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Adam Bahr

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Faculty opponent:
Prof. Thom W. Kuyper
Wageningen University
Department of Soil Quality
Netherlands

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Department of Biology
Microbial Ecology
Ecology Building
SE - 223 62 LUND

Author(s)
Adam Bahr

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Abstract

Almost all northern forest tree species live in symbioses with ectomycorrhizal fungi (EMF). The trees allocate up to half of the photoassimilated C through the roots to EMF. In return EMF deliver nutrients from both inorganic and organic sources in the soil, as well as water. Most of the nutrients trees need are assimilated through EMF, which thus play a potentially important role in retaining nitrogen (N) in the soil. However, it is still not clear to what extent they contribute to the total N retention capacity of northern forest soils. This thesis shows that recently produced (within 4 months) mycelium of EMF was capable to assimilate a substantial amount of inorganic N (6 kg-1 ha-1 month-1). If the total standing biomass of EMF mycelium where to be taken in to account, the total EMF N assimilation would likely be much larger. On the other hand, an abundant network of EMF mycelium was not sufficient to prevent a flush of N leaching that followed immediately after N fertilization, but it may be sufficient to retain more moderate additions of N. Leaching of N has been reported in N saturated northern forests, and further research is needed to reveal the role of N retention by EMF in relation to N assimilation by other microorganisms or directly by the tree roots, under various levels of N input in forest soil.

Northern forest soils have been reported as a major terrestrial carbon (C) pool. This is typically explained by slow decomposition due to low temperatures, and recalcitrant litter from the coniferous trees that dominate these forests. A less discussed source to the C pool is the one originating from roots and root associated microorganisms. Since about half of the photoassimilated C is allocated belowground in coniferous forests, this is a potentially important process affecting the C sequestration. Previous studies have highlighted the potential of EMF as an important contributor to C sequestration. This is based on observations of substantial production of EMF mycelium, and that EMF tissue contains recalcitrant compounds. One of the conclusions in this thesis was that most of the soil organic C (SOC) in late successional stages originates from belowground inputs from roots and root associated fungi. This contrasts the focus on aboveground plant production in other studies where changes in forest C storage have been examined. Other results emphasize the important role of EMF in the build-up of soil organic matter: up to more than half a ton of EMF mycelium was produced during only four months, and the average C sequestration by EMF mycelium in a large scale field survey was calculated to more than 300 kg C ha-1 y-1.

Carbon sequestered by EMF may however be sensitive to additions of N, which typically repress EMF growth. There have been many observations of reduced EMF mycelium production after large additions of inorganic N (typically 100 kg-1 ha-1 y-1), but we show that the moderate (less than 20 kg-1 ha-1 y-1) and continuous addition from elevated N deposition in many northern forest regions is sufficient for the repressive effect to occur. Consequently, C sequestration by EMF may be reduced by N deposition. This opposes the positive effect that N addition has on C sequestration due to other processes that are based on aboveground C inputs and degradation of soil organic matter. Even though previous research indisputably show that large N additions increase the C sequestration in forest soils, the effect is lacking at moderate N inputs. Thus, an important question that still remains to be answered is how low levels of anthropogenic N deposition affect the C cycle in northern forest regions. Further, the belowground contribution to SOC is often neglected, but necessary to include for accurate modeling of the C cycle in northern forests, particularly given the evidence that belowground inputs can make a larger contribution to SOC than aboveground inputs.

Key words:
Ectomycorrhizal fungi, Carbon sequestration, Nitrogen deposition, Nitrogen leakage, Field survey, Boreo-nemoral forest, Boreal Forest, Ergosterol, Free ergosterol, Mesh-bags, Multivariate analysis

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Department of Biology
Microbial Ecology Group
Faculty of Science
Lund University
Ecology Building
SE - 223 62 Lund, Sweden

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Paper II Bahr, A., Ellström, M., Akselsson, C., Ekblad, A., Mikusinska, A., Wallander, H., 2013. Growth of ectomycorrhizal fungal mycelium along a Norway spruce forest nitrogen deposition gradient and its effect on nitrogen leakage. Soil Biology and Biochemistry 59, 38-48.

Paper III Sterkenburg, E., Bahr, A., Brandström-Durling, M., Clemmensen, K.E., Lindahl, B.D., Shift in composition and functioning of the fungal community along a natural boreal forest nutrient gradient (Manuscript)

Paper IV Clemmensen, K.E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., Stenlid, J., Finlay, R.D., Wardle, D.A., Lindahl, B.D., 2013. Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science 339, 1615-1618.

Paper V Bahr, A., Ellström, M., Bergh, J., Wallander, H., Evaluation of soil N retention capacity by ectomycorrhizal fungi in a Norway spruce forest fertilized with nitrogen and phosphorus (Manuscript)

List of contribution

Paper I Contributed to the discussion and writing of the paper, regarding ergosterol methods.

