Plant-Microbe-Soil Interactions and Soil Nitrogen Dynamics in Boreal Forests

Development of Nitrogen Limitation

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Cover: Land uplift chronosequence on Bjuren island, northern Sweden.
(Photo: R. Blaško)

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Abstract

Widespread nitrogen (N) limitation of plant growth in boreal forests is a well recognized phenomenon. Yet, the mechanisms responsible for the development of N limitation are unknown. By exploring the linkage between N cycling and microbial community structure, this thesis examines the role of soil microorganisms in N limitation development.

The first part of the thesis addresses effects of long-term N additions on microbial communities and N cycling in Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.) forests and if the effects are reversible after termination of N addition. The second part of the thesis explores the role soil microbes play in the development of N limitation in primary forest ecosystems. The general structure and biomass of soil microbial communities was assessed by phospholipid fatty acid analyses. Soil and ecosystem N cycling were inferred from gross N mineralisation measurements and ^{15}N natural abundance in soil and foliage. Retention of the ^{15}N label by soil microorganisms was used to infer N retention capacity of the ecosystems.

Despite unique responses in microbial communities and gross N mineralisation to long-term N additions between the two studies, some common patterns emerged. Gross N mineralisation, microbial community structure, and N retention were strongly linked. Microbial biomass decreased but gross N mineralisation increased after N addition. The increased biotic N retention after termination of N addition coincided with increased functional role ectomycorrhizal fungi play in ecosystem N cycle as inferred from changes in ^{15}N natural abundance.

In the land uplift chronosequence, large inputs of N through N_2-fixation resulted in soil N accumulation but a decline in N supply rates. This coincided with increasing microbial N-immobilisation and increasing abundance of ectomycorrhizal fungi suggesting their importance in N retention. I suggest that the strong N limitation typical of boreal forests can develop in about 150 years.

This thesis provides strong evidence that ectomycorrhizal fungi played an important role both in the return of N limitation two decades after termination of N addition and in the development of N limitation in a primary boreal forest.

Keywords: gross nitrogen mineralisation, phospholipid fatty acids, ectomycorrhizal fungi, N limitation, boreal forest, land uplift, long-term N addition, N retention

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Dedication

To my parents and grandparents…and to myself for not giving up.

Dreams are there to be followed…

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List of Publications

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

I Blaško, R., Högberg, P., Bach, L.H., Högberg, M.N. (2013). Relations among soil microbial community composition, nitrogen turnover, and tree growth in N-loaded and previously N-loaded boreal spruce forest. *Forest Ecology and Management* 302, 319-328.

II Högberg, M., Blaško, R., Bach, L., Hasselquist, N., Egnell, G., Näsholm, T., Högberg, P., (2014). The return of an experimentally N-saturated boreal forest to an N-limited state: observations on the soil microbial community structure, biotic N retention capacity and gross N mineralisation. *Plant and Soil* 381, 45-60.

III Blaško R., Holm Bach L., Yarwood S., Trumbore S., Högberg P., Högberg M.N. (20XX). Shifts in soil microbial community structure, nitrogen cycling and the concomitant declining nitrogen availability in ageing primary boreal forest ecosystems. *Manuscript*.

The contribution by Róbert Blaško to the papers included in this thesis was following:

I Participating in planning, field sampling, soil and foliage analyses, data analysis, and writing the manuscript.

II Participating in planning, field sampling, soil and foliage analyses, data analysis, and contributing to the manuscript writing.

III Participating in planning, designing experiments, field sampling, soil and foliage analyses, data analysis, and writing the manuscript.

Abbreviations

m – mili, 10^{-3}
μ – micro, 10^{-6}
n – nano, 10^{-9}
‰ – per mil, parts per thousand
ECM – ectomycorrhizal
FAO – Food and agriculture organisation of the united nations
GPS – global positioning system
m a.s.l. – meters above sea level
PLFA – phospholipid fatty acid (analysis)
qPCR – quantitative polymerase chain reaction
$\delta^{15}N$ – delta values of ^{15}N, the heavier stable isotope of N
$\varepsilon_{f/s}$ – enrichment factor

1 Background

1.1 Widespread nitrogen limitation in terrestrial ecosystems

Human perturbation of the global nitrogen (N) cycle has been substantial in the past century (Canfield *et al.*, 2010; Galloway *et al.*, 2004). Large amounts of reactive N have been added through atmospheric N_2 fixed in fertilizers, cultivation of N_2-fixing crops, and fossil fuel combustion (Fowler *et al.*, 2013; Vitousek *et al.*, 2013; Bobbink *et al.*, 2010). Yet, plant growth in many temperate and boreal forest ecosystems remains limited by N (LeBauer & Treseder, 2008; Tamm, 1991; Vitousek & Howarth, 1991). The underlying mechanisms of this phenomenon are not well understood.

Limitation by N develops in boreal forests over time despite the large N pools in the atmosphere and soil. Fractions of the large atmospheric N_2 and soil N pools become available for plant uptake through N_2-fixation and decomposition of the plant litter and soil organic matter by soil microorganisms. However, plants with the potential of highest rates of N_2-fixation occur usually early in the succession and are less common in the high latitude ecosystems compared to, for example, N-rich tropical ecosystems (Menge *et al.*, 2014; Vitousek *et al.*, 2013; DeLuca *et al.*, 2002; Vitousek *et al.*, 2002). Therefore, the decomposition of organic matter and the release of organic N forms available for plants and microbes and the subsequent mineralisation are crucial processes governed by diverse soil microbial communities (Bobbink *et al.*, 2010; Schimel & Bennett, 2004; Chapin *et al.*, 2002). The highest rates of N release are largely related to the availability of carbon (C) to soil microorganisms (Booth *et al.*, 2005) and microbial community structure (e.g., Binkley & Menyailo, 2005). With predicted alterations in anthropogenic N deposition in many regions of the world (Fowler *et al.*, 2013; Lajtha & Jones, 2013), it thus becomes increasingly important to

study how these changes may affect the linkages between soil microbial community structure and N cycling in boreal forest soils.

1.2 Nitrogen retention in forest ecosystems

Increased deposition or experimental addition of N can lead to N saturation, a state occurring when the N supply exceeds the combined N demand of plants and microbes (Aber *et al.*, 1998; Aber *et al.*, 1989; Ågren & Bosatta, 1988). High N availability but low biological demand cause increased nitrification and denitrification, which in turn may result in substantial losses of N from soils through leaching of nitrate (NO_3^-) into the groundwater and through gaseous losses of denitrification products; N_2 and nitrous oxide (N_2O) – a potent greenhouse gas (Fowler *et al.*, 2013; Aber *et al.*, 1998). Hence, the ability of the ecosystems to retain N is central in this context.

The soil C/N ratio has been used as a proxy for overall N status and retention capacity of the soils or entire ecosystem (Dise *et al.*, 2009; Gundersen *et al.*, 1998), but because it changes only very slowly, it might not be a good predictor of NO_3^- leaching (**Paper II**). Indeed, NO_3^- leaching was found to decrease three years after termination of a high N-addition rate in a boreal pine (*Pinus sylvestris* L.) forest at Norrliden (Johannisson *et al.*, 1999) (**Paper II),** but the soil C/N ratio remained nearly unchanged for another 14 years after termination of the high N addition (Högberg *et al.*, 2011; Tamm *et al.*, 1999). On the other hand, the foliar N concentrations and natural abundance of the heavy isotope of N, ^{15}N, in the foliage decreased and an increased abundance of ectomycorrhizal (ECM) fungi was proposed to drive these changes, which were associated with higher N retention capacity of the ecosystem (Högberg *et al.*, 2011; Johannisson *et al.*, 1999). This suggests that the trees and the soil microbial communities may respond in much shorter time than soil C/N ratio to changes in N supply.

Aber *et al.* (1998) hypothesized that high N retention of un-saturated forests may be associated with abundant ECM fungi that without increasing their biomass assimilate inorganic N and produce extracellular enzymes that form stable N compounds in reaction with humus. In contrast, Högberg *et al.* (2011) hypothesized that the increased ecosystem N retention is due to increased C allocation to ECM roots, mycelium, and associated microorganisms under low N supply (Högberg *et al.*, 2010; Högberg *et al.*, 2003). Soil fungi appeared to be stronger N sinks than bacteria in a natural gradient of a N supply (Högberg *et al.*, 2006). Interestingly, lower N leaching under higher growth of ECM mycelium (measured in mesh-bags) was observed across a N deposition

gradient in Norway spruce (*Picea abies* (L.) Karst.) forests in southern Sweden (Bahr *et al.*, 2013).

Some of the effects of high N deposition or N loading such as NO_3^- leaching (Dise & Wright, 1995), decreased decomposition of organic matter (Janssens *et al.*, 2010; Berg & Matzner, 1997; Fog, 1988), and increased N concentration in foliage (Emmett, 2007) are well established. Importantly, high N deposition or additions of N have been found to cause decreases in fungal and bacterial biomass (Wallander *et al.*, 2011; Treseder, 2008; Wallenda & Kottke, 1998) and changes in the community structure as indicated by decreasing fungi/bacteria and gram negative/gram positive bacteria ratios (Demoling *et al.*, 2008; Högberg *et al.*, 2007). The effects of high N deposition or N additions on the gross N mineralisation are still under debate. Only a few studies explored the reversal of N saturation in forest ecosystems by intercepting the high N deposition or by terminating N addition. In such experiments, NO_3^- leaching declined within few years after removal of high N deposition or after termination of the N addition (Johannisson *et al.*, 1999; Boxman *et al.*, 1998; Bredemeier *et al.*, 1998). Contradictory effects of high N deposition withdrawal or termination of N addition on gross N mineralisation have been observed. It is well known that a high N deposition leads to retarded decomposition of organic matter (Berg & Matzner, 1997), which may explain increasing trends in gross N mineralisation found ten years after the high N deposition had been intercepted by the means of a roof (Corre & Lamersdorf, 2004). However, gross N mineralisation was still elevated and not significantly different from the on-going N treatments 14 years after a termination of high N addition in the same boreal *P. sylvestris* forest as studied in the **Paper II** (Chen & Högberg, 2006).

In terms of microbial community, the ECM fungi in the same experiment (**Paper II**) and their function in tree N uptake recovered from the N addition after termination of the treatment, while the bacterial community did not recover (Högberg *et al.*, 2014; Högberg *et al.*, 2011). It was hypothesized that the recovery of ectomycorrhizal symbiosis and its function was associated with the increased C allocation belowground to ECM fungi and their functional role in N uptake (Högberg *et al.*, 2011). Others did not find a clear pattern in recovery of microbial community although the N retention increased after reduction of N deposition (Dörr *et al.*, 2012).

1.3 Nitrogen mineralisation

Measurements of net N mineralisation assessed in the absence of active plant roots were traditionally used as an estimate of plant-available N (Schimel &

Bennett, 2004, and references therein). Net N mineralisation represents the net accumulation of inorganic N over time and is a result of two co-occurring processes; gross N mineralisation and microbial immobilisation of inorganic N (Hart *et al.*, 1994b). One of the main reasons for using net N mineralisation assays as an estimate of plant-available N was the assumption that microbes win over plants in competition for organic N (Schimel & Bennett, 2004; Lipson & Näsholm, 2001). However, the recognition that net N mineralisation rates were negative in some N-limited ecosystems indicating net immobilisation and that they constituted far less N than plants acquired (Nadelhoffer *et al.*, 1992) prompted measurements of gross rates of N mineralisation and nitrification. Gross N mineralisation represents the total production of NH_4^+, while gross nitrification represents the total production of NO_3^- in soil. Measurements of gross N mineralisation and nitrification revealed that gross rates can be over two orders of magnitude higher than the net mineralisation rates, especially in N-limited ecosystems (Stark & Hart, 1997; Hart *et al.*, 1994a; Davidson *et al.*, 1992).

