The Effect of Simulated Anthropogenic Nitrogen Deposition on the Net Carbon Balance of Boreal Soils

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Abstract

Anthropogenic activities have globally increased nitrogen (N) deposition and carbon dioxide (CO_2) gas emissions. It is proposed that anthropogenic N deposition may increase the size of boreal forest CO_2 sink, because boreal ecosystems are N limited. Despite studies that have helped to clarify the magnitude by which N deposition enhances carbon (C) sequestration in the vegetation, there remains a paucity of studies evaluating how soils respond. This thesis aim to clarify the magnitude to which C sequestration in boreal forests responds to N enrichments, including rates that realistically simulated N deposition ($\leq$ 12.5 kg N ha^{-1} yr^{-1}). This work was conducted in two long-term experiments in northern Sweden. The N treatments consisted of ambient, low N addition (3-12.5 kg N ha^{-1} yr^{-1}) and high N addition (50 kg N ha^{-1} yr^{-1}) rates, in a Norway spruce and a Scots pine forest, maintained since 1996 and 2004, respectively. The organic soil C pool positively responded to N enrichment, especially at the high N addition level. This increase corresponded to a relationship between C sequestration and N addition of 10 kg C kg^{-1} N. Further, low N addition treatments had no effect on microbial biomass and soil respiration (i.e. soil C outputs, microbial activity), while high N addition decreased total microbial, ectomycorrhizal fungal biomasses and soil respiration. The actinomycetes were the only microbes showing an increase with N addition. Annual litter production showed a minor impact on aboveground litter C inputs. Only mosses were the only major litter component showing significant quantitative and qualitative changes in response to N additions. Further, litter quality mediated by N enrichment was not the main driver of litter decomposition, while shifts in soil microbes strongly influence the early stages of litter decomposition. Low N addition rates had little effect on litter and humus decomposition, whereas high N addition rates impeded the early stage of decomposition of both substrates. The decline of litter decomposition appeared to be mediated by shifts in the abundance or community structure of saprophytic organisms, while the decrease of humus decomposition was likely the result of reduced ectomycorrhizal fungi. Altogether, the results of this thesis show that long-term N inputs simulating current atmospheric N deposition in the boreal region are likely to have subtle effects on the soil C balance and therefore on soil C accumulation.

Keywords: boreal forest, carbon sequestration, soil respiration, litter, humus, PLFA, litter bags, ectomycorrhizae, ecological stoichiometry, root-exclosure

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Effekten av Simulerad Kvävedeposition på den Boreala Skogsmarkens Nettokolbalans

Abstrakt

Över hela världen ökar depositionen av kväve (N) och emissionerna av koldioxid till följd av människans aktiviteter. Eftersom tillväxten i boreala skogar är kvävebegränsad, kan kvävedeposition leda till att skogens upptag av koldioxid ökar. Många studier har gjorts för att klargöra hur kvävedeposition bidrar till att kol (C) binds i skogens vegetation, medan det fortfarande saknas studier för att vi säkert ska veta hur själva marken reagerar på kvävedepositionen. Syftet med denna avhandling är att klargöra i vilken utsträckning kolupptaget i boreal skog påverkas av kvävetillförsel i nivåer som realistiskt simulerar kvävedeposition ($\leq$ 12.5 kg N ha^{-1} $år^{-1}$). Arbetet utfördes i två långliggande fältforskningsförsök i norra Sverige. Kvävebehandlingarna var låg (3-12.5 kg N ha^{-1} $år^{-1}$) och hög (50 kg N ha^{-1} $år^{-1}$) kvävetillförsel till två skogsekosystem, ett grandominerat som kvävebehandlats sedan 1996, och ett talldominerat som kvävebehandlats sedan 2004. Resultaten visar att organiskt kol i marken ökade vid kvävetillförsel, särskilt vid hög sådan. Ökningen korresponderade till en ökning av markkol med 10 kg C kg^{-1} N. Låg kvävegiva hade ingen effekt på markens mikrobiella biomassa eller på markrespirationen, medan hög kvävegiva minskade den totala mikrobiella biomassan, biomassan av ektomykorrhizasvampar och markrespirationen. Actinomyceter var de enda markmikrober som ökade med kvävegivan. Den årliga förnaproduktionen var inte särskilt påverkad av kvävegivorna. Mossorna var den enda förnafraktionen som reagerade, och både kvantitet och kvalitet på förnan ändrades av kvävebehandlingarna. Den tidiga nedbrytningen av förna som sker under de två första åren styrdes av kväveinducerade förändringar hos markens mikrobsamhälle och inte av förnakvaliteten. De låga kvävegivorna hade dock endast små effekter på förna- och humusnedbrytningen, medan den höga kvävegivan kraftigt hindrade den tidiga nedbrytningen av bägge substraten. Den minskade förnanedbrytningen verkade bero på förändringar vad gällde abundansen av saprofytiska svampar i marken till följd av den höga kvävebehandlingen, medan den minskade humusnedbrytningen troligen var ett resultat av en reducering av ektomykorrhizasvamparna. Sammanfattningsvis visar resultaten från denna avhandling att långsiktig kvävebehandling som simulerar kvävedeposition i det boreala området troligen bara har små effekter på markens kolbalans och kolackumulering.

Nyckelord: boreal skog, kolbindning, markrespirationen, förna, humus, PLFA, förna nedbrytning, ektomykorrhiza, ekologisk stökiometri

Effet des Dépôts Anthropiques Azotés Simulés sur l´Équilibre Net du Carbone des Sols Boréaux

Résumé

Les activités anthropiques ont globalement augmenté les dépôts azotés (N) ainsi que les émissions de dioxyde de carbone (CO_2). Il a été proposé que les dépôts d´N puissent augmenter la taille du puits en C des forêts boréales car ces écosystèmes sont limités en N. Bien que de nombreuses études aient aidé à clarifier la magnitude à laquelle les dépôts d´N influencent la séquestration du carbone (C) au sein de la végétation, il subsiste encore d'importantes incertitudes sur les capacités de stockage en C des sols. Le but de cette thèse vise à clarifier la magnitude à laquelle la séquestration du C dans les forêts boréales est impactée par l´enrichissement en N, en utilisant des doses qui simulent de façon réaliste les dépôts d'N ($\leq$ 12.5 kg N ha^{-1} yr^{-1}). Ce travail a été réalisé dans deux sites expérimentaux du nord de la Suède, sur une pessière et une pinède fertilisées annuellement depuis 1996 et 2004, respectivement. Les parcelles ont reçu les traitements suivants: contrôle, faibles (3-12.5 kg N ha^{-1} yr^{-1}) et fortes (50 kg N ha^{-1} yr^{-1}) doses en N. Parmi les horizons du sol, seule la fraction en C de l´horizon organique a répondu positivement à l'enrichissement en N avec une quantité en C séquestrée de 10 kg C kg^{-1} N. Seul l'ajout de fortes doses d'N a diminué la biomasse microbienne totale, celle des champignons ectomycorhiziens ainsi que la respiration du sol (i.e. sortie de C, activité microbienne), alors que les actinomycètes ont répondu positivement aux fortes doses d´N. La production en litière a eu peu d´effet sur les entrées de C du sol. Seule la litière de mousses a montré des changements qualitatifs et quantitatifs en réponse à l´N. En outre, la qualité de la litière par l´intermédiaire de l´enrichissement en N ne fut pas le principal driver de la décomposition de la litière, alors que les changements microbiens ont influencé les premières étapes de celle-ci. Seul l'ajout de fortes doses d'N a diminué les premières étapes de décomposition de la litière et de l´humus. Le déclin de la décomposition de la litière était lié aux changements d´abondance ou de structure des communautés des champignons saprotrophes, alors que le déclin de la décomposition de l´humus était lié à la diminution des champignons ectomycorhiziens. Cette thèse démontre que les intrants d´N simulant les dépôts azotés atmosphériques dans la région boréale ont vraisemblablement peu d´effet sur l´équilibre carboné du sol et ainsi sur la séquestration du C.

Mots clefs: forêt boréale, séquestration du carbone, respiration du sol, litière, humus, PLFA, décomposition de la litière, ectomycorhizes, stœchiométrie écologique, exclusion racinaire

Dedication

To everyone who made this journey possible.

Aimer un Papou, un enfant ou son voisin, rien que de très facile. Mais une éponge ! Un lichen ! Une de ces petites plantes que le vent malmène! Voilà l´ardu: éprouver une infinie tendresse pour la fourmi qui restaure sa cité.

Dans les forêts de Sibérie, Sylvain Tesson (2011)

Contents

List of Publications

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

I Maaroufi, N.I., Nordin, A., Hasselquist, N.J., Bach, L.H, Palmqvist, K. and Gundale, M.J. (2015). Anthropogenic nitrogen deposition enhances carbon sequestration in boreal soils. *Global Change Biology* 21(8), 3169-3180. DOI: 10.1111/gcb.12904

II Maaroufi, N.I., Nordin, A., Palmqvist, K. and Gundale, M.J. (2016). Chronic nitrogen deposition has a minor effect on the quantity and quality of aboveground litter in a boreal forest. *PLOS ONE* 11(8), e0162086. DOI: 10.1371/journal.pone.0162086

III Maaroufi, N.I., Nordin, A., Palmqvist, K. and Gundale, M.J. (2016). Nitrogen enrichment impacts on boreal litter decomposition are driven by changes in soil factors rather than litter quality (in review).

IV Maaroufi, N.I., Nordin, A., Palmqvist, K., Hasselquist, N.J., Forsmark, B., Rosenstock, N., Wallander, H. and Gundale, M.J. Shifts in ectomycorrhizal and saprophytic fungal communities mediate litter and humus decay in response to chronic nitrogen enrichment (manuscript).

My contribution to the papers included in this thesis was as follows:

I Major participation in planning, field sampling, experimentation, data analysis and writing.

II Major participation in planning, field sampling, experimentation, data analysis and writing.

III Major participation in planning, field sampling, experimentation, data analysis and writing.

IV Major participation in planning, field sampling, experimentation (except sequencing), data analysis and writing.

Abbreviations and definitions

%	Percentage
°C	Degree Celsius
AM	Arbuscular mycorrhizae
Biotrophs	Organisms that use new photosynthates obtained directly from the plant host as C source, for example, mycorrhizal fungi.
BNF	Biological dinitrogen fixation
C	Carbon
ca.	*Circa* (about)
cm	Centimetre (10^{-2} meter)
CO_2	Carbon dioxide
D-	*Dexter* "right"
DCA	Detrended Correspondence Analysis
DNA	Deoxyribonucleic acid
DON	Dissolved organic nitrogen
e.g.	*Exempli gratia*
EM	Ectomycorrhizae
Fenton reaction	Reaction between hydrogen peroxide (H_2O_2) and iron (Fe^{2+}) that generates highly-oxidizing radicals such as superoxide ($O_2^{\cdot -}$).
g	gram
Gadgil effect	The competition (e.g. for N, water) between saprotrophic and mycorrhizal fungi that leads to the decline of organic matter decomposition rate.
h	hour
Ha	Hectare (10 000 m^2)
HNO_3	Nitric acid
Hylocomium splendens	Glittering wood moss, stair-step moss (husmossa)
i.e.	*Id est*
kg	Kilogram (10^3 grams)
L-	*Laevus* "left"
m^2	Square meter
Mg	Megagram or tonne (10^6 grams)
min	minute
ml	Millilitre (10^{-3} liters)
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide

ng	Nanogram (10^{-9} gram)
NH_4NO_3	Ammonium nitrate
NH_y	Reduced nitrogen
NO_x	Oxidized nitrogen
N_r	Reactive nitrogen
P	Phosphorus
PCA	Principal Component analysis
Pg	Pentagram (10^{15} grams)
Picea abies	Norway spruce (gran)
Pinus sylvestris	Scots pine (tall)
Pleurozium schreberi	Red-stemmed feathermoss (väggmossa)
PLFA	Phospholipid fatty acid
Priming effect	The increase of soil organic matter turnover caused by the addition of easily decomposable organic compounds in the soil (e.g. root exudates).
RDA	Redundant Analysis
RNA	Ribonucleic acid
rpm	Revolution per minute
SLA	Surface leaf area
Tg	Teragram (10^{12} grams)
Vaccinium myrtillus	Bilberry (blåbär)
Vaccinium vitis-idaea	Lingonberry (lingon)
yr	year
µl	microliter
µm	micrometer

1 Introduction

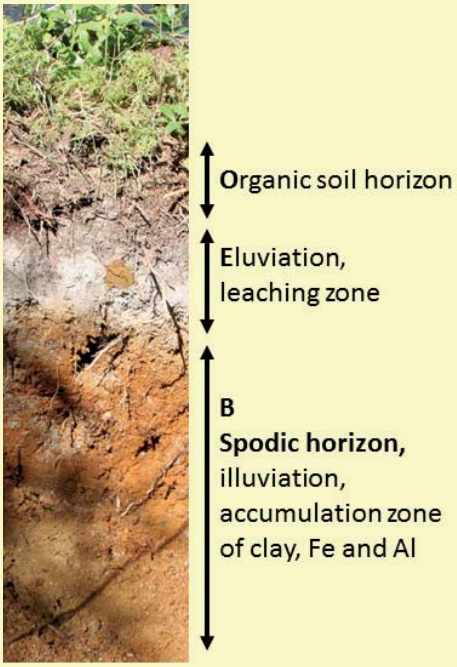
1.1 Boreal forests and nitrogen limitation

Boreal forests cover approximately 10-15% of the terrestrial land surface area and form a circumpolar belt across North America and Eurasia (Lal 2005). The climate is cold and characterized by a short plant growing season with strong seasonal variation (Bonan & Shugart 1989). The plant community is relatively species poor and composed primarily of coniferous trees and to a lesser extent by deciduous tree species, with the understory vegetation mainly composed of ericaceous shrubs, bryophytes and lichens (Arnborg 1990). Most of the boreal zone, including Scandinavian boreal forests, grow on soil in the Spodosol order (i.e. Podzols), which are characterised by a spodic mineral horizon (Brady & Weil 2002), see box 1. Spodosols usually have a low pH, low mineral nutrient content, slow decomposition rates and high surface organic matter content.

Box 1. Spodosol profile

Spodosols are characterized by a spodic horizon where organic C, precipitated oxides of aluminum and iron accumulate which give this typical orange reddish color.

Spodosols occur mainly under coniferous forest or heath vegetation where litter (e.g. needles, ericaceous leaves) is rich in acidic compounds. During litter decomposition, those acidic compounds are released and react with iron and aluminum which are then leached and carried downwards until they precipitate in the spodic horizon. The leached layer, also referred to as the E horizon, is characterized by minerals such as quartz that are resistant to eluviation.



The high degree of stratification in these soils is in part due to the absence of soil fauna capable of mixing and redistributing newly produced surface carbon (C) into the deeper mineral horizons (Buol et al. 1997). Interestingly, boreal forests are the second largest C pool in terrestrial biomes after tropical forests and represents ~38% of the total C pool worldwide (Lal 2005). Boreal ecosystems store a substantial amount of their C in standing vegetation (~88 Pg C); however, to a greater extent they store C in the soil (~471 Pg C) (Malhi et al. 1999; Lal 2005). Such large stores and uptake of C play an important role in the global C cycle (Bonan 2008).