Paper II Main responsibility for the planning of the study. Shared responsibility in setting up the design and performing the fieldwork. Main responsibility for analysis and writing of the paper.

Paper III Responsible for analysis and interpretation of ergosterol. Contributed to statistics, discussion and writing of the paper.

Paper IV Contributed to fieldwork and shared responsibility regarding sample preparation. Main responsibility for analysis and interpretation of ergosterol. Contributed to the discussion and writing of the paper.

Paper V Main responsibility for the planning of the study and setting up the design. Shared responsibility in performing the fieldwork. Main responsibility for analysis and writing of the paper.

ABSTRACT

Almost all northern forest tree species live in symbioses with ectomycorrhizal fungi (EMF). The trees allocate up to half of the photoassimilated carbon (C) through the roots to EMF. In return EMF deliver nutrients from both inorganic and organic sources in the soil, as well as water. Most of the nutrients trees need are assimilated through EMF, which thus play a potentially important role in retaining nitrogen (N) in the soil. However, it is still not clear to what extent they contribute to the total N retention capacity of northern forest soils. This thesis shows that recently produced (within 4 months) mycelium of EMF was capable to assimilate a substantial amount of inorganic N (6 kg^{-1} ha^{-1} $month^{-1}$). If the total standing biomass of EMF mycelium where to be taken in to account, the total EMF N assimilation would likely be much larger. On the other hand, an abundant network of EMF mycelium was not sufficient to prevent a flush of N leaching that followed immediately after N fertilization, but it may be sufficient to retain more moderate additions of N. Leaching of N has been reported in N saturated northern forests, and further research is needed to reveal the role of N retention by EMF in relation to N assimilation by other microorganisms or directly by the tree roots, under various levels of N input in forest soil.

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BACKGROUND

Carbon cycle

Global warming is a widely accepted phenomena, and it has been ascribed to increased anthropogenic greenhouse gas (GHG) emissions (Bernstein et al., 2008). The most important GHG is carbon dioxide (CO_2), which has increased from a preindustrial concentration of 280 ppm to 389 ppm in 2010 (Tarasova et al., 2012). Even though the main cause of the atmospheric increase in CO_2 is combustion of fossil fuel, inappropriate management of boreal forests may result in a considerable elevation of these values, due to degradation and respiration of soil C. Boreal forests cover about 10 % of the land (Bonan and Shugart, 1989; Watson and Change, 2000) and together they constitute one of the largest terrestrial C pools, predominantly due to the large soil C stock (Fig. 1). Actually, the C stored in boreal forest soils has been estimated to be at level with the combined C storage in tropical forest vegetation and soils, even though the latter biome covers a larger area (Watson and Change, 2000). These estimates are based on C content in the upper one meter of the soil (Dixon et al., 1994), and it should be noted that recent findings show that much C is bound to minerals in deep soils. However, the very deep C stocks are more stable (Fang and Moncrieff, 2005) and less sensitive to changes in climate or management than the upper part (Brady and Weil, 2008).