At the same time, other research showed that mycorrhizal plants (Smith & Read, 2008; Read, 1991), arctic plants (Jones & Kielland, 2002; Kielland, 1994), and boreal forest plants can take up soluble organic N forms directly (Kielland *et al.*, 2007; Näsholm *et al.*, 1998) as reviewed by Näsholm *et al.* (2009). Hence, it became clear that plants compete for organic N with microbes and that especially in N-limited ecosystems net N mineralisation rates do not well reflect plant-available N (Schimel & Bennett, 2004; Kaye & Hart, 1997). During the last decades, the view on N cycling has developed and the definition of N mineralisation has broadened (Schimel & Bennett, 2004). The old paradigm viewed N mineralisation as a critical step in the overall N cycling, the new emerging paradigm proposed the depolymerisation of polymers and microsite dynamics as the regulators of overall N cycling (Schimel & Bennett, 2004). Despite some efforts in developing new methods to estimate the gross rate of amino acid production and immobilisation (Wanek *et al.*, 2010), it remains a challenge to quantify the importance of organic N in the plant uptake (Näsholm *et al.*, 2009). Recent methodology using micro-dialysis in the field showed, however, that amino acids can dominate the composition of N species in the soil of N-limited, but also N-loaded boreal forests (Inselsbacher & Näsholm, 2012).

1.4 Soil microbial community structure and N immobilisation

In studies of a short natural gradient of N-supply in a boreal forest, sharp decreases in the abundance of fungi and thus the fungi/bacteria ratio (Högberg

et al., 2006; Nilsson *et al.*, 2005) and retention of ^{15}N label (Högberg *et al.*, 2006) was observed along the gradient from the N-poor to the N-rich ecosystem. It was hypothesized that the decrease in fungi/bacteria ratio was caused by a decrease in below-ground C allocation to ECM fungi in response to high N supply in line with plant C-allocation theory (Waring & Running, 2010; Wallenda & Kottke, 1998; Cannell & Dewar, 1994), which in turn would decrease the capacity of the C-limited microbial community to immobilise N. This hypothesis was corroborated by a dual $^{13}C/^{15}N$ labelling experiments in a nearby *P. sylvestris* forest, in which trees were exposed to $^{13}CO_2$ and ^{15}N was injected into the soil layer dominated by ECM fungi (Näsholm *et al.*, 2013; Högberg *et al.*, 2010). These studies observed high below-ground C allocation to ECM fungi and high N immobilisation in microbial cytoplasm under low N supply. Moreover, in these studies, fungal phospholipid fatty acid (PLFA) biomarkers were the ones most labelled with ^{13}C in contrast to rarely labelled bacterial biomarkers (Högberg *et al.*, 2010) and the ECM root tips were the strongest sink for both photosynthates and labelled $^{15}NH_4^+$ (Högberg *et al.*, 2008). In the same long-term N fertilisation experiment as studied in **Paper II**, ECM fungi and their functional role in the N uptake seemed to be restored and similar to that in the N-limited control plots 15 years after the termination of high N addition (Högberg *et al.*, 2011). It was hypothesized that the decreasing N supply in the terminated treatment stimulated greater C flow below-ground to ECM fungi, which was reflected in ^{15}N-depleted litter and increasing ^{15}N enrichment with the depth of the soil profile. The ^{15}N enrichment in the ecosystems with ECM symbiosis increases with the depth of the soil profile, which was attributed to the retention of the ^{15}N isotope by the ECM fungi. The lighter ^{14}N is to a higher extent passed onto the plant partner and the ^{15}N-depleted plant litter then forms a surface layer that is isotopically lighter than the underlying soil layers (Hobbie & Högberg, 2012; Högberg *et al.*, 2011; Hobbie & Ouimette, 2009; Högberg *et al.*, 1996).

1.5 Interactions between the N and C cycles

While plant growth in boreal forests is N-limited, soil microorganisms are considered to be C-limited. The majority of C entering soil is organic and originates ultimately in photosynthesis, and thus C-limited microbes and N-limited plants are intimately linked. Carbon is supplied to soil microbes mainly via (1) litter production (both above- and below-ground) and via (2) direct allocation of C fixed in photosynthesis to plant roots, root-associated fungi, and associated soil microorganisms. These two C-pathways promote different parts of the soil microbial community. While the litter pathway supports the

heterotrophic community including saprotrophic fungi, the below-ground allocation of C photosynthates to roots will support growth of mycorrhizal mycelium and associated microorganisms (Nazir *et al.*, 2010; Warmink *et al.*, 2009). This explains why the surface layers of soil covered by moss and litter in an N-limited boreal forest are occupied by saprotrophic fungi, while the older F and H layers of the mor humus are predominantly occupied by ECM and ericoid fungi (Clemmensen *et al.*, 2013; Lindahl *et al.*, 2007). Heterotrophic microbes get increasingly C-limited with soil depth because of consumption of available substrates (Paul, 2006), which makes them poor competitors for N against ECM fungi receiving C from their plant hosts, especially under N-limiting conditions.

Substrate properties, particularly with regards to C and N are decisive for the microbial community structure and function (e.g., N mineralisation). Bacteria and fungi have different physiologies and requirements for C and N (Sterner & Elser, 2002). Bacteria require less C per each N atom and in contrast to fungi that respire more C relative to the C converted to the biomass, i.e. C use or growth efficiency of bacteria is lower compared to fungi (Keiblinger *et al.*, 2010). The substrate C/N ratio also determines the balance between mineralisation-immobilisation processes. A soil C/N ratio of 25:1 is commonly regarded as the threshold value indicating whether net N mineralisation or net immobilisation will occur (Chapin *et al.*, 2002). If the soil C/N ratio is above 25:1, N immobilisation will most likely occur because microbes first fulfil their N requirements and absorb additional N from the soil (Chapin *et al.*, 2002). Below a soil C/N ratio of 25, however, microbes are increasingly C-limited and N that is in excess to C is released to soil (Booth *et al.*, 2005; Chapin *et al.*, 2002; Hart *et al.*, 1994a). As reviewed by Booth *et al.* (2005), this is reflected in a negative relationship between soil C/N ratio and gross N mineralisation (when adjusted for differences in soil C) across a wide range of ecosystems. Moreover, fungi/bacteria ratio, i.e. fungal proportions in the microbial community, tends to be higher in soils with higher C/N ratios (Waring *et al.*, 2013; Nilsson *et al.*, 2012).

1.6 When and how does N limitation in a boreal forest occur?

In their review, Vitousek and Howarth (1991) asked the question: How can N limitation on land and in the sea occur? Yet two decades later, we are still lacking solid understanding of this phenomenon. Vitousek and Howarth (1991) proposed four main biogeochemical mechanisms affecting N limitation. First, N is nearly absent in new soils and is mostly derived from the atmosphere. Second, N limitation may result from large losses of N from ecosystems. The

high mobility of some of the N species would potentially promote N limitation. However, the most mobile of the N species is NO_3^- because of its negative charge, but as the latest research shows, NO_3^- is not the dominant form of N in boreal forest ecosystems (Inselsbacher & Näsholm, 2012; Kielland *et al.*, 2007; Nordin *et al.*, 2001). In support of this are gross and net nitrification measurements in boreal forests documenting low nitrification rates (Högberg *et al.*, 2006). Mobilisation of N through disturbances such as fire, wind throw, or clear-cutting could cause substantial losses of N via leakage into groundwater or volatilisation through burning (Sutton *et al.*, 2011; Gundersen *et al.*, 2006; Paavolainen & Smolander, 1998; Smolander *et al.*, 1998). Third, denitrification in sediments can cause N loss from both freshwater lakes and coastal marine ecosystems (Vitousek & Howarth, 1991) and may also occur in some terrestrial ecosystems. Importantly, the fourth mechanism is based on the fact that organic N is directly bond to C in the organic matter forming complex molecules and is not readily available for plant uptake, unless enzymes are engaged in a breakdown of such compounds.

Another question one can ask is: Why does N limitation in older forest ecosystems occur despite historically high inputs from symbiotic N_2-fixation? High energy costs associated with N_2-fixation seem to prevent N_2-fixers from reversing N limitation in the older ecosystems (Vitousek *et al.*, 2013; DeLuca *et al.*, 2002; Vitousek *et al.*, 2002). Another explanation may be limitation of N_2-fixers by another nutrient in disguise such as phosphorus (Vitousek & Howarth, 1991). Phosphorus (P) is derived from the parental material and its availability decreases along with the ecosystem development, which could be the reason why is vegetation with symbiotic N_2-fixers usually confined in the early succession (Vitousek *et al.*, 2013; Vitousek *et al.*, 2002). Although N_2-fixing cyanobacteria living in association with bryophytes occur in the older forest ecosystems (Lindo *et al.*, 2013; Gundale *et al.*, 2011; DeLuca *et al.*, 2002), the rates are far lower compared to N_2-fixation rates of *Frankia* bacteria living in symbiosis with trees, e.g. grey alder (*Alnus incana* (L.) Moench) (Binkley *et al.*, 1992; Huss-Danell *et al.*, 1992; Johnsrud, 1978).

More recently, a potential link between ECM fungi and N limitation has been suggested. It is well known that fungal food webs develop during primary successions (Wardle *et al.*, 2004; Walker & Moral, 2003; Chapin *et al.*, 1994), but the patterns of changes in fungal communities are less clear (Dickie *et al.*, 2013; Jumpponen *et al.*, 2012). In dual $^{13}C/^{15}N$ labelling field-experiments in a boreal *P. sylvestris* forest where ECM fungi dominated microbial community, the high supply of recently fixed C to ECM mycelium coincided with increased N immobilisation under low N supply (Näsholm *et al.*, 2013; Högberg *et al.*, 2010). A C-N exchange model showed that ECM fungi transferred smaller

fractions of N to their plant hosts under conditions of N limitation, but opposite was true when N supply was high (Näsholm *et al.*, 2013). Hence, ECM fungi may potentially aggravate N limitation and the forest ecosystem may become trapped in N limitation (Franklin *et al.*, 2014; Näsholm *et al.*, 2013). Thus, the potential of ECM fungi as a strong N sink and the potential role as a mechanism of N limitation remains to be further explored.

1.7 Objectives and approaches

The overall objective of this thesis was to increase our understanding of the plant-microbe-soil interactions and N cycling in boreal forests and provide insights about mechanisms through which boreal forests progress or return to N-limitation during the ecosystem development or after the termination of long-term N additions.

The effects of experimental N additions on soil microbial community structure and N cycling in otherwise N-limited boreal forests and the linkages between them are discussed in **Paper I** and **II**. The aim was to explore whether the effects of long-term N fertilisation of *P. abies* (**Paper I**) or *P. sylvestris* (**Paper II**) forests are reversible in terms of general microbial community structure and N cycling and how long it takes for these indices to recover from a high N load and become similar to N-limited ecosystems.

