Biomass, community structure and phosphorus uptake of ectomycorrhizal fungi in response to phosphorus limitation and nitrogen deposition

Almeida, Juan Pablo

2019

Link to publication

Citation for published version (APA):
List of papers


Biomass, community structure and phosphorus uptake of ectomycorrhizal fungi in response to phosphorus limitation and nitrogen deposition
Biomass, community structure and phosphorus uptake of ectomycorrhizal fungi in response to phosphorus limitation and nitrogen deposition

Juan Pablo Almeida

LUND UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Science, Lund University, Sweden.
To be defended at Blue Hall, Ecology Building, Sölvegatan 31, Lund, Sweden on the 8th February 2018.

Faculty opponent
Dr. Andrea Polle
High levels of nitrogen (N) deposition might result in a transition from N to phosphorus (P) limitation in high latitude forests. This could have fundamental consequences for forest production, nutrient acquisition and nutrient leaching.

I studied a Norway spruce forest in a region of high N deposition in southwest Sweden and added N, P or N+P to force the system to N or P limitation. I studied tree growth and foliar nutrient concentration. Also, using ingrowth meshbags, I followed ectomycorrhizal (EMF) production, foraging for N and P patches (urea and apatite) and community composition.

I found that tree production was limited by P. Furthermore, P fertilization reduced EMF production indicating that EMF biomass production was stimulated by P-limiting conditions. Apatite had a positive effect on EMF production when the system was P-limited. P fertilization reduced foraging for nutrients by EMF, also for N rich urea. P had a stronger effect on the composition of EMF communities than N, suggesting that P nutrition had a larger impact on belowground carbon (C) allocation than N in this ecosystem. Furthermore, certain EMF species responded positively to apatite under P limiting conditions, which might have increased mobilization of P from this source.

To enhance my understanding of P mobilization from different P compounds by EMF, I studied one species, *Paxillus involutus*, under more controlled conditions in the laboratory. *P. involutus* is adapted to high N deposition levels and has a documented capability to take up P from poorly soluble sources. I found that *P. involutus* was able to take up P from apatite, P bound to goethite and from phytic acid. Moreover, I found that iron-reducing activity was produced when these sources were provided but not when the fungus was provided with soluble P (phosphate). One possible interpretation to this result was that iron (Fe) reduction is a way for the fungus to prevent that newly liberated phosphate ions are captured by Fe³⁺ and become unavailable for uptake.

In conclusion, the high production of EMF found in P-limited forest decline when P is added, probably due to reduced belowground C allocation when less foraging for P is needed. EMF communities are strongly regulated by P in these forests and species better adapted for P foraging are probably selected for under these conditions.

Key words: Phosphorus and nitrogen limitation, nitrogen deposition, ectomycorrhizal fungi, community composition, apatite, *Paxillus involutus*

Classified system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title

ISBN 978-91-7753-952-0

Recipient’s notes

Number of pages

Price

Security classification

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

Date Dec 19 2018
Biomass, community structure and phosphorus uptake of ectomycorrhizal fungi in response to phosphorus limitation and nitrogen deposition

Juan Pablo Almeida
To my family: Micaela, Juan Pablo, Yolita, Gabriel, Belén y Marita
# Table of Contents

List of papers ........................................................................................................... 10  
Author Contributions ............................................................................................. 11  
Abstract ....................................................................................................................... 12  

**Introduction** .................................................................................................................. 13  
Nitrogen and phosphorus nutrition in forested ecosystems ..................................... 13  
Nitrogen deposition and anthropogenic phosphorus limitation ............................ 15  
Nitrogen deposition and phosphorus limitation in Swedish boreal forests .. 17  
Mycorrhizal associations and nitrogen and phosphorus nutrition ...................... 20  

**Belowground carbon allocation and EMF biomass in response to phosphorus limitation and high nitrogen deposition** ................................................................. 23  
Belowground carbon allocation during nitrogen and phosphorus limitation .......... 23  
Below ground carbon allocation during the transition from nitrogen to phosphorus limitation ..................................................................................................................... 24  
EMF biomass production and nutrient amendments ............................................... 30  

**Nitrogen leaching and EMF nitrogen uptake in response to phosphorus limitation and high nitrogen deposition** ................................................................. 33  
Nitrogen uptake and demand ..................................................................................... 33  
Nitrogen retention and EMF biomass ......................................................................... 35  

**EMF community structure in response to phosphorus limitation and high nitrogen deposition** .......................................................................................................... 37  
Effect of phosphorus limitation and high nitrogen deposition on the community structure ........................................................................................................... 37  
Effect of phosphorus limitation and nitrogen high nitrogen deposition on individual species .................................................................................................. 39
Effect of phosphorus limitation on EMF phosphorus uptake from organic and mineral molecules ........................................43
   EMF organic phosphorus uptake ..................................................43
   EMF phosphorus uptake from mineral compounds ........................44
   Phosphorus uptake under low P availability ................................46
   Phosphorus uptake and phosphorus status of the mycelium ..........48
   Limitations of axenic experiments for nutrient uptake studies ....48

Conclusions and future perspectives .............................................55
References .....................................................................................59

Popular science summary ..............................................................71

Acknowledgments .........................................................................75
List of papers

In this thesis the papers are referred to the roman numerals


Author Contributions

**Paper I** Responsible for laboratory work, bioinformatics, statistics, interpretation of data. Minor contribution to writing the text.

**Paper II** Responsible for planning the experiment, fieldwork, laboratory work, statistics, interpretation of data and writing the manuscript.

**Paper III** Responsible for planning the experiment, field work, laboratory analysis, most of the statistical analysis, interpretation of data and writing the manuscript.

**Paper IV** Responsible for designing the experimental set up, performing all laboratory analysis and statistical tests, interpretation of data and writing the manuscript.
Abstract

High levels of nitrogen (N) deposition might result in a transition from N to phosphorus (P) limitation in high latitude forests. This could have fundamental consequences for forest production, nutrient acquisition and nutrient leaching.

I studied a Norway spruce forest in a region of high N deposition in southwest Sweden and added N, P or N+P to force the system to N or P limitation. I studied tree growth and foliar nutrient concentration. Also, using ingrowth meshbags, I followed ectomycorrhizal (EMF) production, foraging for N and P patches (urea and apatite) and community composition.

I found that tree production was limited by P. Furthermore, P fertilization reduced EMF production indicating that EMF biomass production was stimulated by P-limiting conditions. Apatite had a positive effect on EMF production when the system was P-limited. P fertilization reduced foraging for nutrients by EMF, also for N rich urea. P had a stronger effect on the composition of EMF communities than N, suggesting that P nutrition had a larger impact on belowground carbon (C) allocation than N in this ecosystem. Furthermore, certain EMF species responded positively to apatite under P limiting conditions, which might have increased mobilization of P from this source.

To enhance my understanding of P mobilization from different P compounds by EMF, I studied one species, Paxillus involutus, under more controlled conditions in the laboratory. P. involutus is adapted to high N deposition levels and has a documented capability to take up P from poorly soluble sources. I found that P. involutus was able to take up P from apatite, P bound to goethite and from phytic acid. Moreover, I found that iron-reducing activity was produced when these sources were provided but not when the fungus was provided with soluble P (phosphate). One possible interpretation to this result was that iron (Fe) reduction is a way for the fungus to prevent that newly liberated phosphate ions are captured by Fe$^{3+}$ and became unavailable for uptake.

In conclusion, the high production of EMF found in P-limited forest decline when P is added, probably due to reduced belowground C allocation when less foraging for P is needed. EMF communities are strongly regulated by P in these forests and species better adapted for P foraging are probably selected for under these conditions.
Introduction

Nitrogen and phosphorus nutrition in forested ecosystems

Forested ecosystems are important reservoirs that store carbon (C) either in aboveground or into belowground biomass (Hui et al., 2017). Carbon fixation and partitioning by trees is strongly dependent on other essential macronutrients, such as nitrogen (N) and phosphorus (P) (Gill & Finzi, 2016). N is an important component of amino acids, monomers that forms proteins, while P is a structural part of the phospholipid bilayer of cell membranes (Sterner & Elser, 2002). Both elements are key components of nucleic acids and the energy-transfer compound ATP (Terry & Ulrich, 1973; Sterner & Elser, 2002; Hyland et al., 2005). ATP is needed in cellular functions, such as stomatal opening and transfer of organic solutes across membranes (Terry & Ulrich, 1973; Sterner & Elser, 2002). Moreover, biosynthesis and respiration rely on the energy stored in the ATP molecule (Terry & Ulrich, 1973; Sterner & Elser, 2002). Therefore, P and N are critical elements for plant physiology (Sterner & Elser, 2002; Smith et al., 2011). However, N and P are not easily accessible resources in soils; therefore, the proportions required for optimum plant growth are often not sufficiently provided. As a result, tree primary productivity and growth are generally limited by the nutrient present in the lowest supply (von Liebig, 1855).

When a nutrient is limiting tree growth, meaningful nutrient additions are expected to enhance primary productivity and biomass production (Vitousek et al., 2010). In forested ecosystems, N fertilization has been shown to have positive effects on tree growth in high latitude forests (boreal and temperate forests) (LeBauer & Treseder, 2008; Schulte-Uebbing & De Vries, 2018), while tropical forests generally respond more to P fertilization (Elser et al., 2007; Li et al., 2016), suggesting a latitudinal difference in N and P limitation.

This latitudinal trend has been attributed to the geological and climatic processes that differentiate tropical and high latitude forests (Vitousek et al., 2010; Gill & Finzi, 2016). New N inputs in an ecosystem are the result of the fixation of atmospheric N₂, which is an enzymatic process conducted by free living or root associated N-fixing bacteria (Davidson, 2008; Houlton et al., 2008). For terrestrial
ecosystems, an increase in latitude is associated with a decrease in N fixation (Houlton et al., 2008). Decreasing temperatures at higher latitudes might decrease the efficiency of nitrogenases, which are responsible for the conversion of atmospheric N$_2$ into biologically available ammonia (Davidson, 2008; Belyazid & Belyazid, 2013). At low temperatures, efficient use of nitrogenases might require more C investment by plants (Rastetter et al., 2001; Houlton et al., 2008). It is predicted that N foraging and uptake from soils in boreal and temperate forests will be more efficient in terms of C cost than supporting the symbiosis with N fixers (Rastetter et al., 2001). As a result, trees in high latitude forests have developed better strategies to forage and mine for N in soil (e.g., symbiosis with ectomycorrhizas) relative to those in low latitude forests, where fixation is less costly and there is a high turnover of nutrients in soils (Rastetter et al., 2001; Gill & Finzi, 2016). Moreover, in boreal forest soils, N is mainly present in recalcitrant organic compounds that reduce the N uptake efficiency by the plant roots (Sponseller et al., 2016). N fixation in low latitude forests (tropical forest) increases because of the higher temperature and abundance of plant N$_2$-fixing trees (Davidson, 2008; Houlton et al., 2008). The high temperature also increases soil organic matter turnover and plant available N.

Unlike nitrogen, for which there is potentially large input from biological fixation, phosphorus inputs derive almost exclusively from rock weathering. As a result, the phosphorus status of an ecosystem is heavily dependent on the soil parent material (Rosenstock et al., 2016) and age (Wardle et al., 2004). For example, in temperate and boreal forests, where glaciers exposed a great deal of primary mineral surface area less than 15,000 years ago, phosphorus does not generally limit primary production. However, in older ecosystems lacking recent significant geological activity, including much of the world’s tropical forests, phosphorus has become the limiting nutrient (Vitousek et al., 2010). When phosphate is released through weathering of soil minerals, it follows a cycle in which mineralized phosphorus is taken up by soil organisms and plants and cycled rapidly though uptake and organic matter decomposition (Hyland et al., 2005; Belyazid & Belyazid, 2012; Sohrt et al., 2017) and, over time, becomes increasingly less available by binding to secondary mineral surfaces (Turner, 2008; Belyazid & Belyazid, 2012).

