as a reference point to other N addition experiments (Hyvönen *et al.*, 2008), as well as lower N addition rates ($< 12.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) similar to current N deposition rates in the boreal region (Fig. 1, Table 1).

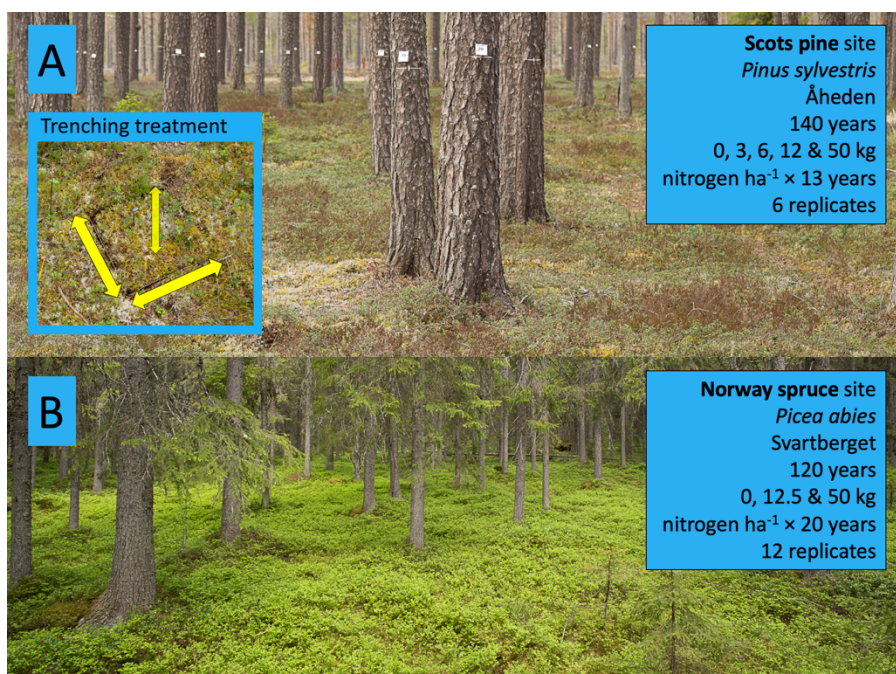


Figure 3: Photographs of the Scots pine (A) and Norway spruce (B) forests where the two nitrogen addition experiments used in this thesis are located. A trenching treatment (insert photograph) consisting of two 50×50×50 cm steel frames was installed in each plot at the Scots pine site in the 11th year of the experiment to study the role of tree roots on soil processes. Photo by the author.

Table 1: Overview of soil properties of the organic layer in the Scots pine and Norway spruce experiment. Values are averages ±standard error (n = 6 at Scots pine site and n = 12 at Norway spruce site).

	Nitrogen (N) treatment				
	Control	Low			High
Scots pine site (<i>Pinus sylvestris</i>)					
Annual N addition rate (kg N ha ⁻¹ yr ⁻¹)	0	3	6	12	50
Cumulative N 2017 (kg N ha ⁻¹)	0	39	78	156	650
C:N	44.0 (±1.27)	41.9 (±1.20)	41.7 (±1.84)	41.2 (±1.14)	35.6 (±1.02)
pH	3.3 (±0.06)	3.2 (±0.04)	3.3 (±0.03)	3.2 (±0.03)	3.1 (±0.04)
Norway spruce site (<i>Picea abies</i>)					
Annual N addition rate (kg N ha ⁻¹ yr ⁻¹)	0			12.5	50
Cumulative N 2016 (kg N ha ⁻¹)	0			250	1000
C:N	39.4 (±1.17)			38.0 (±1.43)	32.1 (±0.85)
pH	3.2 (±0.04)			3.2 (±0.06)	3.1 (±0.04)

Note: pH determined in CaCl₂

3.1.1 Scots pine experiment (2004) - study I-III

The experiment at the Scots pine site is located on well-drained sand sediment and dominated by Scots pine (*Pinus sylvestris* L.) (From *et al.*, 2016), with a forest floor with a sparse field layer of Ericaceous shrubs, including *Vaccinium vitis-idaea* and *Calluna vulgaris*, and *Cladina* and *Cladonia* spp. lichens, and *Pleurozium schreberi* (Brid.) Mitt, and *Dicranum* spp. mosses (Gundale *et al.* 2011). Nitrogen treatments at five levels were assigned to 0.1 ha plots in a randomized block design ($n = 6$) and initiated in 2004 with N additions at 0, 3, 6, 12 and 50 kg N ha⁻¹ yr⁻¹ (hereafter referred to as 0N, 3N, 6N, 12N, and 50N, respectively), where the low doses (3N, 6N, and 12N) simulate a gradient of N deposition from central to southern Fennoscandia (Fig. 1), whereas the high N treatment (50N) simulates upper N deposition rates in central Europe and also serves as a useful comparison with other N addition experiments. A trenching treatment was established in 2014 in a split-plot design to study the role of tree-roots on the response of soil processes to N addition (Hanson *et al.*, 2000, Maaroufi *et al.*, 2019). Each main plot was split into four equal-sized quadrat sub-plots, and two of the sub-plots were assigned to the trenching treatment, whereas the other two remained un-trenched. Each trench consisted of a 50 × 50 × 50 cm steel frame inserted 50 cm into the soil in July 2014 in the middle of each of the two sub-plots assigned to the trenching treatment.

3.1.2 Norway spruce experiment (1996) - study IV-V

The Norway spruce site is located on a podzolized glacial till and dominated by Norway spruce (*Picea abies* Karst.) (From *et al.*, 2016), with an understory dominated by *Vaccinium myrtillus* and less abundantly *V. vitis-idea*, with bottom layer vegetation dominated by *Dicranum* spp., *Hylocomium splendens*, *Pleurozium schreberi* and *Ptilium crista-castrensis* (Nordin *et al.*, 2006). The entire experiment consists of N treatments at two rates at plots ranging in size from 1 to 2500 m², with each N addition rate and plot size replicated 6 times. In this thesis, I have used plots at 1000 m² and 2500 m² to reach a total replication of 12 plots per N addition rate. In addition to the high N treatment, this experiment also includes a low N addition rate at 12.5 kg N ha⁻¹ yr⁻¹ (hereafter referred to as the 12.5N treatment) that is more representative for N deposition rates in the boreal region (SMHI, 2019).

3.2 Soil carbon and nitrogen stocks (study I)

Soil C and N stocks were estimated in the Scots pine forest in 2017. The organic layer and upper 20 cm of the mineral soil were sampled, which corresponds to the majority of the soil organic matter and the main rooting depth in this type of forest (Helmisaari *et al.*, 2007, Maaroufi *et al.*, 2015). The organic layer was sampled by collecting the forest floor down to the mineral soil surface with a 10 cm diameter PVC tube fitted with a serrated blade. Mineral soil samples were then collected from the exposed mineral soil surface using a sharp steel corer at 0-10 cm, and 10-20 cm depth. 10 cores from each depth were pooled to form one composite sample for each depth within each plot. Subsamples of the composite sample were analyzed for C and N concentrations by mass spectrometry (DeltaV IRMS coupled to a Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany). Organic layer C stocks were calculated as the C concentration multiplied with the dry mass of the sample and finally divided with the total area sampled. Mineral soil C stocks were calculated by multiplying the C concentration with the mass of the specific soil volume, based on site-specific bulk density estimates (Persson, 2000).

3.3 Above and below ground litter production (study I-II)

Above and below ground litter production was measured in the Scots pine experiment during 2016-2018. Aboveground litter was collected in five 0.36 m² litter traps per plot from May 2016 to May 2018 (Fig. 4A). All needles were separated from the total above ground litter (including all tree litter up to 20 mm thick) to estimate needle biomass production (Ukonmaanaho *et al.*, 2016). The production of fine-roots was measured in 5 cm wide and 20 cm long ingrowth cores constructed from 2 mm nylon mesh (Fig. 4B). 10 ingrowth cores were installed 20 cm into the mineral soil in each of the 30 plots in July 2016, and half of the ingrowth cores were incubated until October 2017 (15 months) and the other half incubated until October 2018 (27 months). The production of EMF biomass was measured in sand ingrowth cores with a 50 µm mesh (Wallander *et al.*, 2012) (Fig. 4C). EMF constitutes a large part of the microbial biomass in coniferous forest soils but there are no biomarkers that enable separation of EMF from other fungi. Acid-washed quartz sand has been used extensively as a substrate for ingrowth cores, where the absence of C in the ingrowth core select for mycorrhizal fungi that are directly supplied with C from photosynthesis (Ekblad *et al.*, 2013). In study II, tubular mesh bags 2 cm in diameter and 10 cm long were incubated vertically entirely into the mineral soil. In study V, the same type of ingrowth core was used at the Norway spruce site where half of the ingrowth cores amended with the phosphorus (P) containing mineral apatite to

measure fungal exploration of the mineral soil for P. In study III, triangular ingrowth cores were incubated in the organic layer outside and inside the trenches to measure the role of tree roots on ingrowth of fungi.