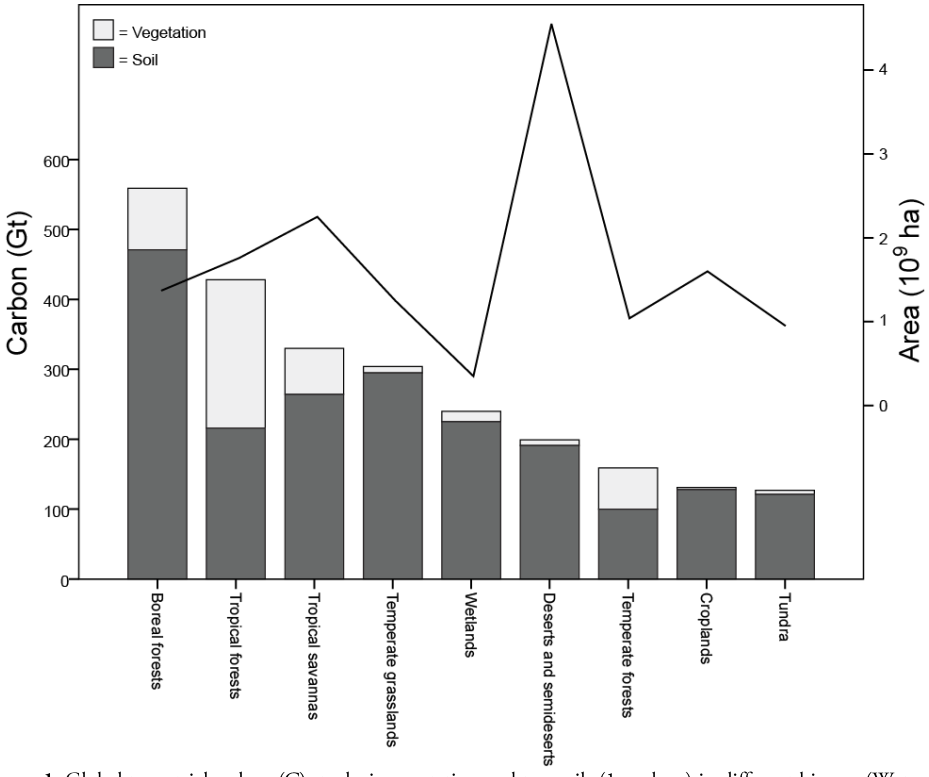

Figure 1: Global terrestrial carbon (C) stocks in vegetation and top-soils (1 m deep) in different biomes (Watson and Change, 2000). The line illustrates the total area covered by the different biomes. Due to ambiguity in the definition of biomes there is considerable uncertainty in the data, but it provides an overview of the distribution of the terrestrial C stocks and highlights the magnitude of C stored in soils.

It is well known that slow decomposition due to low temperatures, low pH and recalcitrant litter from the coniferous trees that dominate the boreal forests are important factors contributing to the build-up of soil C in the boreal region (rev. by Deluca and Boisvenue, 2012). However, a less understood source of the soil C pool is the autotrophic belowground input from roots and root associated microorganisms (Hyvönen et al., 2007). About half of the photoassimilated C is allocated belowground in coniferous forests, but the proportion is highly dependent on nutrient status and relatively less C is allocated belowground during sufficient nutrient supply (rev. by Litton et al., 2007). Thus, it is important to put more emphasis on trying to understand the contribution to the soil C pool by belowground allocated C.

Nitrogen cycle

There has been a growing concern about the environmental effects of anthropogenic alteration of the nitrogen (N) cycle (Gimeno et al., 2001; Hyvönen et al., 2007; Reay et al., 2008; Jacobson, 2009; Rockström et al., 2009), which has been identified, together with climate change and biodiversity loss, as one of totally nine planetary systems where the boundary for a sustainable use has been passed (Rockström et al., 2009). Threats from anthropogenic N emissions include soil acidification, eutrophication and N leakage (Vitousek et al., 1997). Political regulations regarding emissions of air pollutants have resulted in a decrease of deposited N in the end of the 20th century (Bertills et al., 2007). Although, deposition of N can still reach 50-100 kg ha^{-1} y^{-1} in some Central European areas, while remote forests at higher latitudes receive considerably less (rev. by Hyvönen et al., 2007). Elevated N deposition has also been reported from Swedish forests; during the period 2003-2007 N deposition ranged from about 16 kg ha^{-1} yr^{-1} in the south to values close to the natural background deposition of about 2 kg ha^{-1} yr^{-1} in the north (Akselsson et al., 2010). Nitrogen, which is one of the most important nutrients for primary production has a substantial impact on forest growth globally (LeBauer and Treseder, 2008), and is generally the limiting nutrient for productivity in temperate and boreal forests (Vitousek and Howarth, 1991). However, if the N addition rates exceed the boreal forest N retention capacity it may result in N leaching (Aber et al., 1998). Research regarding the N retention capacity of boreal forests is not only important to prevent N leaching and improving the efficiency of possible fertilization in forest management, but also to better understand the C cycle which is highly connected to the N cycle. Since boreal forest trees assimilate most of the N through symbiotic microorganisms (Smith and Read, 2008), it is important to focus on their function to better understand the N retention mechanisms.

ECTOMYCORRHIZAL FUNGI

Anatomy

Almost all boreal and northern temperate forest tree species live in symbiosis with ectomycorrhizal fungi (EMF) (Smith and Read, 2008). The mycelium of EMF attaches to the fine roots of the trees and encases them in a structure called a mantle (Fig. 2). Within the mantle, fungal hyphae penetrate the root cortex and grow as a network, called the hartig net, between the cortical cells. From the outer mantle, the ectomycorrhizal extramatrical mycelium (EMM) extends into the soil, where it assimilates nutrients from both inorganic and organic sources as well as water. Apart from the sporocarps (the mushrooms),