Nitrogen and P biogeochemical cycles are dynamic processes that depend on input sources, biological demand and uptake. Changes in the availability and inputs of one of the nutrients can alter the N:P ratios in soils and plants, and thus change the nutrient regime of an ecosystem (Vitousek et al., 2010). For example, during early succession stages in some boreal forests, N fixers are abundant and the new N inputs surpass the inputs of P, leading to P limitation (Chapin et al., 1994; Vitousek et al., 2010). Moreover, during late successional stages of temperate and boreal forests, an accumulation of N from atmospheric deposition and biological fixation and a decrease in P availability can lead to a declining phase with low
productivity caused by phosphorus limitation (Wardle et al., 2004, 2016; Du & Fang, 2014). Thus, even though P limitation is rare in young ecosystems (northern, glaciated areas), these studies demonstrate that P limitation can occur during both early and late succession in boreal and temperate forests.

Nitrogen deposition and anthropogenic phosphorus limitation

During the post-industrial era, global C, P, and N cycles have changed significantly (Elser et al., 2007; Peñuelas et al., 2013) and anthropogenic N inputs from industrial fertilizers and fossil fuel emissions have increased by more than one order of magnitude in comparison with the biologically fixed N (Fig. 1) (Falkowski et al., 2000; Galloway et al., 2008). This steep increase in anthropogenic C and N inputs relative to P inputs (Fig. 1) can alter plant nutrient stoichiometry and lead to unbalanced nutrition (Peñuelas et al., 2013; Jonard et al., 2015).

Figure 1: Total anthropogenic N and P inputs on a global scale since the industrial revolution (1860). Error bars indicate the range of data reported. Taken from Peñuelas et al. (2013).
The unbalanced N and P inputs in terrestrial ecosystems have increased the N:P ratios in plants and soils, especially in forests from North America and Central and Northern Europe (Peñuelas et al., 2013). For example, in Europe, Jonard et al. (2015) analysed foliar nutrient concentrations based on data from six different tree species collected from forests across the entire continent. They reported that, for all tree species, the N:P ratios were above the level in which a detrimental effect (defoliation) can be expected (Veresoglou et al., 2014). It was suggested that some forested ecosystems in high latitudes are P-limited, or transitioning to P limitation due to an increased N inputs.

Many have corroborated this idea by testing the effects of N fertilization on tree growth. Nitrogen addition experiments in temperate forests in North America (Lovett et al., 2013) and in central Europe (Braun et al., 2010) have reported a lack of response of tree growth to N fertilization, suggesting that N is not the main limiting nutrient. However, based on a meta-analysis of data from fertilization experiments in natural forests and forest plantations around the globe, Schulte-Uebbing & De Vries (2018) reported that there was a significant strong response of tree growth to N fertilization in boreal and temperate forests, suggesting that N limitation remains in most of these ecosystems and that P limitation is not yet widespread across high latitude forests.

Very few studies have examined the effects of P fertilization alone (without concomitant N additions) on tree growth in temperate and boreal forests (see Radwan et al., 1991; Finzi, 2009; Mainwaring et al., 2014), and adult trees do not generally respond to P fertilization in high latitude forests (Finzi, 2009; Mainwaring et al., 2014; Schulte-Uebbing & De Vries, 2018). It has been reported that tree response to fertilization can vary depending on the local soil conditions of a forest stand (Finzi, 2009; Bergh et al., 2014; Mainwaring et al., 2014; Schulte-Uebbing & De Vries, 2018). For example, in forest stands with low pH, occlusion of the P fertilizer to iron oxides can dampen the effects of fertilization. However, in sites with high pH, P fertilization is easily available for use in plant growth (Finzi, 2009). In temperate plantations from North America, Mainwaring et al. (2014) compared the effects of P fertilization on 16 different Douglas fir (Pseudotsuga menziesii) stands with different edaphic characteristics. They found a positive effect of P fertilization on tree growth in stands with a pH higher than 5 that was not found in stands with a pH lower than 5. Therefore, local conditions of a forest stand are important when interpreting the effects of P fertilization in high latitude forests.
Nitrogen deposition and phosphorus limitation in Swedish boreal forests

In Western Europe, boreal forests receive less N deposition than temperate forests (Erisman et al., 2015) and N deposition has decreased in recent decades (Binkley & Högberg, 2016; Pihl Karlsson et al. 2017). However, in some forests from Southwest Sweden, the N deposition values currently range from 7.5 to more than 15 kg N ha⁻¹ yr⁻¹ (Erisman et al., 2015; Pihl Karlsson et al. 2017). These values exceed the N critical loads above in which negative changes in the function and composition of an ecosystem are expected (Kuylenstierna et al., 1998; Pardo et al., 2011; Pihl Karlsson et al., 2017) (Fig. 2).

Several studies have reported N excess in southern Swedish forests. In a study of the effects of nitrogen fertilization on floor vegetation, Hedwall et al. (2013) found that N enrichment had a significant effect on the forest ground flora species composition in central and northern Sweden. In southern Swedish forests, the effects of fertilization were smaller. Forest floor flora is normally sensitive to N addition and has been used to test the effects of N deposition (Hedwall et al.,...
The lack of N fertilization effects on forest floor vegetation in southwest Sweden indicates that the region is probably N saturated.

In a N deposition gradient across Sweden, Akselsson et al. (2010) measured nitrate concentrations and C:N ratios in soil water and modelled nitrogen accumulation based on N inputs (N deposition and N fixation) and outputs (N leaching and tree harvesting) of the system. They found that southwest Sweden, which receives the highest N deposition, showed the highest levels of N accumulation and had the highest risk of N leaching. In addition, Akselsson et al. (2008) analysed how much N and P would be lost if the trees were harvested using data collected from 14,550 sites in Swedish forests. Based on N and P inputs (N and P deposition, N fixation and mineral weathering) and outputs (N and P leaching and tree harvesting), they modeled N and P accumulation and losses after tree harvesting. The results revealed that tree harvesting would result in net losses of P from forests. However, some forests in southwest Sweden will accumulate N in soils even after tree harvest. Based on these findings, the authors concluded that forests in southwest Sweden are transitioning to P limitation.

Despite this evidence, the transition from N to P limitation of some forests in Sweden is still the subject of debate since there has not been evidence of a positive effect on tree growth of P fertilization without concomitant N addition in upland Swedish forests (Binkley & Högberg, 2016). In the experiment described in the second manuscript from this thesis (Paper II), we fertilized a Norway spruce forest (Picea abies) in southwest Sweden with P, and found a significant increase in tree stem growth in P fertilized plots (Fig. 3A). Needle nutrient analysis revealed that the P content in the unfertilized control plots were below the deficiency levels reported by Thelin et al. (1998) (Fig. 3B). Moreover, the N:P ratios in the unfertilized controls plots were above the threshold level at which P is considered to be limiting growth according to Linder (1995) (Fig. 3C). We also found a reduction of acid phosphatase activity after P fertilization in a pilot study in the same research forest (Fig. 3D).
Figure 3: (A) Tree growth (n=6); (B) Needle P concentration (n=3) (the lower and upper lines represent P deficiency and optimal fertilization levels respectively); (C) N:P ratio in needles (n=3) in the fertilization experiments. The line represent the optimal N:P ratio reported by Linder (1995). Modified from Almeida et al. (2018). (D) Acid phosphatase activity in the P fertilized plots (n=3). Bars represent the average value per treatment and error bars correspond to two standard errors. P and C are abbreviations corresponding to the P fertilized plots and the unfertilized (control) plots, respectively.

The tree growth response to P fertilization plus the foliar nutrient concentrations suggest that the trees at this stand are P-limited. The reduction in phosphatase activity suggests that when fertilization alleviated limitation, there was a reduction in the tree and soil microorganism efforts to acquired P from organic compounds. These findings support the case for a transition to P limitation in areas with strong anthropogenic N deposition. However, the effects of N deposition in southwest Sweden have been shown to be variable on a local scale (Akselsson et al., 2010) and the response to fertilization can be dependent on tree species (Bergh et al., 2014). Therefore, this evidence cannot be extrapolated to other forest stands in the region. Nevertheless, this forest is a valuable site for investigation of the transition from N to P limitation and its effects on tree nutrition. In this thesis, I used this forest as a case of study for Papers I, II, and III to investigate the effects of this
transition on several aspects of ectomycorrhizal fungi (EMF), which are root symbionts crucial for nutrition in forested ecosystems (Smith & Read, 2010).

Mycorrhizal associations and nitrogen and phosphorus nutrition

Nitrogen and P limitation have important implications for plant nutrient acquisition and trees have developed different strategies to improve N and P uptake (Smith and Read, 2010). For example, most tree species rely on mycorrhizal associations to obtain N and P from soils (Gadd, 2006; Smith & Read, 2010). Mycorrhizal associations are symbiotic relationships between filamentous fungi and plant roots, in which, under most conditions, the fungal symbiont provides nutrients to their plant hosts in exchange for photosynthetically fixed carbon (Finlay, 2008).

Different kinds of mycorrhizal associations have appeared during different times in the phylogeny of filamentous fungi (Strullu-Derrien et al., 2018), with ectomycorrhizas and arbuscular mycorrhizas being the most abundant (Perez-Moreno, 2003; Bonfante & Genre, 2010; Tedersoo et al., 2014). Despite relatively similar properties concerning plant nutrition (exchange of nutrients for photosynthates), both mycorrhizal types differ in their physiological structures, their main distribution, and their nutrient uptake strategies (Bonfante & Genre, 2010).

The main structural difference between EMF and arbuscular mycorrhizal fungi (AMF) is the features associated with the root: fungus interphase. For EMF, fungal tissue does not penetrate the root cell walls, but instead surrounds them, forming a layer called the Hartig net. For AMF, the fungal tissue goes inside the root cell wall to form vesicles or arbuscules inside the cell (Bonfante & Genre, 2010) (Fig. 4).
In temperate and boreal forests, EMF are the dominant mycorrhizal type (Read & Perez-Moreno, 2003; Tedersoo et al., 2014), while AM can be present in the understory of some temperate forests (Read & Perez-Moreno, 2003). However, in tropical forests, AMF is the dominant mycorrhizal type and very few EMF genera are present (Tedersoo et al., 2014; Mafla, 2018).

In addition to the structural differences, EMF and AMF have different nutrient acquisition strategies. EMF are more specialized to take up N from soils where turnover is low and nutrients can be locked in organic compounds (Marschner & Bell, 1994; Read & Perez-Moreno, 2003). Some EMF species are able to produce different enzymes to release N from soil organic matter (Bödeker, 2012). Other species have versatile capabilities similar to those of saprotrophic fungi. For example, *P. involutus* for example, can produce secondary metabolites to induce a Fenton reaction and produce free radicals that will ultimately act on organic matter and release N (Shah et al., 2015; Op de Beeck et al., 2018). Enzymatic capabilities to release N from organic compounds are absent from AMF, probably because in tropical forests, where the turnover of organic N is fast and N fixation is high,
there is enough available N for fungal and plant consumption (Read & Perez-Moreno, 2003). Despite being adapted to ecosystems generally limited by N, EMF are able to take up P from different sources present in soils (Antibus et al., 1992; Rosling, 2009; Cairney, 2011; Plassard et al., 2011). These findings indicate that EMF have great potential to improve P nutrition in high latitude P-limited forests.