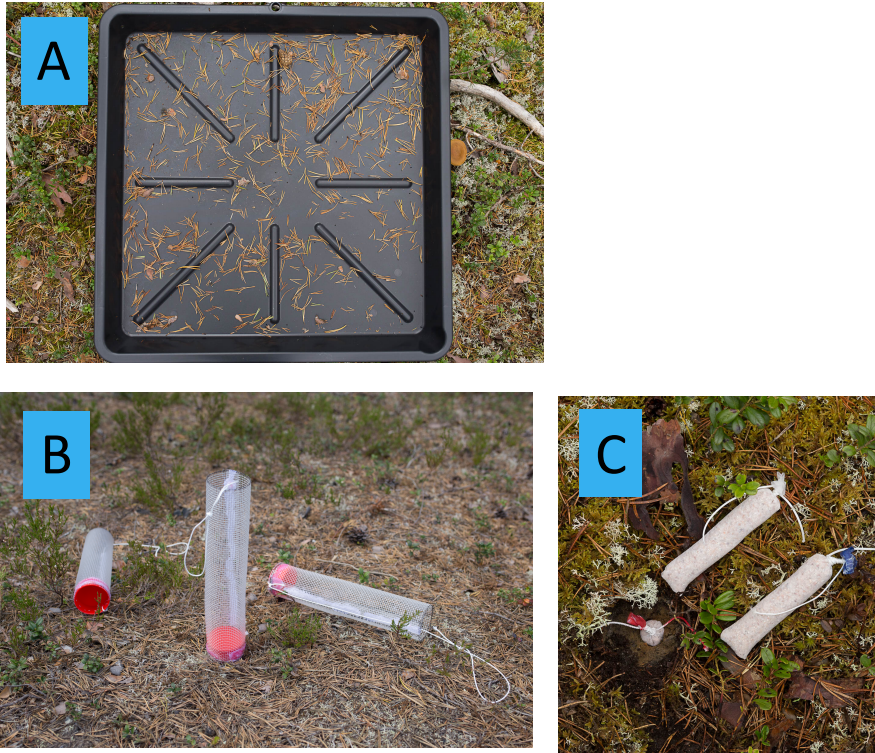


Figure 4: Methods used to measure above and below ground litter input to the soil at the Scots pine site. Aboveground litter was collected in 5 litter traps in each plot (A) and the production of fine-roots in 10 ingrowth cores in each plot, each with 2 mm mesh and filled with root-free soil (B), and the production of ectomycorrhizal fungal mycelium in sand-filled ingrowth with 50 μ m mesh (C).

3.4 Soil respiration (study I-II)

Emissions of C by soil respiration was measured in 2016-2017 in the Scots pine experiment, inside and outside trenches at permanent measurement locations. Each of the measurement locations consisted of a cylindrical 12.5 cm high and 25 cm wide (0.05 m², 5 liters) PVC collar installed six weeks before the first measurement, with five collars installed in each un-trenched sub-plot and two collars, one in each trenched sub-plot, and the collar kept free from photosynthesizing biomass by hand-weeding. This design enabled estimation of N effects on total soil respiration (outside steel frames) as well as on soil without tree roots, i.e. heterotrophic respiration only (inside steel frames), which further enabled estimation of the contribution of tree roots and their associated microbiota, by subtraction (outside – inside steel frames), which we hereafter refer to as autotrophic respiration. This definition of heterotrophic soil respiration excludes C input by tree root litter. The efflux of CO₂ at the soil surface was measured as the build-up of CO₂ in the headspace during 3 minutes after sealing the chambers. Measurements were made at a total of 11 occasions during the growing season of 2016 and 2017.

3.5 Decomposition (study III, V)

Decomposition was measured as the mass loss of C, N, and P in needles and humus in nylon bags with a mesh size of 50 µm during 1-year incubations in the soil. The size of the mesh enables microbial colonization but prevents ingrowth of roots. Needles represent recently shed litter that is rich in labile resources, whereas humus represents late stages of decomposition when labile resources are depleted, energy content is low, and nutrients are tied up in recalcitrant molecules or supramolecular aggregates (Bödeker *et al.*, 2016). Substrates for study III were collected at the same experimental site as they were incubated, whereas the substrates for study V were collected in a P addition experiment, which provided humus and needles that were poor or rich in P.

3.6 Extracellular enzyme activities (study IV)

Enzyme activities were measured in humus samples collected during summer and autumn at the Norway spruce site. The activities were measured in soil slurries in the lab using standard methods according to Saiya-Cork *et al.* (2002) with modifications as in Allison (2012) and Baldrian (2009). The potential activity of a selection of hydrolytic enzymes was measured as the release of fluorescent dye on a microplate fluorometer. The activity of the cellulose and

hemicellulose targeting enzymes β -1.4-glucosidase, endoglucanase, and β -1.4-xylosidase, and the aminosugar targeting β -1.4-N-acetylglucosaminidase (chitinase), and phosphate targeting acid-phosphatase was measured using 4-Methylumbelliferyl (MUB) labeled substrates, and the activity of peptide targeting Leucine-aminopeptidase using 7-amido-4-methylcoumarin (AMC) labeled leucine. The potential activity of oxidative enzymes was measured spectrophotometrically as the absorbance of light after the oxidation of specific substrates to the soil slurry. Laccase activity was measured by oxidation of ABTS (2.2-azino-bis(3-ethylbenzoline-6-sulphonate) whereas Mn-peroxidase activity was measured as the oxidation of DMAB (3-dimethylaminobenzoic acid) MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) and its actual activity calculated as the difference in oxidation with and without Manganese (II) sulphate added to the soil slurry.

3.7 Identification of soil microbes (study II-V)

The abundance of specific molecules in the soil enables quantification of the biomass of different groups of soil microbes. In this thesis, I have measured the abundance of three components of the microbial biomass combined to profile the microbial community broadly, and to target certain groups of soil microbes specifically, including phospholipid fatty acids (PLFA), ergosterol and fungal DNA. The lipid bilayer of the cell consists of short polar chains of C attached to a phosphate group, with some variations in its composition that can be used to broadly classify different organism groups (Bligh & Dyer, 1959). Ergosterol is the fungal equivalent to cholesterol (found in animals), where they determine the fluidity of the cell membranes.

3.7.1 Phospholipid fatty acid analysis

Lipids were extracted using the Bligh and Dyer method (Bligh & Dyer, 1959, McIntosh *et al.*, 2012, White *et al.*, 1979) and the abundance of individual PLFAs was measured on a gas chromatograph (Perkin-Elmer Clarus 500, Mundelein, Illinois, USA) coupled to a flame ionization detector (Waltham, MA, USA) at the Swedish University of Agriculture, SLU Umeå, and expressed in moles per gram soil C. A total of 28 PLFA markers were identified and described using standard nomenclature and classified according to previous work in this study system (Maaroufi *et al.*, 2015). The sum of all PLFA markers was used as a measure of total microbial biomass, and i-15:0, a-15:0, 15:0, i-16:0, 16:1 ω 9, 16:1 ω 7, 16:0, i-17:0, cy-17:0, a-17:0, 18:1 ω 7, and cy-19:0 represented the total bacterial biomass (Frostegård & Bååth, 1996). The

branched PLFAs i-15:0, a-15:0, i-16:0, i-17:0, and a-17:0 represented gram-positive bacteria (Wardle *et al.*, 2013), whereas 10me16:0, 10me17:0, and 10me18:0 were used to estimate actinobacteria¹, and cy-17:0, cy-19:0, and 18:1 ω 7 represented gram-negative bacteria. PLFA 18:2 ω 6 alone represented fungal PLFA (Frostegård *et al.*, 2011, Maaroufi *et al.*, 2019).

3.7.2 Ergosterol

Fungal biomass in fine-roots extracted from root ingrowth cores, in humus samples, and sand ingrowth cores was measured as the concentration of ergosterol, a fungi-specific biomarker (Olsson *et al.*, 2003). Ergosterol was extracted in MeOH according to Bahr *et al.* (2013) by mechanical extraction, centrifugation, and filtration (45 μ m) and ergosterol separated and quantified by liquid chromatographic analysis. Ergosterol in sand ingrowth cores was converted to fungal biomass using conversion factors in Ekblad *et al.* (2013), and C flux assuming a 50 % C content of the fungal biomass.

3.7.3 Fungal DNA

Nucleic acids were extracted from humus, fine-root tissues, and sand ingrowth cores using standard methods, e.g. according to Almeida *et al.* (2018) and Gundale *et al.* (2016). DNA from the ITS2 region of the internal transcribed spacer (ITS) was amplified with fungi-specific primers and sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). The taxonomic identity of the DNA sequences was assigned using the Basic Local Alignment Tool (BLASTN program 2.2.25), and ecological guild (ectomycorrhizal, saprotrophic, ericoid, endophyte) of all taxa for which genera could be assigned was classified with the assistance of the database tool FunGUILD (Nguyen NH, 2016) and based on the most current knowledge of the ecology of known close relatives and according to Tedersoo *et al.* (2010). Read abundances for all OTUs for each sample were summed and the abundance of each OTU expressed as the relative abundance per sample. A matrix containing the rarefied relative abundance of each OTU per sample was then used to analyze differences in community composition.

¹ Actinobacteria were labeled actinomycetes in study III

4 Result and discussion

4.1 Soil carbon stocks

Nitrogen increased soil C stocks in the organic layer in the Scots pine forest (Fig. 5). In pair-wise comparisons with the control treatment, only the effect of the 50N treatment was significant. However, there is no evidence against a linear positive effect of the N additions on the soil C stock (Fig. 6A). In the 50N treatment, 14.5 Mg C had accumulated during the 13 years of N additions, which is equivalent to an annual average C accumulation rate of 1117 kg yr^{-1} , or $22.3 \text{ kg C kg}^{-1} \text{ N added}$ (Fig. 6). This rate is similar to the average in a meta-analysis of temperate forests (Janssens *et al.*, 2010), and in the upper range reported for Scots pine and Norway spruce forests in Sweden and Finland (Hyvönen *et al.*, 2008). This rate is also twice as high as for the Norway spruce forest (study IV-V), where a recent study reported on an average rate of $10 \text{ kg C kg}^{-1} \text{ N added}$ during 16 years (also in the organic layer) (Maaroufi *et al.*, 2015). The higher soil C accumulation response in the Scots pine forest compared to the Norway spruce forest may reflect differences in site fertility, as the relative tree growth responses to N addition often is stronger on nutrient-poor soils (Solberg *et al.*, 2009), and the C:N ratio in the control treatment is higher at the Scots pine site compared to the Norway spruce site (C:N 44.0 versus 39.4), indicating stronger N limitation to growth at the Scots pine site. Taken together, the soil C inventories at these two sites show that N additions increase the soil C sink, and particularly in the organic soil layer, and support the view that N deposition can be a driver of soil C accumulation. Moreover, the low N treatments, simulating current N deposition rates in the boreal region, did not lead to a significant increase in soil C, and a longer duration and higher replication ($n = 6$) might be needed to detect a significant effect at those N addition rates, and to the mineral soil C stocks (Fig. 5).