Many ecosystems in cold climate regions, such as boreal forest, are strongly limited by nitrogen (N) availability (Tamm 1991; Vitousek & Howarth 1991). Nitrogen plays a key role in organism maintenance and growth. For instance, N is an important constituent of proteins, amino acids, nucleic acids (e.g. DNA and RNA), thus N limitation may limit primary production. The sources of N that can be utilized by living organisms are collectively called reactive nitrogen (N_r) and includes inorganic reduced N (NH_y), oxidized N (NO_x, HNO_3, N_2O) forms, and organic N compounds (proteins, amines, urea) (Bobbink et al. 2010; Erisman et al. 2011). In boreal forests, N_r derives from three main sources, biological dinitrogen (N_2) fixation (BNF), litter decomposition, and atmospheric deposition (IPCC 2013) (Fig. 1).

Although N_2 (also called non-reactive N) is the most abundant constituent of the atmosphere (~78% air volume), this pool remains mostly inaccessible to non N_2-fixing organisms. In boreal forest, several bryophytes belonging to feather mosses (e.g. *Hylocomium splendens*, *Pleurozium schreberi*) serve as hosts for epiphytic N_2-fixing cyanobacteria (e.g. *Nostoc* sp.) (Zackrisson et al. 2009). Feather mosses provide a surface for cyanobacteria to grow on, and may potentially supply them with C, although this is yet to be clearly shown. The feather mosses in turn receive N from the cyanobacteria, but it remains unclear whether this is an intentional transfer or whether they receive N solely because of their intimate location (Lindo et al. 2013; Bay et al. 2013; Gundale et al. 2012). Further, BNF is constrained by low temperatures occurring in northern ecosystems, partly due to N_2-fixing enzymes having an optimum activity at 25 °C (Houlton et al. 2008). In boreal forests, current BNF rates have been estimated to 0.01-3.5 kg N ha^{-1} yr^{-1} (Lindo et al. 2013; Stuiver et al. 2015).

Natural atmospheric N_r deposition is also a source of N_r that comes from lighting and wildfire events (i.e. NO_x). In the absence of anthropogenic contribution, N_r deposition rates are low and have been estimated to ~0.5 kg N ha^{-1} yr^{-1} (Erisman et al. 2008).

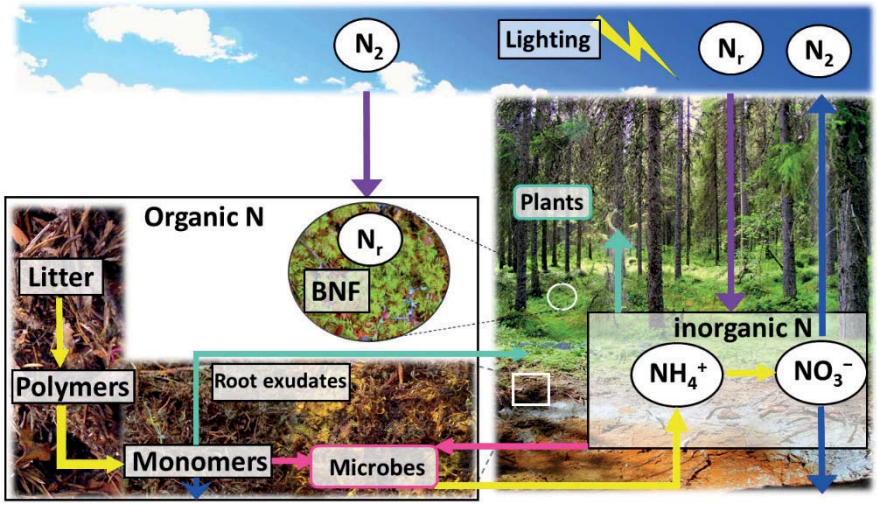


Figure 1. Nitrogen sources and cycling in boreal forests. The left box is a zoom in of fluxes in the organic soil horizon and in the moss layer (i.e. BNF). Yellow arrows represents processes mainly carry out by microbes. Pink arrows represents N uptake by microbes, while turquoise-blue arrows represent plant uptake. Purple arrows represent N inputs through N_2 fixation and atmospheric N_r deposition (natural and anthropogenic). The dark blue arrows are N outputs through denitrification process and leaching events.

Boreal soils hold a high organic matter content, and thus represent a large reservoir of N, mainly found in organic forms (e.g. proteins, amino acids) (Näsholm et al. 2009). A great part of this organic N comes from aboveground plant litter. This litter contains highly recalcitrant compounds such as lignin or phenols derived from coniferous trees (e.g. needles, stems, branches), ericaceous shrubs (e.g. waxy leaves) and feather mosses that have high C:N ratios (DeLuca & Boisvenue 2012), see also Box 2. These inputs can often be easily identified within the soil organic horizon because they decompose so slowly. During intermediate steps of litter decomposition by extracellular microbial enzymes (e.g. depolymerisation processes), large litter compounds are cleaved into monomers and join the dissolved organic N pool (i.e. DON). A part of this DON is thus readily used by plant, or further immobilized or mineralized by microbes (Schimmel & Bennett 2004; Rennenberg et al. 2009). However, a great part of DON compounds are recalcitrant, too complex to be cleaved by a sole enzyme and are too large to cross cellular membranes (Carreiro et al. 2000; Neff et al. 2003). Thus, the depolymerisation rates of these N rich compounds into bioavailable forms is a critical step in the soil N cycle that controls N availability to plants (Schimmel & Bennett 2004). Altogether, low BNF, slow litter decomposition and low natural atmospheric N_r deposition rates are believed to contribute to N limitation in boreal forest ecosystems.

1.2 Anthropogenic nitrogen deposition

1.2.1 Origins of anthropogenic nitrogen deposition at a global scale and in Sweden

During the 20th century, anthropogenic activities have increased the quantity of N_r emitted into the atmosphere, which enter the biosphere via deposition (IPCC 2013). Those anthropogenic N_r emissions derived from the production and use of N fertilizer in managed lands (e.g. crop lands, managed forests) and occur in NH_y forms, whereas burning of fossil fuel by internal combustion engines and industrial activities generate NO_x forms (Fowler et al. 2013). Once in the atmosphere, anthropogenic N_r compounds are highly mobile and have a residence time of a few weeks, with the exception of the long-lived greenhouse gas nitrous oxide (N_2O) that can persist over a century (Lamarque et al. 2013; Fowler et al. 2013). Although there is a portion of N_r compounds that may be transformed within the atmosphere, most of the NO_x and NH_y follow winds and transferred back to the biosphere via N_r deposition (Galloway et al. 2003). It has been estimated that total global NO_x and NH_y deposition have increased by more than 3-fold since the mid-19th century (from 32 to 100 Tg N yr^{-1}), and current predictions estimate total NO_x and NH_y deposition would reach 200 Tg N yr^{-1} by 2100 (Fowler et al. 2013; Galloway et al. 2004).

In Sweden, current N_r deposition rates range from ~0.5-15 kg N ha^{-1} yr^{-1} with an increasing N_r deposition gradient from North to South-West Sweden (Gundale et al. 2011; Pihl Karlsson et al. 2011). In northern areas, NO_x deposition predominates, while in Southern parts of the country, NH_y deposition prevails, but on average a ratio of 1:1 NO_x:NH_y deposition is observed (Lövblad et al. 2000). Interestingly, only ~10-20% of the total NO_x deposition are originated from Sweden, with the rest coming from other EU state members such as Germany, Poland, and Britain. The same pattern is observed with NH_y deposition, except that atmospheric deposition originating within Sweden is highly variable ranging from 10-44% depending upon the region (i.e. a positive gradient north-south) (Lövblad et al. 2000). Since the end of the 20th century, total N_r deposition rates have on average remained unchanged or increased in the northern part of Sweden, while N_r deposition rates declined in the southern half of Sweden by 25% (~2.1 kg ha^{-1} yr^{-1}) (Lucas et al. 2016; Lövblad et al. 2000). This later is consistent with the decreasing trend observed in western central Europe at the same period (Waldner et al. 2014; Binkley & Högberg 2016).

1.2.2 An historical and human perspective on nitrogen fertilizer and fossil fuel combustion

In the early 1900s, the human population was expanding which resulted in an increase demand for food and fiber products. Natural N_r sources were not enough to sustain the intensification of agriculture (Galloway et al. 2002). During the same period, two German chemists, F. Haber and C. Bosh developed a method to artificially convert the almost unlimited atmospheric N_2 stock to ammonia (NH_3) at industrial scale. This Haber-Bosch process was primarily established to face the demand of nitrate to manufacture munitions during the World War I (Galloway et al. 2013). It was only in the second half of the 20th century that this process became widely used to produce synthetic fertilizers, and at that time it even surpassed natural BNF in unmanaged terrestrial ecosystems (Erisman et al. 2008). Today, the production of N fertilizers accounts for feeding approximately 50% of the global human population, although strong inequalities exist among countries due to geopolitical, financial and environmental factors (Erisman et al. 2008; Galloway et al. 2013). In parallel, the production of fossil fuel based energy also increased with the expansion of human population through industrialization and mechanization, however to a lesser extent than food production (Galloway et al. 2004). Until the late 19th century, most of the energy was still produced by biofuel combustion (e.g. wood) which also emits carbon dioxide (CO_2) in the atmosphere. It was only at the beginning of the 20th century that biofuel was replaced by fossil fuels in supplying energy (e.g. coal, oil, gas) (Smil 2004). The society started to become more aware of the various negative impacts on human health and ecosystems (see Erisman et al. 2013). As such, governments started to implement new legislations in an attempt to limit the negative impact of anthropogenic N_r on human health and ecosystems, such as the Gothenburg protocol adopted in 1999 in Europe (Erisman et al. 2008; Fowler et al. 2013).

1.3 Impact of anthropogenic nitrogen on carbon sequestration

Anthropogenic N_r can have a myriad of consequences within the atmosphere, hydrosphere and biosphere during its transfers and transformations (Galloway et al. 2002). A N_r molecule can cause a series of impacts along its biogeochemical pathways, which is also referred to as the "N cascade" (Galloway et al. 2003). In boreal forest, atmospheric N_r deposition can have a multitude of consequences, including a shift in plant species composition towards more fast growing nitrophilous species (e.g. the graminoids, grasses) at the expense of slow-growing species (e.g. ericaceous dwarf-shrubs) (Bobbink et al. 2010). Further, anthropogenic N_r deposition has been reported to impact aboveground

food webs by increasing plant antagonists (pathogens and herbivores) (Nordin et al. 1998), and also soil food webs by shifting the fungal energy channel typical in boreal forest to a bacterial energy channel (Meunier et al. 2015).

Another impact of high N_r deposition rates is an increase of C sequestered in non-agricultural ecosystems (Erisman et al. 2008). In the last decade, there has been intense interest and debate in understanding whether anthropogenic N_r deposition leads to changes in forest C sequestration (Magnani et al. 2007; de Vries et al. 2008). The role of forests as C sinks is of particular interest because C can be stored for a long period in stem wood, and the C:N ratios of woody tissues can be high, which allows large amount of CO_2 to be fixed per unit N taken up by trees (Pan et al. 2011; Thomas et al. 2010). There has been a major focus to quantify the strength of the ratio of C sequestered per unit N_r deposition (i.e. N-use efficiency) especially in northern latitudes, where N_r deposition may alleviate N_r limitation and hence, enhance productivity and C sequestration (Gruber & Galloway 2008; Schlesinger 2009; de Vries 2014; Fernández-Martínez et al. 2014). This is also of particular interest because in parallel of N_r emissions, anthropogenic activities have also increased the emission of greenhouse gas carbon dioxide (CO_2) by more than 40% since the end of the 18th century (from 589.5 to 828 Pg C) due to the increase of fossil fuel combustion, cement production and net land use change (IPCC 2013). As such, anthropogenic N_r deposition may increase the size of CO_2 sinks, such as forest ecosystems, which could be used by industrialized countries to meet their CO_2 emissions mitigation commitments (Reay et al. 2008; Myneni et al. 2001).

Experimental and modelling studies have suggested sequestration rates as much as 108-500 kg of C sequestered per kg of N deposition (Holland et al. 1997; Magnani et al. 2007; Eliasson & Ågren 2011). These estimates are of interest because approximately 15 to 30% of the annual CO_2 emissions are sequestered into an unknown terrestrial sink, and several studies have suggested that N deposition in northern forests may account for a large portion of this missing C sink (Reay et al. 2008; Myneni et al. 2001; Magnani et al. 2007). Few long-term experiments (> 10 years) using realistic levels of N_r deposition in boreal and temperate forests have shown much lower C sequestered to N added, ranging from 5-30 kg C kg N^{-1} (Hyvönen et al. 2008; Pregitzer et al. 2008; Sutton et al. 2008; de Vries et al. 2009; de Vries et al. 2014; Gundale et al. 2014). Although these studies help to clarify the magnitude by which anthropogenic N_r deposition enhances C sequestration in the aboveground plant biomass, there are a lack of studies assessing whether similar changes occur in the soil (but see Högberg et al. 2006; Hyvönen et al. 2008; Pregitzer et al. 2008; Frey et al. 2014). These later studies showed contrasting results, where soil C accumulation was equal or greater than aboveground biomass in northern temperate forests

(Pregitzer et al. 2008; Frey et al. 2014), while the opposite pattern has been shown in boreal forests in response to long-term, high N dose additions (Högberg et al. 2006; Hyvönen et al. 2008). Boreal and temperate forest are composed of plant communities that are likely to respond differently to anthropogenic N_r deposition and, hence may influence how the soil C pools responds. Further, uncertainty remains regarding the magnitude to which N_r deposition enhances C sequestration in soils because earlier studies estimating this relationship have applied N at rates several times greater than upper N deposition rates in each region of study. Furthermore, N addition experiments are often located in areas with high background N_r deposition rates, making difficult to isolate the impact of N addition treatments (de Vries et al. 2009). The following sections will focus on several aspects of N impacts of C dynamics that are relevant for my research focus, namely mechanisms through which anthropogenic N_r deposition may influence soil C dynamics.

1.4 Potential mechanisms

1.4.1 Increase of litter inputs and change in litter quality

One of the several mechanisms by which N_r deposition is thought to enhance soil C accumulation is by increasing plant growth, thereby potentially increasing aboveground litter inputs to soil (Janssens et al. 2010). Several studies have shown that N addition can cause coniferous trees to increase their canopy leaf area, while also reducing the life span of their needles (Schaberg et al. 1997; Nohrstedt 2001; Bauer et al. 2004; Krause et al. 2012). Understory vegetation such as ericaceous dwarf-shrubs and bryophytes also contribute substantially to litter production, and may be impacted by N addition. Several studies have shown that production of these understory components can decline in response to N addition, likely resulting in reduced litter production, that may offset increases of canopy litter (Gundale et al. 2013; Palmroth et al. 2014). A meta-analysis by Janssens et al. (2010) reported that relatively high dose (37-290 kg N ha^{-1} yr^{-1}) N fertilization experiments have highly variable effects on aboveground litter inputs, suggesting substantial uncertainty remains regarding the impact of N deposition on aboveground litter production at realistic N addition levels.