Since EMF are essential for N and P uptake (Gadd, 2006; Smith & Read, 2010) and a significant part of the photo-assimilates goes underground to support the symbiosis (Finlay, 2008), N and P limitation and the transition from one to the other influences C allocation, which can in turn influence EMF growth, nutrient uptake, and the structure of EMF communities. In this thesis, I studied the effects of P limitation in a forest with high N deposition on EMF biomass (Papers II and III), N uptake and leaching (I and II), EMF community composition (papers I and II), and P nutrition (Paper IV).
Belowground carbon allocation and EMF biomass in response to phosphorus limitation and high nitrogen deposition

Belowground carbon allocation during nitrogen and phosphorus limitation

In boreal and temperate forests, trees depend on EMF associations to forage and mine for N, which is commonly the limiting nutrient in these regions (Binkley & Högb erg, 2016). A substantial amount of C is delivered belowground by trees to support EMF symbiosis (Gill & Finzi, 2016). Gill & Finzi (2016) conducted a meta-analysis in which data pertaining to the annual gross primary productivity, total belowground carbon allocation, and annual N and P mineralization rates were collected from different forests around the globe to test belowground C partitioning across biomes. They found that as available N (as indicated by N:P ratios) in soils decreased, there was an increase in belowground C partitioning. They concluded that to access N, which is locked in the soil organic matter, trees in high latitudes deliver more C to the fungal symbionts to enhance foraging and N uptake.

However, an increase of N in the system is predicted to reduce belowground carbon allocation since a high belowground C investment is not cost efficient when the nutrient is abundant (Treseder, 2004; Högb erg et al. 2010; Janssens et al. 2010; Bae et al. 2015). The effect of N availability on belowground C allocation was tested by Bae et al. (2015), who estimated belowground C allocation (based on the difference between C respired in the soil and C in the litter fall) in temperate forest stands with different inherent soil fertilities and ages. They found that belowground C allocation was inversely correlated with soil N availability, independent of stand age. This decrease in belowground C allocation is expected to influence EMF, probably by decreasing the mycobiont biomass (Nilsson & Wallander, 2003; Högb erg et al., 2007, 2010). In an N fertilization experiment,
Högberg et al. (2010) traced the belowground C by providing the plants with $^{13}$C labelled CO$_2$. They found that when N was added the amount of $^{13}$C detected in EMF decreased by almost 50% relative to the unfertilized control plots. Högberg et al. (2007) tested the effects of N fertilization and tree girdling (to block the passage of C to the roots by cutting off the phloem) on EMF biomass in a boreal forest. They found that fertilization and girdling decreased EMF biomass to almost the same degree, supporting the idea that the reduction in belowground C allocation caused by fertilization reduces EMF biomass as well.

Similar to N limitation, P limitation should be expected to increase belowground C allocation and EMF growth to enhance nutrient forging and uptake of P. Keith et al. (1997) showed that P fertilization of a natural eucalyptus (Eucalyptus pauciflora) stand significantly increased aboveground C allocation, indicating that the plants were P-limited. However, belowground C allocation was decreased by fertilization. This decline in delivered C was associated with a decrease in EFM biomass. In a study comparing Norway spruce stands with different P availabilities (caused by the soil parent material P), Rosenstock et al. (2016) reported that in P limiting stands above ground tree biomass was lower while EMF biomass was higher in comparison with P sufficient stands. Thus, P limitation can affect C tree partitioning by decreasing aboveground growth and allocating more C to the fungal symbionts. These findings are consistent with other P fertilization studies that have shown a negative effect of P addition on EMF hyphal length (Baum & Makeschin, 2000) and EMF root colonization (Pampolina et al., 2002).

Below ground carbon allocation during the transition from nitrogen to phosphorus limitation

As mentioned above, evidence suggests that during P limitation, trees will increase belowground C allocation, which will lead to increased EMF production. However, N increase (due to deposition) is expected to have the opposite effect and reduce belowground C partitioning. Therefore, it is less certain how belowground C allocation and EMF growth will be affected when prolonged N addition results in P limitation. The three scenarios described below can be expected (Fig. 5).

First, allocation to EMF decreases as N availability increases until P becomes limiting and C allocation increases again. Wallander & Nylund (1992) tested the effects of nutrient additions on extramatrical mycelium growth of the EMF Laccaria bicolor inoculated in P. sylvestris seedlings in semi-hydroponic culture systems. They found that when seedlings were fertilized with a solution containing all nutrients except for P, L. bicolor produced significantly more extramatrical
mycelium than seedlings growing with full nutrient solution. Moreover, when plants were fertilized with a solution with no P and an excess of N, there was an even higher increase in extramatrical mycelium. These findings could suggest that the excess N exacerbated P limitation, causing more belowground C allocation. A similar effect could be expected in boreal forests transitioning from N to P limitation due to N accumulation from deposition. However, since seedlings respond stronger to fertilization (Mainwaring et al., 2014; Schulte-Uebbing & De Vries, 2018) it is less known if this also occurs under field conditions in mature forests.

Second, allocation of C to EMF is more strongly controlled by N limitation than P limitation, resulting in reduced allocation to EMF at elevated N. Most of the N pool in the leaves constitutes immobile N bound to structural proteins in leaf cells. Foliar P is more mobile and can be transferred to new leaves before foliar abscission (McGroddy et al., 2004). McGroddy et al. (2004) analysed data pertaining to foliar C, P, and N stoichiometry from different tropical and temperate forests around the globe and found that the C:P ratio in leaf litter was higher than that in fresh foliage, indicating P resorption from senescent foliage. This difference was more marked in tropical forests in which P limits growth. However, differences in C:N ratios between leaf litter and fresh leaves were similar across forests from different latitudes, suggesting that N resorption was not enhanced in N-limited forests. Thus, in N-limited forests, trees probably rely more on soil foraging to increase N uptake, which will increase the need for belowground C allocation. Conversely, P resorption from senescent foliage might alleviate P demand in P-limited forests and moderate the C cost of P acquisition from soils (McGroddy et al., 2004; Gill & Finzi, 2016). This could indicate that belowground C partitioning is more strongly controlled by N limitation than P limitation.

Finally, relative allocation to EMF decreases because of N deposition, but tree productivity also increases. In this scenario, total belowground allocation remains constant.
Figure 5: Different scenarios of belowground C allocation to EMF during transition from N to P limitation. First scenario (yellow): allocation to EMF decreases as N availability increases, until P becomes limiting and C allocation increases again. Second scenario (red): allocation of C to EMF is more strongly controlled by N than by P resulting in reduced allocation to EMF at elevated N. Third scenario (green): relative allocation to EMF decreases but tree productivity increases as N availability increases so total belowground allocation remains constant.

To test the effects of P limitation (in a region with high N deposition) on EMF biomass, in Paper II we studied EMF biomass from ingrowth meshbags (see below) incubated in a P-limited Norway spruce forest in southwest Sweden (see section 1.3). We added N or P to push the system to further N or P limitation. Two experiments were performed. In the first experimental site (NP experiment), N alone (200 kg N ha\(^{-1}\) ammonium nitrate) and a combination of N and P were added (200 kg N ha\(^{-1}\) ammonium nitrate and 400 kg P ha\(^{-1}\) superphosphate). In the second experimental site (P experiment), P (400 kg ha\(^{-1}\) of superphosphate) was added. Non-fertilized plots were used as controls in both experiments. Additionally, P foliar content in the tree needles was measured.

The EMF biomass was estimated by incubating ingrowth meshbags belowground (Fig. 6). This method is useful for measuring fungal biomass, especially from EMF mycelium. This technique employs carbon-free substrate (acid washed quartz sand) covered by a nylon mesh that excludes fine roots and allows mycelium ingrowth (Wallander et al., 2001). After 144 days of incubation, EMF biomass was estimated using the fungal membrane component ergosterol as a biomarker. In addition, a visual estimation of the frequency of hyphae counted in the mesh was used as a proxy for mycelial ingrowth (Wallander et al., 2001; Bahr et al., 2015).
The NP experiment described in Paper II did not reveal an effect of N fertilization on ergosterol, although the hyphal visual frequency tended to decrease. However, when the plots were fertilized with both N and P, there was a significant decrease in both ergosterol and visual hyphal frequency. The foliar P concentrations and N:P ratios in the unfertilized controls and the N fertilized plots suggest that this forest is P-limited. The significant decrease in EMF biomass after N+P fertilization was probably caused by alleviation of the host P demand. This is supported by the significant increase in foliar P concentrations and the reduction of foliar N:P ratios to optimal levels after N+P fertilization. The significant EMF biomass reduction in the N+P fertilization treatment in comparison with the
control plots and the N plots suggests that P limitation increases EMF biomass even under high N levels as shown by Wallander & Nylund (1992) for seedlings. However, Bahr et al. (2015) studied EMF biomass in the same forest and found that the effect of N+P additions was not higher than the effect of N alone. This might indicate that EMF growth is still sensitive to N additions. Also, it is possible that by the time the experiment described in Paper II was performed, the effect of N fertilization had diminished. The measurements conducted in Paper II were done 3 years after fertilization while measurements conducted by Bahr et al. (2015) were done 1 year after fertilization.

In the P experiment, although there was a significant effect of P fertilization on foliar P concentrations, foliar N:P ratios, and tree growth, no effect on EMF biomass was detected after P fertilization, contradicting the previous conclusion that P limitation increased EMF biomass.

In Paper II, the EMF biomass measurements are an estimation of the standing biomass in the meshbags after a given incubation period. The standing biomass might not reflect the total EMF biomass production because the turnover (mortality of the mycelium) has not been considered. It has been shown that increases in soil fertility can influence EMF turnover rates and total EMF biomass production (Ekblad et al., 2016; Hendricks et al., 2016). It is possible that the standing biomass measured in Paper II underestimated the effect of P fertilization. To overcome this problem, in Paper III we aimed to estimate EMF turnover to test the effects of P fertilization on the total EMF biomass production.

To estimate turnover and EMF biomass production in Paper III, we used a combination of short sequential meshbag incubations overlapped with longer incubations times (Fig. 7). The ergosterol data obtained from the different incubations were used in an exponential decay model in which the turnover rates were calculated.

Briefly, the sum of the biomass produced during two sequential short incubation periods is expected to exceed the biomass produced in an overlapping longer incubation period. This difference can be accounted for by dead biomass (necromass) during the long period. The biomass produced in the long incubation period should then be equal to the sum of the shorter incubation periods less the amount of biomass that died (Fig. 7).
Figure 7: Scheme of the rationale behind the model used to calculate the turnover rates. Two sequential short incubation periods SIBt1 and SIBt2 are overlapping with a longer incubation period LIB. LIB is equal to the sum of SIBt2 and RSIBt1 (the biomass remaining from SIBt1).

Assuming that EMF biomass is lost at a constant exponential rate, the turnover \( k \) can be calculated using the following equation:

\[
k = \frac{-\ln \frac{LIB - SIBt2}{SIBt1}}{t2 - t1}
\]

Once the turnover rate \( k \) is calculated, total biomass production can be estimated using the function of \( k \) and the standing biomass of a given time (for further details see Paper III).

For a five-month period starting in July 2015 and ending in November 2015, the meshbags were incubated for variable lengths of time (30, 60, 90, 120, and 150 days) in the same fertilized plots that were used for the P experiment described in Paper II.