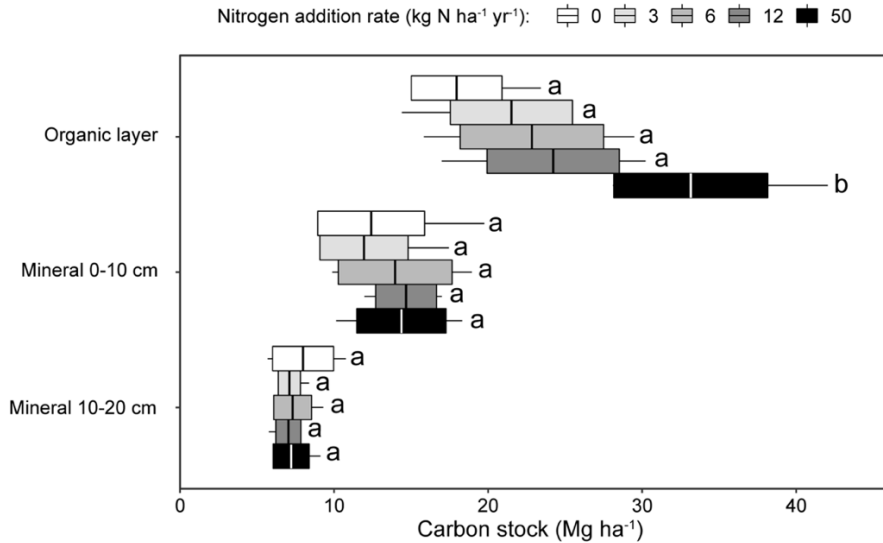


Figure 5: The soil carbon stock in the organic layer, 0 – 10 cm, and 10 – 20 cm mineral soil layers after 13 years of annual nitrogen additions (0, 3, 6, 12, and 50 kg N ha⁻¹ yr⁻¹) in the Scots pine forest. Boxes display the median and one standard deviation above and below the median, and whiskers are the maximum and the minimum (n = 6). Boxes sharing letters within each soil layer are not significantly different at $p < 0.05$ according to Tukey's test.

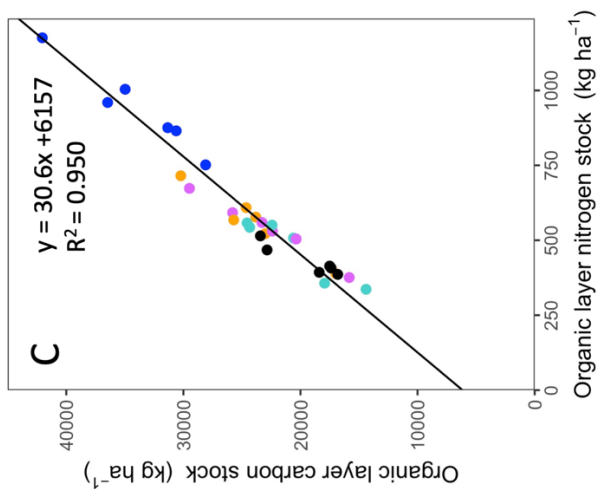
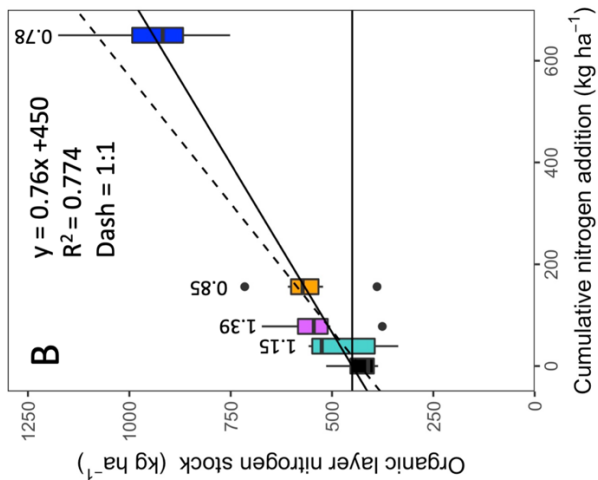
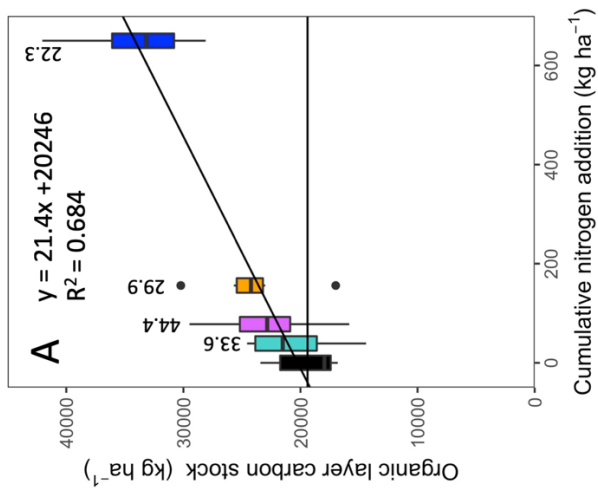
4.2 Soil nitrogen stocks

Soils are strong sinks for N and competition between plants, soil microbes, and abiotic sinks in the soil may limit plant access to added N, and thus also limit the positive effect of N on the soil C balance (Melin *et al.*, 1983, Templer *et al.*, 2012). The N treatments at the Scots pine site increased soil N stocks in the organic layer, significantly in pair-wise comparisons with the control treatment at the highest N addition rate, where N stocks had increased by the equivalent of 78 %, i.e. 507 of the 650 kg cumulative amount of N added per ha (Fig. 6B). Again, N stocks in the low N addition treatments (≤ 12 kg N ha⁻¹ yr⁻¹) was not different from the control in pair-wise comparisons (Fig. 6B). A previous study at the Norway spruce site showed that N stocks in the organic layer had increased by the equivalent of 75 % and 50 % the N added in the 12.5 and 50N treatments, respectively (Maaroufi *et al.*, 2015), which is lower than for the Scots pine site but high compared to the maximum of 47 % reported for ¹⁵N recovery in Tietema *et al.* (1998) and 39 % for organic layers in forests in Templer *et al.* (2012). Even

if the precision in N budgets are relatively low, the steep increase in N stocks in the organic layer indicate that it has a strong capacity to retain N, a result which is consistent with those of more precise methods using stable N isotopes, that have shown that the recovery of N in the organic layer typically is lower in soil with high N inputs (Gundale *et al.*, 2014, Tietema *et al.*, 1998).

A key question for the soil C balance is whether N is sequestered abiotically into soil organic matter (Knicker, 2004, Kögel-Knabner & Amelung, 2014, Nõmmik & Vahtras, 1982) or whether it is assimilated by organisms and entering the soil C pool via biomass turnover (de Vries *et al.*, 2014, Liang *et al.*, 2017). The 78 % of the N added in the 50N treatment at the Scots pine site (507 of 650 kg) was retained in the organic layer, together with an additional 14.5 Mg C, meaning that the N that remain in the organic layer is associated with nearly 30 times as much C (Fig. 6C). The congruent growth of soil C stocks contradicts abiotic stabilization mechanisms where N is accumulating without additional C (Kögel-Knabner & Amelung, 2014, Schmidt *et al.*, 2011). It is, however, possible that this value indicates the amount of C that N can sequester via depolymerization and re-condensation reactions (Knicker, 2004), or the average amount of C per N in supramolecular soil aggregates (Clarholm *et al.*, 2015). The accumulation of C together with N is also consistent with incorporation into some form of biomass with a relatively high C:N ratio (Cleveland & Liptzin, 2007, Wallander *et al.*, 2003), and could then possibly reflect the average C:N ratio of litter inputs at this site (Liang *et al.*, 2017). More studies are needed to clarify the mechanisms for N retention in the organic layer, as its strong relation to C indicates that it is key to understand the soil C sink.

Figure 6 (opposite page): Soil carbon and nitrogen stocks in the organic soil layer at the Scots pine site after 13 years of annual nitrogen additions (0, 3, 6, 12, and 50 kg N ha⁻¹ yr⁻¹). Boxes in panel A and B display the median and the quartile above and below the median, and whiskers the maximum and the minimum (n = 6). Outliers (>1.5 interquartile outside the box) are plotted individually. Horizontal lines denote the average element stock in the control treatment and solid lines, equation, and R² values are linear models. The dashed line in panel B denotes the cumulative nitrogen addition rates in excess of control treatment average. Scatter color in panel C corresponds to color in boxplot A and B. Data from study I and II. Numbers above each box are the average increase in C or N mass per unit mass N added. Only the 50N treatment is significantly different from the control in pair-wise comparison with Tukey's test in panels A and B.