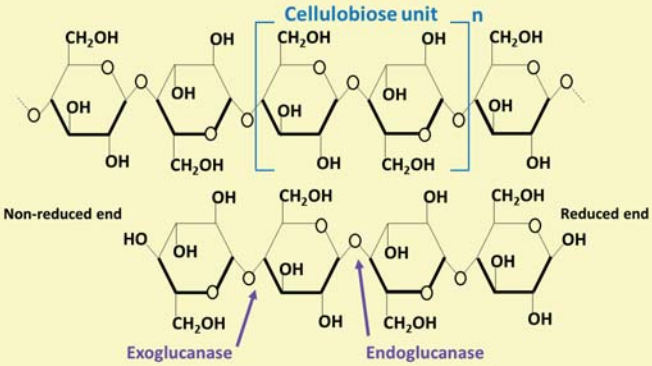
Nitrogen enrichment may also alter the quality of litter entering the soil, which in turn could influence litter decomposition rates, and soil C accumulation and stability (Knorr et al. 2005). In boreal forests, N fertilization has been reported to increase foliar N concentrations, as well as concentrations of other elements (Berg & Matzner 1997). It is also well known that elemental ratios such as C:N control litter decomposition rates (Aerts 1997; Zhang et al. 2008).

Further, lignin and its ratio with N or cellulose have been shown to be good predictors of litter decomposition rates as lignin is a more recalcitrant compounds than cellulose (Entry & Backman 1995; Berg & McClaugherty 2008), see Box 2. Some studies reported a reduction in lignin concentrations in response to N enrichment (Haukioja et al. 1998; Waring et al. 1985), thereby potentially accelerating decomposition and reducing soil C accumulation rates (Cornwell et al. 2008). In contrast, more recent studies argued that this enhancement of litter quality in response to N enrichment may instead increase soil C sequestration, through the increase of degradation efficacy of microbes (Cotrufo et al. 2013; Cotrufo et al. 2015). Soil microbes may degrade and immobilize a greater portion of litter products (e.g. labile compounds) in their biomass, which potentially accumulate and further would stabilize within the mineral soil fraction (Cotrufo et al. 2013; Kaiser & Kalbitz 2012). On a different note, a recent study on deciduous trees reported an increase of leaf N content in response to N enrichment in boreal forest, which resulted in an outbreak of fungal pathogens and more frequent herbivore attacks (Bandau 2016). In this study, the plant antagonists induced an increase of leaf tannin concentration (i.e. phenolics, plant defence compounds), as well as a decrease of surface leaf area (SLA) which also caused leaf litter to decompose at slower rates (Bandau 2016), suggesting N impacts on litter decomposability can be somewhat unpredictable.

These studies demonstrate that uptake of anthropogenic N by trees and understory vegetation may not only impact the quantity of litter produced, but also its chemical quality. However, the direction to which litter chemistry may change is somewhat unpredictable in response to anthropogenic N addition. Furthermore, the direction to which soil C pool may respond to N enrichment remains also unclear.

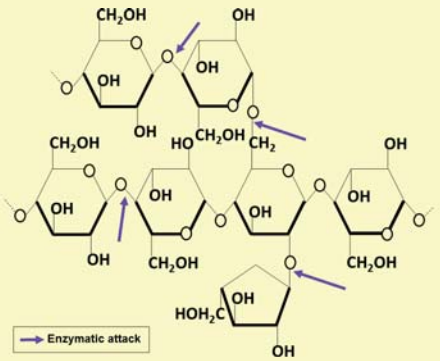
Box 2. Main groups of compounds in aboveground plant litter (1)

Cellulose is the most abundant polymer in boreal plant litter and can constitutes ~10-50% of litter mass. The polymer consists of linear chains organized into fibers of D-glucose units linked to each other by β-1,4 bonds. Cellulose degradation requires three main types of **hydrolytic enzymes**, the endocellulases that randomly cleave β-1,4 bounds within the chains; the exocellulases that split-off a D-glucose from the extremities of the chains; and glucosidases that cleave short water soluble oligosaccharides of few D-glucose units to single D-glucose.



Example of cellulose chain structure and the enzymes involved

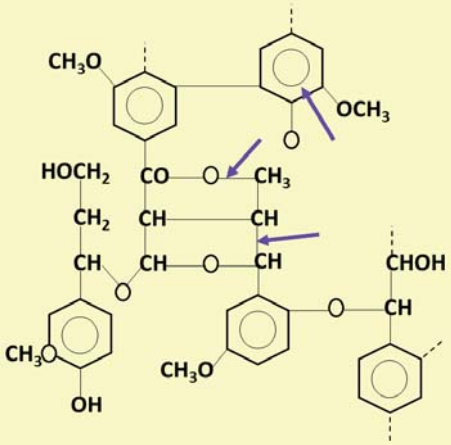
Hemi-cellulose is a more complex carbohydrate polymer that can make up 25-40% of boreal litter mass. The polymer consists of linear and short side-chains composed of pentose and hexoses residues of D-mannose, D-glucose, D-galactose, L-arabinose, D-xylose and D-glucuronic acid units linked by different bonds: β-1,4, α-1,3 and α-1,6 bonds. Hence, hemi-cellulose degradation requires a more complex set of **hydrolytic enzymes** than for cellulose degradation.



Example of hemi-cellulose chain structure

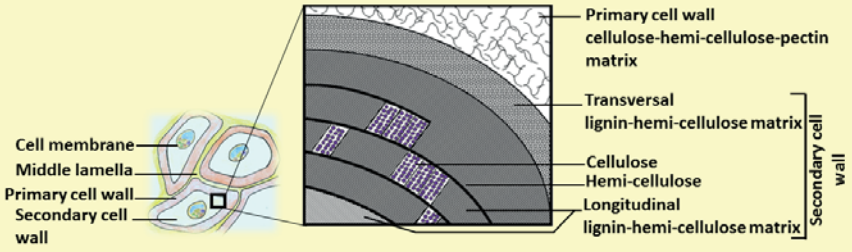
Box 2. (2)

Unlike cellulose and hemi-cellulose that are made of sugars, **lignin** is a non-soluble aromatic polymer of phenolpropanoid units. Lignin content is highly variable ranging from 4 to 50% of boreal litter mass. The polymer consists of a heterogeneous structure of phenolic residues linked by C-C and ether (C-O-C) bonds. Lignin degradation requires a complex set of **oxidative enzymes** that oxidize phenolic and/or non-phenolic lignin units resulting in demethoxylation, aromatic ring cleavage but they also generate secondary reactive compounds.



Example of a lignin polymer

The abundance of these three polymers vary between plant species, but also within a same plant depending upon specific plant organs, and stage of growth. Further, (see Pérez et al. 2002; Martínez et al. 2005; Berg & McClaugherty 2008).



Plant cell and secondary cell wall structure

1.4.2 Decrease of litter and humus decomposition rates

Humus and litter decomposition is a key process that controls soil carbon and nutrient cycling, and is mediated by the soil microbial community (see section 1.4.4.). Litter decomposition has been divided in different stages. In early stages soluble organic substances (e.g. sugars, phenolics) and unshielded cellulose and hemi-cellulose are decomposed. In later stages, the lignin polymer becomes dominant, and other substances interlocked within the lignin fiber may be degraded (Berg & McClaugherty 2008). The remaining plant residues are resistant organic matter that then form the humus (Berg et al. 2015).

Litter decomposition rates vary among plant species. As an example, mass loss of vascular plants and feather mosses litter can vary by ~50-90 and ~5-30 % after 2 years, respectively (Lang et al. 2009). Studies have shown that litter decay of recalcitrant litter (i.e. high lignin content, high C:N ratio) is generally impeded by N enrichment, while the decomposition of high quality litter (i.e. low lignin content, low C:N ratio) is stimulated (Knorr et al. 2005; Prescott 2010). Studies have also shown that these patterns are likely related to the microbial enzymatic activities, where oxidative enzymes involved in lignin degradation are repressed, while hydrolytic enzymes involved in cellulose degradation are stimulated by N addition (Sinsabaugh et al. 2002; Waldrop et al. 2004). Likewise, several studies have reported a decline of humus decomposition in response to N addition due to the incorporation of N in humus aggregates which may reduce lignolytic enzyme activities (Magill & Aber 1998; Sjöberg et al. 2003).

1.4.3 Shift in soil microbial biomass, community, and activity

Soil C and N cycling are primarily driven by saprophytic (i.e. free-living) microbes that degrade the litter and soil organic matter to obtain C and other nutrients, and biotrophic microbes (e.g. mycorrhizal fungi) that use new photosynthates obtained directly from the plant host as C source (Berg & McClaugherty 2008). Both bacteria and fungi are able to degrade polymers such as lignin, cellulose and hemi-cellulose contained in plant litter using an arsenal of hydrolytic and oxidative enzymes (see Box 3). In boreal forests, fungi represent an important fraction of the soil microbial community compared to bacteria, because fungi are favoured by low soil pH and high C:N ratio, which are typical of boreal soils (Högberg et al. 2007; Joergensen & Wichern 2008). Further, bacteria and fungi in general differ in their life history strategy; fungi are considered oligotrophs (i.e. K-strategists) that are efficient decomposer of recalcitrant organic matter in N-limited environments, whereas bacteria are

considered copiotrophs (i.e. r-strategists) with high N requirements and rapid growth response (Andrews & Harris 1986; Fog 1988; Koch 2001).

Box 3. Main saprophytic fungi decomposing plant litter

White-rot fungi are ascomycetes and basidiomycetes that degrade the three main compounds of plant litter namely, cellulose, hemi-cellulose and lignin, notably by using peroxidases. This group of fungi has been named this way because of the whitish appearance of the substrates of decomposed substrates. This bleaching has been attributed to the remaining cellulose still present in the fiber.

Brown-rot fungi (or cubical brown rot) are basidiomycetes that can carry cellulose and hemi-cellulose degradation and to a lesser extent they partially oxidize lignin fibers resulting in a characteristic brown color of the remaining tissues. They often oxidize organic matter using Fenton chemistry. Further, brown-rot fungi are able to degrade the entire cell walls which causes typical cracks of cubic shapes.

Soft-rot fungi are mainly ascomycetes and deuteromycetes and to a lesser extent basidiomycetes (and bacteria) that mainly degrade cellulose and hemi-cellulose using hydrolytic enzymes. They generally occur in environment and substrates that do not favor brown- and white-rot fungi, such as areas with high nutrient availability.
See Blanchette (1995), Martínez et al. (2005) and Berg & McClaugherty (2008).

Saprophytic fungi such as white-rot fungi have been shown to dominate the upper soil organic horizon (e.g. surface litter layer), while mycorrhizal fungi dominate the humus horizon (Lindahl et al. 2007; O'Brien et al. 2005). It has been suggested that saprophytic fungi may outcompete mycorrhizal fungi due to their efficiency to colonize energy rich litter (Lindahl et al. 2007). In boreal forests, a majority of trees form symbioses with ectomycorrhizal fungi (EM), ~95% of coniferous root tips are colonized by EM fungi (Taylor et al. 2000); and ericaceous shrubs often form ericoid mycorrhizal symbioses (Read 1991). Mycorrhizae are fungal symbioses with vascular plants, where the host plant provides photosynthates (e.g. sugars) that serve as a primary C source to the symbiont (Talbot et al. 2008), while mycorrhizal fungi explore the soil through their hyphal network and provide water, and nutrients such as N to the plant host (Smith & Read 1997); although the role of EM fungi in this mutualistic C-N exchange has been recently questioned (Franklin et al. 2014; Näsholm et al. 2013). Interestingly, a study in a boreal forest suggested that EM fungi may aggravate rather than alleviate N limitation of tree growth through N

immobilization in their fungal tissue, especially when soil N availability is low and tree C allocation is high (Näsholm et al. 2013).

Studies have shown that some EM fungi can use proteolytic enzymes to acquire N that is bound in highly recalcitrant organic matter (Chalot & Brun 1998). Further, through this breakdown of highly recalcitrant organic matter, EM fungi may enhance C availability to saprophytic soil organisms through priming effects (Fernandez & Kennedy 2015; Brzostek et al. 2015). Studies suggested that rhizospheric and mycospheric priming effects may be involved though through root and hyphal exudates (Kaiser et al. 2015; Kuzyakov 2002). Further, a recent study conducted in a deciduous temperate forest showed that EM fungi can also be abundant in the surface litter layer, especially during the growing season when the C supply from trees to EM fungi is high (Voříšková et al. 2014). Other recent studies have also suggested that some EM species may be as efficient as saprophytic fungi to decompose the main compounds of plant litter through their ability to produce oxidative enzymes (Rineau et al. 2013; Bödeker et al. 2014). Although there is increased evidence that EM fungi participate in litter degradation, less is known about their contribution to litter decomposition and thus to C cycling in forest soils.

Despite their contribution to soil C losses, as described above, it has also been suggested that EM fungi may contribute to the buildup of soil C. A study conducted in a boreal forest demonstrated that up to 70% of soil C is derived from tree roots and root-associated microorganisms (Clemmensen et al. 2013). This study challenged the current opinion that most of the soil C inputs are derived from aboveground litter inputs (Treseder & Holden 2013). The authors showed that fungal necromass tissues remained deep in the soil organic horizon, notably through the accumulation of recalcitrant polysaccharidic cell wall compounds (i.e. chitin) (Clemmensen et al. 2013). On a different note, other studies suggested that mycorrhizal fungi may compete with saprophytic organisms for resources (e.g. N, water), which may slow down decomposition rates resulting in an increase of C accumulation (Averill et al. 2014; Averill & Hawkes 2016). This antagonistic interaction is also known as the Gadgil effect (Fernandez & Kennedy 2015 and references therein). As such, uncertainty remains regarding how changes in microbial abundance and community structure in response to anthropogenic N addition would impact soil C dynamics.

Reduction in microbial biomass

One of several mechanisms by which N_r deposition is thought to enhance soil C accumulation is by negatively impacting soil microbial biomass (Treseder 2008; Janssens et al. 2010). Several studies have reported a reduction of microbial biomass in response to high doses of N fertilizers (50-150 kg N ha^{-1}

yr^{-1}), with a decline of EM fungi (Nilsson & Wallander 2003; Frey et al. 2004; Hasselquist & Högberg 2014), a concomitant decrease in fungal and bacterial biomass (Treseder 2008; Blaško et al. 2013) or a greater decrease in fungal than bacterial biomass (Högberg 2007; Demoling et al. 2008). In contrast, few studies examined responses to low chronic N addition rates (< 50 kg N ha^{-1} yr^{-1}). One of the few studies that has showed an increase in EM sporocarp production in response to 20 kg N ha^{-1} yr^{-1} (Hasselquist & Högberg 2014), although sporocarp production is often poorly related to hyphal biomass within the soil. Thus, further work is needed to understand how EM fungal biomass responds to low chronic doses of N deposition.

A variety of mechanisms have been proposed to explain the decline in mycorrhizal biomass that is frequently reported in response to high doses of N fertilizer. For instance, several studies have suggested that alleviation of N limitation through N fertilization results in reduced belowground tree C allocation to support mycorrhizae (Haynes & Gower 1995; Högberg et al. 2010; Kaiser et al. 2010). The optimal allocation or functional equilibrium models suggest that both plants and mycorrhizal fungi are limited by soil nutrients availability (Fig. 2), yet the threshold of N limitation is likely to be lower for mycorrhizae than for plants (Treseder & Allen 2002; Johnson et al. 2003; Johnson et al. 2013). As such, EM biomass could potentially increase when soil N availability increases as long as trees are still N limited, while EM biomass should decline when trees N limitation is eventually alleviated, as this would result in reduced C allocation belowground that would cause EM fungi to become C limited (Treseder & Allen 2002).