Boreal forest ecosystems differing in ground age and N supply were studied along a land uplift chronosequence (**Paper III**). The aim was to address when and how N limitation occurs during the development of a boreal forest ecosystem and to explore the associated changes in the structure of soil microbial communities, N and C cycling.

Specific questions addressed in the thesis

How are soil microbial biomass and structure, gross N mineralisation, and N retention interlinked and do these parameters respond in the same way to long-term N additions in boreal *P. abies* and *P. sylvestris* forests? (**Paper I and II**)

What changes occur in structure and biomass of soil microbial communities and N cycling in boreal forests during ecosystem development or after termination of long-term N additions? When and how do boreal forests progress or return to N-limitation in spite of the high N load and large inputs from N_2 fixation? (**Paper I, II, III**)

Does microbial community structure, and ECM fungi in particular, play a specific role in the development of N limitation? (**Paper I, II, III**)

2 Materials and Methods

2.1 Study sites

2.1.1 Long-term N fertilisation experiments (Paper I, II)

The study sites located at Stråsan (**Paper I**) and Norrliden (**Paper II**) are part of the long-term forest nutrition experiments set up by prof. Carl O. Tamm and co-workers across Sweden (Högberg & Linder, 2014) to study the effects of nutrient additions and acidification on the forest growth (Tamm *et al.*, 1999; Tamm, 1991; Tamm *et al.*, 1974). Both experiments are a full factorial combination of different treatments. However, experiments in **Paper I** and **II** were solely focused on N treatments. The longevity and rates of N additions in long-term fertilisation experiments vary among studies. Four decades of N additions at three different levels in the experiments at Stråsan and Norrliden make them unique in their longevity, rate, and total amount of N additions. At the same time, the discontinuation of some of the treatments in the 1990´s opened up for a unique opportunity to study whether the microbial community structure and N cycling recovered two decades after the termination of N treatments (Table1).

The study site in the **Paper I** was a *P. abies* forest (experiment E26A, *Figure 1*) near Stråsan in central Sweden (60˚55'N, 16˚01'E, *Figure 4*). The site was located at an elevation of 360-410 m a.s.l., which is well above the highest coastline after the last glaciations (Tamm *et al.*, 1974). The annual mean temperature is 3.1˚C and the mean annual precipitation 745 mm, while the background N deposition is < 5 kg N ha^{-1} $year^{-1}$. The soil is a glacial till dominated by medium and fine sand fractions. The previous forest was cut in 1955 and the current forest was planted in 1958 following prescribed burning in 1957. The forest was thinned in 1983.

Figure 1. A soil core of the mor humus with F and H layers from fertilised plot in Norway spruce (*Picea abies*) experimental forest at Stråsan, central Sweden. Photo: R. Blaško.

Table 1. *Average rates and total N load in the fertilisation experiments at Stråsan and Norrliden. The duration of N loading and recovery times are shown. Recovery time was defined as the time since the termination of the treatments.*

	Treatment			
	N0	N1	N2	N3
Stråsan				
Average rate (kg ha^{-1} yr^{-1})	0	34	73	108
Duration (yrs)		42	23	25
Recovery time (yrs)			19	17
Total N load 1967-2009 (kg ha^{-1})	0	1450	1760	2820
Norrliden				
Average rate (kg ha^{-1} yr^{-1})	0	34	68	108
Duration (yrs)		39	38	20
Recovery time (yrs)			1	19
Total N load 1971-2011 (kg ha^{-1})	0	1350	2520	2160

The study site for the **Paper II** was a *P. sylvestris* forest at Norrliden (experiment E55, *Figure 2, 3*) located at an elevation of 260-275 m a.s.l. in northern Sweden (64˚21´N, 19˚46´E, *Figure 4*). Annual mean temperature is 1.2 °C, and the mean annual precipitation 595 mm. Background N deposition in the area is about 2 kg N ha^{-1} $year^{-1}$. The soil is a glacial till with fine sand as the dominant fraction and the soil profile is classified as Haplic Podzol (FAO classification) or Typic Haplocryod (US Soil Taxonomy). The forest was established by planting 2-yr-old seedlings in 1953, after a clear-felling of the old forest in 1951 and a prescribed burning in 1952.

Figure 2. Control treatment in the experimental Scots pine (*Pinus sylvestris*) forest E55 at Norrliden, northern Sweden. Photo: R. Blaško.

In these experiments, four treatments (N0, N1, N2, and N3) were replicated on two (**Paper I**) or three (**Paper II**) 30 x 30m plots organised in a random block design. The N in form of ammonium nitrate (NH_4NO_3) had been added for four decades at three different rates (Table 1); the overall average rates of N additions were 35 (N1), 70 (N2), and 110 kg ha^{-1} $year^{-1}$ (N3). The N0 plots served as control and did not receive any other N additions in excess to atmospheric deposition. At Stråsan **(Paper I)**, the N2 and N3 treatments were terminated in 1990, and 1992, respectively (Table 1). At Norrliden (**Paper II**), the N3 treatment was terminated in 1990 (Table 1) and the N2 in 2008, which

was only one year prior to our study and thus this treatment was considered to be still on-going. The average rates for particular fertilisation experiment and treatment, as well as the total N loads are shown in Table 1.

Figure 3. Scots pine (*Pinus sylvestris*) forest in fertilised plot in the E55 experiment at Norrliden, northern Sweden. Photo: R. Blaško.

2.1.2 Land uplift chronosequence of a boreal forest ecosystem (Paper III)

Following the last deglaciation around 10 000 years ago, isostatic rebound causes the crust once pressed down by the thick glaciers to emerge from the sea. The rate of this land uplift along the Gulf of Bothnia is among the highest in the world and is currently about 8 mm yr^{-1} in the area of the study sites (Vestøl, 2006; Ekman, 1996). The study sites were located within a nature reserve on Bjuren island (N 63°44′, E 20°35′) in the Gulf of Bothnia, c. 15 km SE of Umeå, Sweden (*Figure 4*). The mean annual temperature was 5°C and the mean annual precipitation c. 550 mm (2003-2012, Swedish meteorological and hydrological institute, SMHI webpage). The mean annual N deposition in this area was < 3 kg ha^{-1} (2003-2012, SMHI webpage).

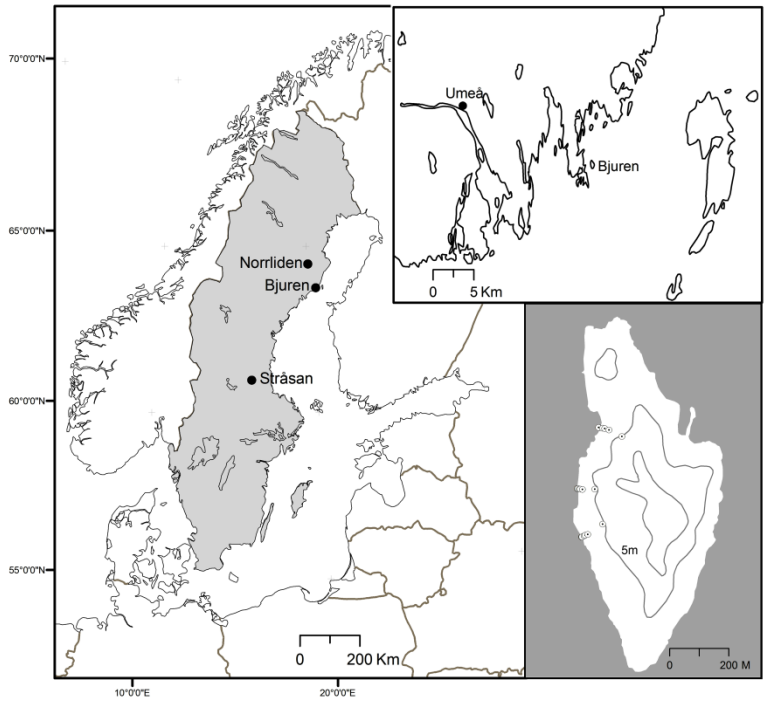

Figure 4. Location of the study sites used for **Papers I-III** across Sweden. The map of Bjuren island, the study site of **Paper III**, is enlarged and shows the location of the studied ecosystems and transects.

Five sites, representing ecosystems different in ground age and vegetation cover (*Figure 5*), were selected and replicated along three parallel transects running perpendicular to the shoreline (*Figure 4*). The ground age of these five sites was estimated from the elevation above the sea level (Trimble R6 GPS Rover, Trimble Navigation Limited, California, USA, Sweref99 coordinate

system) and the land-uplift rate (8 mm yr^{-1}) in this region (Vestøl, 2006; Ekman, 1996). The elevation measured by GPS was related to the average sea level (RH 2000 measuring system, SMHI) and the actual sea level on the day of measuring for the closest station at Ratan that is part of the sea observations network of the SMHI. The accuracy of the GPS measurements given by the manufacturer is ± 2-5 cm, which would translate to c. ± 2-6 years in this study.

The youngest, 25-yr-old ecosystem (*Figure 4*) was represented by recently exposed ground (25 ± 12 years old, mean ±1 SE) with meadow vegetation (e.g., *Agrostis stolonifera* var. *bottnica* Hyl. and *Juncus balticus* Willd.). The 115-yr-old ecosystem (112 ± 13 years) was dominated by *A. incana* vegetation. A distinct borderline between *A. incana* vegetation and an emerging dense *P. abies* representing the 150-yr-old ecosystem (151 ± 15 years) was discernible. A young *P. abies* stand dominated the 215-yr-old ecosystem (215 ± 24 years). The oldest, 560-yr-old coniferous ecosystem (564 ± 31 years) was dominated by old *P. abies* and *P. sylvestris*.

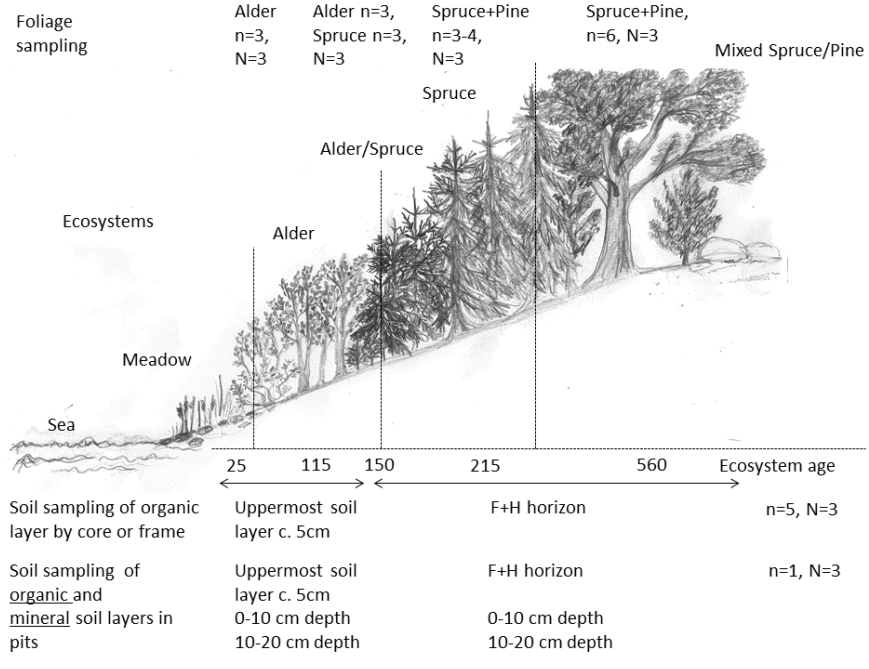

Figure 5. Land uplift chronosequence representing five ecosystems differing in ground age and vegetation composition on Bjuren island in northern Sweden. Dominant tree species were: grey alder (*Alnus incana*), Norway spruce (*Picea abies*), and Scots pine (*Pinus sylvestris*). The ground, or ecosystem, age was estimated from the elevation of the sites above the sea level and the land-uplift rate (Vestøl, 2006; Ekman, 1996). The figure shows sampling design for the soil and foliage and number (n) of samples forming the mean values for every transect (replicate = N). Note that the scale is approximate.