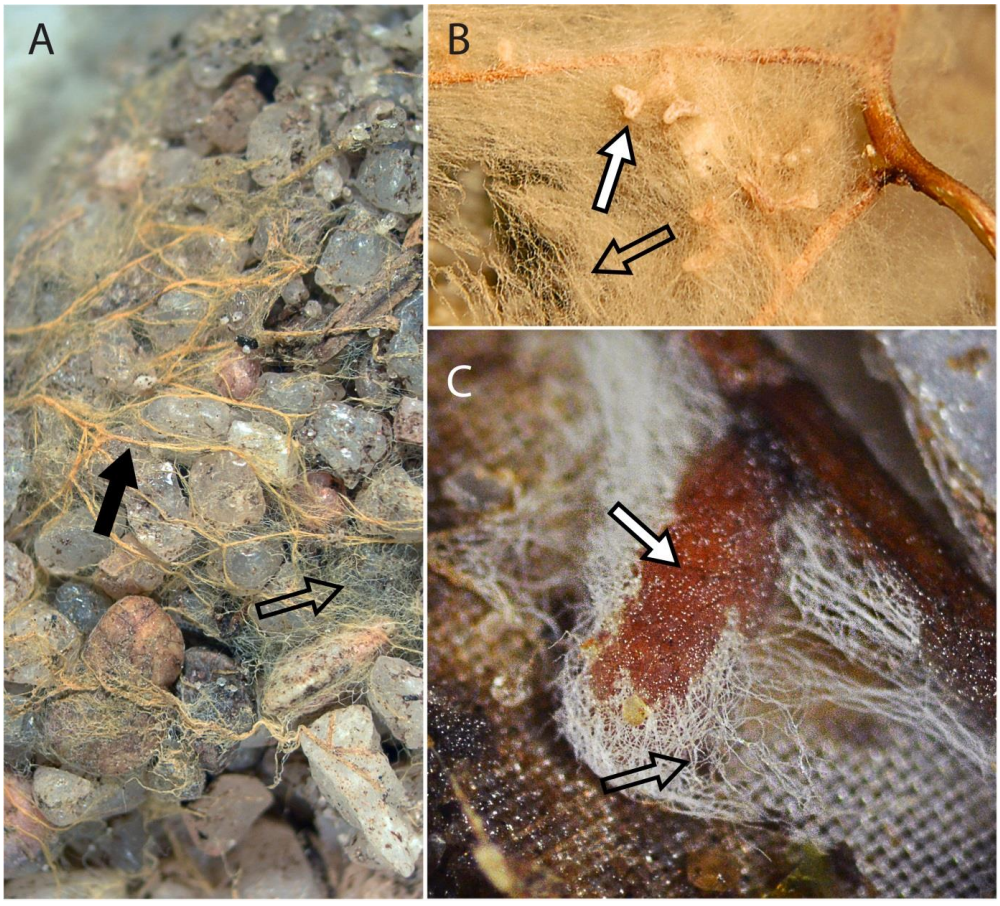

Figure 2: Mycelium of ectomycorrhizal fungi colonize fine tree roots and encapsulates them in a structure called the mantle (white arrows). From the mantle the extramatrical mycelium of the fungi extends into the soil with fine hyphae (transparent arrows) and rhizomorphs (black arrow). Picture C shows a colonized fine root on the outside of a mesh-bags, the mesh in the background has a pore size of 50 µm and prevents in-growth of tree roots while the fine hyphae of the fungi can penetrate into the bag. Picture A shows the interior of a harvested bag with abundant mycelium aggregating the sand.

EMF consists of the EMM, mantles and hartig nets. It has been estimated that within one single gram of humus soil hundreds of meters of EMM can be found (Söderstrom, 1992), and that the absorptive surface area of the tree roots can be increased by two orders of magnitude by the symbiosis (Smith and Read, 2008). The formation of EMM varies between different species, and Agerer (2001) divided EMF into the following exploration types depending on the structure of the EMM: contact, short-distance, medium-distance and long-distance. The contact exploration type have a very limited growth of EMM and is hypothetically less C demanding than the distance exploration types which have thick bundles of hyphae (rhizomorphs) reaching far out in the soil (Fig.3) (Hobbie and Agerer, 2010). The difference in biomass and distance, of the EMM connected to one mantle, between different exploration types can be 15-fold (Weigt et al., 2012).

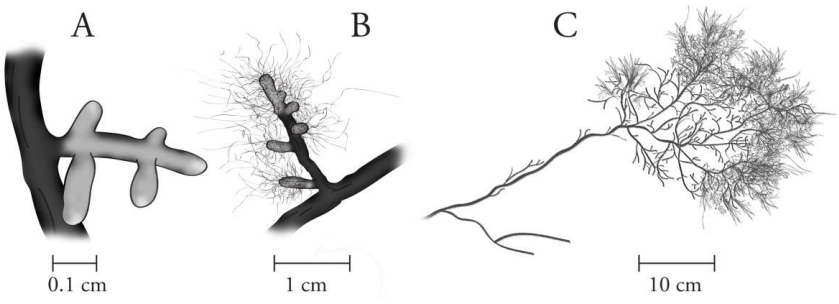

Figure 3: Agerer (2001) divided ectomycorrhizal fungi (EMF) into the different exploration types depending on the structure of the extramatrical mycelium (EMM). Contact exploration types (A) mainly form mantles and have almost no EMM. Short distance exploration types (B) have fine hyphae extending into the close surrounding area of the mantles. In contrast to the previous types long distance exploration types (C) have rhizomorphs (thick aggregated bundles of hyphae, likely more involved in transport than in uptake and only produced by some EMF species) stretching far into the soil. From the rhizomorphs, fine hyphae typically forms fan like structures.