The results from Paper III showed that P fertilization had a negative effect on the standing biomass in most incubation times in contrast with what was observed in Paper II. The reason for the stronger effect of P fertilization in Paper III is not known; however, the fact that more incubation periods and a larger number of bags were used makes the present study more reliable. Thus, the standing biomass of one given incubation time might not truly reflect the effects of fertilization.
The turnover rates did not significantly differ between the P fertilized and control plots. However, the EMF biomass production was decreased by P fertilization as expected from a P-limited forest. This reduction in ECM biomass production as a result of P fertilization can be interpreted as a decreased carbon allocation by the tree when limitation is relieved. The decrease in EMF biomass production in the P fertilization treatment shown in Paper III supports the hypothesis that EMF growth increases when P becomes limiting as a result of N deposition. Accordingly, these results might suggest that C allocation to EMF in forests with high N deposition decreases until P becomes limiting and C allocation to EMF increases again (Fig. 5, first scenario).

EMF biomass production and nutrient amendments

Ingrowth meshbags have been intensively used to capture EMF growth (Wallander & Nylund, 1992; Bahr et al., 2015; Ekblad et al., 2016; Rosenstock et al., 2016; Almeida et al., 2018) because roots and most saprotrophic fungi can be excluded (Wallander et al., 2001; Wallander et al., 2013). However, the main advantage of the use of ingrowth meshbags is the potential to add the nutrient rich substrates into the bags to assess preferential colonization of different nutrient sources. If nutrient limitation in the forest increases EMF production as a strategy to enhance nutrient foraging and uptake, nutrient rich substrates should enhance EMF production depending on the nutrient status of the forests.

There is evidence of an increase in EMF biomass in the presence of P rich sources, such as apatite in P deficient forests (Hedh et al., 2008; Berner et al., 2012; Rosenstock et al., 2016). Rosenstock et al. (2016) reported that the difference in EMF biomass in meshbags between P-limited and P sufficient stands was exacerbated in the apatite amendment meshbags. Bahr et al. (2015) found that ingrowth meshbags amended with apatite presented higher EMF biomass than unamended meshbags. However, in N+P fertilized plots, the effect of apatite on EMF biomass disappeared as P limitation was alleviated.

As mentioned above, biomass estimates obtained from a given incubation time are the result of the total biomass production and turnover in the mycelium colonizing the meshbag. Experiments analysing EMF colonization on nutrient substrates have shown that the residence time of the mycelium is affected by the nature of the substrate analysed (Bending & Read 1995; Rosling et al., 2004; Ekblad et al., 2016). In Paper III, we analysed the effects of nutrient amendments on EMF biomass production and turnover. Apatite and urea-amended meshbags were incubated along with pure-quartz bags as described in the previous section.
We found that mycelial turnover was not affected by the nutrient amendments but total EMF production was. As expected, apatite amendment only increased EMF biomass production (in relation to the pure-quartz bags) in the control plots, where P limits growth. In the fertilized plots, where limitation was alleviated, there was no effect of apatite on EMF production (Fig. 8).

Phosphorus fertilization significantly decreased EMF production in the urea-amended meshbags (Fig. 8). These findings suggest that the decrease in EMF biomass production caused by P fertilization also affects foraging for N, which would be expected if P fertilization resulted in a general decline in belowground carbon allocation. The fact that P fertilization had such a strong effect on EMF production in N amended bags further supports that P fertilization reduces EMF production. Moreover, the strong effect of N amendment on EMF production, even when N is not limiting growth, suggests that the EMF production (as a measurement of EMF production in soils) is underestimated in the pure quartz meshbags, probably because they are void of nutrients. This potential limitation in the ingrowth meshbag method could be overcome by adding ion exchange resin to improve the nutrient holding capacity in the meshbags as done by Wallander et al. (2011) and increase EMF colonization.

![Figure 8: EMF biomass production estimates between the control and P-fertilized plots and between the meshbag amendments. The bars correspond to the standard deviation of the mean.](image-url)
Nitrogen leaching and EMF nitrogen uptake in response to phosphorus limitation and high nitrogen deposition

Nitrogen uptake and demand

In temperate and boreal forests, organic N is the main N source for nutrition (Näsholm et al., 2009); however, increased N availabilities are expected to shift N demand from organic to mineral N compounds (Zhou et al., 2019; Allison et al., 2008). It has been reported that increased N availability enhanced ammonium uptake in comparison with amino acid uptake by EM plants (Wallander et al., 1997; Zhou et al., 2019). In a fertilization study of a boreal forest in Alaska, Allison et al. (2008) found that N fertilization reduced soil enzymatic activity related to chitin and protein breakdown. It was suggested that N deposition in boreal forests could have negative effects on organic N cycling because of the decrease in N enzymatic activity by EMF and other soil organisms.

To test the effects of N fertilization on EMF N uptake from organic matter, in Paper I, we amended ingrowth meshbags with composted maize leaves and incubated them in the same plots from the NP experiment described in Paper II. After 17 months of incubation, the chemical changes in the maize amendment resulting from fungal colonization were characterized using infrared and X-ray absorption spectroscopy.

We found that in meshbags incubated in the non-fertilized plots, there was a reduction in heterocyclic N compounds from the maize compost material relative to non-incubated compost material, suggesting that fungi had utilized these compounds and probably transferred them outside the meshbags. In the N-fertilized plots, the reduction of heterocyclic N compounds was lower. Heterocyclic N compounds are components of organic molecules, such as nucleic acids, carbohydrates, and chlorophyll, which comprise up to 35% of the total organic nitrogen in natural soils (Schulten & Schnitzer 1997; Talbot & Treseder...
This higher reduction of heterocyclic N compounds in the non-fertilized plots support previous findings showing that elevated N might reduce N fungal mining from soil organic matter. However, it was also found that there was an increase in the C:N ratios in the maize amendment from meshbags relative to the C:N ratios of the non-incubated compost material, irrespective of N fertilization. This could suggest that N was transferred out of the meshbag by EMF and probably transported to the host trees. Moreover, there was an increase in the amounts of carboxylic compounds in the meshbag compost material in comparison with the non-incubated compost material, suggesting enhanced oxidative degradation of the organic material. The N uptake from the organic matter even after N fertilization might indicate that the N demand in this forest persists. An increase in N availability caused by N deposition can lead to N leaching from the system. Areas with high levels of N deposition are reported to have enhanced N losses through leaching (Stevens et al., 1993; Gundersen et al., 1998; Högberg et al., 2010; Bahr et al., 2013). Gundersen et al. (1998) analysed data regarding N fluxes (N inputs and outputs) from more than 300 different forest stands across Europe and found that those receiving more than 10 kg N ha\(^{-1}\) year\(^{-1}\) as deposition and with a soil C:N ratio less than 25 C:N have a high risk for N loss through nitrate leaching. Enhanced N leaching can be a result of less N uptake and retention by EMF (Hogberg et al., 2010). In forests transitioning to P limitation, a decrease in N demand and an increase P demand by trees and EMF are expected to lead to diminished N uptake and to increased N leaching (Stevens et al., 1993; Blanes et al., 2012). Indeed, P fertilization has been shown to be able to enhance N demand, increase N uptake by trees (Blanes et al., 2012; Mayor et al., 2015), and reduce nitrate concentrations in soil solution (Stevens et al., 1993). In a Spanish fir (Abies pinsapo) forest receiving 12 kg N ha\(^{-1}\) year\(^{-1}\) of throughfall N deposition, Blanes et al. (2012) reported that P fertilization increased above growth biomass production and reduced phosphatase enzymatic activity in the mycorrhizal roots as a result of alleviated P limitation. Moreover, P fertilization increased N retention in the roots and litter layer (as measured by \(^{15}\)N recovered). As described in Paper II, we found an increase in N concentration in the needles following P fertilization, suggesting that an increase in tree growth generated N demand and increased N uptake. However, the fungal colonization in urea-amended meshbags reported in Paper III was not enhanced, and was actually significantly decreased by P fertilization, which suggests that fungal demand for N was not enhanced by P fertilization. Blanes et al. (2012) found that P fertilization decreased EMF root colonization but increased fine root production. It was suggested that an increase in P availability would reduce the dependence of plants on EMF, and that the increased N uptake observed was a result of direct plant uptake. Thus, P fertilization seems to increase tree N demand, enhance N uptake and decrease N
leaching. However, because P fertilization also has a negative effect on EMF, the enhanced N uptake could be accomplished directly by the plant.

**Nitrogen retention and EMF biomass**

In addition to the great capacity of EMF to take up N in different forms (Näsholm et al., 2009), the large underground mycelial networks can contribute to N retention in boreal forests (Read et al., 2004). Therefore, a decrease in EMF biomass as a result of N excess might also enhance N leaching (Bahr et al., 2013).

Bahr et al. (2013) analysed the correlation between EFM biomass from ingrowth meshbags and environmental data, such as throughfall N deposition, soil water N content, and humus C:N ratio from 29 Norway spruce stands distributed across southern Sweden. The forests stands had varying N deposition levels and soil water N contents. The N deposition ranged from 0.95 to 24.6 N ha\(^{-1}\) year\(^{-1}\). Multivariate correlation analysis revealed that the EMF biomass (assessed as the visual estimation of hyphal growth) was negatively correlated with soil water N. However, a direct correlation between EMF biomass and leaching could not be found.

In paper II, we tested the correlation between N leaching and EMF biomass. We investigated N incorporation and leaching through the fungal mycelia colonizing the meshbags from the NP and P experiments described in previous sections.

Using a syringe, we injected \(^{15}\)N as ammonium-nitrate on the top of the meshbags two days before meshbag harvesting. The meshbags contained ion resin beads at the bottom to collect the \(^{15}\)N that had leached through the ECM mycelia (Fig. 9).

**Figure 9:** Scheme of the \(^{15}\)N addition to the ingrowth meshbags from Paper II (Made by Adam Bahr).
After harvesting, the mycelium was isolated from the sand inside the meshbags and the $^{15}$N incorporated in the ECM mycelium was quantified as the fraction of the total amount of tracer added that was recovered in the mycelium. To estimate the amount of $^{15}$N that had leached through the ECM mycelium, the amount of $^{15}$N tracer recovered in the resin beads was also measured and quantified (see Paper II for details regarding the methodology).

We found that the amount of $^{15}$N leaching to the resin beads in the bottom of the meshbags was negatively correlated with fungal biomass in the meshbag (ergosterol) and with the total $^{15}$N incorporated in the ECM mycelia, suggesting that the increase in N leaching was related to a decrease in biomass (Fig. 10).

The total $^{15}$N incorporated in the ECM mycelia and the $^{15}$N concentration in the mycelia biomass was not affected by N fertilization. We expected a decrease in the concentration of $^{15}$N in the meshbag mycelium in the N fertilization treatment because of a lower expected N demand. However, these results suggest that leaching in the meshbags was caused by a reduction in ECM biomass rather than reduced N demand and uptake by the fungi.

Therefore, we predict that leaching can be increased in response to a reduced EMF biomass in N saturated forests. However, if N deposition induces P limitation, an increase in EMF biomass production might help reduce N leaching in these forests.