The soils were sandy and silty glacial deposits (FAO) with large amounts of boulders in the recently exposed and young ecosystems and large fraction of sand in the mineral layers. The continuous organic horizon of a mor humus type with F and H layers (Fisher & Binkley, 2000) started to form in the 150-yr-old ecosystems under *P. abies* and from there onward soils were classified as Haplic Podzol (FAO classification).

2.2 Methodological considerations

2.2.1 Soil sampling and handling

In **Papers I** and **II** and in the coniferous ecosystems of **Paper III**, the microbial community structure and N cycling rates were estimated in the soil F and H layers (equivalent approximately to Oe and Oa horizon) where most of the biological activity in boreal forests occurs. Moreover, F and H layers in this type of boreal forests were expected to be dominated by ectomycorrhizal fungi (Clemmensen *et al.*, 2013; Lindahl *et al.*, 2007) and ectomycorrhizal N uptake was shown to occur in these layers in a boreal *P. sylvestris* forest in this region (Lindahl *et al.*, 2007). In the two youngest ecosystems of the chronosequence (**Paper III,** *Figure 5*), the mor humus was not developed and the organic layer was not continuous, thus; the upper soil to about 5 cm depth was sampled using spade and a frame of known area.

The mean plot (**Paper I,** $N = 2$**; Paper II,** $N = 3$) or ecosystem age (**Paper III,** N = 3) values were based on five composite samples each consisting of 2 - 7 randomly collected soil cores within the inner 20 x 20 m area of the N0-N3 plots (**Paper I and II**) or within the 10 x 15 m area of the different ecosystems (**Paper III**). The five composite samples consisting of 2 - 7 soil cores should have ensured that the plot (**Paper I and II**) or ecosystem age (**Paper III**) means were representative of the heterogeneity in soil microbial processes and N cycling.

The ECM fungal mycelium (and associated microorganisms) in the soil sample collected by soil core is disconnected from its plant host and thus its vital C supply. Hence, the fungal PLFA biomarker 18:2ω6,9 abundance decreases in the first few days after sampling and storage between 4.5 and 25˚C (Petersen & Klug, 1994). This PLFA biomarker degrades over prolonged period of time if stored at temperatures higher than 15˚C, but remains rather stable at 5˚C for several months (Bååth *et al.*, 2004). However, the time since sampling in this study is not specified, which complicates the comparison with the findings by Petersen and Klug (1994). Degradation of ECM fungal mycelium should alter microbial community structure and stimulate the growth of heterotrophs (Lindahl *et al.*, 2010), which could in turn alter gross N

mineralisation. Therefore, to minimise the putative sampling effects, soil samples were: (i) kept on ice during the sampling and transport to the laboratory where they were stored at 4˚C and (ii) the soil for gross and net N mineralisation assays were conducted within one and a half day from sampling. Subsamples of homogenized soil for PLFA analysis, qPCR, radiocarbon, and soil chemistry (except pH) analyses were freeze-dried also within one a half day from sampling and kept at -20˚C until the analysis.

2.2.2 Microbial biomass and community structure

In the past, soil microbial biomass was mainly estimated by fumigation-incubation method (Jenkinson & Powlson, 1976). This method was failing in strongly acidic soils and required 10 days of incubation (Jenkinson *et al.*, 2004). Hence, new and rapid fumigation-extraction methods for measuring microbial biomass C (Vance *et al.*, 1987) and N (Brookes *et al.*, 1985) were developed and calibrated to the incubation method. One of the problems of fumigatione-extraction resides in the efficiency of extraction of the microbial biomass C and N, denoted as k_{EC} or k_{EN}. These constants are different for different groups of soil microorganisms, soil types and also depend on the calibration of the fumigation-extraction (Jenkinson *et al.*, 2004). Brookes et al. (1985) calibrated fumigation-extraction against fumigation-incubation. They found that chloroform ($CHCl_3$)-N after one day of fumigation represented 79 % of the N mineralised during the 10-day fumigation-incubation, in which 68 % of the original microbial biomass N was mineralized, and hence the $k_N = 0.68$ (Shen *et al.*, 1984). Thus, a $k_{EN} = 0.54$ was derived from this relationship (Brookes *et al.*, 1985):

Biomass N = ($CHCl_3$-N/0.79)/0.68, which equals to:
Biomass N = $CHCl_3$-N/0.54.

In a similar manner, the k_{EC} derived from calibration with fumigation-incubation yielded $k_{EC} = 0.38$ (Vance *et al.*, 1987). In the present study (**Paper III**) however, $k_{EC} = k_{EN} = 0.4$ were used and were based on the study from a boreal *P. sylvestris* forest in Finland with a similar soil type, in which the $CHCl_3$-labile C and N were calibrated against direct microscopic counting of bacteria and fungi (Martikainen & Palojärvi, 1990). The mean ratio of $CHCl_3$-C to C obtained in the microscopic counts did not correlate with fungi/bacteria ratio (obtained in microscopic counts) suggesting thus that it can be applied to a range of different soil types (Martikainen & Palojärvi, 1990). The microbial biomass C and N in the **Paper III** were calculated as:

$C(N)_{mic} = C(N)_f/k_{EC(EN)}$,

where $C(N)_f$ = (organic C (N) extracted from fumigated soil) − (organic C (N) extracted from unfumigated soil) (Högberg & Högberg, 2002; Brookes *et al.*, 1985).

Phospholipid fatty acid (PLFA) analysis was used to assess the biomass and general structure of soil microbial communities (Frostegård *et al.*, 1993). Phospholipids are building units of all cell membranes in living organisms and the fatty acids derived from them were shown to be specific for different taxonomic groups (Frostegård *et al.*, 2011; Zelles, 1999; Frostegård & Bååth, 1996; Tunlid & White, 1992). The turnover of PLFAs is fast and thus the PLFA profiles reflect the living microbial biomass, which is an obvious advantage of this method (Frostegård *et al*. 2011, and references therein) . The PLFA 18:2ω6,9 was found to correlate with ergosterol, a fungi specific compound, in a wide range of natural ecosystems (Wallander *et al.*, 2013; Högberg, 2006; Frostegård & Bååth, 1996) and this PLFA is commonly used as a fungal biomarker (Wallander *et al.*, 2013; Frostegård *et al.*, 2011; Frostegård & Bååth, 1996). However, it seems to be an invalid biomarker in disturbed ecosystems (Demoling *et al.*, 2008; Högberg, 2006). The other commonly used biomarker for fungi is 18:1ω9 PLFA, which is also present in some bacteria (Frostegård *et al.*, 2011; Ratledge & Wilkinson, 1988). The 18:1ω9 PLFA correlates well with the 18:2ω6,9 PLFA but these two biomarkers did not correlate with ergosterol in the N-loaded *P. sylvestris* forest at Norrliden (Högberg, 2006). In addition, neither 18:1ω9 PLFA, nor ergosterol responded to tree girdling that stopped belowground C-allocation of photosynthates to ECM roots in a similar *P. sylvestris* forest (Högberg, 2006) to the one at Norrliden (**Paper II)**. While ergosterol did not respond to N treatment, 18:1ω9 PLFA decreased in the N1 - N3 treatments but less so than 18:2ω6,9 PLFA (Högberg, 2006). Hence, ergosterol and 18:1ω9 PLFA fungal biomarkers may not be universally good biomarkers for ECM fungi. In contrast, 18:2ω6,9 PLFA correlated strongly with the ECM sequences in the *P. sylvestris* forest at Norrliden where it decreased by c. 50% after N loading in the N1 and N2 treatments and decreased by 45% after tree girdling in a similar *P. sylvestris* forest (Högberg *et al.*, 2011; Yarwood *et al.*, 2009; Högberg, 2006). Although 18:2ω6,9 PLFA may derive from the roots, these contributions are negligible (Kaiser *et al.*, 2010) and were further minimized by removing roots larger than 1 mm in diameter (**Paper I-III**). Algae are also known to contain the fungal 18:2ω6,9 PLFA, although it is unlikely that they have occurred in the dark soil in **Papers I** and **II** but have contributed to this biomarker in the youngest ecosystem in **Paper III**. Quantitative polymerase

chain reaction (qPCR) enabled quantification of targeted fungal genes using the method described previously (Yarwood *et al.*, 2010; Fierer *et al.*, 2005). The pattern of the fungal gene quantities obtained from the qPCR was in agreement with the pattern of fungal 18:2ω6,9 PLFA abundances and corroborated that 18:2ω6,9 PLFA biomarker was of fungal origin across all ecosystems in the chronosequence (see Supplementary material **Paper III**).

For the above mentioned reasons, only the 18:2ω6,9 PLFA was used as a fungal biomarker in this thesis, while 18:1ω9 PLFA was included in saturated/monounsaturated PLFAs ratio. The ratio of fungal/bacterial PLFA biomarkers (Frostegård & Bååth, 1996) has been widely used to describe the proportions of major taxonomic groups of microorganisms in the community (Frostegård *et al.*, 2011). Here, the ratio of 18:2ω6,9 PLFA to ten bacterial PLFA biomarkers was used as an indicator of ECM fungal and bacterial proportions in the microbial community. The other microbial indices used were gram negative/gram positive (G-/G+) bacteria, saturated/monounsaturated (sat/mono), and cyclopropyl/precursors (cy/pre).

2.2.3 Internal N cycling

While measuring net rates in agricultural soils (Jansson, 1958) and in some forest ecosystems can give a relatively good estimate of N mineralisation (Hart *et al.*, 1997), this is usually not the case in N-limited ecosystems because gross N mineralisation can exceed net estimates by two orders of magnitude (Booth *et al.*, 2005; Chapin *et al.*, 2002; Hart *et al.*, 1994b). On the other hand, assessment of gross N mineralisation rates requires the use of a more advanced pool dilution technique and ^{15}N stable isotopes at high enrichment levels (Stark, 2000; Kirkham & Bartholomew, 1954).

The principle of the pool dilution method is the labelling of soil NH_4^+ pool (product pool) with highly enriched label, such as 99 atom% ^{15}N-NH_4Cl used in this thesis. The rate of NH_4^+ production (Schimel, 1996) is then calculated from the dilution of the ^{15}N enrichment of the soil $^{14+15}NH_4^+$ pool by $^{14}NH_4^+$ mineralised from indigenous organic N. The pool dilution method rests on three major assumptions: (1) that no fractionation during NH_4^+ production occurs, i.e. there is no preferential use of either ^{14}N or ^{15}N (see e.g. Figures 4 and 5 in Powlson & Barraclough 1993); (2) microorganisms immobilise but do not remineralise added ^{15}N; and (3) and the mineralisation rates are constant throughout the incubation (Hart *et al.*, 1994b; Kirkham & Bartholomew, 1954). The error caused by fractionation would be small taking into account the high enrichment of the labelled pool (Murphy *et al.*, 2003; Davidson *et al.*, 1991). To avoid remineralisation of the ^{15}N label, incubation between one and three

days is recommended (Hart *et al.*, 1994b; Bjarnason, 1988). In this thesis, one-day incubation was used to avoid the remineralisation of added label.