4.3 Changes in carbon input and output

4.3.1 Above-ground litter input

The input of C by aboveground tree litter was measured in study I and II at the Scots pine site. These measurements identified a clear increase in aboveground litter production by +46 % at the highest N addition rate, whereas the low N addition rates ($\leq 12 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) had no effect on aboveground production (Fig. 7A). This result is consistent with two previous studies on the tree growth response in this experiment that has shown that the highest N addition rate has a strong positive effect on aboveground growth, whereas the low N treatments have no effect (From *et al.*, 2016, Lim *et al.*, 2017). Several explanations have been suggested for the low responsiveness of the low N treatments. Failure to detect a growth response is one possibility, considering the heterogeneity in tree ages and sizes of the study system (From *et al.*, 2016). However, the response is consistent with competition for N with understory vegetation, particularly mosses which both have been shown to down-regulate their N_2 fixation rates already at relatively low N addition rates and thus offset N inputs at low rates (Gundale *et al.*, 2011) and serve as a strong sink for N (Gundale *et al.*, 2014). Ericaceous shrubs, notably *Vaccinium vitis-idaea* and *Calluna vulgaris* may also be strong sinks for added N in these treatments and produce recalcitrant litter. Moreover, a lower growth response in these treatments is also consistent with competition for N with soil microbes (Kaye & Hart, 1997), including EMF (Näsholm *et al.*, 2013), which can use C more efficiently to produce biomass as N availability increases, as long as the supply of labile C in the root zone persists (Franklin *et al.*, 2014).

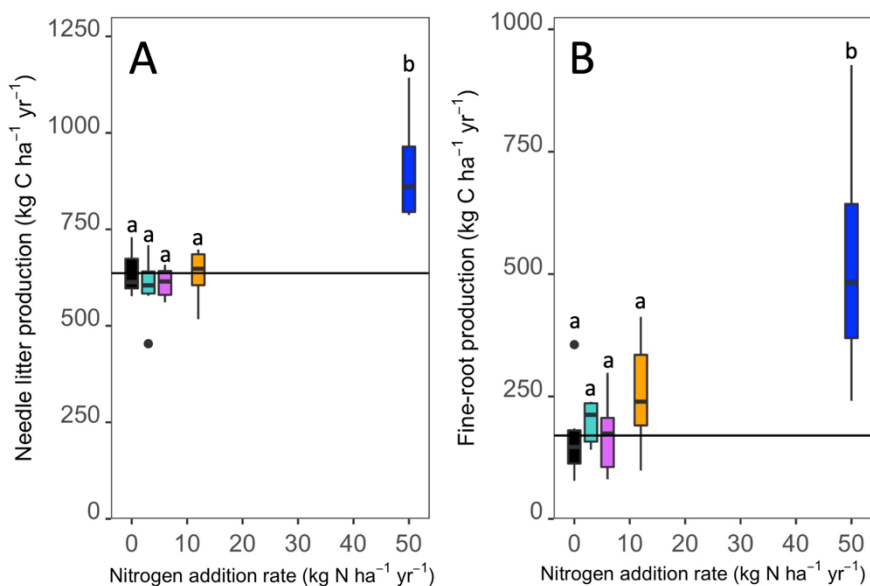


Figure 7: Above ground needle litter production (A) and production of fine-roots in the upper 20 cm of the mineral soil (B) at five levels of annual N additions (0, 3, 6, 12, and 50 kg N ha⁻¹ yr⁻¹) in the Scots pine experiment. Data from study II.

4.3.2 Fine-root litter input

The measurements of fine-root biomass and production at the Scots pine site in study II revealed an increase in fine-root production at the highest N addition rate (Fig. 7B), but no effect of N on the standing biomass. The N effect on fine-root production was similar to the response of the needle biomass production, in that both increased significantly at the highest N addition rate, and thus indicate that N increases the production of biomass both above-, and below-ground, and that the production of biomass above ground did not lead to a reduction in production below ground (Fig. 7). As the standing biomass of fine-roots was unresponsive to N, the longevity of the fine-root biomass had decreased, from approximately 4 years in the control treatment to approximately 1 year at the highest N addition rate. A high longevity of fine-root biomass is expected in cold and nutrient-poor ecosystems (Eissenstat *et al.*, 2000, Kleja *et al.*, 2008, Yuan & Chen, 2010), although the exact value may be overestimated to some degree due to the disturbance effect at the beginning of the incubation with the ingrowth core method (Addo-Danso *et al.*, 2016, Brunner *et al.*, 2013). Increased fine-root production was unexpected based on the result of a meta-analysis of temperate

and boreal forest responses to N (Janssens *et al.*, 2010), and how more fertile Scots pine and Norway spruce forests have relatively more root biomass compared to aboveground biomass (Helmisaari *et al.*, 2007). Moreover, in a global meta-analysis, Yuan and Chen (2012) found that N additions increased fine-root production but less than above ground litter production. In another global meta-analysis, Liu and Greaver (2010) found that N additions increased above ground litter production, but not fine-root litter production.

However, a few studies have reported similar findings to my data. As an example, Stober *et al.* (2000) reported on increased fine-root production in ingrowth cores amended with N at the same Scots pine site, before the N addition experiment was set up there. Similarly, fine-root production also increased in an experiment with fertilization applied by irrigation in a Norway spruce forest, 20 km from the Scots pine site (Leppalammi-Kujansuu *et al.*, 2014, Majdi, 2001). The change in fine-root longevity with N shows that litter C inputs to soils depend on nutrient availability, and a better understanding of the drivers of fine-root dynamics and the input of C by fine-root litter production is needed to better predict changes in soil C inputs (Fernandez-Martinez *et al.*, 2014, Ľupek *et al.*, 2016).

4.3.3 Soil respiration

Soil respiration was measured in study I at the Scots pine site outside and inside trenches to estimate the contribution of tree roots to the total release of C. These measurements showed that the total soil respiration, measured as the areal CO₂ efflux outside trenches, decreased non-significantly at the highest N addition rate (Fig 8A), whereas soil respiration inside trenches, i.e. in the absence of tree roots, generally increased by N additions (Fig 8C). However, higher soil C stocks in N treated plots (Fig. 5) also contributed to differences in respiration, and expressing respiration on a per unit mass soil C shifted the total respiration to a -31.2 % decrease in the 50N plots (Fig 8B), and shifted respiration inside trenches at the same plots to a non-significant decrease by -11.1 % (Fig 8D). The overall effect of N on soil respiration is therefore generally negative, as has been shown in numerous previous studies (Janssens *et al.*, 2010), including at the Norway spruce site (Maaroufi *et al.*, 2015). At the Scots pine site, this effect occurred mainly as a consequence of a reduction in root-associated respiration (Fig 8E), although the heterotrophic respiration, expressed on a per mass unit soil C, also tended to decrease. Again, significant effects of the low N treatments were rarely detected in pair-wise comparisons with the control.

Coupled to the higher input of C by fine-root biomass production (Fig. 7B), the decrease in autotrophic soil respiration at the highest N addition rate (Fig.

8E) implies that the production of fine-roots to some degree occurred by down-regulation of respiration, either in the root tissue or in the microbial community depending on root exudates. This can potentially be explained by a higher plant biomass production efficiency, which has been hypothesized to occur by decreasing C allocation to roots in general (Fernandez-Martinez *et al.*, 2014) or more specifically by decreasing C supply to root-associated fungi (Vicca *et al.*, 2012). Such shifts in plant C use are therefore likely to influence the microbial communities and their decomposition of litter and humus, as is discussed in the following sections.