Figure 10: Correlation between A) ergosterol and leaching ($^{15}$N recovered in the resin beads) B) total recovery of $^{15}$N in the mycelia and leaching ($^{15}$N inorganic nitrogen recovered in the resin beads). The red squares correspond to the NP experiment. The green triangles correspond to the P experiment. Modified from Almeida et al. (2018).
EMF community structure in response to phosphorus limitation and high nitrogen deposition

Effect of phosphorus limitation and high nitrogen deposition on the community structure

Different ectomycorrhizal species differ in their abilities to utilize N and P, in the amount of C needed from the host and in the tolerance to the excess or deficiency of a nutrient (Lilleskov et al., 2002a; Simard et al., 2015; Zavišić et al., 2018). Therefore, changes in the amount of C delivered by the host under different nutrient conditions can alter the structure and composition of the EMF communities (Allison et al., 2008). Indeed, several studies have shown that N deposition significantly influenced EMF community structure (Lilleskov et al., 2002a; Allison et al., 2008; Kjøller et al., 2012; Suz et al., 2014). In Paper II, we tested the effect of N and P addition on EMF communities in a region with high N deposition. A meta-barcoding survey of EMF was performed in the fertilized plots described in the previous sections. Community analyses in meshbags were conducted using apatite-amended and pure-quartz meshbags incubated in the NP experiment (N and N+P fertilization treatments) only. Community analyses in soils were conducted using soil samples collected from both NP and P experiments (see details in Paper II).

In the meshbag EMF communities, apatite amendment significantly influenced EMF communities in the meshbags in the control and N fertilized plots; however, the effect of apatite on the communities disappeared when the plots were fertilized with N + P. These findings suggest that the presence of a P-rich mineral source strongly regulates the EMF community composition under P limiting conditions. Species that efficiently take up P from minerals might be rewarded with more C from the trees in P-limited forests. The lack of effect of apatite when P demand is alleviated by N+P fertilization might indicate that the host trees allocate less C to EMF species adapted to P uptake from these sources.
In the soils EMF communities, N+P fertilization had a stronger effect on ECM communities than N fertilization alone, indicating that the soil ectomycorrhizal community composition was more sensitive to changes in P than to N availability. Indeed, P fertilization significantly altered the ECM community composition (Fig. 11).

These results stress the importance of P in this forest and indicate that EMF community assemblage is strongly regulated by this nutrient, suggesting a dynamic interaction between EMF fungi and the nutritional status of forests and soils.

**Figure 11**: Response of EMF soil communities to fertilization treatments. NMDS ordination analysis of EMF communities in the humic soil for the NP experiment (A) and the P experiment (B). Differences between treatments were calculated based on Bray-Curtis dissimilarity and plotted in non-metric multidimensional scaling plots (NMDS). Modified from Almeida et al. (2018).
Effect of phosphorus limitation and nitrogen high nitrogen deposition on individual species

EMF species are diverse in structure and function and form different extramatrical structures (fungal mycelium extending from the root tip into the soil) adapted to soil exploration and nutrient uptake (Agerer, 2001; Hobbie & Agerer, 2010). These structures vary from short hydrophilic emanating hyphae to long hydrophobic rhizomorphs (Agerer, 2001). Based on these features, EMF species can be categorized into exploration types depending on the distance the emanating hyphae extend into the surrounding soil (Agerer, 2001). Different exploration types are expected to differ in the amount of C needed from the host and in their strategies to take up N and P from the soils (Lilleskov et al., 2002b; Allison et al., 2008). Thus, when there is an increase in N in soils and a concomitant reduction in belowground C allocation, EMF exploration types with low C requirements should be benefited and exploration types that require high C investment to produce long distance structures are predicted to be reduced (Lilleskov et al., 2011).

Suz et al. (2014) surveyed EMF community structure and species functional traits along N deposition gradients in 22 oak (Quercus spp.) temperate forest stands across nine countries in western and central Europe. They found that species with restricted soil exploration ranges, such as Lactarius quietus, increased in abundance in response to N deposition, while the abundance of species from the genera Cortinarius, Piloderma, and Tricholoma, which have medium exploration types, were negatively affected by N deposition. In a survey of an N deposition gradient from a boreal forest in Alaska, Lilleskov et al., (2002a) found that Cortinarius and Piloderma species responded negatively to N deposition and Lactarius increased with N deposition, suggesting that some species could be used as bioindicators for N deposition (Suz et al., 2014). However, the distance the mycelium lengthened from the root tips did not always correlate to N deposition, and some short exploration type species have been reported to decline under high N deposition (Lilleskov et al., 2011; Kjoller et al., 2012; Almeida et al., 2018), suggesting that not all EMF short distance species have the same response to decreased belowground C allocation. Allison et al. (2008) analysed EMF community structure in another N deposition gradient in Alaska and found that, for some EMF species, the abundance of sporocarps and the relative abundance of the fungus in soils were significantly reduced as an effect of N deposition. However, other species significantly reduced sporocarp formation as an effect of N deposition while the relative abundance of the fungus in soils remained constant. This might be an indication that some species can reduce C investments in reproductive structures and persist under low C allocation.
Long exploration types have also been shown to vary in their response to N deposition (Lilleskov et al., 2011; Suz et al., 2014). In the EMF communities from ingrowth meshbags studied in Paper II, the long exploration type *Boletus badius* (currently known as *Imleria badia*) significantly increased in abundance after N fertilization. Considering the evidence regarding P limitation in these stands, this could suggest that N fertilization exacerbated P limitation and that the increase in abundance of this long exploration species is a strategy to enhance P foraging and uptake. Indeed after N+P fertilization, the abundance of this species decreased significantly (Fig. 12). Moreover, when the meshbags were amended with apatite, the abundance of this species was further enhanced, suggesting that the growth of this species is stimulated by apatite and it is possible that superior P uptake from this mineral compared to other species was rewarded by larger C flux to the fungus.

![Relative abundance](image)

**Figure 12:** Relative abundance (DNA sequences reads) of *Boletus badius* (currently known as *Imleria badia*) in the apatite and quartz meshbags in the fertilization treatments from the NP experiment. Bars represent average values per treatment (n=12), error bars correspond to two standard errors. Modified from Almeida et al. (2018).

It has been postulated that, during the transition from N to P limitation, the EMF community composition will change from nitrophylic taxa to taxa specialized and more efficient for phosphorus uptake (Lilleskov et al., 2002a). In an N deposition gradient in a boreal forest from Alaska, Lilleskov et al. (2002a), reported that among the species adapted to excess N, at the end of the gradient *Paxillus involutus* significantly increased in abundance. *P. involutus*, is able to access P from poorly soluble sources in axenic cultures as well as when growing in symbiosis with plants (Wallander et al., 1997; Leake et al., 2008; Rosling, 2009;
Smits et al., 2012). In addition, it has been shown to be efficient at taking up P from mineral compounds (Adeleke et al., 2012) and to have fast phosphate uptake when compared with other EMF species (Van Tichelen & Colpart, 2000). Therefore, Lilleskov et al. (2002a) proposed that *P. involutus* is a P efficient species and its increase might respond to an increase in P demand caused by N deposition.

In Paper II, we suggest that *B. badius* might be a species efficient at P uptake that responds with higher abundance when P demand from trees increases. Kottke et al. (1998) analyzed nutrient storage capacity in 17 different EMF species from root tips collected from the black forest in Germany and found that *B. badius* had high amounts of large intracellular polyphosphate granules and the highest concentration of P in the fungal sheath when compared with the other species analyzed. It was suggested by the authors that *B. badius* was potentially important for tree P nutrition, which supports our finding of increased abundance of this fungus in response to apatite under P-limiting conditions.
Effect of phosphorus limitation on EMF phosphorus uptake from organic and mineral molecules

Even though P can be abundant in soils, its uptake is challenging for plant nutrition because only a small fraction is present as free inorganic phosphates (PO$_4^{3-}$, HPO$_4^{2-}$ and H$_2$PO$_4^-$), which are the primary forms available for plant uptake (Plassard et al., 2011). Significant amounts of P may be locked in organic molecules or poorly soluble minerals, or bound to iron oxides (Belyazid & Belyazid, 2012; Nehls & Plassard, 2018). Plants overcome these challenges through EMF associations. EMF can enhance P uptake and plant nutrition by using different strategies in which phosphate is released from minerals and organic molecules (Finlay, 2008). Therefore, it is expected that increases in plant P demand will favour EMF species adapted to P limitation by being efficient for P uptake from the different P sources in the soil (Lilleskov et al., 2002a; Almeida et al., 2018). Understanding the mechanisms responsible for accessing P pools in soils by EMF is important to determining the impacts that N deposition and P limitation have in boreal and temperate forests (Peñuelas et al., 2013). In this chapter, I provide a short physiological insight into the mechanisms EMF use to access P from different pools in soils and briefly mention the factors that regulate P uptake.

EMF organic phosphorus uptake

Organic P compounds constitute a large pool of phosphorus in soils (Cosgrove, 1967; Richardson, 1994; Belyazid & Belyazid, 2012; Nehls & Plassard, 2018) and are expected to contribute significantly to the total phosphorus uptake by trees in forest ecosystems (Rennenberg & Herschbach, 2013; Vincent et al., 2013). For instance, in boreal forests, up to 70–90% of soil phosphorus may be bound to organic substrates (Cosgrove, 1967; Vincent et al., 2013). The most abundant organic source is phosphomonoesters, which can comprise more than 50% of the organic phosphorus pool in soils (Makarov et al., 2002). Inositol phosphates, such
as phytic acid (six phosphate molecules bound to an inositol ring) (Mittal et al., 2011), are probably the most abundant phosphomonoesters (Gerke, 2015). Phytic acid, which is mainly present in plant seeds as P reserve (Graf et al., 1987; Lott et al., 1995), is an important P source in natural soils since it accumulates when the seed does not germinate (Cairney, 2011; Becquer et al., 2014). The conversion of organic phosphorus into available phosphates depends on phosphatase enzymatic activity (Plassard et al., 2011). For example, phytates (phosphate monoesterase enzymes) are responsible for the hydrolysis of phytic acid and the release of phosphate (Plassard et al., 2011; Sanz-Penella & Haros, 2014; Antibus et al., 1992). Experiments in axenic cultures have shown that several ectomycorrhizal fungi are able to subsist on phytic acid as the sole P source (Antibus et al., 1992). In an enzymatic essay using beech mycorrhizal roots, Bartlett & Lewis (1973) confirmed the hydrolysis of myo-inositol hexaphosphate and an increase of orthophosphates in the medium. Norisada et al. (2006) reported that EM Pinus densiflora seedlings provided with inositol phosphate as a P source had comparable P content in the needles as plants provided with phosphate. Several studies have shown that plants have poor capacity to solubilize phytates (Irshad et al., 2012; Becquer et al., 2014), suggesting that ectomycorrhizal fungi might play an important role in plant nutrition from phytic acid.

**EMF phosphorus uptake from mineral compounds**

In soils, P can be part of poorly soluble minerals (Cairney, 2011). The most common P-bounded minerals in soils are calcium phosphates, such as apatite (Osman, 2012). Many studies have shown that EMF are able to take up phosphorous from apatite sources when growing in symbiosis with plants or in axenic cultures (Wallander et al., 1997; Leake et al., 2008; Rosling, 2009; Smits et al., 2012). When using apatite as the sole phosphorus source, Wallander et al. (1997) showed that the foliar P concentrations were higher in mycorrhizal pine seedlings than in non-mycorrhizal seedlings. Laparye et al. (1991) tested the capacity of 11 different EMF species to solubilize P from apatite suspended in solid agar (with or without phosphate) and found that the majority of species were able to solubilize apatite, regardless of the presence or absence of phosphate in the medium.