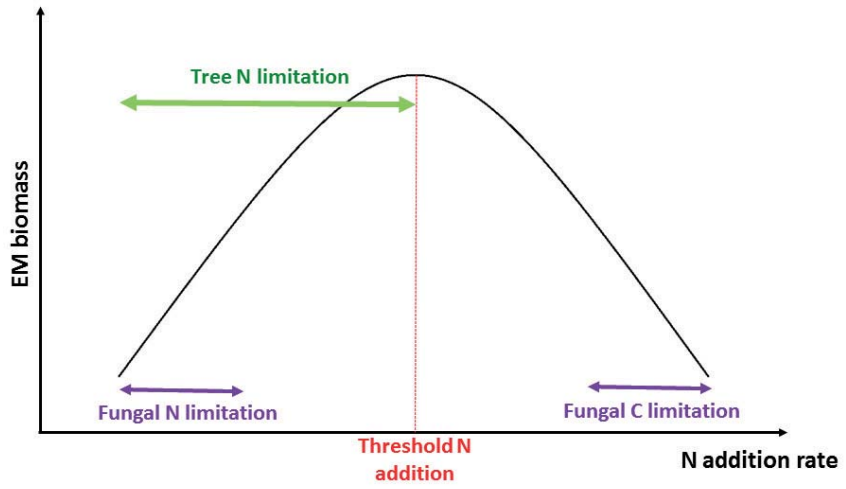


Figure 2. The optimal allocation model. Adapted from Treseder & Allen (2002).

Decreased fungal diversity and activity

While it is well established that high N addition levels cause a decline of total fungal biomass (i.e. saprophytic and EM fungi), studies also propose that a decrease of fungal functional diversity may explain the increase of soil C accumulation with increasing N addition due to the decline of litter decay (Eisenlord et al. 2013). The decomposition of complex recalcitrant litter polymers requires several enzymes that target different parts of the molecule. However, these enzymes are produced by different fungal species and therefore a certain fungal assemblage is needed for litter decomposition to be most efficient (Hanson et al. 2008; Eisenlord et al. 2013). Further, N enrichment has been reported to change microbial communities towards more N-demanding microbes (i.e. copiotrophic; Fog 1988; Ågren et al. 2001). For instance, studies have showed that with increasing N additions there is a corresponding increase of actinomycetes (i.e. actinobacteria) which are not able to completely metabolize lignin to CO_2 than fungi, which could in turn increase soil C accumulation (Godden et al. 1992; Berrocal et al. 1997; Zak et al. 2011).

An additional mechanism that may explain the greater sensitivity of fungi relative to bacteria is that N has been shown to slow down or inhibit several fungal lignolytic enzymes (Waldrop & Zak 2006; Ekberg et al. 2007; Kaiser et al. 2010; Freedman et al. 2015). This impairment of enzyme activity could reduce the ability of fungi to decompose recalcitrant compounds rich in lignin (e.g. spruce needles), resulting in a reduction in fungal biomass and an increase in soil C (Waldrop et al. 2004; Zak et al. 2008).

Abiotic stabilization and condensation

Additionally, abiotic condensation and stabilization mechanisms caused by N addition may also be responsible for the decline of fungal biomass. When litter decay begins, lignin molecules incorporate N through condensation reactions, producing recalcitrant N compounds that may act as barriers for more labile organic compounds to be degraded (e.g. cellulose) (Berg & McClaugherty 2008 and references therein). Thus, added N may stabilized organic matter to highly recalcitrant compounds resisting further transformation and degradation from microbial decay (Neff et al. 2002; Swanston et al. 2004), thereby potentially impacting fungal metabolism. Abiotic stabilization is a key mechanisms controlling C residence time in the soil. Soil C pools have been classified into two groups, the light and heavy fractions which may respond differently to N enrichment. The light soil fraction has a turnover time on decadal time scales, and it has been proposed that N addition may accelerate its turnover or enhance its stabilization into mineral forms. The second group, the heavy fraction has a longer residence time of decades to century and is, highly recalcitrant C associated with soil minerals (Trumbore & Harden 1997; Gaudinski et al. 2000).

1.4.4 Decrease in soil respiration

Total soil respiration consists of the sum of autotrophic respiration (i.e. plant root and root-associated microbe respiration) and heterotrophic (i.e. saprotrophic respiration) (Högberg & Read 2006). After gross primary productivity (i.e. rate of CO_2 fixed by photosynthesis), soil respiration is the second largest annual CO_2 flux in the global C cycle (Goulden et al. 1996). Soil respiration releases annually approximately ~98 Pg C yr^{-1} to the atmosphere, by comparison anthropogenic CO_2 emissions release ~10 Pg C yr^{-1} to the atmosphere (Bond-Lamberty & Thomson 2010; IPCC 2013). As such, any alteration of soil respiration rate would likely impact soil C accumulation rates. The mechanisms previously described in section 1.4 are related to soil respiration as they can influence soil respiration rates. A decrease of soil respiration has been proposed as one mechanisms by which N_r deposition is thought to enhance soil C accumulation (Janssens et al. 2010).

Several studies in temperate and boreal ecosystems have reported that the applications of high doses of N fertilizers (> 50kg N ha^{-1} yr^{-1}) can decrease soil respiration by ~21% on average (Bowden et al. 2004; Olsson et al. 2005; Janssens et al. 2010). Interestingly, recent studies have shown that relatively low rates of N addition can have contrasting effects relative to higher N doses. For example, a study has shown that the addition of relatively low quantities of N (20 kg N ha^{-1} yr^{-1}) over short time scales can have positive effect on autotrophic soil respiration (Hasselquist et al. 2012). These studies highlight that low chronic

N addition rates (i.e. <20 kg N ha^{-1} yr^{-1}), which are typical of N_r deposition in the boreal region, may have different effects on the activity of soil biota (see section 1.4.4), and subsequently on C accumulation rates, compared to the higher N addition rates that have been used in a majority of previous long term forest fertilization experiments.

2 Objectives

The overall aims of this thesis are to clarify the magnitude to which realistic N_r deposition rates enhances soil C sequestration in boreal forest soil and expand our understanding of the different mechanisms via which anthropogenic N_r deposition impact soil C accumulation (Fig. 3). Thus, **paper I** focuses on estimating the magnitude and response pattern of soil C sequestration in response to long-term N addition and to explore three of the proposed mechanisms, namely the decrease in soil respiration, a decrease of soil microbial biomass, and a shift in microbial community. **Paper II** explores whether annual aboveground litter production increases and how litter quality (i.e. total C, N, P and cellulose, lignin and hemi-cellulose) is altered after 17 years of simulated N enrichment. **Paper III** investigates the importance of litter quality *versus* soil factors on litter decomposition of two common boreal forest species, *Picea abies* and *Vaccinium myrtillus*. Finally, **Paper IV** seeks to determine how EM fungi mediated by a gradient of chronic N addition affect soil C dynamics, and how any shifts in microbial community may impact litter and humus degradation, consequently affecting the soil C pool.

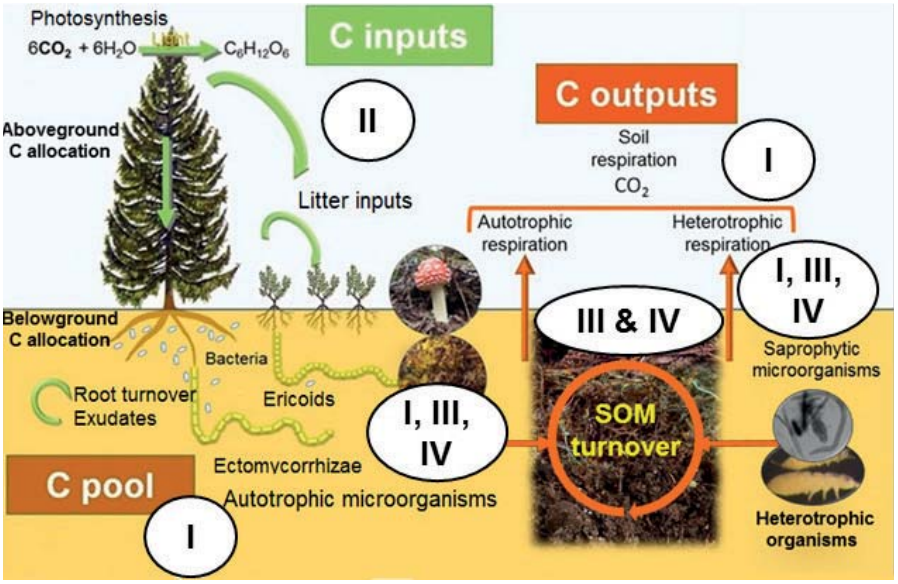


Figure 3. Soil carbon balance in boreal forest. Roman numerals relate to the four papers of this thesis. SOM: soil organic matter.

3 Material and Methods

3.1 Study system

All studies were performed at Svartberget experimental forest (64°14´N, 19°46´E) in the middle boreal zone of northern Sweden (Ahti et al. 1968). Background atmospheric N_r deposition in this region is approximately 2 kg N ha^{-1} yr^{-1} (Pihl-Karlsson et al. 2009). The mean annual precipitation and temperature at the sites are approximately 583 mm and +1.0°C, respectively.

3.1.1 Norway spruce boreal forest, Svartberget

Research in **papers I-III** was conducted in a late successional (~120 years old) Norway spruce forest (*Picea abies* (L.) Karst) with occasional Scots pine (*Pinus sylvestris* L.), Birch (*Betula pendula* Roth) and Aspen (*Populus tremula* L.) throughout (Fig. 4). The plant community at the site is classified as a mesic-dwarf shrub type (Arnborg 1990). The understory layer is dominated by ericaceous species, *Vaccinium myrtillus* L. and to a lesser extent by *V. vitis-idaea L.* and the grass species, *Deschampsia flexuosa (L.)* Trin, *Maianthemum bifolium* (L.) F. W. Schm., *Melampyrum sylvaticum* L., *Solidago virgaurea* L. and *Trientalis europaea* L. (Nordin et al. 1998). The moss-mat consists primarily of *Hylocomium splendens* (Hedw.) B.S.G, *Pleurozium schreberi* (Bird), and *Ptilium crista-castrensis* (Hedw.) (Fig. 5). Soils at the site are podzols developed from glacial till.

Figure 4. Norway spruce (*Picea abies*) forest at Svartberget, Vindeln. Photo: N. Maaroufi

Figure 5. Understory vegetation with the dwarf shrubs *Vaccinium myrtillus* and *V. vitis-idaea*, the mosses *Hylocomium splendens* and *Pleurozium schreberi* and the grass *Deschampsia flexuosa*. Note the purple spots on *V. myrtillus* leaves that correspond to the infection by the fungi *Podosphaera myrtillina* and *Valdensia heterodoxa*. Photo: N. Maaroufi

A long-term N addition experiment was set up at this site in 1996, consisting of three levels of N addition treatments, 0 N added (control), 12.5 (low N treatment) or 50 kg N ha^{-1} yr^{-1} (high N treatment) (Strengbom et al. 2002; Nordin et al. 2005). The experiment consists of six blocks, with two plot sizes for each N addition level within each block (0.1 and 0.25 ha), where the larger plot size were used for destructive sampling. We excluded one of the six blocks within the experiment from our sampling because it occurred on an area without free drainage, resulting in a deep peat layer that was not present in the other blocks. Thus, two plot sizes within five blocks were utilized for this study. The low N addition treatment was chosen to simulate upper level N_r deposition rates in the southern boreal region, whereas the high N addition treatment is more similar to many long-term forest fertilization experiments. Each N addition treatment has been applied annually since 1996 by manually spreading solid granules of ammonium nitrate (NH_4NO_3) directly after snow melt.

3.1.2 Scots pine boreal forest, Åheden

Research in **paper IV** was conducted in a naturally regenerated ~170-year-old Scots pine forest (*P. sylvestris* L.) (Fig. 6). The plant community at the site is classified as xeric-to-dry dwarf-shrub type (Arnborg 1990). The understory layer is dominated by ericaceous shrub species, *V. vitis-idaea* L. and *Calluna vulgaris* (L.) Hull. The bottom layer is composed mainly by the bryophytes *P. schreberi* (Bird) and to a lesser extent by *Dicranum sp*. and, by the lichens *Cladonia rangiferina* (L.) Weber and *C. arbuscular* (Wallr.) Flot (Fig. 7). Soils at the site are typic haplocryods developed from fine sandy and silty glacial outwash sediments (FAO, Cambic Podzol).

Figure 6. Scots pine (*Pinus sylvestris*) forest at Åheden, Vindeln. Photo: B. Forsmark

Figure 7. Understory vegetation with a *P. sylvestris* seedling in the foreground, the dwarf shrubs *Calluna vulgaris*, *Vaccinium vitis-idaea* and *V. myrtillus*, the moss *Pleurozium schreberi* and the lichens *Cladonia sp*. Photo: N. Maaroufi

A long-term N addition experiment was set up at this site in 2003, consisting of five levels of N addition treatments, 0 N added (control), 3, 6, 12 (low N

treatment) or 50 kg N ha^{-1} yr^{-1} (high N treatment). The experiment consists of a fully randomized design, and 0.1 ha plots. The low N addition treatment was chosen to simulate current N_r deposition rates in the boreal region, whereas the high N addition treatment is more similar to many long-term forest fertilization experiments. Each N addition treatment has been applied annually since 2004 by manually spreading solid granules of NH_4NO_3 directly after snow melt.

3.2 Soil carbon estimation

In **paper I**, the amount of soil C present in each plot in the Norway spruce dominated forest was estimated by sampling soil during the growing season of 2012 within the 0.1 ha plots (n=5). Soil cores were taken from five locations within each plot. At each of these locations, a soil core was collected spanning the entire organic horizon, as well as from two 10 cm depth intervals within the mineral soil layer. After collection, the depth of each core was measured. The mineral soil was sampled from the same location. Sampling of mineral soil initially included 20-30, 30-40, and 40-50 cm depth intervals, but this sampling was terminated after determining that parent material existed at these depths in a majority of sampling locations.

Soil samples were immediately transported to the laboratory and sieved in order to separate roots, soil, and coarse material. The organic horizon and mineral soil samples were sieved at 4 mm and 2 mm, respectively. All sieved soil samples and roots were dried (70°C for 48h), weighed, and homogenized for nutrient analyses. The soil dry mass and core volume were used to estimate soil bulk density (i.e. mass divided by volume). Root C and N concentrations were determined and reported in a previous study (Gundale et al. 2014).

In order to determinate the % of stones in the mineral soil and thus correct the soil C estimation in the mineral soil, one soil pit (50×50×30 cm depth) in each block was excavated in order to determine the average ratio of soil (< 2 mm particle size) to stones and boulders (i.e. > 2 mm). To estimate the stone volume from each pit, the soil from each pit was first sieved (2 mm), and then the coarse material removed by the sieve was placed in a large plastic box containing water. The volume of water displaced by this material was quantified. The soil volume was estimated by subtracting the stone and boulder volumes from the total pit volume.

The quantity of soil C and N present in each plot 16 years after the start of the experiment was estimating by multiplying the mass of each soil core by the C or N concentration. These values were then scaled up to a surface area basis (Mg C ha^{-1}) using the core surface area, and then corrected using the stoniness constant (32.6% stones), as described above.

3.3 Soil respiration

In **paper I**, the total soil respiration was measured (i.e. autotrophic and heterotrophic respiration combined) to investigate whether soil CO_2 efflux decrease in response to N addition. Soil respiration measurements took place every three weeks between May 30th and October 10th 2013, resulting in a total of seven sampling events. Soil respiration was measured between 9:00 am and 4:30 pm and the order in which plots were measured was changed at each sampling time with the intention of evenly dispersing any diurnal variation in these measurements evenly across treatments (Betson et al. 2007).