Studies on gross N mineralisation have used a variety of approaches. For example, pool dilution was applied in intact soil cores incubated *in situ* in the field to test, whether a homogenous distribution of the ^{15}N label in the sample is necessary (Davidson *et al.*, 1991). In this study, authors concluded that perfectly homogeneous distribution of label is not necessary if the microbial processes are randomly distributed (Davidson *et al.*, 1991). However, significant errors in gross rate estimates could arise when the label distribution is not uniform, while the microbial activity is non-randomly distributed in the soil sample (Davidson *et al.*, 1991). Other studies used also different variations of *in situ* incubations of intact cores (Booth *et al.*, 2006; Westbrook & Devito, 2004; Verchot *et al.*, 2001). However, microbial processes in N-limited ecosystems are often non-randomly distributed (Schimel & Bennett, 2004), and hence, the errors arising from non-uniform label distribution could be significant. To ensure homogeneous distribution and mixing of the label in the sample, sieved (< 5 mm), root free, and homogenized soil was used in gross N mineralisation assays in **Papers I-III**. Mixing of soil may, on the other hand, stimulate release of the substrate for microbial processes and thus increase gross rates (Booth *et al.*, 2006; Schimel *et al.*, 1989). However, the largest errors in gross N mineralisation estimates could most likely arise from the fact that the degradation of ECM mycelium representing 30 – 40% of microbial biomass in boreal forest (Högberg *et al.*, 2010; Högberg & Högberg, 2002) would promote growth of heterotrophic microbial community and alter N turnover. Therefore, the short time between sampling and ^{15}N pool dilution assay along with storing the soil samples at around 4˚C prior to pool dilution may be crucial in this context. In the presented studies, the extraction of the soil and ^{15}N pool dilution assay was commenced within 36 hours from the sampling (**Papers I-III**).

The time between sampling and pool dilution can vary greatly among studies. For example, soil samples were preincubated for a week at 15 - 20˚C prior to the pool dilution in some studies (Zeller *et al.*, 2007; Vervaet *et al.*, 2004), while soil in other studies was extracted within 24 hours from sampling (Christenson *et al.*, 2009; Boyle *et al.*, 2008; Zeller *et al.*, 2008). The incubation procedures and times in gross N mineralisation studies also vary greatly. One-day incubation used here (**Paper I-III**) was in the line with recommendations to avoid remineralisation of added ^{15}N (Hart *et al.*, 1994b; Bjarnason, 1988; Kirkham & Bartholomew, 1954). In some studies, soil moisture was adjusted to eliminate the variability in soil moisture among samples (Christenson *et al.*, 2009; Vervaet *et al.*, 2004; Merilä *et al.*, 2002a). In

the gross N mineralisation assays in **Paper I-III**, soil moisture was not adjusted and a constant temperature during the incubation period (17˚C) was maintained. The amount of the added ^{15}N label was on average 1.3 μg N g^{-1} organic matter and the volume of the label represented on average 8.1% ± 4.4 (SD) of the soil water content. The gross N mineralisation was calculated using equations by Hart *et al.* (1994b) (see **Paper III**).

2.2.4 N retention

Calculation of N retention was based on the retention of ^{15}N label added to the soil NH_4^+ pool at the time zero (t_0) in the ^{15}N pool dilution studies (see methods for gross N mineralisation in **Papers I-III**). The recovered ^{15}N in the salt extractable NH_4^+ pool within 30 s (t_0) from injection of the label is:

Recovery (%) = 100% * (^{15}N atom% in excess of natural abundance at t_0 *$[NH_4^+]t_0$ / ^{15}N injected).

The fraction of ^{15}N retained was calculated as follows:
Retention (%) = 100 (%) – Recovery (%).

2.2.5 Functional role of ECM fungi in tree N uptake inferred from ^{15}N in foliage and soil

The difference between the foliar and soil $\delta^{15}N$ values expressed as the enrichment factor, $\varepsilon_{f/s}$, can be used as an indicator of N availability (Garten & Miegroet, 1994) and a functional role of ECM fungi in the N uptake (Högberg *et al.*, 2011). Under N-limiting conditions, N-transport from the ECM fungal mycelium discriminates against the heavier ^{15}N isotope and the lighter ^{14}N is to a slightly greater extent passed to their plant host. Thus, the foliage becomes ^{15}N-depleted relative to the soil (Högberg *et al.*, 2011; Hobbie & Ouimette, 2009; Högberg, 1997). Consequently, ^{15}N-depleted litter forms a surface layer with lower $\delta^{15}N$ than the underlying soil layers (Högberg *et al.*, 2011; Högberg, 1997). The increasing enrichment of the soil profile with depth is associated with the residues from turnover of ^{15}N enriched ECM mycelium and reflects movement and transformations of N spanning from decades to centuries or most likely millennia in some soils (Clemmensen *et al.*, 2013; Hobbie & Ouimette, 2009; Wallander *et al.*, 2009; Högberg, 1997; Högberg *et al.*, 1996)

2.2.6 Statistics

In all statistical analyses (i) plot means of five composite samples (Stråsan, N = 2, n = 8; Norrliden, N = 3, n = 12, see 2.2.1), (ii) treatment means (N = 2

at Stråsan and N = 3 at Norrliden for each treatment, one plot in each of three blocks, see 2.1.1), or (iii) ecosystem age means (N = 3, n = 15) were used as specified below. In *Figure 6* and *Figure 7* in the thesis, relationships between variables were tested by linear regression (n = 8 and 12, respectively, $p < 0.05$). Nitrogen treatments were assigned randomly to plots within the block, so each block contained the control (N0), N1, N2, and N3 treatments. ***Stråsan (Paper I)***. This experiment was used because of its unique longevity of 42 years and because it is situated in a boreal *P. abies* forest. However, the experimental design did not allow testing for the significance of N-treatment effects in analysis of variance (ANOVA). Thus, the trends in the mean values are reported. To complement and to enhance the understanding of the results mean values and variance for each plot (n=5) are reported in the Supplementary material of **Paper I** in Table S1 and S2. The Pearson product moment correlations (n = 8) were used for testing relationships ($p < 0.05$) among the variables. ***Norrliden (Paper II)***. Effects of N treatments and blocks on variables were tested by two-way ANOVA. Holm-Sidak post hoc test for pairwise comparisons followed the ANOVA when the treatment effect was significant ($p < 0.05$). The strength of correlations among variables was tested by Pearson product moment (n =12 plots). ***Bjuren (Paper III)***. The mean values over three replicate transects (*Figure 4*) were used to test for the significance of differences among the ecosystem ages (*Figure 5*). Correlations among variables were tested in Pearson product moment (N = 3, n = 15, $p < 0.05$).

3 Results and Discussion

In this thesis, the main results from the individual studies are summarised and linked to the objectives as expressed in the beginning of the thesis. The overall theme of the three studies was to explore the role of soil microorganisms in the development of N limitation in boreal forests.

One of the main objectives of the studies in **Papers I** and **II** was to investigate the links among soil microbial biomass and structure, gross N mineralisation, N retention, and tree growth in the studied boreal *P. abies* and *P. sylvestris* forests and how these variables respond to long-term N additions Furthermore, these studies aimed to examine whether these N-loaded ecosystems returned to a state of N-limitation two decades after termination of N additions in the sense that the processes became similar or not significantly different from the control.

3.1 Long-term N fertilisation experiments (Papers I and II)

Overall, the results shown in **Paper I** and **II** suggest that microbial community structure, gross N mineralisation, and N retention are strongly linked in both *P. abies* and *P. sylvestris* boreal forests. Despite a few unique responses to long-term N additions and the termination of high N addition, some general trends emerged: First, gross N mineralisation increased after additions of N. Second, the lowest rate of N addition (N1 treatment) had the most positive effect on gross N mineralisation and on stem wood production. Third, gross N mineralisation was negatively correlated to N retention and was linked to the general structure of microbial communities. Fourth, increasing N retention corresponded to a trend of putative increasing functional role of ECM fungi for tree N uptake. Fifth, microbial biomass per gram organic matter decreased in the N-addition treatments. However, many of the responses to

long-term N addition and its termination in the studied *P. abies* and *P. sylvestris* forests were more complex and specific.

3.1.1 Experimental *P. abies* forest at Stråsan

The experimental design in *P. abies* forest (**Paper I**) with only two replicate plots per treatment did not allow statistical testing of N-treatment effects (see 2.2.6). However, it was possible to test for the relationships among variables and their response to N addition as well as to the termination of N additions (*Figure 6*, Table 3 in Paper I).

Additions of N had a stimulating effect on total stem volume in all the treatments (Fig. 1 in **Paper I**). The addition of N at all three rates had the same effect on the wood production rate during the first c. 20 yrs of the experiment. However, thereafter the wood production rate in the N3 treatment decelerated by the addition of 108 kg N ha^{-1} yr^{-1} resulting in a lower mean value for total stem volume in 2010 relative to the N1 and N2 treatments but still higher compared to control treatment (Fig. 1 in **Paper I**). This highlights that the addition of N at an average rate of 108 kg N ha^{-1} yr^{-1} over 25 yrs and an accumulated N load of 2820 kg N ha^{-1} had no further beneficial effect on wood production (Tamm, 1991). Two decades after termination of N additions, the mean N concentrations (%) in needles in the N2 and N3 treatments decreased to the levels comparable with those in the control (N0) plots (Fig. 2a in **Paper I**) which suggests decrease in soil N availability and tree N uptake.

The microbial biomass as indicated by the total abundance of PLFA biomarkers per gram of organic matter decreased in the on-going N1 treatment and remained low in the terminated N2 and N3 treatments relative to the control N0 almost two decades after termination of N additions. The decrease in the microbial biomass following N addition is a common response observed for forest soil (Demoling *et al.*, 2008; Treseder, 2008). However, a more complex picture emerged when the abundance of PLFA biomarkers was expressed per unit area, which provides information that is more relevant from the ecosystem and tree perspective. The abundance of PLFAs per m^2 was unaffected by N additions with an exception of higher mean values in the N2 treatment reflecting the highest organic matter content per m^2 and thickest mor layer in this treatment (Table 2 in **Paper I**). Both bacterial and ECM fungal abundances changed roughly in the same proportions and thus the mean ECM fungi/bacteria ratio was rather constant across the N treatments (Table 2 and Fig. 5 in **Paper I**).

The $\delta^{15}N$ of *P. abies* needles was correlated to the retention of the ^{15}N label ($R = 0.89$, $p < 0.01$) but not to the ECM fungal biomarker, which corresponded to the rather constant proportions of ECM fungi in the microbial community in

this forest soil (**Paper I**). Despite the lack of an apparent effect of N addition on the ECM fungi/bacteria ratio, increasing $\varepsilon_{f/s}$ in the terminated N2 and N3 treatments (2.4 and 3.8‰, respectively compared to 4‰ in N0 and 0.2‰ in the on-going N1) suggested lower N availability and increasing functional role of ECM fungi in tree N uptake two decades after termination of N additions.