Among the mechanisms suggested for the release of P from apatite by EMF is the excretion of low molecular weight organic acids (LMWOA). Due to their acidifying and complexing properties, they are important agents for mineral weathering in soils (Landeweert et al., 2001). The carboxylic groups of LMWOA donate protons to the soil solution, which acidify the medium, protonate mineral surfaces, and increase P solubility (Gadd, 1999; Rosling et al., 2007). In addition,
organic acid anions interact with calcium from the surface of the mineral and displace phosphate, increasing P release rates (Zhang et al., 1997; Landeweert et al., 2001). There are a variety of low molecular weight organic acids, including oxalic, citric, and fulvic acid (Van Hees et al., 2000; Plassard et al., 2011). Oxalic acid is one of the most studied compounds in phosphorus release from minerals because of its strong chelating and acidifying capacities (Landeweert et al., 2001; Bindschedler et al., 2016; Nworie et al., 2017). When oxalic acid is excreted, the oxalate ions bind with calcium (forming calcium oxalate) and release phosphorus (Cannon et al., 1995; Rosling et al., 2007). Accumulation of calcium oxalate crystals in ectomycorrhizal roots and under hyphal mats in forest soils has also been observed (Cromack et al., 1979; Malajczuk & Cromack, 1982), especially in the presence of apatite (Wallander et al., 2003), corroborating the role of oxalic acid in the release of P from minerals.

The production and accumulation of oxalic acid varies greatly between different ectomycorrhizal species (Plassard et al., 2011). Species such as Paxillus involutus and Rhizopogon roselous are known to produce large amounts of oxalic acid. Other species, such as Hebeloma cylindrosporum and Laccaria laccata (Lapeyrie et al., 1991), as well as the genera Amanita, Cenococcum, Tylospora, and Thelephora, have been shown to be poor oxalic acid producers (Casarin et al., 2004; Plassard et al., 2011). The ecological meaning of such interspecific variation remains unclear (Plassard et al., 2011). It would be useful to study the EMF community’s composition in response to P limitation to determine if species that produce high amounts of oxalic acid are selected for in forests soils with low P availability. It is possible that such species might be rewarded by enhanced carbon allocation by the host in environments in which phosphorus is not readily available and where enhanced phosphorus acquisition is worth the costs of producing and excreting high amounts of oxalic acid.

It is important to consider what concentration of LMWOA is high enough to affect apatite weathering in soils. Oxalate is usually estimated in µM concentrations in soil solution (Van Hees et al., 2005), and efficient apatite dissolution has been measured in the mM range (Rosling et al., 2007). However, hyphae could produce enough oxalic acid to reach concentrations as high as 30 mM if the oxalic acid is released in small pores or cavities in the soil (Van Hees et al., 2005). Thus, the capacity of EMF to release P from apatite might not only depend on the total amount of oxalic acid released, but also on the ability of the fungi to explore and colonize small cavities in the soil while looking for minerals.

Phosphate can also be tightly bound to secondary minerals, such as iron oxides (Belyazid & Belyazid, 2012; Nehls & Plassard, 2018). Due to its stability, Goethite is the most common iron mineral in soils (Torrent & Schwertmann, 1992; Scheinost, 2005). Phosphate ions can be absorbed on its surface by ligand
exchange between the phosphate and OH groups from the mineral (Celi et al., 2000; Martin et al., 2002; Fink et al., 2016). It has previously been suggested that EMF can access P bound to Fe minerals. Casarin et al. (2004) reported improved P nutrition of P. sylvestris in symbiosis with Rhizopogon roseolus growing on soils containing low amounts of available P because of high P absorption on Fe oxides. Adeleke et al. (2012) found that P. involutus in symbiosis with B. pendula was the most efficient among three EMF species tested at taking up P from Fe minerals containing 90% Fe oxides and several P-bearing minerals, such as apatite.

Different mechanisms to mobilize P from iron oxides have been proposed; namely, surface displacement of phosphate ions from the Fe oxide surface mediated by strongly absorbing molecules, such as LMWOA and siderophores (Casarin et al., 2004; Rosling et al., 2007; Rosling & Rosenstock, 2008), by pH reduction mediated by LMWOA and proton exudation (Rosling et al., 2007; Adeleke et al., 2012), and by reductive dissolution mediated by Fe-reducing compounds (Baldwin & Mitchel, 2000; Chacon et al., 2006; Zhang et al., 2014).

Proton exudation, LMWOA and siderophores production have been proposed and studied as the main mechanisms to release phosphate from Fe-mineral surfaces by EMF (Casarin et al., 2004; Rosling et al., 2007; Rosling & Rosenstock, 2008 Adeleke et al., 2012). Fe reductive dissolution as a mechanism to release P has been reported mainly by Fe-reducing bacteria in soils and water sediments (Baldwin & Mitchell, 2000; Chacon et al., 2006; Zhang et al., 2014). However, the role of Fe-reducing activity by EMF in response to P nutrition needs further exploration, although P. involutus has been reported to produce Fe-reducing metabolites to induce Fenton reaction and improve N nutrition from organic matter (Shah et al., 2015). In Paper IV, we investigated whether the capacity of P. involutus to reduce Fe can be also used as a mechanism to improve P nutrition (see below).

Phosphorus uptake under low P availability

Some studies have shown that P availability in medium (either soil or axenic cultures) influences EMF phosphatases activity (Cairney, 2011; Plassard et al., 2011). For example, Alvarez et al. (2006) investigated the activity of phosphate monoesterase bound to P. involutus hyphae in axenic cultures using enzymatic fluorescent labelling. They found that the monoesterase activity peaked when mycelium was growing in the lowest P concentration. In soils, it has been reported that increased soil P availability reduces EMF phosphatase activity (Alvarez et al., 2006; Blanes et al., 2012), and increased EMF phosphatase activity in soils is expected to enhance P uptake from organic compounds.
In **Paper I**, we amended ingrowth meshbags with maize compost and incubated them in a P-limited spruce forest to investigate chemical changes of the compost material after EMF colonization. C:P and C:N analysis of the maize compost revealed that, after incubation in the soil, the EMF community removed substantial amounts of P in addition to the N removal from maize amended meshbags reported above (Table 1). An increase in P demand in this forest might have caused a decrease in soluble P availability in soils, leading to enhanced P uptake from organic matter by EMF. This suggests that, in forests undergoing P limitation, P mining by EMF might be important to the decomposition of soil organic matter.

**Table 1**: C:N and C:P ratios of the meshbag contents at the beginning of the experiment (initial material) and after 17 months of incubation in the soil humus and mineral layers of the control and N-fertilized plots at the Norway spruce forest. Standard error is in parentheses (n = 3 for the C:N ratios; n=6 for the C:P ratios). Values followed by different lowercase letters indicate significant difference between treatments and the initial material (p < 0.05). Modified from Nicolás et al. (2017).

<table>
<thead>
<tr>
<th>Initial material</th>
<th>Humus layer</th>
<th>Humus layer</th>
<th>Mineral layer</th>
<th>Mineral layer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>N-Fertilized</td>
<td>Control</td>
<td>N-Fertilized</td>
</tr>
<tr>
<td>C:N 11.9 (0.1) a</td>
<td>14.7 (0.5) b</td>
<td>14.3 (0.3) b</td>
<td>14.8 (0.6) b</td>
<td>14.0 (0.1) b</td>
</tr>
<tr>
<td>C:P 53.8 (6.2) a</td>
<td>137.6 (12.7) b</td>
<td>139.2 (13.2) b</td>
<td>159.0 (13.9) b</td>
<td>139.8 (12.1) b</td>
</tr>
</tbody>
</table>

Regarding P uptake from mineral P compounds, such as apatite, low P availability has been reported to increase production of LMWOA by EMF (Van Schöll et al., 2006; Rosling et al., 2009; Smits et al., 2012) and to increase P uptake from this mineral (Wallander, 2000; Casarin et al., 2004; Leake et al., 2008; Smits et al., 2012). Leake et al. (2008) grew *P. sylvestris* seedlings inoculated with *Paxillus involutus* and fertilized them either with a full nutrient solution or a solution containing no phosphate. The roots and mycelium were grown in an agar-chamber where either apatite or quartz (no-containing-P mineral) was added to test if apatite could improve the host P nutrition. When no phosphate was provided in the nutrient solution, the P content of the host increased significantly in the presence of apatite. However, when the seedlings were provided with a full nutrient solution, no difference in the seedling P content was observed between apatite and quartz additions. These findings clearly demonstrate the capability of EMF to increase P uptake from mineral sources, such as apatite, when the P availability in soils is low.
Phosphorus uptake and phosphorus status of the mycelium

In axenic cultures, low intracellular P concentrations of the mycelium have been reported to increase P absorption from the growth medium (Cairney & Smith, 1992; Torres-Aquino et al., 2017). Cairney & Smith (1992) studied Pisolithus tinctorius uptake of $^{32}$P-labelled phosphate after the fungus was exposed to different P concentrations in the growth medium, and they found a negative correlation between the intracellular phosphate concentration and the total amount of $^{32}$P taken up. Similarly, Torres-Aquino et al. (2017) found that after a period of P starvation, P uptake by Hebeloma cylindrosporum was enhanced and high amounts of poly-P granules were produced when the fungus was resupplied with phosphate. The amount of polyphosphate registered in the starved hyphae was as high as the amount registered in the non-starved hyphae. The production of poly-P granules is suggested as a mechanism to reduce free-phosphate concentrations in the cytoplasm, which might activate phosphate transporters in the membrane, thereby increasing P uptake (Cairney & Smith, 1992; Cairney, 2011). Moreover, the production of polyphosphate was not restricted to the amount of phosphate resupplied. Indeed, starved hyphae could produce similar amounts of polyphosphate granules, even when resupplied with a 10-fold lower phosphate concentration than the concentration originally resupplied. These findings suggest that the P status of the hyphae is an important factor that regulates P uptake and storage.

Limitations of axenic experiments for nutrient uptake studies.

Regarding P uptake from poorly soluble P sources, the results from culture experiments are not always consistent with those from experiments in which the fungus is growing in symbiosis with a plant (Rosling, 2009). In a symbiotic system, the host acts both as a sink for nutrients and as a carbon source. It has been proposed that the P taken up in the mycelial front is translocated to the fungus: root interphase in which P is accumulated (Cairney & Smith, 1992; Cairney, 2011). This phosphate efflux maintains low concentrations of intracellular P at the hyphal front, which might enhance P uptake (Cairney & Smith, 1992). Therefore, EMF growing in pure culture systems lack the nutrient sink that allows the reciprocal translocation of C and P. Moreover, in pure culture experiments, glucose and P are generally both provided in the growing medium, which
suppresses the formation of fungal structures adapted to nutrient foraging, uptake, and translocation (Smits et al., 2012).

To overcome some of these problems, in **Paper IV**, I developed an experimental set up designed to resemble a symbiotic system in which the C and P source come from different locations. To accomplish this, I grew *P. involutus* in a divided petri dish system in which carbon and P were provided in the different compartments of the plate (Fig. 13). Using this set up, I examined the capacity of the fungus to take up P from poorly soluble forms. Additionally, to test the effects of P status of the mycelium on P uptake, half of the fungal cultures were previously exposed to a starvation growth phase in which no P was supplied in the growing media (see details in **Paper IV**). Starved and non-starved mycelia were then provided with the following P sources: phosphate, apatite, P-bound goethite, phytic acid or no P addition (as a control). Apatite and P-bound goethite were provided as solid particles on a piece of cellophane paper laying on top of the upper glass bead layer at approximately 1 cm from the dividing wall of the plate (Fig. 14). After 2 weeks of incubation, the part of the mycelium growing on the C compartment was collected to measure total P taken up and transferred from the P compartment. The part of the mycelium growing in contact with the P source was then collected and used for gene expression analysis (data not presented).