Nutrient assimilation

The EMF are dependent on photo-assimilated C allocated belowground by the trees (Smith and Read, 2008). In exchange, they provide the trees with nutrients and water that are efficiently assimilated from the soil solution by extensive hyphal networks of EMM (Smith and Read, 2008). The relationship may however be more or less mutual (Karst et al., 2008), since the assimilated N may be retained in the EMF mycelium (Näsholm et al., 2013) and invested in the production of EMF biomass rather than allocated to the host plant. EMF can directly assimilate inorganic nutrients and simple forms of organic nutrients (such as amino acids), and also release extra-cellular enzymes that facilitate the release and uptake of nutrients bound up in macromolecules (such as proteins) or organic compounds (such as plant cell walls) (Smith and Read, 2008). Typically, more than 90 % of the fine tree roots are ectomycorrhizal (Taylor et al., 2000) and thus, most nutrients that are assimilated by the trees go through EMF. The trees allocate 10-50 % of the photoassimilated C (Simard et al., 2002) to the EMF. It has several times been shown that EMF affects both the C cycle and N cycle of boreal and temperate forests (rev. by Smith and Read, 2008; Ekblad et al., 2013), but still there is a lack of knowledge regarding the extent to which they contribute to the globally important C sequestration of boreal forests as well as the N retention.

Carbon sequestration

Respiration by EMF is generally considered autotrophic since it is driven by photosynthetically assimilated C, and EMF are dependent on the symbiosis with a plant to acquire C (rev. by Ekblad et al., 2013). Heterotrophic C assimilation by EMF through degradation of soil organic matter (SOM) has been found in laboratory experiments (Read and Perez-Moreno, 2003), but typically only at one order of magnitude less than saprotrophic fungi. At least some species of EMF have the ability to modify and perhaps solubilize SOC (Rineau et al., 2012) by exudation of extra-cellular enzymes with the likely purpose of scavenging for other nutrients (Rineau et al., 2013). Autotrophic soil respiration may in contrast to heterotrophic respiration reflect

allocation of C to the soil, since it constitutes a portion of recent photoassimilated C allocated belowground.

Carbon allocation to EMF is regulated by environmental parameters affecting the production and the root:shoot ratio of the trees, i.e. there have been many reports of repressed EMM production after addition of inorganic N to the soil (e.g. Arnebrant, 1994; Nilsson and Wallander, 2003; Kjøller et al., 2012). Up to 50 % of the photoassimilated C is allocated through the roots to EMF (Simard et al., 2002). Even though 16-71 % of that C is directly respired by EMF (rev. by Leake et al., 2004), a large part is incorporated into fungal biomass (Bidartondo et al., 2001), of which about 10 % consists of the cell wall component chitin (Ekblad and Näsholm, 1996). Chitin is generally considered recalcitrant to soil degradation processes (rev. by Ekblad et al., 2013) and thus have the potential to accumulate in the soil. This was however questioned in a recent study (Fernandez and Koide, 2011), indicating that other components and factors such as low N content (Koide and Malcolm, 2009), hydrophobicity and rhizomorph abundance rather influence the recalcitrance of EMF residues (Ekblad et al., 2013). Still, further research is needed to understand the properties affecting the turnover of EMF.

There have been many reports pointing out that N addition leads to enhanced C sequestration in boreal forests, due to enhanced primary production, increased litter fall and reduced degradation of SOM by heterotrophic microorganisms (rev. by Janssens et al., 2010). There are however still uncertainties about a possible N induced C sequestration, especially in regard to the contribution from belowground inputs of C from roots and root associated microorganisms. The belowground allocation of C links activity in the forest canopy to the activity in the soil, and provides a flow of organic C from shoots to soil via fine roots and mycorrhizal hyphae. The pathways by which this organic C can contribute to soil organic carbon (SOC) are complex, involving biomass turnover (Godbold et al., 2003), biomass grazing (Setälä et al., 1999) and turnover of low molecular weight exudates from roots and fungal hyphae (van Hees et al., 2005). The determination of the pools and fluxes of biomass inputs from fine roots and mycorrhizal fungi provides a major scientific challenge. Some studies (e.g. Wallander et al., 2004) suggest that biomass pools and inputs from fine roots and mycorrhizal hyphae are in the same order of magnitude. It is crucial to assess the role of EMF in the buildup of SOC in boreal and temperate forest soils, and to quantify their relative contribution to SOC in relation to litter and plant roots. If EMF is a main player in the C sequestration of northern forest soils, a different approach in forest management may be needed to maintain the large C pool. However, estimates of fungal inputs rely on methods and conversion factors with large uncertainty that needs to be considered, and precise measurements of standing biomass, production and turnover of EMM are essential in order to accurately describe the C cycle of terrestrial ecosystems.

Effect of nitrogen

Several studies have shown that the net primary production of boreal forests is enhanced by N deposition or N fertilization (Bergh et al., 2008; Brockley, 2010; Jacobson and Pettersson, 2010), while added N has been observed to have a negative influence on EMF biomass, growth and colonization in pot/microcosm studies (e.g.