Retention of ^{15}N label (%) in boreal forest soil is thought to be of biotic origin and indicative of the ecosystem capacity to immobilise N (*cf.* Högberg *et al*. 2011, Högberg *et al.* 2006). The highest mean retention of ^{15}N label in the control plots suggested N-limitation of the microbial community (*Figure 6*). There was an increasing trend of N retention in the terminated N2 and N3 treatments relative to the on-going N1 treatment (*Figure 6*), however, N retention in the N2 and N3 treatments was comparably lower than in the control plots (Table 4 in **Paper I**). Similarly, high N retention was found in a natural N supply gradient in a boreal *P. sylvestris* ecosystem where low N supply corresponded to a high ECM fungi/bacteria ratio (Högberg *et al.*, 2006). On the other hand, the lowest mean values for N retention in the on-going N1 corresponded to the N retention in a productive ecosystem with high N supply but low ECM fungi/bacteria ratio (Högberg *et al.*, 2006). In the study **I**, retention of N was related to the general structure of microbial community as described by scores obtained in non-metric multidimensional scaling (NMS) analysis (*Figure 6*, $R^2_{adj} = 0.65$, $p = 0.01$). From this relationship (*Figure 6*) as well as from the cluster analysis (**Paper I**, Supplementary material), it is apparent that the terminated N2 and N3 were in a transition phase in between the N3 and N0 plots. This suggests that neither microbial community structure nor N retention had recovered 19, and 17 years, respectively, after termination of N additions in the N2 and N3 treatments (*Figure 6*, Table 2). Nitrogen retention was negatively correlated with gross N mineralisation rates (per gram organic matter, $R = -0.93$, $p < 0.001$, per m^2 see Fig. 4b in **Paper I**) suggesting that microbial communities were N-limited and immobilised more N under low N supply as observed previously in boreal forests of this region (Högberg *et al.*, 2006). The tight linkage between gross N mineralisation and the general structure of microbial community as described by NMS ordination method was depicted in Fig. 6b of **Paper I**. Following long-term N addition, gross N mineralisation rates increased and remained consistently high in the terminated N2 and N3 treatments almost two decades after termination of N treatments (Fig. 3 and Table 4 in **Paper I**).

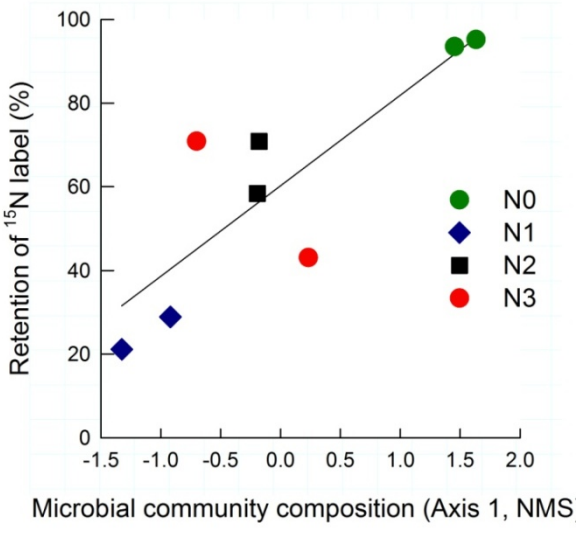

Figure 6. Retention of ^{15}N label in relation to the general microbial community structure (linear regression, R^2_{adj} = 0.65, p = 0.01, n =8) in the long-term N fertilisation experiment at Stråsan. Axis 1 scores were obtained from non-metric multidimensional and explained 93% of the variation in the data describing microbial community structure. Values are plot means (see 2.1.1).

3.1.2 Experimental *P. sylvestris* forest at Norrliden

In general, N fertilisation in the *P. sylvestris* forest had a beneficial effect on tree growth except in the N3 treatment (Fig. 1 in **Paper II**). The high rate of N addition in the N3 treatment caused a decline in the rate of stem wood production and after about 20 yrs, the total stem volume was lower than in the control N0 (Fig. 1 in **Paper II**). The addition of N at an average rate of 68 kg ha^{-1} yr^{-1} in the N2 treatment also decreased the stem wood production rate resulting in the lower total stem volume in 2010 compared to the on-going N1 treatment but higher than in the N0 treatment (Fig. 1 in **Paper II**). However, higher concentrations of N (%) in needles suggested higher tree N-availability in the N2 than in N1 and continuously higher tree N-availability in N3 following the termination of N additions (Fig. 2a in **Paper II**).

Total abundance of PLFA biomarkers per gram organic matter decreased significantly (Fig. 4a in **Paper II**) in response to long-term N additions in the on-going N1 and N2 treatments. Two decades after termination of N addition in the N3 treatment, however, the total PLFA abundance per gram of organic matter increased and was not significantly different from the control (Fig. 4a in **Paper II**) suggesting that microbial biomass had recovered from the effects of N additions. A different picture emerged when the total abundance of biomarkers was expressed per unit area (m^2). In this case, N additions did not

have a significant effect on microbial biomass in the on-going N1 and N2; however, microbial biomass was significantly higher in the terminated N3 relative to the control and the on-going N1 and N2 treatments (Fig. 4c in **Paper II**). This result might be explained by the highest organic matter contents in the N3 plots (Table 2 in **Paper II**) and most likely by the recovery of microbial biomass after termination of N addition. The abundance of ECM fungi per m^2 in the N3 treatment was not different from the control suggesting recovery of the ECM fungi (Fig. 4c in **Paper II**). However, the increase in bacteria was disproportionally higher than in ECM fungi, and thus, the ECM fungi/bacteria ratio was the same as in the on-going N1 and N2 treatments and significantly different from the N0. This was in line with the previous studies at Norrliden, which found decreased ECM fungi/bacteria ratio following N addition (Högberg *et al.*, 2007).

The results of foliar N% and $\varepsilon_{f/s}$ suggested continuously elevated soil N-availability in the N3 treatment (Fig. 2a, b in **Paper II**). The $\varepsilon_{f/s}$ in the N3 treatment was significantly different from the N0 ($p < 0.01$, two-way ANOVA, data not shown) but not from the on-going N1 and N2 treatments. However, the increase of $\varepsilon_{f/s}$ by 2‰ since the termination of the N3 treatment in 1990 could indicate increasing functional role of ECM fungi for tree N uptake (Högberg *et al.*, 2011). An increasing importance of ECM fungal mycelium in tree N uptake was also supported by a strong negative correlation of $\delta^{15}N$ in the needles and the abundance of ECM fungal mycelium per unit area (m^2) (*Figure 7b*), which was in line with previous observations in this forest (Högberg *et al.*, 2011).

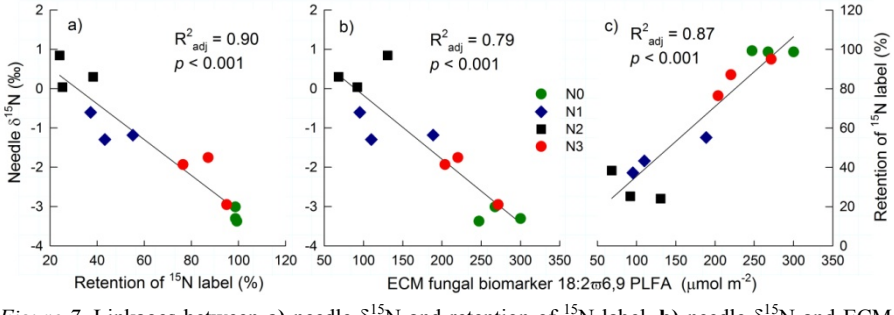

Figure 7. Linkages between **a)** needle $\delta^{15}N$ and retention of ^{15}N label, **b)** needle $\delta^{15}N$ and ECM fungal PLFA 18:2ω6,9 (µmol m^{-2}), **c)** retention of ^{15}N label and ECM fungal PLFA 18:2ω6,9 (µmol m^{-2}) in a Scots pine (*Pinus sylvestris*) forest at Norrliden (linear regression, n = 12, $p <$ 0.05). The figures show that ^{15}N-depletion in needles coincided with higher retention of ^{15}N label and abundance of ECM fungi. Moreover, higher retention of ^{15}N label occurred when the abundance of ECM fungi was higher.

Furthermore, $\delta^{15}N$ in the needles was in negative correlation with N retention (*Figure 7a*) and N retention was in turn positively correlated to the abundance of ECM fungi (*Figure 7c*) and microbial community structure ($R = 0.83$, $p < 0.001$). Thus, these results collectively add evidence to the hypothesis that ECM fungi might play a crucial role in N immobilisation and retention in ecosystems returning to N-limitation after termination of N addition, such as the ecosystems in the terminated N3 treatment plots.

Recovered capacity of the ecosystem to retain N in the terminated N3 treatment was further corroborated by the increased retention of ^{15}N label relative to the on-going N1 and N2 treatments while being not significantly different from the N retention in the N0 control (Table 3 in **Paper II**). Furthermore, the retention of N was negatively related to gross N mineralisation ($R = -0.65$, $p = 0.02$) and gross N mineralisation was linked to the general structure of the microbial community as described by scores obtained in the NMS ordination ($R = 0.65$, $p = 0.02$). These relationships were common for both experimental forests studied. Overall, these results highlighted the important role of microbial community structure in the N retention and gross N mineralisation.

In line with the previous findings at the study site Norrliden (Chen & Högberg, 2006), the highest rates of gross N mineralisation occurred in the N1 treatment, which received the lowest rate of N addition, and differed from the rates measured in the N0 control (Table 3 in **Paper II**). In contrast to the previous findings of consistently high gross rates 14 years after termination of the N3 treatment (Chen & Högberg, 2006), gross N mineralisation per unit area in the N3 treatment differed neither from the N1 nor from the N0 treatments. However, gross N mineralisation rate per gram of organic matter in the N3 treatment differed from the N1, but not from the N0 treatment (Table 3 in **Paper II**) lending a support to the idea that the N3 treatment with regards to gross N mineralisation returned to N-limiting conditions 19 years after cessation of N additions. The increase in root N uptake in the N3 relative to N0 treatment since 1992 and the absence of a significant difference in root N uptake between these two treatments (Fig. 3 in **Paper II**) suggests that the trees in N3 have become N-limited during the two decades since the last N addition in the N3traetment.

The increase in gross rates following N additions was in contrast to other studies that reported stable or declining gross N mineralisation rates (Cheng *et al.*, 2011; Christenson *et al.*, 2009; Venterea *et al.*, 2004; Fisk & Fahey, 2001). Increased gross N mineralisation in the experiments using roofs to intercept existing high N-deposition was interpreted as a result of increased decomposition of organic matter and subsequent release of N (Corre &

Lamersdorf, 2004). However, in strongly N-limited ecosystems, such as the ones studied in **Paper I** and **II**, an increase in N supply should cover microbial and plant demands and mitigate thus the competition for available N (Schimel & Bennett, 2004). Lower competition and demand for N should in turn stimulate gross N mineralisation (Schimel & Bennett, 2004; Hart & Stark, 1997). The significant correlations among gross N mineralisation, soil C/N ratio, and microbial community structure (Fig. 4 and 6 in **Paper I**) in both experiments further highlight that the soil properties in regards to C and N contents, microbial community structure, and gross N mineralisation rates were closely interlinked.

The results discussed above suggest that not all measured variables returned to conditions characteristic for the N-limited control plots in the studied *P. abies* and *P. sylvestris* forests (Table 2) after termination of long-term N additions.