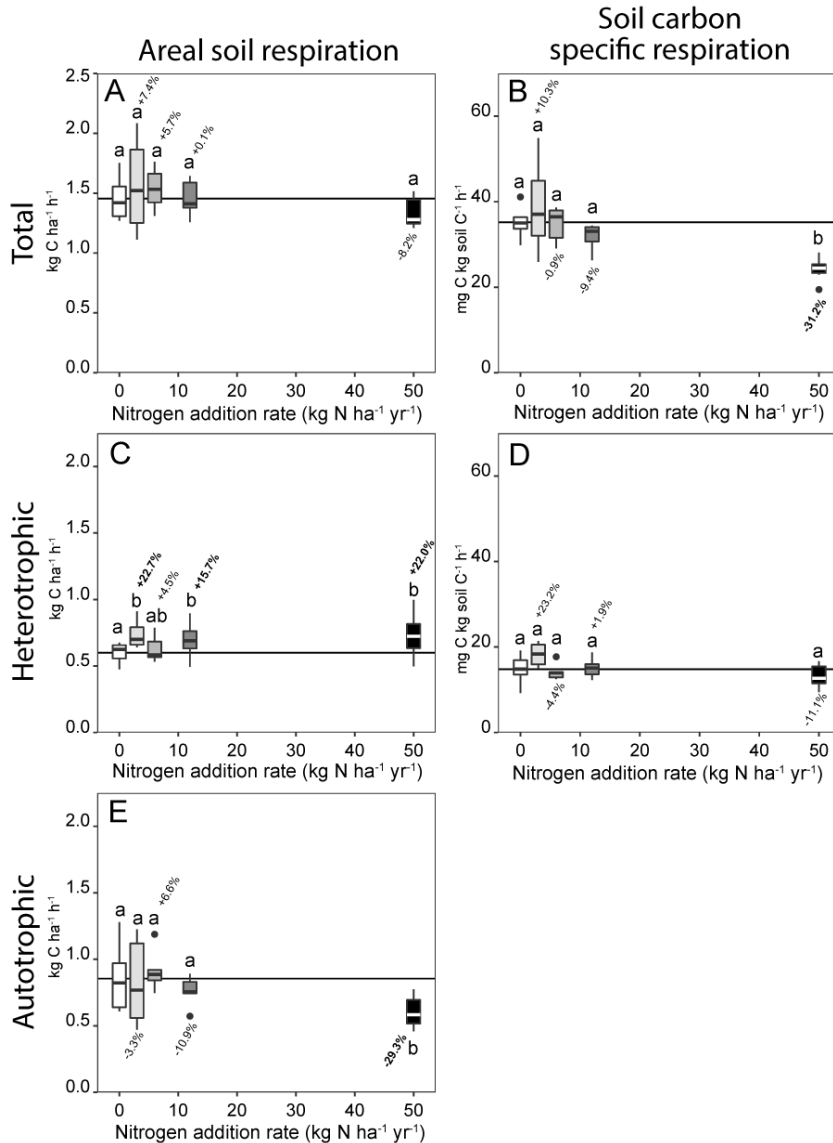


Figure 8. Soil respiration averaged for 11 measurement occasions during the growing season after 12 to 13 years of annual nitrogen additions (0, 3, 6, 12, and 50 kg N ha⁻¹ yr⁻¹) inside and outside trenches at the Scots pine site. The total (A-B) and heterotrophic (C-D) respiration expressed on an area (left) and per mass unit carbon down to 20 cm in the soil (right). Autotrophic soil respiration was measured as the difference between total and heterotrophic soil respiration, i.e. outside minus inside trenches and thus measure respiration associated with tree roots. Boxes display the median and the quartile above and below the median, and whiskers the maximum and the minimum (n = 6). Outliers (>1.5 interquartile outside the box) are plotted individually. Boxes sharing letters within each panel are not significantly different at $p < 0.05$ according to mixed effect models (areal respiration) or Tukey's test (soil carbon-specific respiration). Horizontal lines denote mean respiration in the control treatment and values above or below boxes represent percentage difference in mean respiration compared to the control treatments. Data from study I.

4.4 Decomposition

The mass loss of C in organic substrates incubated in the soil serves as a complementary measure of soil C losses, and changes in N and P can inform on which specific elements decomposition processes are targeting. Decomposition was measured in study III and V using needles and humus, where needles correspond to a relatively high-quality substrate primarily exploited by saprotrophs, whereas humus was used to study late stages of decomposition, where EMF may be more active. The trenching treatment at the Scots pine site enabled an assessment of the role of tree roots and their associated microbial communities to decomposition processes. In addition to mass loss of these elements during a 1-year incubation, measurements on the activity of eight soil enzymes targeting organic molecules with the elements were made during summer and autumn at the Norway spruce site in study IV, and these results are discussed in parallel below.

4.4.1 Mass loss and gain of carbon

Incubation of needles and humus in N treated plots increased the final mass of C compared to control treatments at both the Scots pine (study III) and the Norway spruce site (study V). At the Scots pine site, N decreased mass loss of C across the two substrate types (needle and humus) and the trenching treatment. The effect was mainly driven by the high N treatment that decreased mass loss of C in needles, and a stronger negative effect of N on mass loss of C inside than outside trenched plots. A similar effect of N was observed at the Norway spruce site (study V), where N additions decreased mass loss of C in needles and increased the mass gain of C in humus. Enzymes involved in mass loss of C include hydrolytic enzymes involved in the controlled decomposition of cellulose releasing C for ingestion, as well as oxidative enzymes that degrade recalcitrant forms of soil organic matter. These two classes of enzymes responded in opposite directions to N, where the three enzymes involved in the cellulose decomposition increased with N (Fig. 9A-C), indicating increased uptake of C from soil organic matter, whereas the activity of one of the oxidative enzymes, Mn-peroxidase, were down-regulated by N, and the other, Laccase, was unresponsive to N (Fig. 9 G-H).

Taken together, the decreased respiration discussed above, decreased mass loss, or increased mass gain of C during incubation of needles and humus in the soil, and decreased activity of Mn-peroxidase, are all consistent with the observed accumulation of C in the organic layer (Fig. 5). The increased activity of cellulases is the only response that contradict the overall positive impact of N on retention of C in the soil, but it should be noted that the release of C by these

enzymes are tightly coupled to assimilation (Allison *et al.*, 2011), and a larger fraction of assimilated C can be expected to be used for microbial growth, instead of respiration at higher N availability (Manzoni *et al.*, 2012). Notably, these effects were driven by the highest N treatment, and there was little support for a stimulating effect of the low treatments N on mass loss of C (de Vries *et al.*, 2014, Knorr *et al.*, 2005).

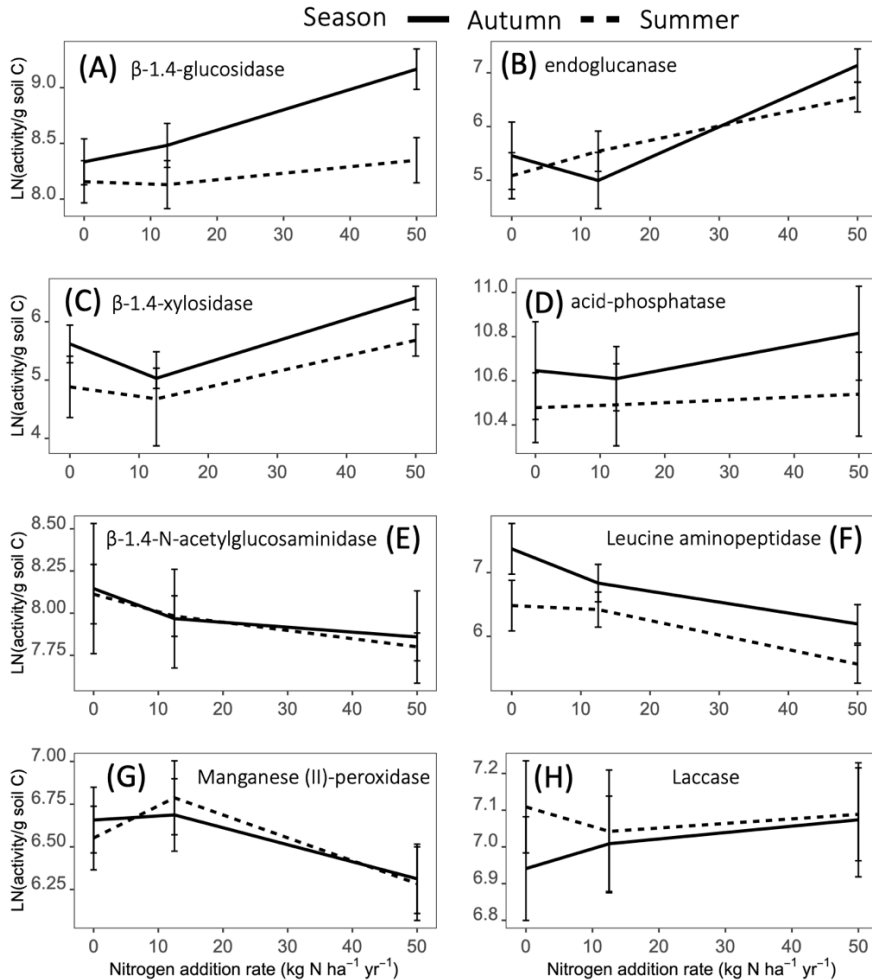


Figure 9: Interaction plot of potential enzyme activity across three levels of nitrogen addition (0, 12.5, and 50 kg N ha⁻¹ yr⁻¹ × 20 years) during summer (dashed line) and autumn (solid line) at the Norway spruce site. Enzyme A-F are hydrolytic and A-C is involved in cellulose degradation, D in the release of phosphate from organic matter, E-F release organic nitrogen and G-H are oxidative enzymes involved in the degradation of recalcitrant organic matter. Values are the natural logarithm of molar cleavage of enzyme-specific substrates standardized to the amount of carbon in each sample (molar substrate cleavage h⁻¹ g C⁻¹). Error bars represent standard errors of the mean (n = 12). Data from study IV.

4.4.2 Mass loss and gain of nitrogen

Similar to the impact of N addition on mass loss of C, the final mass of N increased with increasing N addition rate at both sites, again with slightly different responses depending on trenching treatments and substrate types. Nitrogen was net imported in needles and net exported from humus at the Scots pine site, and trenching strongly increased the mass gain of N in needles, and decreased the mass loss of N in humus. At the Norway spruce site, N was exported from the needles incubated in the control treatment but switched to import at the highest N addition rate, whereas N always was net imported into the humus at this site. Thus, the N content of the substrates was consistently higher at the end of the incubation for substrates incubated in an N enriched plot. This effect was expected, and has been observed in several previous studies, and is likely driven by a combination of increased ingrowth of microbial biomass, coupled to a higher N concentration of that microbial biomass. The accrual of N further corresponded with a general decrease in the activity of enzymes involved in the uptake of organic N from aminosugars (glucosaminidase) and peptides (Leucine aminopeptidase) (Fig. 9E-F) (Sinsabaugh, 2012), indicating that mineral N addition down-regulates the uptake of organic N by soil microbes.

4.4.3 Mass loss and gain of phosphorus

In contrast to mass loss and gain of N in the substrates, P was consistently unresponsive to N treatments at both sites but was highly responsive to the trenching treatment at the Scots pine site. Similar to the effect of substrate type on mass loss and gain of N, P was net imported into the needles, and net exported out of the humus. Moreover, the effect of substrate type on P was also similar as for N, wherein P mass gain increased non-significantly in the needles and P mass loss decreased significantly in the humus. Phosphorus was net lost from both needles and humus at the Norway spruce site, and as expected, the effect was highly dependent on P amendment, wherein substrates with high P also lost a larger fraction of the P during the incubation. Notably, no effect of N treatments on mass loss or gain of P was detected at any of the sites. Moreover, measurements of the activity of acid-phosphatase, a hydrolytic enzyme involved in the release of P was also unaffected by added N (Fig. 9 D).