**Figure 13:** The divided plate set up used in Paper IV explained in detail. The fungus was inoculated at the dividing zone between the two compartments. In the P compartment, liquid growth medium was provided. Different P sources were provided depending on the treatment. On this side of the plate, the fungus grew on three layers of glass beads. In the C compartment, agar and growing medium were added and all of the nutrients but P were provided. Grey circles represent a glass bead system while the beige area represents agar-added growing medium.
Figure 14: Photographs of *P. involutus* cultures and the different phosphorus source treatments at the end of the experiment: phosphate (A), phytic acid (B), goethite (C), and apatite (D) and no-phosphorus (E).
We found that _P. involutus_ was able to access P from all of the sources provided (apatite, phytic acid, and P-bound goethite), although not to the same extent as the easily available phosphate. The fungus was able to translocate P to the part of the mycelium that had access to C. These results support previous evidence that _P. involutus_ is able to take P up from both mineral and organic sources. Furthermore, we provide for the first time direct evidence of EMF uptake from P that has been absorbed into a Fe-oxide surface. The capacity of _P. involutus_ to release P bound to goethite, which is one of the most abundant Fe mineral in natural soils (Torrent & Schwertmann, 1992), suggests that this species is of ecological importance for P nutrition.

In **Paper IV**, we also examined whether the capacity of _P. involutus_ to reduce Fe$^{3+}$ was related to P uptake and influenced by P source and P starvation. For this purpose, we collected liquid medium from the fungal cultures at the end of the experiment and determined the Fe-reducing capacity.

The Fe-reducing capacity was measured using a Ferrozine assay (Goodell _et al._, 2006). Ferrozine is a compound that reacts with divalent Fe (Fe$^{2+}$) to produce a colour reaction. The concentration of Fe$^{2+}$ in the solution can be then measured based on the absorbance of the sample using a spectrophotometer. We added Fe$^{3+}$ (in the form of Fe Cl$_3$) to the liquid medium collected. If _P. involutus_, had produced Fe-reducing metabolites during the experiment, the added Fe$^{3+}$ will be reduced. The Fe-reducing capacity of the medium was estimated as the amount of added Fe$^{3+}$ that was reduced to Fe$^{2+}$ during the assay.

In the P-starved mycelium cultures, we found significant Fe-reducing capacity for all of the P treatments (phytic acid, apatite, goethite and no-P) with exception of the phosphate treatment (Fig. 15). Fe-reducing activity by _P. involutus_ could be a mechanism to enhance phosphate uptake from poorly soluble sources. The release of P from apatite and phytic acid in soil is probably very slow and the released phosphate ions may easily be captured by Fe$^{3+}$ present in the soil (Turner, 2008; Rosling, 2009; Plassard _et al._, 2011). However, by producing Fe-reducing compounds, EMF may increase the possibility for assimilation of the newly liberated phosphate ions.

Furthermore, the P source also affected Fe-reduction capacity in which phosphate registered the lowest Fe-reducing capacity and apatite registered the highest. In our experiment, low concentrations of phosphate in the medium solution might have induced Fe-reducing activity as a response to improve phosphate uptake. Phosphate released from the apatite was likely rapidly taken up by the fungus, resulting in low phosphate concentrations in the medium, which may have induced continuing exudation of Fe-reducing metabolites. In **Paper IV**, we report for the first time that Fe-reducing capacity by EMF may be related to P nutrition and dependent on the P source provided. Fe-reduction as a mechanism to improve P
uptake from organic and mineral P sources is a strategy not reported for EMF and should therefore be further investigated.

Contrary to what was expected, we did not see an increase in P uptake in the starved mycelium in comparison with the non-starved mycelium when growing with any of the P sources. However, we reported that the capacity to reduce Fe was increased in the starved mycelium, especially when the fungus was provided with apatite or growing without any P source. Moreover, the total amount of oxalate produced by the fungus was significantly increased in the starved mycelium relative to the non-starved mycelium, but only when no P source was provided (results not shown in Paper IV) (Fig. 16). Thus, we show that the P status of the mycelium in P. involutus is important to regulation of mechanisms that improve P uptake from poorly soluble sources, even though an effect on total P uptake could not be detected.
Figure 16: *P. involutus* oxalate production for all of the P treatments in starved and non-starved mycelium. Error bars correspond to two standard errors of the mean (n=4). Phos, no-P, Phyt, Goe, and Apa are abbreviations of the phosphate, no-phosphorus, phytic acid, goethite, and apatite treatments, respectively. Asterisks (*) represent significant differences between the starved and non-starved mycelium according to ANOVA tests.

As shown here, *P. involutus* has the capacity to release P from different poorly soluble sources (including P bound to Fe oxides surfaces) and to produce Fe-reductants that facilitate uptake of the newly released P. This might confer this species with an adaptive advantage over species in the EMF community in soils, especially under P limiting conditions. In a forest with high P demand, *P. involutus* could be rewarded with a higher C allocation from the host in return for P taken up from minerals and organic compounds. This supports the results reported by Lilleskov *et al.* (2012a), who found that this species is favoured under high P demand conditions, probably because of its high P uptake capacity. *Boletus badius* is another species that has been reported to increase in abundance in response to P limitation (Paper II) and suggested to be efficient for P uptake in forest soils (Kottke *et al.*, 1998). In a pilot study, we isolated *B. badius* from the P-limited spruce forest described in previous sections. Preliminary analyses showed that *B. badius* exhibited significant levels of Fe-reducing capacity that were enhanced by P starvation. These findings corroborate the idea that Fe reductive dissolution could be a mechanism to avoid Fe-P binding, and that this strategy is being used in natural soils by EMF.
In conclusion, in this chapter, I show why EMF association can be important for P nutrition in high latitude forests. EMF can access P from different P pools via different strategies, and this is enhanced by low P availabilities. This can be crucial in forests facing P limitation. Other EMF species responsive to P limitation in high latitude forests should be studied to test the potential mechanisms by which species deal with nutrient limitation and how EMF communities adapt to shifts in nutrient regime.
Conclusions and future perspectives

In this thesis I highlight the importance of P over various aspects of EMF at a northern conifer forest receiving high N deposition levels. I show that P regulates EMF biomass production and EMF communities’ assembly. Also, P limitation favors the abundance of EMF species better adapted to P uptake from non-easily available P sources, which are abundant in natural soils.

N has been considered the most important nutrient limiting plant growth and regulating EMF growth and EMF communities’ assemblage in high latitude forests. However, since N deposition can lead to a shift in nutrient limitation increasing P demand, P can become an important element for high latitude forest nutrition. The shift from N to P limitation has been widely discussed for these forests (Vitousek et al., 2010; Peñuelas et al., 2013; Jonard et al., 2015), yet studies showing a positive effect of P fertilization on tree growth are scarce in temperate forests and boreal forests. In Paper II, I provide strong evidence about the effect of P fertilization on tree growth and foliar P concentration and I show that regions receiving high N deposition levels can transition from N to P limitation. The forest analyzed in our studies has more elevated N deposition levels in comparison to other northern conifer forests, though these levels are low in comparison temperate regions in central Europe. Even so, under these conditions a strong effect of P was detected. Other P fertilization experiments are needed especially in temperate forests to evaluate the response of EMF to the transition from N to P limitation as done in this study.

Under high soil N availability the host trees reduce belowground C allocation, which results in reduced EMF biomass production. In this thesis I show that an increase in P availability also decreases EMF total production in forest where N deposition has resulted in P limitation. P-rich sources like apatite have a stronger positive effect on the total EMF production but only under P limiting conditions. This corroborates that in this forest the P status of the trees regulates EMF production and suggest that P regulates belowground C allocation to the fungal symbionts. Interestingly, EMF foraging on N rich sources (urea) was also decreased by an increase in P fertility, which may indicates that a general reduction of belowground C allocation results in a reduction for foraging to other nutrient sources.
Here I have suggested that the effect of P on EMF biomass production is a result of a decreased belowground C partitioning by the host. However, I have measured only EMF biomass of mycelium colonizing the meshbags and I have not estimated the total belowground C allocation since I have no data on root production, respiration and EMF root colonization. Therefore, it is difficult to state which nutrient N or P is more important for belowground C partitioning. N could be more important regulating fine root production while P can be more important regulating EMF extramatrical production as suggested by Ekblad et al. (2016) and Blanes et al. (2012). More studies on root respiration, fine root production and EMF root colonization and are needed to identify how N and P nutrient limitation affects belowground C allocation to different components of the rhizosphere and soils. Additionally, EMF total production seems to be a more robust tool to assess extramatrical mycelium responses to nutrient limitation than measurements of the standing biomass of one given incubation time in meshbags or the standing biomass from soils.

P had a stronger effect on EMF community’s composition than N. Moreover, P strongly regulates EMF community’s structure especially in presence of apatite in P limited plots. This clearly indicates that EMF community composition is related to the trees nutrient necessities and species adapted for P foraging and uptake are favored by a higher C allocated by the host when P demand increases.

Besides the overall main effect on the EMF community structure, P limitation strongly favored single species like Boletus badius (especially responsive to apatite in meshbags under P limiting conditions) that have been shown to be efficient to take up and store P from soils. The increase in abundance of B. badius indicates a potential role of this species in tree P nutrition, similar to what was suggested for other species responsive to an increase of P demand in N saturated forests like P. involutus.

It is necessary to do more studies on the ecology and the physiology of individual species that respond to an increase of N deposition and so P demand, such as B. badius and P. involutus, to determine the importance of these species for forest P nutrition. Studies that test the EMF capacity to take up P from organic and mineral compounds, the rate of P uptake in comparison with other species, the ability to subsist with minutes amounts of P, or the ability to transfer P to the host according to the host needs, are necessary to evaluate how EMF will adapt to increase P demand in high latitude forests. Here, I have isolated B. badius from the forest soils and I aimed to test P uptake from different sources by this potential P efficient species in axenic cultures and symbiosis experiments.

I have shown that P. involutus has the capacity to take up P from poorly soluble sources like phytic acid, apatite and P bound to secondary minerals like goethite that are abundant in many soils. Furthermore, I observed that P. involutus induces
Fe-reduction when provided with these sources especially when it grows with apatite. This Fe-reducing capacity could be a strategy by the fungus to enhance phosphate uptake probably by preventing binding between the phosphate release by the mineral and Fe$^{3+}$ present in the soils. A follow up study could be to analyze which reducing metabolites are involved in the Fe-reduction activity registered for *P. involutus*. Involutin has been detected as a secondary metabolite involved in N mining form organic compounds. I observed that *B. badius* also presents Fe-reducing activity in response to P starvation. It would be important to isolate the compounds and metabolites (as done by Shah *et al.* (2015)) produced during P starvation to test the metabolites involved in P uptake from these two boletal species.

The P status of the hyphae of *P. involutus* increases Fe-reducing capacity when the fungus is provided with apatite or when the fungus was growing without any P source. The total oxalate production followed a similar pattern as the Fe reducing capacity. This suggests that the P status of the hyphae regulates some strategies to weather poorly soluble P sources. The majority of studies about physiological responses of EMF to low P availabilities have focused on low external phosphate concentration as a proxy for P limitation. It is important to study how the P status of the mycelium affects P uptake and regulate strategies to increase P uptake. Understanding how the P status of the hyphae influence on P uptake will allow us to understand how P limitation in forests affect P nutrition from the perspective of the fungus own needs.