Table 2. *Recovery status of the central indicators of microbial community structure and N cycling at Stråsan (Paper I) and Norrliden (Paper II) based on the comparison with the control treatment. The N2 and N3 treatments at Stråsan were terminated 17 and 19 years, and the N3 at Norrliden 19 years before the studies commenced (see* Table 1*). The low number of replicates (n=2) in the 42-yr-old P. abies experiment at Stråsan did not allow to evaluate differences among the treatments statically; hence, trends among treatment mean values were considered to suggest the recovery status and should be taken with caution. In P. sylvestris experiment at Norrliden, the statistical difference ($p < 0.05$) between the terminated N3 and control (N0) was considered. Positive (+) signs indicate recovery, negative (-) signs indicate no recovery.*

Experiment	*P. abies*		*P. sylvestris*
Terminated treatment	N2	N3	N3
N retention	-	-	+
ECM fungi (mmol m^{-2})	-	-	+
Bacteria (mmol m^{-2})	-	-	-
ECM fungi/bacteria ratio	*	*	-
Functional role of ECM ($\varepsilon_{f/s}$)	-	+	-
Gross N mineralisation (mg m^{-2}day)	-	-	+
Root N uptake	n.a.	n.a.	+

* no change

The ECM fungi/bacteria did not suggest recovery of ECM fungi; however the abundance of ECM fungi recovered in the N3 treatment in *P. sylvestris* 19 yrs after termination of the N addition. Despite there was still significant difference in $\varepsilon_{f/s}$ values between the N0 and N3 treatments in *P. sylvestris*, the $\varepsilon_{f/s}$ increased by 2‰ since the termination of N3 suggested recovering function of ECM fungi. There was no effect of N addition on the mean values of ECM fungi/bacteria ratio in *P. abies* forest (Table 2). Furthermore, the abundance of

ECM fungi and bacteria per unit area in *P. abies* forest was higher in the terminated N2 treatment compared to other treatments (Fig. 5c in **Paper I**), which was attributable to the highest thickness and organic matter contents (Table 2 in **Paper I**). However, the abundance of both ECM fungi and bacteria per gram of organic matter (Fig. 5b in **Paper I**) decreased after N addition and remained low suggesting that ECM fungi and bacteria were not recovered two decades after termination of N addition (*Table 2*). The mean values of N retention in the terminated N2 and N3 in *P. abies* increased by c. 60% but were not as high as in the control plots. The finding of only 8% higher N retention in the N2 treatment (Table 4 in **Paper I**) was in strong contrast to the N3 treatment given the two yrs longer recovery time and 1000 kg ha^{-1} lower total N-load in the N2 treatment (Table 1).Despite the higher N retention in the N2 treatment but higher gross N mineralisation and total N-load in the N3 treatment (Table 1), the mean values of $\varepsilon_{f/s}$ between N0 and N3 differed only by 0.2 ‰ (**Paper I**) suggesting recovered functional role of ECM fungi. Furthermore, these findings suggested resilience of the forest ecosystems in the N3 treatment in spite of the substantial N-load (Table 1).

3.2 The development of the N cycle in boreal forests (Paper III)

Nitrogen availability progressively declined with ecosystem age in the boreal forest studied here despite N_2-fixation and substantial accumulation of total N in soil (Table 1 in **Paper III**). The most pronounced accumulation of soil N and organic matter occurred between the 115-yr-old *A. incana* ecosystem and the 150-yr-old ecosystem dominated by young *P. abies* forest. The total soil N accumulated during 39 years (see 2.1.2) of soil development between these two ecosystems amounted to 900 kg ha^{-1} (Table 1 in **Paper III**). The apparent accumulation rate was 23 kg N ha^{-1} yr^{-1}; based on an assumption that all N accumulated in the soil came from N_2-fixation. An important role of *A. incana* vegetation in accumulation of soil N was recognized also in other primary boreal forests (Chapin *et al.*, 1994; Bormann & Sidle, 1990; Walker, 1989) and N_2-fixation rates of 20 kg ha^{-1} yr^{-1} are not uncommon in the literature (Myrold & Huss-Danell, 2003; Binkley *et al.*, 1992; Johnsrud, 1978). Almost three times higher N concentrations (%) in *A. incana* leaves in the 115-yr-old ecosystem compared to N% in conifer needles in the oldest ecosystem (Table 1 in **Paper III**) indicated high inputs from N_2-fixation by *Frankia* actinobacteria living symbiotically in the nodules of *A. incana* roots (Põlme *et al.*, 2014). The significant decline in N% in needles of conifers by about 20% from the 150-yr-old to the 560-yr-old ecosystem suggested decrease in plant available N with ecosystem age (Table 1 in **Paper III**). Foliar N concentrations

in these coniferous ecosystems (150 yrs old and older) were in the range found in the control plots of N-limited *P. abies* (**Paper I**) and *P. sylvestris* (**Paper II**) ecosystems in several-thousand-year-old boreal landscapes. Moreover, the range of foliar N% in the coniferous ecosystems along the studied chronosequence are characteristic for high latitude ecosystems (Reich & Oleksyn, 2004).

High rates of gross N mineralisation and large extractable pools of NH_4^+ corresponded to high external inputs of N via N_2-fixation in the young ecosystems (Table 1, 2, and Fig. 4 in **Paper III**). Noteworthy, extractable NO_3^- was detected only in the 115-yr-old *A. incana* ecosystem, whereas the pool was small relative to the NH_4^+ pool (Table 1 in **Paper III**). With substantial accumulation of the organic matter and total N, the gross N mineralisation rates per unit area declined sharply by about 40% between 115-yr-old *A. incana* and 150-yr-old *P. abies* ecosystems and by 70% between *A. incana* and 215-yr-old *P. abies* or 560-yr-old mixed coniferous ecosystems. Although the differences in gross N mineralisation were large across the studied ecosystems, they were not significant because of the high variance caused by heterogeneity within the ecosystems and differences among the three studied transects. The apparent discrepancy between the gross N mineralisation rates expressed per gram of soil C or per m^2 in the youngest meadow ecosystem (Table 2 in **Paper III**) resulted from the large differences in the organic matter contents along the chronosequence. Small amounts and large spatial heterogeneity in the distribution of organic matter along with low soil C/N ratio, highest abundance of PLFA biomarkers, and highest microbial biomass N (and C) per gram of soil C, and a microbial community dominated by bacteria, coincided with the highest gross N mineralisation when expressed per gram of soil C but not when expressed per unit area (m^2). This result, in conjunction with low concentrations of recently fixed C (Fig. 2 in **Paper III**), suggested a C limitation of the microbial communities in the young ecosystems (Bardgett *et al.*, 2007), resulting in higher amount of N mineralised per gram of soil C. Bacteria require less C atoms per N atom assimilated and are thus physiologically better adapted for C-limiting condition (Waring *et al.*, 2013). Hence, the nutrient stoichiometry and bacteria-dominated microbial community could explain why the highest gross N mineralisation rate per gram soil C occurred in the youngest ecosystem.

The most pronounced shifts in the microbial community composition occurred during the first 150 years of the ecosystem development, whereas the microbial communities in the coniferous ecosystems were similar and differed from the microbial communities in the younger ecosystems dominated by meadow and *A. incana* vegetation (*Figure 8*). Moreover, the microbial

communities in the 115-yr-old *A. incana* ecosystem differed from those in the youngest meadow ecosystem (*Figure 8*).

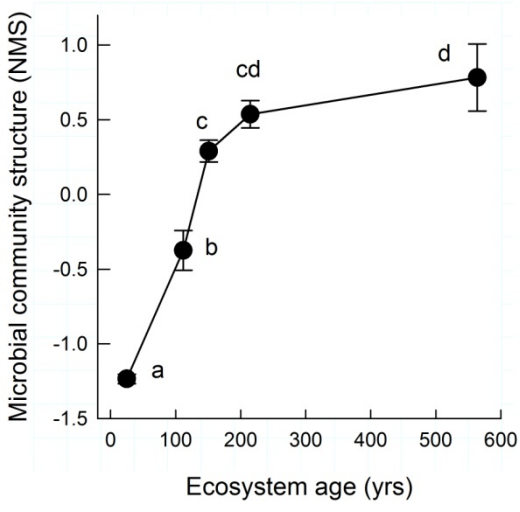

Figure 8. Shifts in microbial community structure as described by scores (describing 77 % of variation in microbiological data) obtained from non-metric multidimensional scaling analysis (NMS) in relation to age of boreal forest ecosystems in the land uplift chronosequence at Bjuren island, northern Sweden. Mean ± 1SE, N = 3.

Total PLFA abundance per gram of soil C decreased along the chronosequence, but the opposite was observed when expressed per unit area (m^2). The latter reflected the build-up of organic layer along the chronosequence. Fungi abundance increased relative to bacteria with ecosystem age and the ECM fungi/bacteria was thus highest in the oldest coniferous ecosystem (Fig.3c in **Paper III**). However, the fungi/bacteria ratio in the *A. incana* ecosystem was nearly as high as in the oldest 560-yr-old ecosystem (Fig.3c in **Paper III).** Roughly the same fungi/bacteria ratio was found in a 12-15 yr-old old soil from underneath *A. sinuata* in a glacier foreland (Bardgett & Walker, 2004). Lower fungi/bacteria ratios were measured in the ecosystems dominated by *A. incana* along chronosequences on the east coast of Finland consistent with the ratios in the older *P. abies* ecosystems (Merilä *et al.*, 2010; Merilä *et al.*, 2002b). Microbial biomass N (and C) contents also increased per unit area as the boreal forest ecosystem aged. The increase in microbial biomass corresponded with the increase in total abundance of PLFAs per unit area and these two estimates of microbial

biomass were in good agreement (R = 0.92, $p < 0.001$, n=15). The proportion of microbial cytoplasm C out of total soil C declined from 3 to 1.3% while at the same time the microbial cytoplasm N out of total soil N increased 10 times from 0.5 to 5.5% with increasing ecosystem age. This coincided with higher concentration of recently fixed C in the coniferous ecosystems compared to the two youngest ecosystems (Fig. 2 in **Paper III**). The low microbial biomass C/N ratio in the *A. incana* ecosystem (Fig. 3c in **Paper III**) likely reflected high N availability in this ecosystem. This was supported by the low N retention (20%) in the *A. incana* ecosystem, which has been observed previously only in exceptionally N-rich areas (Högberg *et al.*, 2006), and in ecosystems subject to high N additions, such as the N1 plots in **Paper I** and the N2 plots in **Paper II**. Retention of ^{15}N label in the other studied ecosystems was around 90% and corresponded to N retention in strongly N-limited control plots in the N fertilisation studies in **Paper I** and **II**. In line with the results of **Paper II**, N retention was not related to cation exchange capacity and thus was most likely of biotic cause (Table 1 in **Paper III**), which was supported by the increased N contents immobilised in microbial cytoplasm as the ecosystem aged.