Beckjord et al., 1985; Wallander and Nylund, 1992; Arnebrant, 1994; Runion et al., 1997) as well as in field studies (e.g. Arnebrant and Söderström, 1992; Nilsson and Wallander, 2003; Nilsson et al., 2007; Högberg et al., 2011; Kjøller et al., 2012). Addition of N in N limited forests typically results in a reduced root:shoot ratio (Ericsson, 1995), since the plant invest more C in above ground production, while relatively less is allocated belowground to facilitate the N assimilation by roots and EMF.

Reduced belowground allocation of C at high N availability may result in a shift of the EMF community towards less C demanding exploration types (Fig. 3). In support for this, Nilsson and Wallander (2003) found reduced growth of EMM in N fertilised plots where the amount of fungal biomass on roots were unchanged, and Kjøller et al. (2012) found that relative abundance of contact exploration types increased with N deposition while long distance exploration types decreased.

Most of the field experiments on the effect of N addition on EMM production have been designed to examine the consequences of fertilization, with additions of about 50-100 kg ha^{-1} y^{-1} (e.g. Nilsson and Wallander, 2003; Wallander et al., 2011). Even though deposition of N can reach such high levels, remote forests at higher latitudes receive considerably less (reviewed by Hyvönen et al., 2007). Moderate N deposition (0.27 – 2.44 kg ha^{-1} 60 d^{-1}) has been found to affect the composition of the EMF community (Lilleskov et al., 2002), but there is still a lack of studies on the effects of continuous low dose addition of N on EMM production.

Nitrogen retention

Even though N is generally considered well retained in the N poor boreal and boreo-nemoral forest soils (Gundersen and Rasmussen, 1995), leaching of N may occur if the N retention capacity is exceeded due to extensive N input (Aber et al., 1998; Gundersen et al., 2006; Akselsson et al., 2010). Elevated N leaching has been recorded in boreo-nemoral forests as a result of N deposition (Nilsson et al., 2007; Akselsson et al., 2010), N fertilization (Berden et al., 1998) and clear-cutting (Akselsson et al., 2004).

Since most of the plant nutrients are assimilated through EMF (Smith and Read, 2008), it is likely that high abundance of EMM increase the N retention capacity of the soil. The coincidence of elevated N leaching and impaired EMM production found after N deposition or fertilization has led to suggestions that EMF growth prevents N losses (Nilsson et al., 2007; Nilsson et al., 2012). However, the capacity of EMF to reduce N leaching still remains uncertain since the effect of impaired EMM production, typically seen after N addition (Arnebrant and Söderström, 1992; Nylund and Wallander, 1992; Nilsson and Wallander, 2003; Nilsson et al., 2007; Högberg et al., 2011; Kjøller et al., 2012; Nilsson et al., 2012), has not been separated from the direct effect from an increased N pool in the soil as well as other environmental variables.