4.4.4 Role of tree roots in decomposition

Mass loss of C and nutrients in needles and humus differed depending on if they were incubated outside or inside trenches, consistent with the hypothesized role of tree C allocation influence on these processes (study III). Specifically, the total mass loss of needles was higher if they were incubated inside trenches compared to needles incubated outside trenches (Fig. 10A). The observation that mass loss of C was greater in the absence of tree roots indicates that roots and their associated microbiota somehow inhibit decomposition. Similar effects have been observed after removal of tree roots in *Pinus radiata* stands in New Zealand (Gadgil & Gadgil, 1971) and mixed coniferous forests in Sweden (Sterkenburg *et al.*, 2018), and other places, but the mechanisms behind this phenomenon and its generality remain poorly understood (Fernandez & Kennedy, 2016). It has been suggested that the selective extraction of N from soil organic matter by EMF fungi leaves organic matter N depleted, and thus induces N limitations on otherwise superior saprotrophic decomposers, resulting in an inhibition of decomposition (Averill *et al.*, 2014, Orwin *et al.*, 2011). Indeed, more N and P were present in the remaining substrate by the end of the incubation if they were incubated inside trenches, compared to outside, which indicates that tree roots and their associated EMF extract nutrients, and thus potentially induces nutrient limitations on the saprotrophic community. However, other factors may also contribute to this effect (Fernandez & Kennedy, 2016).

In the Scots pine experiment, with the trenching treatment crossed with different N addition rates, suppression of saprotrophic decomposition could be expected to decrease with increasing N addition rate if this interaction was mediated by competition for N. In contrast, we found that N had a generally negative effect on decomposition and that the effect of N was stronger inside trenches than outside (Fig. 10). This indicates that N also suppresses saprotrophic decomposition in the absence of tree roots, and also that the suppression of saprotrophic decomposition by tree roots was mediated by some other factor than N limitations. The Scots pine site is located on well-drained sand sediment and is relatively dry, and water may limit decomposition more strongly in the presence of tree roots due to their removal of water via transpiration. Indeed, soil moisture was significantly higher inside the trenches ($p < 0.01$), and may thus support a higher saprotrophic activity. Moreover, the input of C by EMF colonization of the litter would also contribute to higher final mass by the end of the incubation outside trenches, compared to the inside (Godbold *et al.*, 2006, Liang *et al.*, 2017). In conclusion, I suggest that the negative effect of tree roots and their EMF on saprotrophs are mediated by some factor other than N and that N deposition thus is unlikely to lead to loss of soil C by enhancing saprotrophic decomposition.

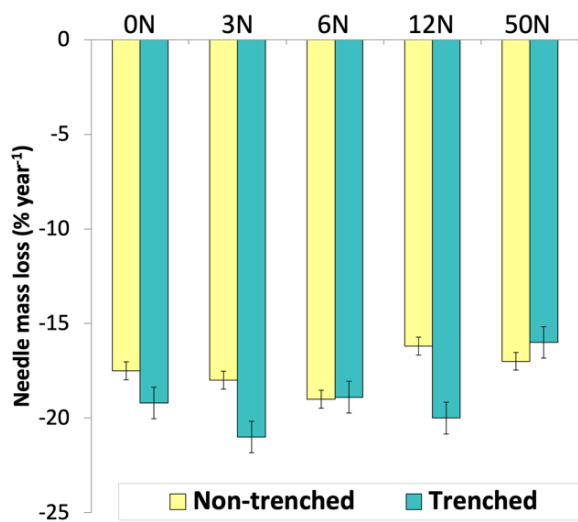


Fig. 10. Total mass loss of needles incubated for 1 year in the organic layer outside (non-trenched) and inside the trenching treatment (trenched) at the Scots pine site at five levels of N addition. Trenching significantly increased total mass loss of needles and N decreased mass loss of litter significantly inside the trenching treatment but not outside. Data from study III.

4.5 Changes in microbial community composition

Nitrogen decreased soil respiration, particularly respiration associated with tree roots, and decreased mass loss of C during decomposition, and had different effects on soil active enzymes, as outlined above. In the following section, I discuss changes in the abundance of specific markers for the microbial biomass that may contribute to explaining these responses. Nitrogen addition had significant effects on the microbial community composition, both in the broad profiling of the microbial community using PLFA markers and more specific profiling targeting the fungal community using DNA sequencing, with some consistent shifts at both the Scots pine (study III) and Norway spruce site (IV). In addition to N, I also assessed the microbial community outside and inside the trenches at the Scots pine site, and during summer and autumn at the Norway spruce site, to assess the influence of tree root C inputs to N responses.

4.5.1 Phospholipid fatty acid analysis

The highest N addition rate decreased the total PLFA by -29 % outside trenches at the Scots pine site, and by -25% on average across the summer-to-autumn sampling at the Norway spruce site. This decrease in total PLFA was reflected in all sub-classes of PLFA markers, except for actinobacteria which increase significantly by +36 % outside trenches at the Scots pine site, and was unaffected by N at the Norway spruce site. Actinobacteria harbor some capabilities to degrade recalcitrant soil organic matter, however not as efficiently as fungi (Zak *et al.*, 2011). Moreover, they may be faster growing in N rich environments and thus rapidly produce microbial necromass, and it has also been suggested that they are antagonists to fungal decomposers and their increase thus suppressing fungal decomposition (Jayasinghe & Parkinson, 2008). Thus, the relative increase in actinobacteria may contribute to soil C accumulation both by producing more litter, and by decreasing decomposition rates.

Most bacterial markers decreased in the high N treatment at both sites, but this effect was more pronounced for gram-negative bacteria than for the gram-positive, wherein the actinobacteria belong. Differences in N responses within the bacterial community can possibly be explained by differences in feeding habits, where gram-negative bacteria are more affiliated to the root-zone, presumably being more dependent on root exudates than gram-positive bacteria (Fierer *et al.*, 2007, Kramer & Gleixner, 2008, Söderberg *et al.*, 2004). A recent study showed that the abundance of gram-positive relative to gram-negative bacteria increased with ecosystem age in a boreal forest chronosequence, as well as with the removal of tree roots and shrubs (Fanin *et al.*, 2019). The higher sensitivity of gram-negative bacteria to N additions may therefore indicate a

decreased supply of labile C in the root-zone, whereas the increase in gram-positive 10me16 and 10me17 markers may reflect alleviation of N limitation, as these markers have been reported to increase after N additions (Demoling *et al.*, 2008, Maaroufi *et al.*, 2019, Schleuss *et al.*, 2019) or have been shown to be more abundant in the fertile end of a natural fertility gradient (Högberg *et al.*, 2007). At the Scots pine site, tree root removal by trenching increased the abundance of gram-positive bacteria and actinobacteria, but did not decrease the abundance of gram-negative bacteria, which is inconsistent with the notion that this group of bacteria is dependent on root C supply, at least that derived from trees. However, Ericaceous shrubs that remained inside the trenches could have supplied the soil with labile C by root exudation and thus supported a population of root-associated microbes. Indeed, the abundance of ericoid mycorrhizal fungi increased after trenching (Fig. 11 G), which indicates that the loss of tree roots to some degree was compensated for by an increase in Ericaceous roots.

Fungal biomarkers constitute a large part of the total PLFA and the -35 % decrease in these biomarkers at the highest N addition rate outside trenched at the Scots pine site, and the -38 % decrease in these markers at the Norway spruce site, contributes heavily to the decrease in total PLFAs observed. The negative effect of N on fungal biomass is well described in the literature (Janssens *et al.*, 2010, Treseder, 2008), and it has been proposed that decreased allocation of C to EMF underlie some part of this decline. Consistent with the role of tree roots in supporting the fungal biomass in the soil, and the role of changes in tree C allocation in response to N, the fungi:bacteria ratio was lower inside compared to outside trenches, and the fungal biomass decreased with N more strongly outside than inside trenches. However, a significant negative effect of the highest N addition rate on the fungal biomass was also detected inside the trenches, which indicates that although tree roots contributes strongly to the decrease in fungal biomass, it can also occur in their absence, and other factors may be at play.

4.5.2 Regulation of the ectomycorrhizal symbiosis

Autotrophic soil respiration decreased while the production of fine-root biomass increased, indicating that a major shift in C use in the soil occurred in response to N. Ectomycorrhizal fungi (EMF) are ubiquitous in this type of forest soils and are an important sink for photosynthates, play a key role in N acquisition, and in soil C cycling by immobilizing C into their biomass and in decomposing soil organic matter. Therefore, to better understand how N affects the supply of C to EMF colonized roots and their extramatrical mycelium, and between fine-root production and autotrophic respiration, study II-III used the trenching treatment and ingrowth cores to measure the production of EMF mycelium and the colonization of EMF on tree roots, and the standing biomass of fungi in the soil.

Trenching decreased the abundance of the fungal PLFA marker 18:2 ω 6 in the organic soil layer (study III, Fig. 11E), and reduced the production of fungal biomass to approximately half, measured as the accrual of ergosterol in sand ingrowth cores (Fig. 4C) during a one-year incubation in the organic layer (study III, Fig. 11F). Moreover, DNA sequencing of the fungal communities colonizing these ingrowth cores showed that the abundance of known EMF decreased significantly (study III, Fig. 11G). The trenching treatment thus shows that tree roots are an important source of C for the fungal community colonizing these ingrowth cores (Wallander *et al.*, 2001), and also supports a higher standing biomass of fungi in the soil.