An increasing role of ECM fungi in tree N uptake with increasing ecosystem age was suggested by continuously increasing $\varepsilon_{f/s}$ (*Figure 9*). While the $\delta^{15}N$ signatures of *A. incana* leaves were characteristic for plants with N_2-fixation symbiosis (Hobbie *et al.*, 2000; Högberg, 1997), the increasing $\varepsilon_{f/s}$ in the coniferous ecosystems suggested low N availability and increasing capacity of the ecosystems to retain N. The difference in $\delta^{15}N$ of the needles and the mineral soil at 10-20 cm depth reached 9‰ in the oldest, 560-yr-old ecosystem (*Figure 9*). A similar difference of 10.3‰ between the organic horizon and *Picea spp.* foliage was observed in an 165-yr-old ecosystem dominated by conifers in chronosequence at Glacier Bay, Alaska (Hobbie *et al.*, 2000). In comparison, smaller differences were measured in other N-limited boreal forests (Högberg *et al.*, 1996) and in the control plots at Stråsan (**Paper I)** and Norrliden (**Paper II)** (Högberg *et al.*, 2011). Smaller $\varepsilon_{f/s}$ were also reported in a study of a range of forest ecosystems differing in tree composition, elevation, and N availability (Garten & Miegroet, 1994).

Overall, the results from **Paper III** suggest that N limitation occurred after about 150 years of ecosystem development and that ECM fungi might play an important role in the development of N limitation in this primary boreal forest.

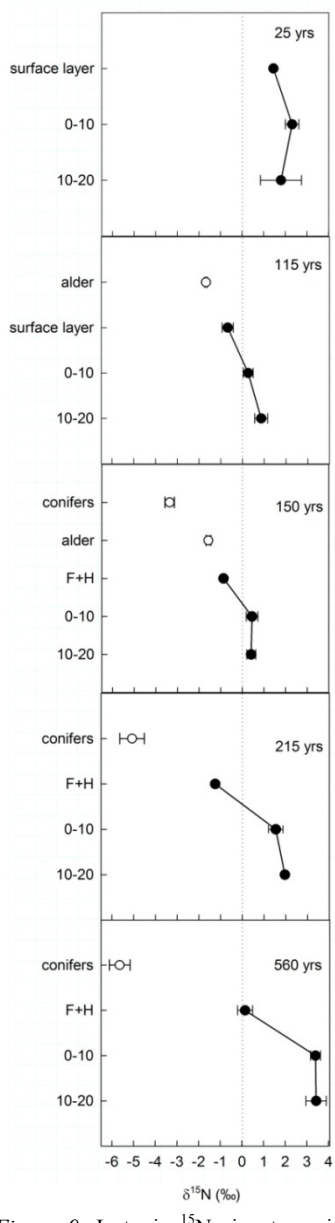

Figure 9. Isotopic ^{15}N signatures in soil profiles and foliage of dominant tree species in a chronosequence of a primary boreal forest on Bjuren island, Sweden. The soil profile consisted of F and H layers of the mor humus (F+H), and mineral soil in 0 - 10 cm (0-10) and 10 - 20 cm (10-20) depth below the mor layer. The values are means ± 1 SE, N = 3. For details on number of samples see *Figure 5* above.

3.3 What are the mechanisms of N limitation development in boreal forests?

Some of the main questions raised in this thesis (**Paper I-III**) concern the mechanisms through which N saturated forests return to N-limitation after termination of N addition and through which mechanisms boreal forest ecosystems progress to N-limitation despite large inputs from N_2 fixation. Do microbial community structure and ECM fungi in particular play a specific role in the development and re-establishment of the N limitation?

One explanation proposed to be responsible for large losses of N in some ecosystems is leaching of highly mobile and negatively charged NO_3^- (Vitousek & Howarth, 1991; Vitousek & Reiners, 1975). However, in this thesis, NO_3^- was detected only in the on-going N fertilisation treatments and in the *A. incana* ecosystem with large N inputs through N_2-fixation. Moreover, measured NO_3^- pools constituted only a fraction (3%) of the NH_4^+ pools. Although gross nitrification can be substantial despite low NO_3^- pools in undisturbed coniferous forests (Stark & Hart, 1997), previous studies on nitrification in this region showed negligible gross nitrification rates except in the soils with high pH and soil N (Högberg *et al.*, 2006). This pathway of losses is most likely minor except in local patches of the boreal landscape presumable in discharge areas where nitrification and therefore denitrification rates could be high under certain conditions (Högberg *et al.*, 2006). Indeed, the high N accumulation of 900 kg ha^{-1} between 115-yr-old *A. incana* and 150-yr-old *P. abies* ecosystem indicated that N losses are small and accumulation of N prevailed.

Given the large accumulation of soil N it appears logical that symbiotic N_2-fixers should have the potential to reverse N limitation. One reason why N limitation develops despite these large inputs could be that plants such as *A. incana* with significant rates of symbiotic N_2-fixation in the high latitude biomes occur mostly early in the succession (Menge *et al.*, 2014; Vitousek *et al.*, 2013). Fixation of N_2 through bryophytes-cyanobacteria associations in the older ecosystems provides appreciable but considerably smaller amounts of N (Gundale *et al.*, 2011; DeLuca *et al.*, 2002).

Another reason for the decline in N availability during the ecosystem development may be the accumulation of N in humus where it is bound with C in complex compounds unavailable for plant uptake unless microbes and enzymes are engaged in breakdown of such compounds (Vitousek & Howarth, 1991). Berg and McClaugherty (2003) showed that litter with higher N contents, such as *A. incana* litter, can initially be decomposed faster, but progressively more N is bound in compounds that turn over slowly and with time the limit value for decomposition decreases, which in turn results in

decreased decomposition rates and accumulation of organic matter (Berg & McClaugherty, 2003). This reasoning was supported by the substantial accumulation of organic matter and soil N in the *A. incana* ecosystem, observed in this and other studies of ecosystems dominated by *Alnus sp.* in primary successions (Chapin *et al.*, 1994; Bormann & Sidle, 1990; Walker, 1989).

A common denominator of progression (**Paper III**) and return after termination of N addition (**Paper I** and **II)** to N limitation from a state of high N availability was the combination of increasing N retention, decreasing gross N mineralisation, and increasing importance of ECM fungi for tree N uptake as discussed above. It was previously shown in boreal forests of this region that greater N immobilisation coincided with higher proportions of ECM fungi in the microbial community and lower N supply (Högberg *et al.*, 2006). On the other hand, allocation of recently fixed C to the ECM fungi was higher under lower N supply (Högberg *et al.*, 2010), whereas greater C supply to ECM fungi coincided with higher N immobilisation in ECM root tips and in soil microorganisms (Näsholm *et al.*, 2013). The higher immobilisation and retention of N by ECM fungi was explained as the decrease in N transferred to tree hosts relative to the amount of N immobilised by ECM fungi (Näsholm *et al.*, 2013). However, when N fertiliser was applied two weeks before the addition of ^{15}N tracer, the transfer ratio increased and ECM fungi transferred more N to trees (Näsholm *et al.*, 2013). The authors hypothesized that this could create a feedback loop in which more C is allocated to ECM fungi while more N is retained by ECM fungi, leading in turn to even greater allocation of C to ECM fungi and further decrease in N availability to plants. Hence this could aggravate rather than alleviate N-limitation in a boreal forest (Näsholm *et al.*, 2013). In another experiment conducted at the same study site as in the **Paper II**, the functional role of ECM fungi in tree N uptake and retention inferred from $\delta^{15}N$ in needles and soil surface, F, and H layers analysed separately was recovered only 15 yrs after termination of high N addition in the N3 treatment (Högberg *et al.*, 2011). The authors hypothesized that the restored ecosystem capacity to retain N was likely because C flow belowground to ECM fungi increased in response to decreasing N supply (Högberg *et al.*, 2011; Högberg *et al.*, 2010).

4 Conclusions and future perspectives

The results presented in this thesis suggest tight linkages among soil microbial communities, gross N mineralisation, and N retention in boreal forests.

The decrease in total microbial biomass, ECM fungi, and bacteria per gram of organic matter following N-additions was common for both *P. abies* and *P. sylvestris* experiments. Unlike in *P. sylvestris* forest where the ECM fungi/ratio was lower in the on-going N1 and N2 treatments but also in the terminated N3 relative to the control N0, ECM fungi/bacteria ratio tended to be rather constant across the treatments in *P. abies* forest. It is possible that the *P. abies* forest at Stråsan was more N-limited and thus the effect of N additions on ECM fungi was not so pronounced as in the *P. sylvestris* at Norrliden.

Another common feature in both experiments were the signs of increasing role of an ECM fungi in tree N uptake and soil N retention in the terminated treatments inferred from the ^{15}N isotopic signatures in needles and soil. The implied increasing role of ECM fungi in tree N uptake and retention of N by ECM fungal mycelium coincided with increasing retention of ^{15}N label after termination of N additions. Concurrently, gross N mineralisation rates decreased in *P. sylvestris* forest at Norrliden, whereas these rates were not significantly different from the rates in the N-limited control plots. Overall, microbial processes and N cycling in the terminated treatments seemed to be returning to a state characteristic for the N-limited forest ecosystems in the control plots. Hence, based on the presented evidence I propose that the functional role of ECM fungi in N uptake and retention was restored two decades after termination of N additions, presumably because of a restored flow of C belowground to ECM fungi in response to decreasing N supply (Högberg *et al.*, 2011; Högberg *et al.*, 2010).

The results from the land uplift chronosequence presented in the **Paper III** suggest that this boreal forest ecosystem with initially high inputs from N_2

fixation progressed to N limitation after about 150 years of the ecosystem development. The sharp decline in gross N mineralisation rates along with the decrease in foliar N% began to occur in the emerging 150-yr-old *P. abies* ecosystem and both continuously decreased with increasing ecosystem age. Substantial accumulation of both organic matter and total soil N was most pronounced between the 115-yr-old *A. incana* and 150-yr-old *P. abies* forest ecosystems. The decline in N availability was associated with a high N retention in the coniferous forest ecosystems corroborated by increasing N immobilisation in microbial cytoplasm. In conclusion, I propose that the primary boreal forest ecosystem studied here progressed to N-limitation after about 150 yrs of development and that the increased abundance and functional role ECM fungi played in tree N-uptake (*Figure 9*) created potentially a large N-sink driving this ecosystem into even stronger N-limitation as the ecosystem aged (Franklin *et al.*, 2014; Näsholm *et al.*, 2013; Högberg *et al.*, 2011). This condition should persist until a major disturbance such as windstorm, fire or forest harvesting would occur resulting in detrimental effects on the ectomycorrhizal symbiosis and thus a loss of a large ecosystem N-sink.

The results in **Papers I and II** suggested that N saturated forests can return to N limitation when N addition is stopped, which could have implications for forest management practises. The decline of strong microbial N sinks following large N loads can lead to substantial increases in gross N mineralisation, nitrification, and subsequently NO_3^- leaching into the groundwater. When the N additions are stopped, forests gradually return to a state of N limitation although some processes may require several decades to recover.

Another important question is what effects will climate change have on N limitation development in boreal forest ecosystems? Several field studies have reported on an increased plant growth under elevated CO_2, however, at the expense of aggravating N-limitation of trees with time known as progressive N limitation (Johnson, 2006; Luo *et al.*, 2004). The mechanism of positive feedback loop proposed by Näsholm *et al.* (2013) suggested that under low N supply higher C allocation leads to higher N immobilisation and retention by ECM fungi. This in turn leads to an even higher C allocation to ECM, which would further contribute to progressive N limitation. In the context of globally increasing CO_2 concentrations, N limitation in boreal forests could potentially be further exacerbated if the increasing CO_2 supply would not be counterbalanced by increased N supply.

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