The measurements were made by establishing five cylindrical collars in each 0.25 ha plot (n=5). The collars (25 cm diameter, 10 cm high) were permanently inserted *ca.* 1 cm into the soil organic horizon, and aboveground vegetation inside and 5 cm around the collar were removed in order to eliminate plant respiration. The collars were allowed to equilibrate for three days after they were initially set up before the first measurement. Prior to the first measurements, the height from soil surface to rim was measured in four cardinal dimensions within each collar in order to calculate the headspace volume. Soil respiration (CO_2 efflux) was measured from the linear rate of CO_2 accumulation within sealed cylindrical headspaces. For each measurement, soil collars were covered by a removable plastic lid that contained an opening for the placement of a solid-state CO_2 sensor (CARBOCAP model GMP 343, Vaisala, Finland). The buildup of CO_2 within the headspace was monitored for 3 min, where after the lid was removed. The lid was equipped with a fan to avoid the development of a vertical CO_2 gradient within the headspace. Individual respiration measurements were corrected to account for differences in air temperature and headspace volume among the different collars. Immediately following measurements of soil respiration, measurements of soil temperature (Model E514, Mingle Instrument, Willich, Germany) were taken inside each collar.

3.4 Litter quality and quantity

3.4.1 Estimation of vascular plant and moss litter production

In **paper II**, the annual litterfall in Svartberget was measured during one complete year (17 years after the start of the N addition experiment). In spring 2012, six litter tray traps were systematically established within each plot on the forest floor five meters from the plot centre. Litter was collected between the start of June 2012 and the end of May 2013. Litter traps were emptied once a month during the growing season until October, and emptied again following snow melt at the end of May. Each litter tray trap had a surface area of 0.22 m^2

and was raised at *ca.* 2 cm above the forest floor to avoid direct contact with the soil. Small drainage holes covered in nylon mesh (1 x 1 mm) were present in the base of the litter traps in order to prevent water accumulation. These tray traps were used to collect litter fall from the following categories: *P. abies* needles, *P. sylvestris* needles, deciduous tree leaves, *V. myrtillus* leaves, coniferous reproductive organs (male cones, seeds) and twigs with a diameter < 0.5cm.

In addition to litter tray traps, six 2 x 2 m sub-plots were also systematically established within each plot and designed to estimate the input of dead branches ≥ 0.5cm. These sub-plots were placed seven meters from the plot centre, marked with sticks, and cleared of any pre-existing branches within this diameter class. This allowed for identification and collection of any new branch material that landed in these plots.

Within each plot, litter collected from different sub-plots and collection periods were combined in order to provide an estimate of annual litter production for each plot. The litter was dried at 60°C for 72h, sorted into each litter category, and the mass was measured. Finally, the annual litter produced in each plot was estimated by scaling up the mass of each litter category to a surface area basis (kg or Mg ha^{-1} yr^{-1}).

Because the litter tray traps were not an effective design to quantify litter production of *V. myrtillus* or bryophytes, their litter production were instead quantified indirectly by estimating their annual aboveground production, which is approximately equal to their litter production since their biomasses have remained relatively stable for the last 10 years of the experiment (Nordin et al. 2009). Aboveground annual production of *V. myrtillus* was estimated by harvesting leaves in late June 2013 from five sub-plots sized 0.20 x 0.60 cm distributed within each treatment plot. The collected plant material was dried (60°C for 72h), and biomass from the five sub-plots were pooled, resulting in a single measurement per treatment plot. *Vaccinium myrtillus* leaf litter production per unit area (i.e. assumed to be approximately equivalent to *V. myrtillus* biomass production) was estimated by scaling up the total biomass from all sub-plots to a per hectare basis.

3.4.2 Moss litter production

Litter production of the moss layer was estimated indirectly by evaluating vertical moss productivity (Nakatsubo et al. 1997; Skre & Oechel 1979). This indirect measurement of moss tissue production could be used because moss biomass has remained stable in the different N treatments for the past 7 years (Nordin et al. 2009), which means that apical moss growth is balanced by distal moss litter production (Skre & Oechel 1979; Callaghan et al. 1978). The annual moss production was estimated by systematically selecting five intact moss

patches within each plot 12 meters from the plot center, on which I established 20×20 cm quadrats (i.e. 5×0.04 m^2 sub-plots per plot). For each of these sub-plots I positioned a plastic mesh screen (1×1cm mesh size) horizontally on the moss carpet to serve as a time zero reference. Mosses were able to freely grow through the mesh material, and in June 2014 (2 years after setting up the mesh quadrats), all moss biomass above the mesh quadrats was harvested, in order to estimate moss production. The collected moss material was dried (60°C for 72h), and biomass from the five sub-plots were pooled, resulting in a single measurement per plot. Moss litter production per unit area (i.e. assumed to be approximately equivalent to moss biomass production) was estimated by first scaling up the total biomass from all sub-plots to a per hectare basis. These values were then multiplied by the percent cover of mosses in each plot, as previously reported by Gundale et al. (2013, 2014). These values were further converted to moss litter production per year by accounting for the two years over which moss biomass production was measured.

Chemical analyses

For **paper II** each litter type collected from litter traps, was first dried, combined to from a composite sample and homogenized before, and sub-samples were used for chemical characterization. For mosses, I used the newly produced biomass for chemical analysis because mosses do not exhibit discreet senescence as vascular plants do; whereas senesced tissues were used for all other litter categories. Sub-samples of each tissue type from each plot were ground using a ball mill (Retsch MM 301; Haan, Germany). The C, N, P, lignin, cellulose and hemi-cellulose concentrations were expressed as mg g^{-1}. For all litter categories, I estimated their annual chemical (i.e. lignin, cellulose, hemi-cellulose) and elemental (i.e. C, N and P) fluxes by multiplying the annual mass per unit area of tissue type by their corresponding chemical and elemental contents.

3.5 Soil microbial structure and composition

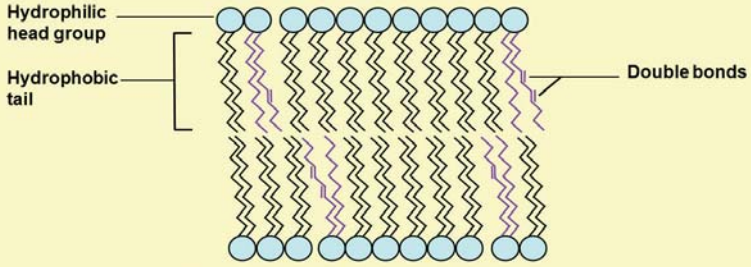
In **paper IV**, in order to separate the contribution of EM fungi from the rest of the microbial community, two 0.25 m^2 root-EM fungal exclosure (Wallander et al., 2001) plots were established within each 0.1 ha N treatment plot during July 2014 (i.e. 10 years after the start of N addition). Root-EM fungal exclosures consisted of square steel collars (50× 50 × 50 cm) that were permanently inserted into the soil in order to prevent any ingrowth of tree roots and EM hyphae. Tree seedlings were also removed within each collar in order to prevent EM fungi networks to establish within the exclosure plot. After the sub-plots were established, they were left to equilibrate for 9 weeks, in order to allow EM fungi

to die, and fine roots to decompose. Paired non-exclosure sub-plots (0.5 x 0.5 m) were established adjacent to each exclosure sub-plot.

3.5.1 Phospholipid fatty acids method (PLFA)

Box 4. Phospholipid fatty acids

PLFAs are the most abundant lipids present in the **cell membrane** (i.e. cytoplasmic membrane) and are arranged in a bilayer. The PLFAs are characterized by a **hydrophilic head group** composed of alcohol, phosphate, and glycerol groups and a **hydrophobic tail** composed of fatty acid chains.



Phospholipid bilayer of a cell membrane

Fatty acids are chains of carbon with or without double C bonds present (C=C), which is referred to as saturation. Two major families of fatty acids exist, those with **straight** or **branched** chains. The branched fatty acids are divided in subgroups called iso (i.e. **i-**) and anteiso fatty acids (i.e. **α-**) which refer to the position of the branch. The branched fatty acids can be composed by methyl (**Me-**) or cyclopropane (**Cy-**) groups. As an example, the nomenclature of the gram negative PLFA 18:1ω7 indicates that the fatty acid chain is composed of 18 C long with one double bonds located at the C n°=7 from the methyl end of the chain (**ω**).

PLFAs have a relatively short residence time in the soil as they are synthetized during microbial growth and degraded very quickly with microbial death. Thus, PLFA analyses can provide an estimation of the living microbial community structure in soil. Different types of PLFAs represent diverse functional groups, such as gram positive, gram negative, fungi and actinomycetes.

The aim of **paper I** and **IV** were to investigate whether low *versus* high N addition rates impact soil microbial communities using PLFA markers (Box 4). For **paper I**, three organic horizon samples were taken from each 0.1 ha plot,

while for **paper IV**, organic soil samples were taken from each both within and outside trenched sub-plots, and pooled into to a single composite sample per plot. Before sieving the soil samples on a 4 mm mesh, all samples were immediately frozen and then were freeze-dried. The PLFAs were extracted from 1g (wet mass) subsamples of each soil sample using a modified method originally described by Bligh and Dyer (Bligh & Dyer 1959; White et al. 1979; Mcintosh et al. 2012). The abundance of PLFAs was quantified using a Perkin Elmer Clarus 500 gas chromatograph (Waltham, Massachusetts, USA), and was converted to micromoles or nanomoles PLFA per gram of organic matter using conventional nomenclature (Tunlid et al. 1989). Different types of PLFAs represent diverse functional groups. Total bacteria were represented by i-15:0, α-15:0, 15:0, i-16:0, 16:1ω9, 16:1ω7, 16:0, i-17:0, cy-17:0, α-17:0, 18:1ω7, and cy-19:0 bacterial PLFAs (Bardgett et al. 1996). Gram-positive bacteria were represented by branched fatty acids i-15:0, α-15:0, i-16:0, i-17:0, and α-17:0, while cy-17:0, cy-19:0, and 18:1ω7 were used as a measure of gram-negative bacteria. The branched fatty acids 10me16:0, 10me17:0, and 10me18:0 were used to estimate actinomycetes contribution (Kroppenstedt 1985; Wardle et al. 2013). The PLFA 18:2ω6 was used to estimate the contribution of fungi, and further, this marker is well known to correlate with ectomycorrhizal biomass in Swedish boreal forests (Frostegård et al. 2010; Yarwood et al. 2009). Further, PLFA marker 16:1ω5 was used (in **paper IV)** to estimate arbuscular mycorrhizae (Frostegård & Bååth 1996; Olsson 1999).

3.5.2 Mycorrhizal ingrowth bags

In **paper IV**, in addition to PLFA, the biomass of EM fungi was determined by a different approach using nylon ingrowth mesh bags (Wallander et al. 2001). Triangular ingrowth bags of 50 µm mesh size (9 × 9 × 12.5 cm sides) were filled with 30 g acid-washed and burned quartz sand (0.5 - 1 mm) (Fig. 8). This mesh size was chosen to allow fungal hyphae but not root ingrowth, while the substrate was chosen because it allows easy extraction of fungal biomass, and it does not contain any organic C, which reduces ingrowth of saprophytic fungi. In September 2014, the ingrowth bags were gently placed at the interface between the mineral soil and the organic horizon, where ectomycorrhizal fungi have been shown to be the most abundant (Lindahl et al. 2007). The ingrowth bags were buried within trenched and paired untrenched sub-plots (i.e. 120 ingrowth bags in total). The buried mesh bags were collected after 12 months (11 years after the start of N addition), and were stored at -20°C until chemical analysis was performed.

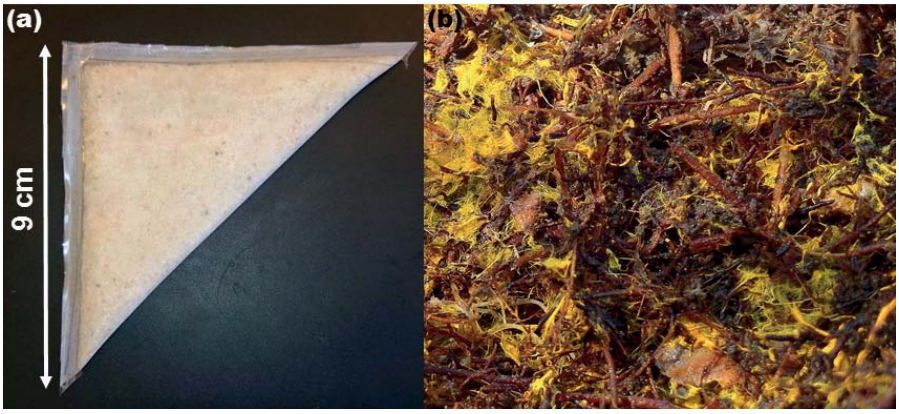


Figure 8. Ingrowth mesh bags filled with quartz sand (a). Picture of *Piloderma* sp., an ectomycorrhizal fungus in the organic horizon (b).

Ergosterol analysis

Fungal biomass was estimated by analysing the ergosterol concentration within the ingrowth bags. Ergosterol is a compound found in fungal cell membrane and used as a biomarker in order to measure living fungal biomass (i.e. free ergosterol) using a modified method of Nylund and Wallander (Nylund & Wallander 1992; Clemmensen et al. 2006). The ingrowth bags were freeze-dried and the contents from bags derived from the two sub-plots within each main plot were pooled, yielding a single sample from inside or outside the root-EM fungal exclosure per main plot. These samples were then homogenized and the ergosterol was extracted from 5 g sand subsamples in 5 ml methanol. After 3 min of sonification at 10 rpm (Multi Reax, Heidolph, Germany) and sand sedimentation, 10 µl of supernatant were centrifuged for 3 min at 1300 rpm. The supernatants were then filtered through a 0.45 µl titan syringe filter (44504-NPC, Sun Sri, USA), analyzed in a reversed-phase column (Chromolith C18 column, Merck and an Elite LaChrome C18 pre-column, Hitachi, Japan) and a high pressure liquid chromatograph (auto sampler L2130 with UV detector L2400, Hitachi, Japan). The ergosterol peak was detected at 280 nm with a flow rate of 1 ml min^{-1}. Ergosterol concentrations were expressed as ng g^{-1} sand.

3.6 Litter and humus decomposition

The impact of N enrichment on litter and humus decomposition were investigated in **paper III** and **IV**.

The aim of **paper III** was to investigate the importance of litter quality *versus* soil factors on litter decomposition of two common boreal species, *P. abies* and

V. myrtillus, using a reciprocal transplant experiment. Both litter species originated from the litter collected in **paper II**.

Picea abies and *V. myrtillus* litter were placed in nylon cloth material (8×7 cm) with mesh size of 0.3×0.1 and 0.5×0.3mm, respectively (Veen et al. 2015; Ayres et al. 2009). The smaller mesh size was necessary for *P. abies* to avoid unintended litter loss through the mesh. Previous studies have shown that these differences in mesh sizes do not influence decomposition rates and microclimate differences are minimal (Bokhorst & Wardle 2013; Knorr et al. 2005). The litter bags contained a dry mass of 2.00 and 1.00 g dry weight of *P. abies* and *V. myrtillus* litter, respectively.