Nitrogen had no effect on the production of fungal biomass measured in ingrowth cores incubated for up to 16 months in the mineral soil (study II, Fig. 11A), or for 12 months in the organic soil layer outside and inside trenches (study II, Fig. 11F). However, the abundance of EMF reads more than doubled between the control treatment and the highest N treatment outside trenches, but not inside (study III, Fig. 11G). This increase was particularly driven by an increase in *Xerocomus ferrugineus* and *Tylospora astherophora*. Notably, species that are frequent in the organic layer and play key roles in C and N dynamics in this type of forest soil, such as members of the genus *Cortinarius* (Clemmensen *et al.*, 2015, Lindahl *et al.*, 2007), occurred in very low abundance in these ingrowth cores, despite that they most likely constitute a major part of the fungal community based on previous studies at this site (Högberg *et al.*, 1999, Taylor *et al.*, 2000), and the corresponding amplicon sequencing at the nearby Norway spruce site (see below). This is consistent with observations from other forests where some taxa, including the *Cortinarius* are underrepresented as mycelium in ingrowth cores compared to root-tips (Kjøller, 2006), and accordingly, it is possible that the production of EMF is higher and more responsive in the bulk soil than in ingrowth cores I used to assess fungal communities.

Fungal colonization of fine-roots retrieved in root ingrowth cores (Fig. 4B) showed a weak tendency to increase in the low N treatments ($< 12 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), but no significant effects on fungal biomass in fine-roots were detected, despite a clear increase in the N:C ratio in these tissues at the highest N addition rate (study II, Fig. 11B-D). Similar observations were reported by Leppalammi-Kujansuu *et al.* (2013) who found that the production of EMF mycelium, as well as the degree of EMF colonization, was unresponsive to fertilization, although fertilization in their experiment was applied by irrigation.

Thus, in conclusion, the evidence for N impacting the ectomycorrhizal symbiosis at the Scots pine site is mixed. No effect was detected on the production of extramatrical mycelium in the mineral soil or in the organic layer, but the increase in EMF read abundance in ingrowth cores in the organic layer indicate increased production of some specific taxa. The concentration of the fungal PLFA marker in the organic layer was higher outside trenches, and decreased more steeply in response to N outside, compared to inside trenches, which suggests a decreased standing biomass of root-associated fungi in N enriched soils.

Figure 11 (opposite page): Compilation of results from study II and III related to the regulation of the ectomycorrhizal symbiosis across five levels of N addition (0, 3, 6, 12, and 50 kg N ha⁻¹ yr⁻¹) at the Scots pine site. The production of fungal mycelium in sand ingrowth cores incubated in the mineral soil (A), and the fungal colonization and N:C ratio of Scots pine fine-roots (B-D). The abundance of fungal phospholipid fatty acid marker in the organic layer inside and outside trenches (E). The production of fungal mycelium in sand ingrowth cores in the organic layer inside and outside trenches (F) and the composition of the fungal community colonizing these ingrowth cores.



4.5.3 Shifts in fungal community composition

Nitrogen changed the fungal community in sand ingrowth cores at the Scots pine site (study III) and in humus and fine-roots at the Norway spruce site (study IV). As noted above, the abundance of EMF reads in the sand ingrowth cores increased with N addition rate outside the trenches at the Scots pine site, with *Xerocomus ferrugineus* and *Tylospora asterophora* increasing in N enriched plots. These species increased, whereas several other taxa decreased, including *Sebacinales* species, *Piloderma sphaerosporum*, *Tomentellopsis echinospora*, *Thelephora terrestris*, *Suillus variegatus*. At the Norway spruce site, *Tylospora fibrillosa* and *Russula griseascens* increased with N in the humus community, while *Oidiodendron pilicola*, *Cenococcum geophilum* and *Cortinarius caperatus* decreased. In the fine-root community, N increased the abundance of *Phialocephala fortinii*, *Piloderma byssinum*, *Pezoloma ericae*, and *Meliniomyces variabilis*, while the abundance of *M. bicolor* and *Cenococcum geophilum* decreased (Fig. 12).

The present results on the microbial community composition show that external N inputs in boreal forest soils restructure the microbial communities, notably by decreasing the abundance of fungi in general, and shifting the dominance among taxa within the fungal community. The relative abundance of EMF reads did not change with N at either the Scots pine or the Norway spruce site, however, shifts among EMF occurred, with *Tylospora* species increasing in relative abundance at both the Scots pine and Norway spruce site, whereas other species decreased. Future studies could link these species to ecological functions, via e.g. morphological traits such as growth forms (Agerer, 2001), and chemical composition of fungal tissues (Fernandez & Koide, 2012), and catalytic capabilities with different enzyme systems (Kohler *et al.*, 2015), which may provide useful links between fungal species and species interactions to key ecosystem processes such as nutrient mobilization and C accumulation.

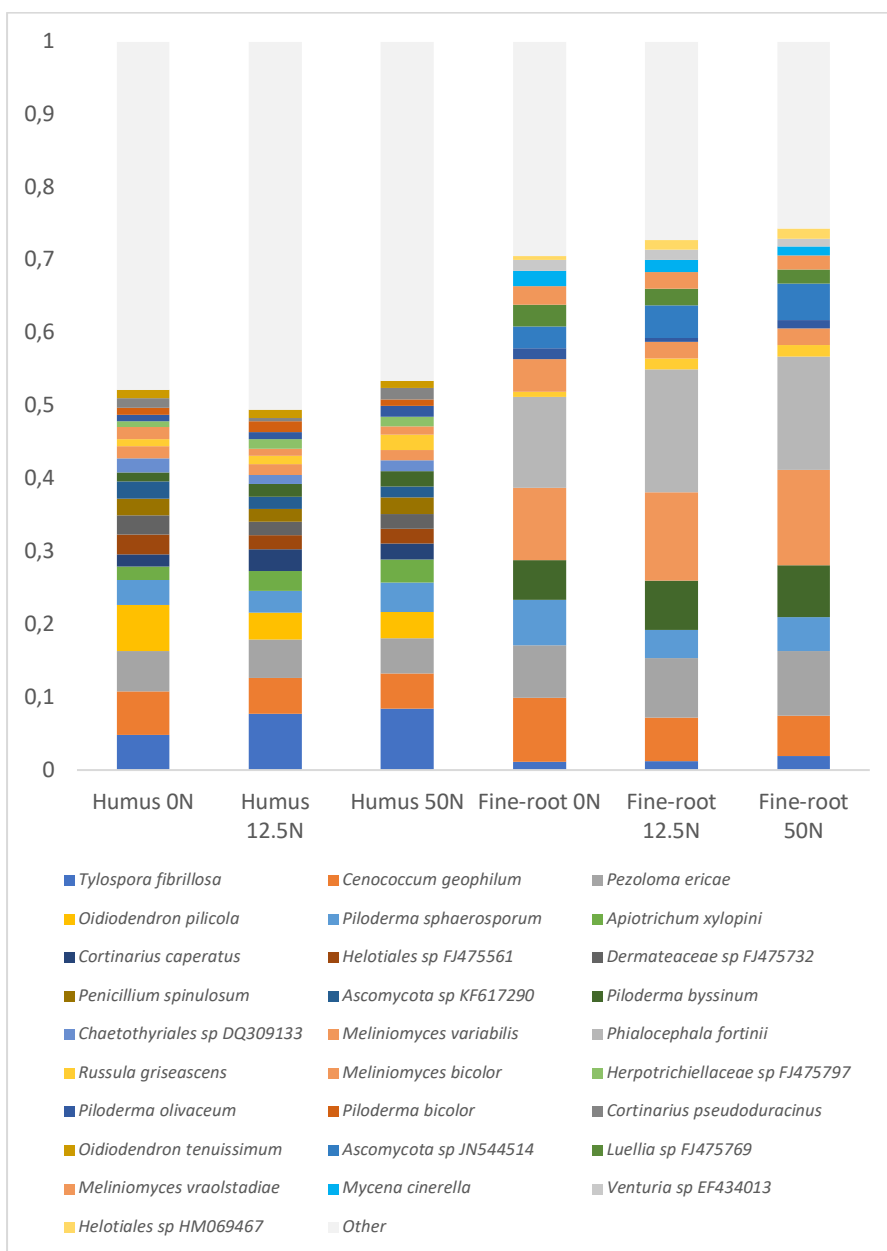


Figure 12: The relative abundance of fungal DNA reads in humus and fine-root samples collected in the organic soil layer at the Norway spruce site during summer and autumn after 20 years of annual N addition at three rates (0, 12.5, and 50 kg N ha⁻¹ yr⁻¹, n = 12). Displayed are the 28 operational taxonomic units (OTU's) with a relative abundance >1 % listed by row in descending order after their relative abundance in humus samples. Each value is the average of two sampling occasions including summer and autumn. In total, 1052 fungal OTU's were identified.

4.6 Nitrogen deposition and soil phosphorus

Phosphorus limitation has been proposed to be induced by N deposition (Jonard *et al.*, 2015), and thus constrain C capture in forests temporarily relieved of N limitations by N deposition (de Vries *et al.*, 2014, Goll *et al.*, 2017). Several studies have found that P concentrations in plant biomass have decreased in areas with high N deposition, which has been interpreted as a sign of incipient P limitation (Braun *et al.*, 2010, Jonard *et al.*, 2015). If long-term N enrichment leads to P limitations, it is likely to have consequences for the C balance in these forests.

The effect of N addition on soil P was studied in Study III, IV, V as the mass loss of P in decomposition bags, fungal colonization of mineral soil apatite sources, and enzyme activities. Nitrogen had no effect on the mass loss of P during decomposition (study III, V), did not increase the stimulating effect of apatite amendment of fungal colonization of ingrowth cores (study V), and did not affect the activity of acid phosphatase in the organic soil layer (study IV). Thus, none of these measurements detected an effect of N on P in the soil. These results may indicate that P limitations have not developed in these study systems, or that mechanisms other than the mobilization of P from soil organic matter and fungal exploration of apatite P minerals are sufficient to ameliorate P limitations, including more efficient resorption of P during abscission of the needles.