The similar pattern between oxalate production and Fe-reducing capacity might suggest a synergetic effect of these compounds during mineral weathering. A similar synergistic effect has been suggested for Fe binding molecules (siderophores) and oxalate during organic matter degradation (Clarholm *et al.*, 2015) and iron oxides dissolution (Reichard *et al.*, 2005). The high Fe-reducing capacity and oxalate production when apatite is provided as a P source requires further studies. A trascriptome study on the mycelium in contact with the P sources provided in the experiment from Paper IV is being developed to analyze the genes involved in apatite weathering and to test if apatite provided to starved mycelia leads to an up regulation of genes involved in Fe-reduction compounds and oxalate production.


Popular science summary

Mycorrhizal associations

Mycorrhizal fungi are organisms that live in association with plant roots and provide plants with water and nutrients in exchange for the sugars obtained during photosynthesis. In forested ecosystems mycorrhizal fungi are crucial for tree nutrition and trees rely heavily on these associations to take up key elements like nitrogen and phosphorus. As result, big amounts of the atmospheric CO$_2$ converted in sugars by trees are delivered to the soils to support the fungal partners.

In boreal and temperate forests, nitrogen is present in the soils in forms that are generally not available for tree consumption. Due to the cold conditions in these ecosystems, the cycling of nutrients in the soils is slow and nitrogen accumulates in complex organic molecules (slowly degrading dead plant and animal tissues) with no nutritious value for the trees. However, mycorrhizal fungi have developed different strategies to access the nitrogen locked in such molecules. For that reason, a great amount of carbon is sent to the fungal partners to enhance nitrogen uptake that is in high demand. Indeed, temperate and boreal forests soils are one of the most important reservoirs for carbon in the world.

Mycorrhizal associations, nitrogen deposition and phosphorus demand

Nitrogen pollution caused by human industrial activities deposits from the atmosphere onto forest soils, increases the availability of nitrogen and reduces the need of the trees to maintain the fungal partners. As a consequence, less carbon is delivered to the soils and roots causing a decrease in the growth of mycorrhizal fungi, which might impact ecosystem functioning. A reduction in mycorrhizal biomass can lead to less nitrogen uptake, which will be leached through the soils.

Initially, excess nitrogen favors tree growth, but eventually the trees stop growing because a new nutrient is now in high demand and is required to keep sustaining growth. This nutrient is phosphorus.

Like nitrogen, phosphorus in soils can be present in form of organic molecules. Besides, phosphorus is also found in soil minerals and ores. Mycorrhizal fungi have the ability to release phosphorus from organic molecules and also from ores and minerals. Therefore, when phosphorus tree demand increases in forests,
mycorrhizal fungi growth is expected to be stimulated to enhance phosphorus foraging and uptake from the soils.

In this thesis I measured ectomycorrhizal growth in a forest where nitrogen deposition has resulted in high phosphorus demand. Mycorrhizal growth was measured using ingrowth meshbags, which are bags made of a 25 micrometers mesh containing sand. The size of the mesh prevents the growth of the plants fine roots but allows the growth of the fungal filaments (series of attached cells that extend into the soil). This method allows the study of mycorrhizal fungi colonizing the inside of the bags in the search for nutrients. Moreover, I added nutrients rich in nitrogen and phosphorus inside the meshbags to see how nutrient foraging is affected in forests with high P demand. The meshbags were then placed underground in the forest where they were colonized internally by mycorrhizal fungi.

I found that when phosphorus demand increases in the forest, there is also an increase in mycorrhizal growth suggesting that trees rely again on these fungal associations and send carbon belowground to support mycorrhizal fungi and improve phosphorus nutrition. The increase in mycorrhizal growth was significantly enhanced when the meshbags contained either nitrogen or phosphorus rich nutrients. This suggests that higher mycorrhizal growth due to P demand influences nutrient foraging for phosphorus but also for nitrogen. Thus, the increase in mycorrhizal growth can help the ecosystems to retain more nitrogen and prevent leaching. Moreover, enhanced carbon deliver to the soils can increase carbon sequestration, which is important to mitigate CO₂ emissions.

**Phosphorus demand and mycorrhizal communities**

Hundreds of different mycorrhizal species inhabits the forest soils and more than tens of different species are associated with the root system of the same individual tree. These species have different abilities to take up phosphorus from soils and to endure the conditions where the trees allocate less carbon due to high nutrient availability.

In this thesis I also studied how the communities of mycorrhizal fungi species are affected when phosphorus is in high demand. I extracted DNA from soils and meshbags collected in the forest described above. Based on the DNA sequences abundance, I estimated mycorrhizal fungi species abundance and the structure of the mycorrhizal fungal communities. I found that phosphorus demand had a strong effect on mycorrhizal communities and stimulated mycorrhizal species more efficient to release phosphorus from organic molecules and soil minerals.

*Boletus badius* is one of the species that increased in abundance during phosphorus demand conditions. Previous studies showed that *Boletus badius* has the ability to take up and storage high amounts of phosphorus from soils.
Moreover, it thrives next to phosphorus rich mineral sources especially in high phosphorus demand conditions suggesting that it has the ability to release phosphorus from minerals.

In a laboratory experiment I showed that *Paxillus involutus* (another species previously reported to thrive under P demand conditions) had the ability to utilize phosphorus from different compounds that form part of the soil phosphorus pool. Furthermore, I demonstrated the elegant chemical mechanisms that this species has to extract phosphorus from soil minerals and ores. It can produce molecules called organic acids that have strong affinity for mineral surfaces and when released they bind the mineral and displace phosphorus, which can be taken up by the fungus. Moreover, *Paxillus involutus* has the ability to produce molecules that donate electrons to the iron present in certain minerals bound to phosphorus. When iron gains this electrons it gets reduced and phosphorus is released and becomes available for plant and fungi consumption.

In conclusion, mycorrhizal growth in forests is a dynamic process regulated by nitrogen and phosphorus availability. These dynamic processes are very important for carbon delivery and storage in the soils. Mycorrhizal community composition assemblage is also dependent on the nutrient status of the trees, and mycorrhizal species adapted to take up phosphorus from different sources can be important to help trees endure phosphorus deficiency.
Acknowledgments

Håkan: Thank you very much for all your support during these four years! You have been a fantastic supervisor. You always had time to meet, discuss and help me. You have always cared not only for the progress of the thesis but also for my development as a person and as a scientist. You are a great scientist but more important an amazing person!

Nick: Thanks for giving me the opportunity to work with mycorrhizal fungi. Your help with the community analysis and during the hard fieldwork has been very valuable. Thanks also for the language editing of the manuscripts.

Anders: Thank you for your help and support as my examiner during these 4 years. Also thanks for helping me during the writing process of the last manuscript.

Per P: Thank you for helping me understanding and interpreting the data from the last manuscript.

Tomas: Thank you for the help with the sequencing data and writing process of the last manuscript. Also thank you very much for introducing me to the microbial ecology group.

Alf: Thank you for your help with the turnover analysis and during the writing of the third manuscript.

Lena: Thank you for the oxalate measurements.

Pål Axel O: Thank you very much for being my mentor and thank you for your advices during the PhD meetings.

Benjamin: One of my first and closest friends here in Sweden. It was very nice to work together with you in the field and in the lab.

César: Gracias por ser un buen amigo y colega! Fue muy divertido trabajar contigo en nuestro artículo. Gracias por enseñarme sobre espectroscopia.

Björn: Thank you very much for all your help with the sequencing data and bioinformatic analyses.

Tao: I was very lucky to have you as my office mate. You have been so kind and patiently answered my endless questions about chemistry; and always with a smile.
on your face. We have had so many interesting conversations about life and science and it made my life as PhD student much more fun! Thanks for been a great colleague but more important an amazing friend!!

**Dimitri:** Thank you for all the interesting discussions about fungi, evolution and life in general. It was so much fun having conversations with you. Thank you for valuable input for my fungal culture experiment. Thank you for always listening to my problems and offering good advices.

**Margarida:** Mi primera amiga aquí en Suecia!! Gracias por todos estos años de amistad. Hemos pasado por muchas cosas juntos… hemos sido compañeros de casa, compañeros de maestría y después compañeros de PhD. Esperemos que en el futuro sigamos compartiendo mucha cosas!! Obrigado!!

**Saeed:** Amigo! It has been very nice to be your friend during these last four years! We have shared a lot, supported each other during the hard times and had lots of fun during the good times.

**Ainara:** Gracias por ser mi amiga durante estos años. Tu buen ánimo es siempre contagioso. Hemos compartido muchos momentos divertidos y serios. Siempre te has preocupado por mi y espero yo también estar siempre para ti.

**Carlos:** Muchas gracias por las interminables tertulias sobre arte, ciencia, cultura. Ha sido un placer compartir tantas cosas contigo!! Muchísimas gracias por las discusiones y el brainstorming para mi último experimento.

**Lelde:** Thank you for being a great friend and for all the help with the goethite preparation.

**Michiel:** Thank you for helping me with the fungal cultures and for setting up the protocols and lab conditions so the fungal culture experiments run smoothly. Thanks for all the discussions about my fungal culture experiment.

**Shakil:** Thanks for being a good friend and for the nice talks about music.

**Tian:** Thanks for being a good office mate when we shared office at the beginning and at the end of my PhD.

**Firoz:** Thanks for your help with the Ferrozyne essay.

**Harald W:** Thank you very much for all your help in the greenhouse.

**Johannes:** Thanks for all the help with the ergosterol measurements and for always being kind and having a cheerful attitude.

**Per B:** Thank you for the help with the $^{15}$N measurements.

**Adam:** Thank you for your help in my first field trips and for some nice meshbag pictures.
Inga B: Thank you for your help with the phosphatase analysis.

Fabio, Giulia, Elena, Sonia: It was very fun to work with you and to help you with your project in the beautiful Aosta Valley in the Italian Alps!!

People from Microbial ecology and MEMEG: Thanks for making the job environment in the Ecology building so much kind and fun!!

Lotta P: I would not be here if it wasn’t for your help! Thank you so much for helping me to come here to Sweden and being part of the fantastic ecology department!

Other friends in the biology department: Mariana, Elsie, Pallavi, Oscar, Alex, Julian, Philip, Hamid, Elsa, Weerachon, John, Utku, Leidys, Vinardas, Kristaps, Martin, Mild, Xi, Albert, Aravin, Andrea A, Yihan, Samantha, Jerker, Beatriz, Jesús, Miguel, Jacob, Anna N, Anna D, Qinyang, Kristin A. Thanks for making these last four years so fun!!

Familia: Muchas gracias por el apoyo incondicional y por siempre creer en mi. Aunque estemos lejos siempre los llevo en mi corazón.

Micaela: Este trabajo no habría podido ser finalizado sin tu ayuda, tanto personal como profesional. Me has dado tanto apoyo durante los momentos difíciles, me has hecho creer en mi mismo y enfrentar las adversidades con valentía. Siempre encuentras la manera de animarme y alegrarme la vida y siempre tienes razón cuando dices: “todo va a estar bien”. Tu dulzura, humildad, inteligencia e integridad siempre me motivan a seguir adelante y ser una mejor persona. Eres una mujer increíble y soy inmensamente afortunado de que estemos juntos. Tú le das color a mi vida! Espero estar a tu lado por muchos más años y compartir juntos cada aventura, disfrutando siempre de las alegrías por venir!
Biomass, community structure and phosphorus uptake of ectomycorrhizal fungi in response to phosphorus limitation and nitrogen deposition

JUAN PABLO ALMEIDA
DEPARTMENT OF BIOLOGY | LUND UNIVERSITY

List of papers