In autumn 2013, a reciprocal transplant experiment was set up, where *P. abies* and *V. myrtillus* litter bags originated from each N treatment (control, low, and high N treatments). Two litter bags of each species were then placed back in the plot they originated from, as well as within each of the other N treatments within the same block. The litter bags were pinned to the forest floor with plastic sticks in October, and were collected at one and two years of incubation (i.e. autumn 2014 and 2015). This, resulted in a total of 180 litter bags (2 species×3 litter origins×3 soil destinations×2 times). After collection, the litter bags were oven dried at 60°C for 48h to prevent further decomposition and weighed. Litter mass loss for each species was expressed as relative mass loss (% mass loss).

In order to investigate whether litter mass loss was correlated with initial litter chemistry and soil biota biomass (i.e. fungi and bacteria) the initial litter chemistry studied in **paper II** was used with the soil biota characteristics investigated in **paper I**.

To model the quantitative impact of N addition on litter decomposition in the Norway spruce forest, I used the % litter mass loss from the bags that decomposed during two years for each individual species and each litter origin per soil treatment (3 litter origin × 5 plots per soil N treatment × 3 soil N treatment × 2 species; n=90) was used. Then all data points were subtracted by the mean litter mass loss (%) of the control plots for each species. All values were further divided by the number of years where the litter bags decomposed (2 years), giving the % mass loss change relative to the control. Finally a linear regression analysis was performed using N addition treatment rates as the independent variable and relative litter mass loss per year as the dependent variable (α=0.05). The slope of this regression indicated the change of the % litter mass loss per unit N added.

Unlike paper III, **paper IV** sought to understand the contribution of EM fungi to litter and humus degradation in response to a N addition gradient. In summer 2014, organic horizon soil (i.e. humus) and freshly senesced *P. sylvestris* litter were collected from unfertilized plots in the vicinity of the experimental site.

Soil and plant litter were chosen because they provided an old versus new C substrate for microbial decomposition, respectively. Soil samples were sieved as described in section 2.2, then homogenized and oven-dried at 60°C for 48h. The collected litter was air-dried and thoroughly homogenized. Sub-samples of litter were dried at 60°C for 48h to determine their dry weight and thus correct the initial litter bag biomass for moisture content. Sub-samples of both humus and litter were also taken and ground for chemical characterization (i.e. %C, %N, % P, C:N, C:P, and N:P ratios). The remaining humus and litter was used to make decomposition bags, following the same design as the ingrowth mesh bags (see section 2.2). The mesh bags were filled with 3.00 g dry weight of humus or *P. sylvestris* needles. The humus and litter bags were buried within the trenched and paired untrenched subplots (i.e. 240 decomposition bags in total). The buried bags were collected after 12 months (11 years after the start of N addition).

After collection, the mesh bags were oven dried at 60°C for 48h to prevent further decomposition, and then weighted. Humus and litter mass loss was expressed as relative mass loss (% mass loss). The content of the two sub-replicate mesh bags of each type removed from each plots were composited, ground, and analyzed as the initial soil and litter samples.

3.7 Elemental analyses

In paper I, II and IV, soil and litter samples were ground and analyzed for C and N content by dry combustion using an elemental analyzer (LECO TruSpec CN analyzer; St. Joseph, MI, USA), and litter P content by acid digestion using nitric-perchloric acid analyzed by inductively coupled plasmography. In **paper II**, litter tissues were also analyzed for lignin, cellulose and hemi-cellulose contents by acid digestion and calcination (performed by the Soil, Water and Plant Testing Laboratory, Colorado State University, USA).

3.8 Statistical analyses

In paper I, one-way analysis of variances (ANOVAs) were used to test for the effects of N addition levels (0, 12.5, 50 kg N ha^{-1} yr^{-1}) on soil C and N contents, soil bulk density, organic horizon depth (see appendix), mean soil respiration, and PLFA. Nitrogen addition levels were used as a fixed factor and block as a random factor whenever significant. A blocked repeated-measures ANOVA was performed to test whether the treatments had significant effects on respiration and soil temperature averaged across all sampling times.

Further, to model the quantitative impact of N addition on C sequestration in the organic horizon, all plot data were first substracted by the mean organic

horizon C pool per ha of the control plots. These values were then divided by the number of years where the treatments were applied, providing kg C sequestered ha^{-1} yr^{-1} relative to the control. A linear regression was performed using N addition rate as the independent variable, and relative C sequestration rate per year as the dependent variable. The slope of this regression indicated the quantity of C sequestered in the organic horizon per unit N added.

For PLFA data, a detrended correspondence analysis (DCA) was performed to be able to choose between linear and unimodal methods. The gradient length was 0.32 SD-units and thus linear methods were used. I first performed a redundancy analysis (RDA) using a Monte Carlo Permutation test (n=999, α=0.05) in order to determine whether multivariate differences in the overall PLFA signatures occurred in response to the N addition treatments. Multivariate PLFA signatures were described using principal component analysis (PCA). The first and second PCA sample scores of this ordination, as well as total PLFA, fungal, bacterial, fungi:bacteria, gram positive, gram negative, actinomycetes PLFAs were then compared between the N addition treatments using one-way ANOVA, as described above.

In **paper II**, one-way ANOVAs were performed, with N addition serving as a fixed factor and block as a random factor on litter chemistry, litter quantities, and elemental and C chemistry. Tree species abundance (i.e. % of trees from a same species per plot) was initially used as a covariate factor but all analyses were subsequently re-run without this co-variate because it never improved the fit of the model.

In **paper III**, a three-factor repeated-measures mixed model ANOVA was used to test for the effects of soil destination, litter origin, and species on litter mass loss, these later were used as fixed factor in the model. Block was used as random factor. Correlation between litter mass loss and litter chemistry (%C, %N, % P, C:N, C:P and N:P ratios), soil biota (PLFAs) were performed using Pearson correlation test (α=0.05).

In **paper IV**, two-way mixed model ANOVAs were used to test for the effects of N addition treatment (0, 3, 6, 12, 50 kg N ha^{-1} yr^{-1}), subplot serving as main factors, with subplot nested within N addition treatment on microbial and fungal composition (PLFAs). One-way ANOVA was used to test for the effect of N addition treatment on the ergosterol content. Three-way mixed model ANOVAs were performed to test for the effects of N addition treatment, substrate (humus, litter), subplot (trenched, non-trenched) were fixed factors, with subplot nested within N addition treatment on litter and humus decomposition as well as the total C, N, P, and C:N:P ratios. Correlation between each substrate mass loss and fungal PLFA biomass for each trenching treatments, and between EM fungal PLFA biomass (i.e. the difference between

fungal PLFA biomass in the untrenched and the trenched plots), were performed using Pearson correlation test (α=0.05). When the variables did not meet the requirements of normality, non-parametric spearman correlation test was used (α=0.05).

Further, multivariate analyses were performed on the PLFA data. First, a DCA was performed in order to indicate whether linear or unimodal methods should be utilized. The gradient length was 0.71 SD units, suggesting linear methods should be used. We then performed Monte Carlo permutation tests (n=999, α=0.05) to evaluate whether multivariate differences occurred in the overall PLFA signatures in response to the N addition treatments. Secondly, I described multivariate PLFA signatures using a RDA to determine how N addition and trenching treatments affects microbial community variability.

For all ANOVAs, when significant differences were detected (α=0.05), *post hoc* pairwise comparisons between treatments were conducted. All response variables were transformed whenever necessary to meet the assumption of normality and homoscedasticity. Variables that did not comply with these assumptions after transformation were tested using Kruskal-Wallis non-parametric test. All statistical analyses were performed using SPSS (Chicago, USA; version 22.0), SigmaPlot (Systat, San Jose, USA; version 10.0) or XLSTAT (Addinsoft, 1995-2015; version 2015.2.02). Multivariate analyses were performed using CANOCO (Biometris, Wageningen, NL, USA; version 5.0).

4 Results and Discussion

The chapters contained in this thesis aimed to explore how anthropogenic N_r deposition impacts the soil C balance in boreal forests. In particular, I sought to understand whether realistic chronic levels of N_r deposition ($\leq$ 12.5 kg N ha^{-1} yr^{-1}) impacted soil C, which is in contrast to previous research that has evaluated much higher N addition rates. Below, the main results are presented and discussed.

4.1 Increase of carbon and nitrogen sequestration in the soil organic horizon in response to long-term nitrogen addition

4.1.1 Soil carbon and nitrogen pools

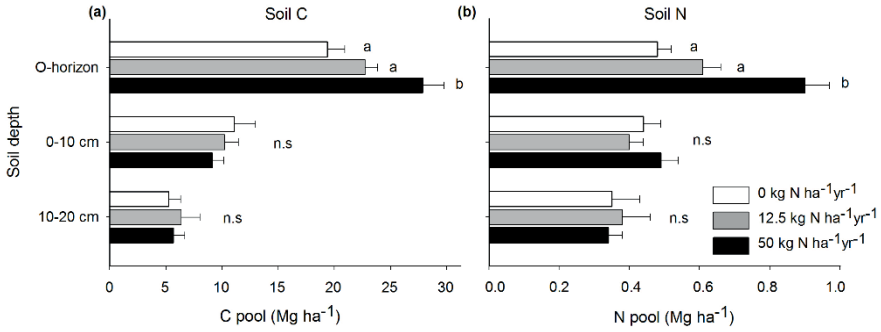


Figure 9. Mean (+SE) soil C (a) and soil N (b) pools at different soil depths (organic horizon and mineral soil) in response to long-term N additions (0, 12.5 and 50 kg N ha-1 yr-1). The data were tested with one-way ANOVA. Different letters (a or b) next to each group of bars indicate significant differences between treatments using *post-hoc* tests. n.s. non-significant. Figure from Maaroufi et al. (2015).

Soil carbon pool

The long-term N addition treatments (16 years) applied in Svartberget increased the soil C pool in the organic horizon by ~30% (8.5 Mg ha^{-1}) on average in the higher N treatment (50 kg N ha^{-1} yr^{-1}) and non-significantly increase by ~15% (3.4 Mg ha^{-1}) in the low N treatment (12.5 kg N ha^{-1} yr^{-1}) relative to the control (Fig. 9). This result was in line with a previous study conducted in a temperate hardwood forest, where the authors showed that 10 years of chronic N enrichment (30 kg N ha^{-1} yr^{-1}) increased the organic soil horizon C by 6.9 Mg ha^{-1} (Pregitzer et al. 2008). The mineral soil C pools were not responsive to the N addition treatments, nor was there a change in total C root pools with increasing N additions. The increase of the soil C pool in the organic horizon

was likely caused by the combined increase of the C concentration, the bulk density (i.e. mass per volume) and the humus thickness.

Soil nitrogen pool

The long-term N addition treatments increased both the total N root and the total N soil pools in the higher N addition treatment. The increase observed in the total soil pool was due to the significant increase of N observed in the organic soil horizon. Interestingly, approximately three-quarter (0.15 Mg ha^{-1}) and half (0.40 Mg ha^{-1}) of the total N added over the course of the experiment in the low and high N treatments respectively, were still present in the organic soil horizon. These results suggest that boreal soil can serve as long-term sink for anthropogenic N_r deposition (Templer et al. 2012). The authors suggested that a portion of the N lost from the soil compartment may be taken up by the vegetation, when the competition with soil microbes declines in response to N enrichment (**paper I**; Treseder 2008; Demoling et al. 2008). Alternatively, other studies that used the same N addition levels reported the same magnitude of N losses, and the authors proposed that the decrease of soil N retention under high N addition levels (> 25 kg N ha yr^{-1}) relative to low N_r deposition (10-25 kg N ha yr^{-1}) levels may be due to N losses by leaching and denitrification, see Fig.1 (de Vries et al. 2014 and references therein).

4.1.2 Relationship between carbon sequestered and nitrogen added

The relationship between C sequestered in the organic horizon per N addition followed a linear relationship (Fig. 10), with a slope indicating 10 kg of C were sequestered per kg of N added after 16 years of N addition. In contrast with the ANOVA analysis that did not detect a significant increase of C sequestration in response to the low N treatment (12 kg N ha^{-1} yr^{-1}), this relationship suggest that soil C sequestration in the organic horizon may be promoted even at low N_r deposition rates. This relationship is in line with a meta-analysis that gathered results from N addition experiments and found a C sequestration rate of approximately 11 kg C kg N^{-1} in response to N addition rates ranging from 30 to 200 kg ha^{-1} yr^{-1} (Hyvönen et al. 2008).

4.2 Long-term nitrogen addition effects upon the quality and quantity of aboveground litter

4.2.1 Litter quality

The chronic N addition treatments (12.5 and 50 kg N ha^{-1} yr^{-1}) had significant effects on element stoichiometry (i.e. C:N:P) of litter only for bryophytes and

reproductive organs of the trees, while the carbon chemistry (i.e. lignin, cellulose and hemi-cellulose) was not affected by N addition (**paper II**).

Carbon, nitrogen and phosphorus litter concentrations

The absence of N addition effects on most of the litter categories contrast studies conducted on green leaves in the same study system, where an increase of N concentrations in *V. myrtillus* and *P. abies* in the high N treatments relative to the control was observed (Gundale et al. 2014; Nordin et al. 2005). Moss N concentrations significantly increased in both low and high N treatments relative to the control by ~18% and ~81%, respectively. The lack of increased N concentration of *V. myrtillus* and *P. abies* litter may indicate their ability to resorb N prior to leaf senescence (Berg & Matzner 1997; Flanagan & Van Cleve 1983). Furthermore, these findings suggest that vascular plants may likely remain N limited in Svartberget even after 17 years of fertilization at a rate of 50 kg N ha^{-1} yr^{-1}, which is 25 times higher than ambient N deposition rates in the region of study. The suggested persistent N limitation even at high N addition rates may have different causes. First, as described above, results from **paper I** showed that approximately half of the N added over the course of the experiment for both N addition levels was still present in the organic soil horizon and may likely remain inaccessible to plant uptake. Second, results from **paper II** indicate an increase of N concentration in moss tissues which is consistent with studies that showed bryophytes can immobilize a large portion of ambient N_r deposition in their tissues, and thus act as a competing sink at the expense of vascular plants, such as trees (Gundale et al. 2011; Gundale et al. 2014; Turetsky 2003). Further, bryophytes are known to produce recalcitrant litter and thus would stabilize N into a stable soil organic N pools which are unavailable for vascular plant uptake (Lindo et al. 2013; Gundale et al. 2011; Du et al. 2014). Moss P concentration significantly decreased in response to the high N addition treatment, and both C:P and N:P ratios increased. This could be indicative of bryophyte inefficiency to balance their P stoichiometry when N is in excess. This result is in line with Phuyal et al. (2008) who reported no change in moss P assimilation in response to N addition, although an increase of phosphatase activity was observed in their tissues. Contrary to some vascular plants and microbes that increase their phosphatase activities in the surrounding soil, when N availability increases (Treseder & Vitousek 2001; Marklein & Houlton 2012), mosses gain P via precipitation and litter interception (Chapin et al. 1987; Eckstein 2000).

All element concentrations of coniferous reproductive organs decreased in response to the two N addition treatments, with a significant increase in the C:N ratio in the low N treatment relative to the control. The literature has generally reported the opposite trend of higher N concentrations in litter in response to N

addition, resulting in an increase of litter decomposition and a decrease of soil C accumulation (Knorr et al. 2005). However, findings presented in this section showed that the litter stoichiometry of many vascular plants may not always be responsive to N, and in some cases may even become less N enriched. This suggests that aboveground plant litter production and chemistry may not always be as impacted by N as is often suggested by the literature (Berg & Matzner 1997), and may have relatively little influence on soil C dynamics.