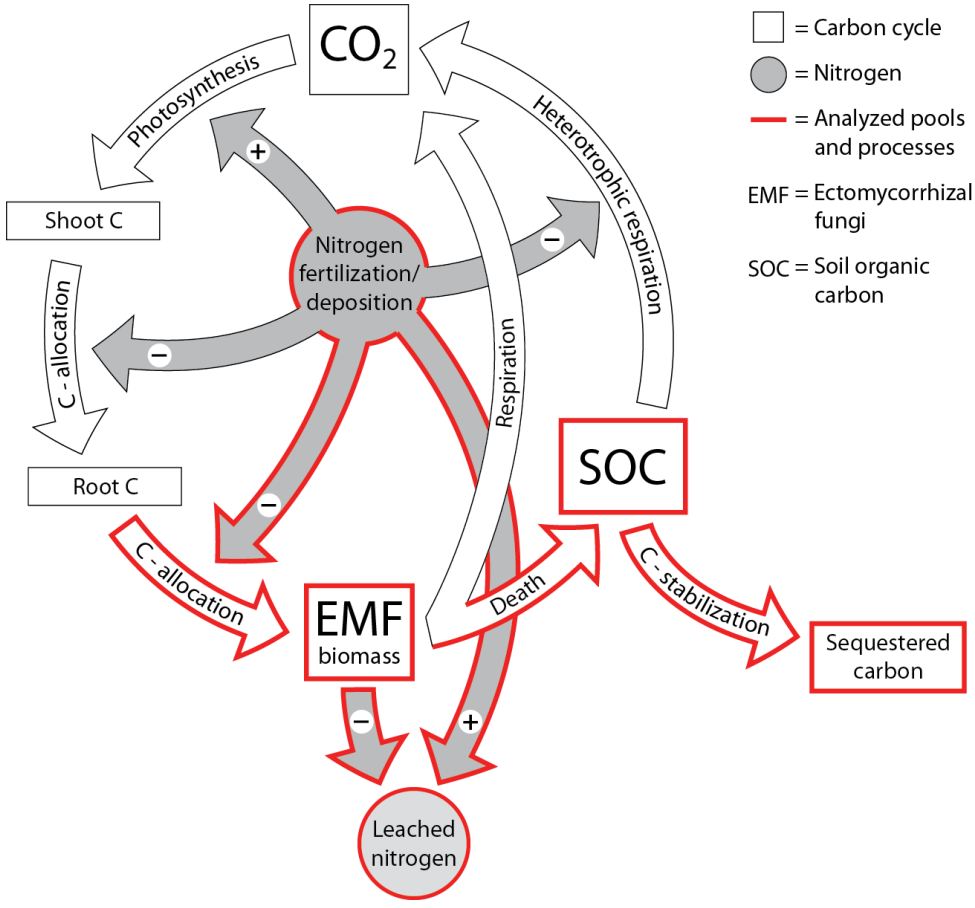

Figure 4: An overview of the main processes (arrows) and pools (boxes and circles) of carbon (C) and nitrogen (N) that affects or are affected by the production, standing biomass and turnover of ectomycorrhizal fungal (EMF) mycelium. Addition of N have several times been shown to increase the primary production of boreal forests resulting in increased photosynthesis and reduced belowground allocation of C to roots and EMF (grey arrows on the left side). However, N addition to severely N limited forests may result in a larger total amount of C being allocated belowground even though the relative allocation belowground decrease, due to an extensive increase in primary production. Nitrogen deposition has the potential to increase N leaching through two processes: (1) directly by enhancing the pool of inorganic N in the soil and indirectly (2) by repressing the EMF production and thus the assimilation capacity in the soil (grey arrows in the bottom). Elevated levels of N have been shown to slow down the heterotrophic degradation of complex molecules in the soil organic carbon (SOC) (grey arrow in the upper right) but, on the other hand, it may also reduce the production, and thus the necromass, of EMF mycelium and shift the EMF community to exploration types with different recalcitrance. Analyzed processes and pools in the current thesis are highlighted with red boarders.

AIMS

The present thesis focuses on the role that EMF plays in the C and N cycles in northern forests (Fig. 4). My aim has been to analyze how EMF growth is affected by additions of inorganic N, and if EMF contribute significantly to the N retention and C sequestration in forest ecosystems.

Briefly, in my studies I expected to find that: (1) EMM is an important contributor to the accumulation of SOC in northern forest soils. (2) Nutrient assimilation by EMM is an important process for the N retention capacity of northern forest soils. (3) A continuous low dose addition of N through deposition is sufficient to repress the production of EMM.

METHODS

This is a brief overview and discussion of the main methods I have worked with during my PhD; a thorough review of methods for analyzing EMF is given in **Paper I**. I have studied different processes in field experiments and surveys to analyze the applicability and ecological importance of previous findings and hypotheses when other environmental variables were taken into account. Performing controlled laboratory experiments is necessary to find and explain underlying mechanisms, while the inclusion of the natural variation in the field enables a more realistic testing of ecological theories (rev. by Underwood, 2009). However, the large variation in environmental variables, due to lack of control and regulation, in field studies makes it necessary to put particular attention to the experimental design.

Field sites

The projects were restricted to Swedish boreal and boreo-nemoral forests (Fig. 5). Given the large south to north geographical distribution, Sweden covers a large portion of the climate, fertility and anthropogenic deposition found in boreal and boreo-nemoral forests globally.

The Swedish Throughfall Monitoring Network (SWETHRO) - Production of EMM in managed Norway spruce forests along a gradient of anthropogenic N deposition was analyzed in a large scale experiment, stretching over more than 600 km, in the south part of Sweden (**Paper II**).

Central Sweden pH gradient - The effects of soil fertility and pH on EMM production and community were analyzed in unmanaged Norway spruce and Scotch pine forests, only exposed to natural background deposition of N, in central Sweden (**Paper III**).

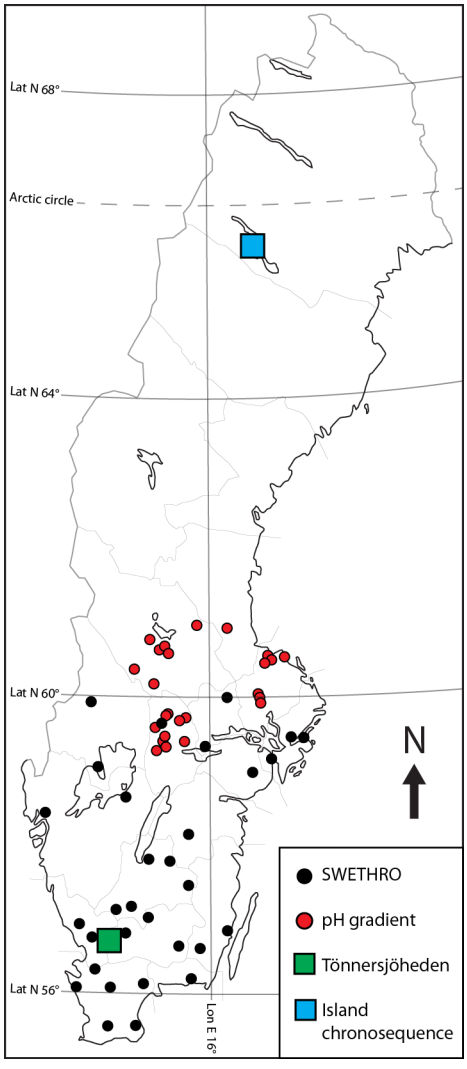

Figure 5: A map of Sweden with location of the field sites of the different experiments in this thesis.