4.7 The ecosystem carbon sink

A complete assessment of the impact of N deposition on the ecosystem C balance requires consideration of both the above and below ground responses to N additions. A recent study estimated the woody biomass production to N additions at the Scots pine site, which enables an assessment of the ecosystem C sink at the Scots pine site (Lim *et al.*, 2017). In that study, a maximum N use efficiency for woody biomass production of $14 \text{ kg C kg}^{-1} \text{ N added}$ was attained at a theoretical N addition rate of $35 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, i.e. close to the highest N addition rate. Combined with the $21.4 \text{ kg C kg}^{-1} \text{ N}$, averaged across all N addition rates (Fig. 6A) in the organic layer gives an ecosystem C sink of $35.4 \text{ kg C kg}^{-1} \text{ N}$. This value should be considered a maximum, as the above-ground tree response to the low N treatments was considerably lower per unit of N, as is reflected in the needle litter production data (Fig. 7). Moreover, including other components of vegetation, such as mosses and ericaceous shrubs, which frequently decrease in response to N, would further reduce the response of the vegetation (Gundale *et al.*, 2014). These values can be compared with the corresponding values for the Norway spruce site, where the vegetation, including understory vegetation, accumulates on average 16, or including only

tree biomass, gives 24 and 20 kg C kg⁻¹ N added in the 12.5N and 50N treatment, respectively (Gundale *et al.*, 2014). Together with the 10 kg C kg⁻¹ N that accumulates in the organic layer, the ecosystem C sink has been estimated to be 26 kg C kg⁻¹ N for that site, or including only woody biomass production in the 50N treatment gives 36 kg C kg⁻¹ N added (Gundale *et al.*, 2014, Maaroufi *et al.*, 2015), which is similar to the 35.4 kg C kg⁻¹ N for the Scots pine site.

These results show that the soil C accumulation response may be relatively more important than accumulation of C in aboveground biomass when the input of N is low (<12 kg N ha⁻¹ yr⁻¹) and short-term (< 13 years), although continued input of N is likely to increase the fraction of N that is available for vegetation. Other limitations are likely to constrain the uptake and storage of C in the vegetation, including self-shading due to high stem density, or elemental limitation such as of P (but see section 4.6). However, these limitations do not appear to lower soil C accumulation response to N deposition as the N treatments continued to decrease mass loss of needles, and increased mass gain of humus, still after 20 years of N additions up to 50 kg N ha⁻¹ yr⁻¹, despite that the above-ground growth response leveled off (From *et al.*, 2016). In summary, this thesis provides empirical support for the positive effect of N deposition on the ecosystem C balance, at a rate that is nearly one order of magnitude lower than the highest estimates (Holland *et al.*, 1997, Magnani *et al.*, 2007), and instead, more within the range of 20-40 kg C kg⁻¹ N reported in a broad meta-analysis of N addition experiments and N deposition gradients by de Vries *et al.* (2009).

5 Conclusions

There have been lengthy discussions about the size of the C sink of the northern hemisphere (Ciais *et al.*, 2019, Holland *et al.*, 1997, Myneni *et al.*, 2001, Pan *et al.*, 2011, Reay *et al.*, 2008), and how various global change drivers affect it (Winkler *et al.*, 2019, Zhu *et al.*, 2016). This thesis investigated the impact of N deposition on the C balance of soils in northern coniferous forests and found support for that N additions can increase their C stocks. However, only the highest N addition rate increased C stocks significantly, whereas the lower N addition rates, simulating current N deposition rates, had no effect. The significant effect of N on soil C stocks was associated with a suite of responses that contributed to increase the input of C to the soil, and decrease the outputs.

Study I and II showed that N increased the production of fine-root and needle biomass, and decreased the autotrophic soil respiration, which indicates that N enables a higher efficiency in plant biomass production. Study III showed that decomposition decreased with N as an overall effect across substrate types (needle and humus) and trenching treatments (presence and absence of tree roots), but decreased with N more strongly inside than outside trenches. This indicates that changes in tree-root C use is not the only mechanism leading to soil C accumulation in response to N. The standing biomass of both fungi and bacteria generally decreased with N both inside and outside trenches, which correspond to the decrease in decomposition. A decline in microbial biomass outside trenches can be explained by changes in C supply, however, the decline in microbial biomass in the absence of tree-roots needs further explanation. The abundance of actinobacteria frequently increases with N additions, which correspond with a decreased decomposition. Their role as antagonists to other soil microbes in response to N, and their capacity generate microbial litter products in N enriched environments, warrant further study.

Study IV and V show that N decreased the activity of enzymes involved organic N uptake in recalcitrant organic matter, and increased the activity of enzymes involved in cellulose degradation, which corresponded with an

increased mass gain of N during decomposition, and higher final mass of C in both needle and humus substrates. The activity of enzymes involved in P acquisition and the mass loss of P in needles and humus was unaffected by N, and similarly, the microbial exploration of mineral soil P sources were not enhanced by N, which shows that P cycling was relatively unaffected by N additions.

Thus, the overarching hypothesis that a reduction in tree C allocation to roots in response to N enrichment would enable a higher production of above-ground biomass, and decrease the input of C via roots needs to be modified. Rather than an overall decrease in belowground C allocation, N appears to have changed the relative allocation between fine-root production and EMF biomass production and led to a decrease in their collective respiration. The shift in C allocation thus occurred primarily in the root-zone, rather than between above and below ground, and may have led to a decrease in the supply of labile C to soil fungi. This effect can, however, not alone explain the decrease in microbial biomass and decomposition as N decreased these both inside and outside root trenches, and other factors are likely at play.

The rate of C accumulation per unit of N added in the two long-term, low N addition experiments used in this thesis is one order of magnitude lower than the highest published estimates (Holland *et al.*, 1997, Magnani *et al.*, 2007) but within the range of estimates published more recently (de Vries, 2009, de Vries *et al.*, 2014, Sutton *et al.*, 2008). As N deposition is relatively low in most of the boreal region, it is likely to have a minor impact on the global C balance, and other factors such as management practices, and changes in precipitation and temperature, are likely to be relatively more important than N deposition for the C balance of the boreal forest.

6 Outlook

This thesis has shown, as has several other studies, that the standing microbial biomass in the soil decrease with N additions, despite the high demand of N in microbial biomass and the low availability of N in the coniferous soil environment. Improving estimates of the production of microbial biomass directly in the field would be a top priority as this aspect of the microbial biomass is likely to be more dynamic than the standing biomass, and even a relatively small standing biomass can produce large amounts of litter. More studies are needed to clarify how changes in N availability affect the synthesis of various microbial products, including biomass components with slow turnover in soils, as well as e.g. enzymes and organic acids that contribute to the degradation of soil organic matter.

Nitrogen additions increases soil C stocks but we do not know in what form and what specific mechanisms preserve this C. The rate of C accumulation per unit of N is frequently within a range compatible with microbial necromass, however, microbial biomass frequently decreases and several indices instead point towards inhibition of lignin decomposition. The mechanisms matter, because if it is mainly microbial residues that store N in relatively easily accessible forms, it may act as a nutrient reservoir and support higher productivity for a long time. If C and N is stored mainly in recalcitrant forms, N may rapidly become limiting again when N deposition declines, and the eventual mobilization of N from this organic matter may lead to loss of the associated C. This effect may become even stronger as the atmospheric CO₂ concentrations continue to increase, and thus potentially exacerbating N limitations. Decomposition was lower in the presence of tree roots and changes in activities of tree roots, such as of higher CO₂, may suppress decomposition even more, while other factors such as windthrow or harvest may have the opposite effect. Most of the added N was retained in the organic layer together with C, and these are susceptible to forest fires and management practices, whereas it may be preserved more permanently by interactions with mineral soil particles deeper in

the soil. More studies are needed on the factors controlling the movement of C from the organic layer into the mineral soil, where it may be preserved for millennia. It is also relevant to study the movement of C and nutrients from forest soils into freshwaters, as it may have undesirable impacts on water quality. Freshwaters has become browner in Europe and North America where acid and N deposition has decreased, and one question that warrants study is whether the decrease in N deposition may lead to leaching of soil organic matter and brownification of freshwaters.

One of the experiments used in this thesis was established already in 1996 to study the impacts of N on ground flora. This has made it possible to test novel hypotheses in a long-term perspective. Collective efforts to maintain experiments like these are crucial to test future research questions. Similarly, long-term climate and environmental monitoring are important to support and develop to be able to investigate trends and relationships that occur at scales beyond what may be feasible to test experimentally. More studies at landscape scale would be useful to provide a more diverse and complete view of how e.g. changes in N input, climate, and management decisions affect nutrient and greenhouse gas balances at larger scales. Watershed study systems such as Krycklan in Sweden and Hubbard Brook in the USA are particularly useful, as they have clear boundaries yet are large enough to be representative for a relatively large area of the Earth's surface. Collective efforts to close C, nutrient, and energy budgets with top-down and bottom-up approaches can be useful to cross-validate methods and to identify processes that may be important at landscape scale but not visible in isolated sub-ecosystems such as a forest or a lake.

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This thesis explores the role of nitrogen deposition in enhancing the carbon sink of coniferous forest soils. Using nitrogen addition experiments, the result presented in this thesis show that nitrogen deposition contributes to enhancing the soil carbon sink. This effect occurs via a suite of responses, including increased input of both above and below ground tree litter, a decrease in soil respiration and decomposition, and shifts in microbial community composition.

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