4.2.2 Litter quantity

Litter biomass was generally not responsive to the two N addition levels (12.5 and 50 kg N ha^{-1} yr^{-1}), except for moss biomass (**paper II**). Moss biomass significantly decreased by ~73% (0.47 Mg ha^{-1} yr^{-1}) and non-significantly decreased by ~33% (0.21 Mg ha^{-1} yr^{-1}) in the high and in the low N treatments, respectively. Several studies reported a decline of moss biomass under high N_r inputs (Gundale et al. 2013; Van Der Wal et al. 2005; Nordin et al. 2006). When N is taken up in excess (predominantly in the form of NH_4^+), this N is accumulated in moss cells as amino acids, and excess of N has been shown to have a toxicity effect on bryophytes (Nordin et al. 2006; Nordin et al. 1998). Further, the decline in moss biomass has also been attributed to light competition with vascular plants (e.g. trees and ericaceous shrubs) (Van Der Wal et al. 2005). Coniferous needle litter was the most abundant sources of aboveground litter (i.e. *P. abies* and *P. sylvestris* combined) and showed only a marginally statistically significant increase of ~40% (0.46 Mg ha^{-1} yr^{-1}) and ~53% (0.61 Mg ha^{-1} yr^{-1}) in response to the high and the low N treatments relative to the control, respectively, when the two coniferous species needles were combined. This finding is consistent with a study where 23 kg N ha^{-1} yr^{-1} was applied over 14 years, which resulted in an increase of needle biomass and needle leaf area (Krause et al. 2012). My finding is also consistent with another study that reported increasing needle litter in response to 50-150 kg N ha^{-1} yr^{-1}, although in this study higher litter production was attributed both to greater tree mortality and a decline of needle life span (Frey et al. 2014).

4.2.3 Litter element and carbon chemistry fluxes

Aboveground litter fluxes of C, N, P and C chemical forms (i.e. lignin, cellulose and hemi-cellulose) were in general not responsive to N additions, except for *V. myrtillus* leaves and moss litter. *Vaccinium myrtillus* C fluxes significantly increased by ~36% (0.40 Mg ha^{-1} yr^{-1}) in the low N treatment relative to the control, while the C flux showed no differences in the high N treatment relative to the control. In contrast, moss C, N, and P fluxes significantly decreased in the high N treatment relative to the control. Moss C flux was non-significantly

reduced by 34% (0.11 Mg ha^{-1} yr^{-1}) and significantly reduced by 74% (0.24 Mg ha^{-1} yr^{-1}) in the low and high N treatment relative to the control, respectively. When combining all C fluxes from understory and overstory litter categories, the N addition treatment had no effects on the total C flux, although the high N treatment caused a significant decrease of the understory C flux by 59% (0.23 Mg ha^{-1} yr^{-1}) relative to the control. The significant decrease observed in total understory C flux was mainly attributable to reduced moss C flux.

The decrease of moss biomass was larger than the increase of *V. myrtillus* C flux observed in the low N treatment relative to the control. These results highlight that the element fluxes, like the C fluxes of specific litter categories can be responsive to N addition, while the total fluxes may be unresponsive because of the counteracting effect of contrasting litter categories.

While these findings presented in **paper II** may be true in this particular study system, (i.e. a late successional spruce forest subjected to 17 year N enrichment), these results are only representative of the current litter production. Empirical and modelling studies have reported a higher increase of tree production in response to N fertilization when forest are young whereas tree growth tends to slow down with increasing forest stand age (Eliasson 2007; Pettersson & Hogbom 2004). Further, higher tree production in response to the high N treatment was observed during the first 10 years of the experiment, while after that tree and understory vegetation production reached a plateau and have remained stable since (From 2014). The initial higher tree growth observed in the high N addition treatment may have corresponded with higher litter production rates during that period that may have contributed to the increased soil C observed in these plots today (**paper I**). Thus, there is little evidence for N addition causing an increase in contemporary litter production, which suggests alternative processes likely underpin this observed accumulation of soil C.

4.3 Long-term effects upon soil microbial community structure and composition

The main goal of **paper I** and **IV** was to investigate whether chronic N addition rates simulating current N_r deposition (from 3-12.5 kg N ha^{-1} yr^{-1}) in the boreal region and higher N addition rate (50 kg N ha^{-1} yr^{-1}) that have been used in previous N addition studies impact soil microbial community.

Significant differences occurred between treatments at both Svartberget and Åheden experimental forests (i.e. Norway spruce and Scots pine forests, respectively), but relatively more PLFA markers were responsive to the treatments in the Scots pine forest. However, at both sites, a majority of PLFA markers were unresponsive to the lower N addition rates that simulate current N_r

deposition rates in the boreal region (i.e. 3, 6, and 12 kg ha^{-1} yr^{-1}). In both forests, the total and fungal PLFA marker significantly decreased by ~30 and ~40%, respectively in response to the highest rate of N addition compared to the control. In the rest of the cases, only bacteria, gram negative PLFAs and the fungi:bacteria ratio showed a non-significant decline in response to the highest N addition treatment relative to the control in Svartberget. At Åheden, bacteria, gram positive and negative PLFA markers showed a significant decrease in response to the highest N treatment relative to the control, the AM fungal PLFA significantly decreased in response to the high N treatment, but also at 12 kg N ha^{-1} yr^{-1} relative to the control. In contrast, the actinomycete PLFA markers significantly increased in the 12 and 50 kg N ha^{-1} yr^{-1} relative to the control. Multivariate analyses indicated that the fungal 18:2ω6 and some of the bacterial (16:0, 16:1ω7), gram positive (i-15:0) and negative (18:1ω7, cy-19:0) PLFA markers were strongly related to the control plots, while the gram positive (i-16:0) and actinomycete (10me16:0, 10me17:0) PLFA markers were strongly related to the high N treatment plots in Svartberget. Similar patterns were observed in **paper IV**.

The decline in total and fungal PLFAs in response to high N addition treatment coincided with the increase of soil C accumulation in Svartberget (**paper I**), suggesting that the decline in microbial activity and change in microbial community composition likely contributed to the increase of soil C sequestration in the organic soil horizon. Microbes differ in their life history strategy, and previous studies have proposed that N addition may release copiotrophs with high N requirements at the expense of oligotrophs (e.g. fungi) more competitive in N-limited environments (Fog 1988; Ågren et al. 2001). Data from the Scots pine forest support this view, where we observed an increase of actinomycetes in the high N treatments at the expense of fungi, AM fungi and certain bacteria groups. Actinomycetes are saprotrophs that have been shown to be antagonists of soil fungi through the production of antibiotics (Jayasinghe & Parkinson 2008), but also by increasing their abundance and litter decay activity in response to N fertilization (Fierer et al. 2012; Zak et al. 2011). However, results from the Norway spruce forest did not support the previous suggestions (i.e. life strategy), as actinomycete PLFA remain unchanged and bacterial PLFA non-significantly decrease in response to N enrichment. Instead of competition between saprotrophs and biotrophs, fungi constitute an important fraction of the soil microbial community and through their high enzymatic activity decompose soil litter and organic matter which can enhance C availability to other saprotrophic soil organisms (Högberg & Read 2006). Further, the fungal PLFA 18:2ω6 has been shown to correlate well with EM fungal biomass (Frostegård et al. 2010). Thus, these results are instead more consistent with fungi, and

especially EM fungi, having a priming effect through the breakdown of recalcitrant litter residues (Janssens et al. 2010).

4.3.1 Mycorrhizal fungi

One of the goals of setting up root-trenching plots in Åheden was to get soil where EM fungi were excluded and thus distinguish how EM fungi and saprophytic fungi respond to N, and how these responses impact soil C dynamics.

As expected, the root-trenching treatment significantly decreased the fungal PLFA marker, revealing the saprotrophic fungal background within each N addition treatments. Interestingly, the fungal PLFA biomass in the high N treatment was very similar to the fungal PLFA biomass in all the root-trenching plots (i.e. on all N addition treatment plots), suggesting that both the root-trenching and the 50 kg N ha^{-1} yr^{-1} addition treatment had similar impact on EM fungal biomass. The high N treatment may be enough to alleviate N limitation to trees such that EM fungi become C limited. Thus, reduced belowground tree C allocation may be one mechanism explaining the drop of EM biomass in the high N treatment. Further, it was noticeable that EM fungi were unresponsive to the low N treatments applied during 11 years showing that current N_r deposition rates occurring in the boreal region may not alleviate N limitation to trees. Similarly it has been reported in the same study system that ericoid mycorrhizal fungi were unresponsive to these N additions (Ishida & Nordin 2010).

In contrast of the fungal PLFA measurements, the estimation of fungal biomass by ergosterol measurements in the ingrowth sand bags showed no significant effect of any of the N addition treatments, although a non-significant increase of EM biomass at 12 kg N ha^{-1} yr^{-1}, with a large error bar was observed. The EM fungal biomass was, however, responsive to the root-trenching treatment and showed a similar pattern as the fungal PLFA marker. This discrepancy between EM biomass measured as PLFA and ergosterol biomarkers has been previously reported in the literature. Ergosterol content measured in poor organic C substrates may not reflect fungal abundance present in the organic soil layer since it may underestimate certain fungal species e.g. *Cortinarius* spp. (Kjøller 2006; Wallander et al. 2013). In addition traces of ergosterol can also be present in yeasts, protozoa and microalgae (Newell et al. 1987; Pasanen et al. 1999), which would lead to overestimating EM fungal biomass. Previous studies also reported cases where ergosterol was not responsive to treatments e.g. fungicide, fumigation, N fertilization or tree girdling (Zhao et al. 2005; Högberg 2006). For instance Högberg (2006) reported a strong decrease of fungal PLFA in response to long-term N fertilization (34-108 kg N ha^{-1} yr^{-1}), while ergosterol was unresponsive. The author suggested

that ergosterol analyses may not always detect negative effects when soil microbial communities are exposed to long-term perturbations.

The decline of EM fungi in the trenching treatment coincided with the decrease of total PLFAs, fungi:AM fungi, gram positive:gram negative and fungi:bacteria PLFA ratios. Further, the decline in EM fungi coincide with the unresponsiveness of the gram negative PLFA, while it significantly increased the AM fungi, the gram positive, the actinomycete. This increase of PLFAs may likely be due to the competitive release of these microbes at the expense of microbial groups that decline in response to the root-trenching treatment. Further, Söderberg *et al.* (2004) showed that gram positive bacteria are relatively more abundant in the bulk soil than in the rhizosphere, which could have made them less sensitive to the decline of belowground tree C root allocation.

4.4 Long-term nitrogen addition effects upon humus and litter decomposition

4.4.1 Soil environment *versus* litter quality mediating litter decomposition in response to nitrogen enrichment

The main goal of **paper III** was to investigate the importance of litter quality and soil environment from plots subjected to ambient (~2 kg N ha^{-1} yr^{-1}), and simulated N_r deposition (12.5 and 50 kg N ha^{-1} yr^{-1}) on litter decomposition of two vascular plant species, *V. myrtillus* and *P. abies* during two years.

Litter mass loss was significantly affected by the main effect of soil destination, plant species, time and the interactive effects of time and plant species. Decomposition of both *V. myrtillus* and *P. abies* litter decreased when placed in the high N plots which contributed to the main effect of soil destination. In contrast, litter decomposition was not affected by litter origin, suggesting that any shifts in litter quality that may occur in response to N enrichment were too small to have an impact on litter decomposition.

The regression analysis showed the presence of a significant linear relationship between the relative mass loss (i.e. litter bags that decomposed during two years) and the annual N addition rate (R^2=0.08, P=0.008). The slope indicated that litter decomposition decreased by 0.06% per kg of N added per year.

The correlation analyses between litter mass loss and litter chemistry (i.e. %C, %N, %P and ratios) or soil biota variables (i.e. PLFA biomarkers) provided several significant correlations. A majority of significant correlations were between litter mass loss and soil biota variables. For instance, *P. abies* litter mass loss positively correlated with total and fungal PLFAs, and *V. myrtillus* positively correlated with bacterial, gram positive and negative PLFAs, both

during the first year of decomposition. The only significant correlation between litter mass loss and litter chemistry variables was a positive correlation between *V. myrtillus* litter mass loss and the litter %C at year 1. Those relationships suggest that different microbial groups (e.g. fungi, bacteria) are important for decomposition of the two plant species studied during the early stages of decomposition.

4.4.2 Implication of mycorrhizae to litter and humus decomposition in response to chronic nitrogen addition

One of the goals of **paper IV** was to investigate whether EM fungi are the drivers mediating humus and litter decay in response to low-intermediate (3-12 kg N ha^{-1} yr^{-1}) and high N addition rates (50 kg N ha^{-1} yr^{-1}).

Both substrates decomposed slower in response to the high N addition treatment, with litter always decomposing faster than humus (~62% faster). Further, the results suggest that different mechanisms were involved depending upon substrate types. The decline of EM fungi observed in the trenching plots did not coincide with any responses of litter decay, since litter decay was not altered by the trenching treatment, where EM fungi were excluded. Further, litter mass loss was significantly correlated to the fungal PLFA marker within the trenched plots (i.e. saprotrophic fungi; R^2=0.32, P=0.035), but not outside the trenched plots (R^2=0.18, P=0.160). These results suggest that the decline in litter decomposition in response to N addition was likely mediated by changes in species level composition of saprotrophic microbes (fungi and possibly bacteria), suggesting that reduced activity of these microbial groups may contribute to the accumulation of organic matter. Another mechanisms that could explain the decline of microbial biomass in response to high N addition is the impairment of fungal enzymatic activities (Deforest et al. 2004; Freedman et al. 2015) which may reduce the ability of fungi to decompose organic matter (Waldrop et al. 2004; Zak et al. 2008). Further, nitrogen enrichment has been reported to increase actinomycetes, which cannot completely process lignin to CO_2 as white rot fungi can (Godden et al. 1992; Berrocal et al. 1997; Zak et al. 2011), that may alter organic matter decay.

In contrast with litter response, the data showed that humus decay was closely linked to EM fungal abundance. Humus decomposed slower in the trenched plots, where EM fungi were excluded. Further, outside the trenched plots, humus decomposition declined in response to N enrichment similarly to the abundance of the fungal PLFA markers (i.e. saprophytic and EM fungi combined). Further, humus mass loss was significantly correlated to the fungal PLFA marker outside the trenched plots (i.e. saprophytic and EM fungi; R^2=0.31, P=0.044), and EM fungal PLFA (i.e. difference between fungal PLFA marker outside and inside

the trenching plots; R^2=0.46, P=0.004), but not within the trenched plots (i.e. saprophytic fungi, R^2=0.10, P=0.294). Consequently, the decline in humus decay in response to N addition was likely caused by the decrease of EM fungi.

Beside the decline of humus and litter decay in response to the high N addition treatment, litter and humus stoichiometry (i.e. C:N:P) was responsive to the trenching treatment, but not to the N addition treatments. The litter and humus N and P concentrations (i.e. mg g^{-1} litter) were always higher in the trenched-plot, which coincided with the shift in microbial community. Several studies have shown that N and P concentrations increase (i.e. immobilization phase or humification process) during initial litter decay followed by a phase of release (Melillo et al. 1982; Berg & McClaugherty 1989). The patterns observed in this experiment have been previously reported in other studies where the authors suggested that saprophytic fungi may redistribute N from well decomposed substrates (e.g. decomposed litter) to new substrates (e.g. fresh litter) (Boberg et al. 2014; Bödeker et al. 2016). This is consistent with the greater increase of N concentration for litter than humus observed especially in the trenched plots, where saprotrophic microbes are supposed to dominate. Another mechanisms that has been proposed is that as decomposition of recalcitrant compounds increases (e.g. lignin), they begin to incorporate N in their molecular structures, resulting in stabilization mechanisms (Berg & Matzner 1997; Neff et al. 2002). Further, phosphorus concentration was also enhanced in litter, while P was depleted from humus bags regardless of N addition and trenching treatments. As with N, litter becomes more enriched in P at early stages of decomposition, as P is likely to get immobilized into the substrate due to its limitation (Staaf & Berg 1982; Berg & McClaugherty 1989).

4.5 Impact of nitrogen enrichment on soil respiration

One of the goals of **paper I** was to investigate whether the increase of soil C accumulation observed in the organic soil horizon (see section 3.1) in response to N addition coincide with a decline of soil C outputs through soil respiration (i.e. autotrophic and heterotrophic combined).

Soil respiration was significantly affected by the main effect of N addition treatment and time. The data showed a significant effect of time probably due to the higher soil C efflux from July to September compared to the rest of the measuring campaign. The N addition treatments had no effect on soil respiration at any individual sampling time, nor for the low N treatment averaged across all sampling times. However, when averaged across all sampling times the high N

treatment significantly decreased respiration by ~11% (176 g C ha^{-1} h^{-1})[1]. This result is consistent with several previous studies that estimated a decline of soil respiration in response to N addition. Reduced respiration in response to N reported in these studies was *ca.* 30% greater than found here; however, those studies used much higher N application rates (60-180 kg N ha^{-1} yr^{-1}) (Franklin et al. 2003; Olsson et al. 2005). In contrast, several other studies have shown that low N addition rates (20 kg N ha^{-1} yr^{-1}), and even high N addition rates (50-150 kg N ha^{-1} yr^{-1}) applied for a short period of time, may increase soil respiration (Bowden et al. 2004; Hasselquist et al. 2012). Further, the study by Bowden et al. (2004) measured soil respiration after a decade of N addition in the same experiment and then observed a substantial decrease of soil respiration. The authors suggested that the transient increase of soil respiration observed may likely be due to an increase of tree productivity which temporarily increases C allocation to roots, thereby stimulating soil microbial activity.

1. The soil respiration values in manuscript I were miscalculated, and the value in brackets is the corrected value (multiplied by a factor 44).

5 Conclusions and implications

Over the last decade, there has been intense debates in understanding whether increased anthropogenic N_r deposition leads to an increase of C sequestration in boreal forests, especially since forest ecosystems in northern latitudes are N limited. For this reason, the purpose of this thesis was: (i) to evaluate the magnitude to which realistic N_r deposition rates enhance soil C sequestration in boreal forest and (ii) to explore potential mechanisms that may explain any shifts of the soil C pool in response to N enrichment.

The results presented in **paper I** show that the relationship between soil C sequestration and N addition in the organic horizon was 10 kg C kg^{-1} N. This finding complements the C accumulation rate of 16 kg C kg N^{-1} estimated in the same system for the aboveground plant biomass (Gundale et al. 2014). Thus, the total C sequestration increases in response to N enrichment in this boreal forest was 26 kg C kg N^{-1} (Fig. 10). This estimate is of interest because boreal forests represent the second largest C pool, where two-third of the C is stored in the soil (Lal 2005). Some studies suggested that the impact of anthropogenic N_r deposition on C sequestration in boreal and temperate forests may account for a large portion of the unidentified terrestrial C sink for annual anthropogenic CO_2 emissions. However, these authors assumed C accumulation rates as high as 108-500 kg C kg^{-1} N (Magnani et al. 2007; Reay et al. 2008; Eliasson & Ågren 2011). Fertilization studies are often located in regions with high background N_r deposition which makes it difficult to separate the impact of chronic N enrichment. Further, previous studies have also estimated this relationship but have applied N at levels several times greater than upper level N_r deposition rates in each region of study, which makes the magnitude of this relationship uncertain. The Svartberget Experimental Forest used in this thesis is to my knowledge the longest running experiment in the boreal region using levels of N fertilization that simulate upper level N_r deposition rates in the boreal region. While this estimate has to be taken cautiously as this relationship between C gained per unit N added may vary among plant communities, forest age and soil types (Pregitzer & Euskirchen 2004; Thomas et al. 2010), the results presented here are consistent with the most recent modelling and experimental studies on the relation between N enrichment and C sequestration in forest ecosystems (de Vries et al. 2014 and references therein; Hyvönen et al. 2008). Hence, the work presented in **paper I** provides a substantial empirical contribution to the growing consensus that N_r deposition in the boreal region has a relatively small impact on the global C cycle, at least in comparison to what has been previously proposed (Eliasson & Ågren 2011; Reay et al. 2008; Magnani et al. 2007).

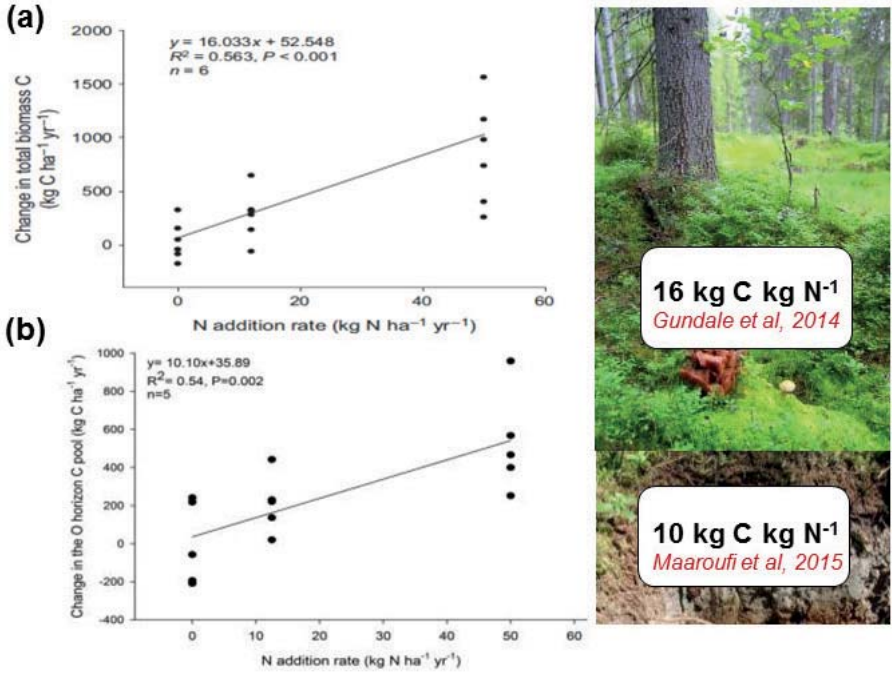


Figure 10. Regression relationship between carbon sequestration (kg C ha^{-1} yr^{-1}) in the standing vegetation (a) or in the soil organic horizon (b) and simulated N_r deposition treatments (0, 12.5 and 50 kg C ha^{-1} yr^{-1}). From Gundale et al. (2014) and Maaroufi et al. (2015).

Furthermore, the results of this thesis also provide insights upon potential mechanisms that may explain the increase of soil C sequestration in response to N enrichment in the boreal region. Enhanced inputs of aboveground litter has been proposed as a potential mechanisms that may explain an N induced increase in soil C. A meta-analysis reported that anthropogenic N addition can have either positive or negative effect on plant litter inputs, but on average the effect was neutral (Janssens et al. 2010). However, most of the data utilized in this meta-analysis were derived from experiments using much higher N doses than the upper N_r deposition rates in the regions of study, leaving some uncertainty regarding the impacts of more realistic chronic N deposition rates. The annual litter inputs estimated in **paper II** showed a minor impact on aboveground litter C inputs when N addition was applied at more realistic N_r deposition rates (~12 kg N ha^{-1} yr^{-1}). In contrast, higher N addition rates (50 kg N ha^{-1} yr^{-1}) caused understory litter C inputs to decline, especially for feather mosses. Thus, there is little evidence for N addition causing an increase in contemporary litter production, which suggests other alternative processes that could underpin this observed accumulation of soil C.

An additional mechanism that likely contributes to enhanced soil C sequestration in response to N enrichment is a shift in microbial mediated soil processes. Microbial responses to N enrichment vary across forest type (plant communities, soil types, tree age), but in both forests low N addition treatments simulating current N deposition rates had no effect on microbial biomass (**paper I and IV**). High N addition rates, on the other hand, decreased total microbial biomass and fungal biomass (i.e. EM fungi). In support of this decline, soil respiration was also altered and has been previously used as a proxy of soil activity (**paper I**). Interestingly, actinomycetes were the only microbes assessed by the PLFA method to increase with N additions, suggesting that they may increase with anthropogenic N enrichment (**paper IV**).

Organic matter decomposition is a key process that influences soil C accumulation. In this thesis, litter quality mediated by N enrichment was not the main driver of litter decomposition while shift in soil properties, especially soil microbes, strongly influence the early stages of litter decomposition (**paper III**). Regardless of litter quality, litter decomposition was negatively affected by N additions, with decomposition rates decreasing by 0.06 % per unit of N added.

Further, **paper III** and **IV** show that current N addition rates in the boreal region may have no or little effect on litter and humus decomposition, whereas high N addition rates (50 kg N ha^{-1} yr^{-1}) impeded the early stage of decomposition of both litter and humus. **Paper IV** also suggest that different mechanisms were involved for these two substrates. The decline of litter decomposition appeared to be mediated by shifts in the abundance or community structure of saprophytic organisms, while the decrease of humus decomposition with high N enrichment was likely the result of reduced EM fungi. In addition, findings from **paper IV** suggest that EM fungi were unresponsive to the low N treatments applied during 11 years showing that N_r deposition rates occurring in the boreal region may not alleviate N limitation to trees.

Altogether, the results of this thesis (Fig. 11) show that long-term N inputs approximating upper level atmospheric N_r deposition rates in the boreal biome are likely to have subtle effects on the soil C balance and therefore on soil C accumulation. Thus, previous suggestions that anthropogenic N_r deposition caused large quantities of C to be sequestered in the boreal region were likely vastly overstated (Magnani et al. 2007; Holland et al. 1997; Reay et al. 2008; Eliasson & Ågren 2011).

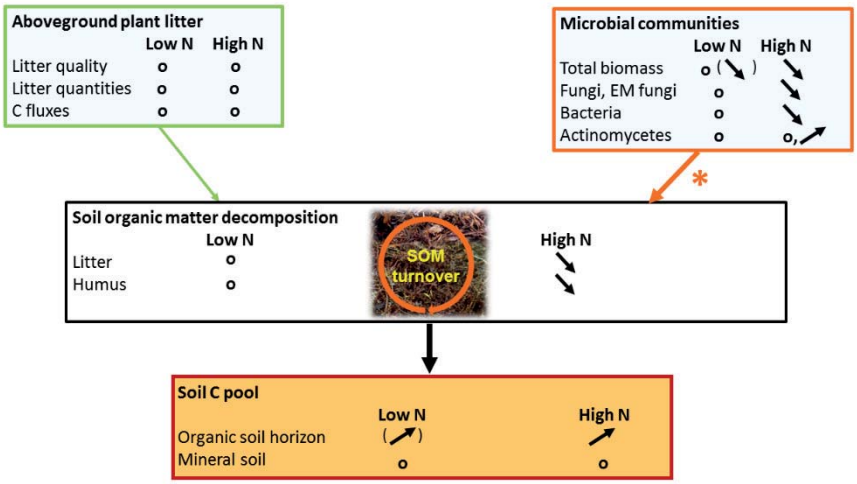


Figure 11. Main findings presented in this thesis. Open circle indicates neutral effect, arrows indicate an increase or a decline, arrows in brackets indicate non-significant results, the star indicates a significant effect of microbial communities mediating soil organic matter decay. Low and high N represent the impact upon low (3-12.5 kg N ha^{-1} yr^{-1}) and high (50 kg N ha^{-1} yr^{-1}) N deposition rates, respectively.

6 Perspectives and future research

Although the findings presented in this thesis improves our understanding of the impact of anthropogenic N_r deposition on the soil C balance in boreal forest as well as on the global C cycle, there are still questions and issues that merit further attention:

- There is an increasing demand of predicting how multiple factors driving climate change interact with each other. Thus, I would recommend to add new factors to the N addition experiments, such as manipulate temperature, since mean air temperature is predicted to rise by 1-3°C at the end of this century (IPCC 2013).
- Caution must be exercised on upscaling the results from my thesis because only two field experiments were utilized to conduct this work. Meteorological and geographical considerations (such as precipitation, temperature, topography) may impact the final results. Thus, there also remains uncertainty whether the effects observed at a specific area are representative across the entire boreal biome.
- Belowground litter inputs (i.e. root turnover, root exudates, hyphal necromass) have been recently demonstrated to contribute to long-term soil C accumulation (Clemmensen et al. 2013). Thus, further research is needed to better understand how N additions affect belowground litter inputs, which also gives an indication of root C allocation.
- In this thesis ergosterol and fungal PLFA marker data gave contrasting results. It raises questions whether another design or duration of incubation would help reconcile the two approaches. Further, it is worth exploring whether other methods would produce even clearer responses such as high throughput sequencing, or measuring ergosterol in ingrowth bags containing soil instead of low organic C substrate.
- Ectomycorrhizae are extensively studied, while studies focusing on ericoid symbioses (i.e. fungal association with ericaceous shrubs) are scarce, despite the fact that ericaceous shrubs are an important component of understory vegetation in boreal ecosystems. A recent study suggest that ericoid fungi may facilitate long-term C sequestration through their production of recalcitrant hyphal tissues (Clemmensen et al. 2015). Thus, I would suggest to study ericoid fungi more extensively, and specifically how they are impacting by anthropogenic N deposition and other climate change factors.
- In this thesis, I measured soil respiration as a proxy of soil microbial activity. The impairment of enzymatic activity may be an underlying mechanisms that could explain the decline of litter decay and soil

respiration. Thus, I would suggest to measure oxidative and hydrolytic enzymatic activities as well as gene expressions to target enzymes that are involved in litter decay.

- It is commonly assumed that boreal and temperate forests are N limited, but increasing anthropogenic N_r deposition may shift the limitation towards P (Elser et al. 2007). So it would be interesting to investigate whether boreal forests are initially co-limited by N and P and/or whether high N enrichment shift the limitation towards P.

On a global basis, fertilizers and the combustion of fossil-fuels have greatly increased the quantity of N_r released into the atmosphere. Swedish forests and Scandinavian forests in general play an important economical role through the production of wood and paper. While this thesis suggests an increase of soil C sequestration in response to N pollution, on a different note, forests are also at the heart of Swedish culture, through literature, arts, but also through production of edible goods. The “Allemansrätten” (i.e. everyman´s right) is a public right that allow people to have access to nature at the exception of the vicinity of houses, it also allows to pick fruits and mushrooms and even camp. The forest can be seen as a common garden, where berry (e.g. bilberries, lingonberries, cloudberries) and mushroom (e.g. boletes, chanterelles) picking gives rhythm to the year. As such, this thesis increases also our knowledge on the impacts of anthropogenic N_r deposition on soil biota (e.g. fungi) and plant (e.g. ericaceous shrubs) resources that are culturally relevant in boreal landscapes.

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