Northern Sweden island chronosequence - The role of roots and root associated microorganisms in the buildup of a humus layer was studied in forested islands, largely unaffected by human activities, in North Sweden. The forests constituted a successional chronosequence, ranging from a few hundred years to several thousand years (**Paper IV**).

Tönnersjöheden fertilization project - To verify the patterns found in the SWETHRO survey (Paper II) of EMM N retention and a repressive effect from both N and P on EMM production, we manipulated the nutrient regimes of a managed Norway spruce forest in South Sweden. Apart from EMM production, we analyzed N leakage as well as N assimilation by EMM (**Paper V**).

Production of ectomycorrhizal fungi

In general, it is problematic to analyze soil microbial processes without exposing the natural soil environment to a major disturbance. This becomes even further complicated when analyzing the activity of EMF mycelium, since they are dependent on the symbiosis with trees (i.e. if a soil sample is taken the EMF growth cease due to broken connections to the trees). Further, a key problem in the determination of mycorrhizal hyphal production is a lack of methods to distinguish growth of mycorrhizal hyphae from that of saprotrophic fungi. As EMF fungi do not form a monophyletic clade (Hibbett et al., 2000; Tedersoo et al., 2010) no single biochemical or DNA based marker can be found to quantify this group in the complex soil environment. PCR and qPCR can be used to identify known EMF species from DNA or RNA, but further development of molecular methods are needed to enable a stable quantification of the EMF biomass (**Paper I**).

Mesh-bags

One solution to analyze the production of EMM in the field is to incubate sand filled ingrowth mesh-bags (Fig. 6 and 7) in the soil (Wallander et al., 2001). A fine nylon mesh (pore size: 50 µm) allows for EMM ingrowth while it prevents tree roots from penetrating into the bag (Fig. 2) (Wallander et al., 2001). A lack of organic C in the sand filled mesh-bags hypothetically reduces ingrowth of saprotrophic fungi, while the EMM has an external C source from the trees. The fungal communities that colonize the mesh bags are usually dominated by mycorrhizal hyphae which has been verified in trenching experiments (Wallander et al., 2001) and with DNA-analyses (Kjøller, 2006; Korkama et al., 2007; Parrent and Vilgalys, 2007; Hedh et al., 2008; Wallander et al., 2010). Ingrowth mesh-bags were used to determine the EMM production in **Paper II** and **V**.

In the SWETHRO survey (**Paper II**), triangular mesh-bags (side: 6 cm) were installed in the interface between the mineral soil and the organic horizon, where mycorrhizal growth is usually most active (Lindahl et al., 2007). They were incubated for one year in the soil during two consecutive years.

In the fertilization experiment at Tönnersjöheden (**Paper V**) the mesh-bags were cylindrical (length: 16 cm, diameter: 2 cm) to cover most of the active EMM region. This form of bag simplifies extrapolation of EMM production to a per area basis that covers a specific soil depth. The design also allows the comparison of adjacent soil and/or root samples taken with the same volume. Further, it is suitable for sequential harvests since the mesh bags can be replaced in the same hole, with minimal disturbance. In this project sequential harvesting was done with incubation periods of 4 months during a period of 16 months.

Possible shortcomings of the mesh-bag method are an initial delay before the EMM has the potential to extend into the whole bag and that the sand constitutes an unoccupied space where competition is lacking. The former would lead to an underestimation of EMM production while the latter could cause an overestimation.

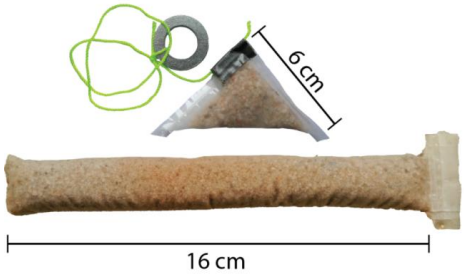

Figure 6: Ingrowth mesh-bags were installed in the soil during different periods to analyze production of ectomycorrhizal mycelium (**Paper II** and **IV**).

Further, there has been criticism about the use of pure quartz sand in the mesh bags since they contain a substrate that in many cases is different from the surrounding soil. It has been shown that mycelial in-growth can be higher when natural soil is used rather than pure sand (Hendricks et al., 2006), indicating that the use of pure sand likely gives an underestimation of the production. This has to be considered in regard to quantification and extrapolation of the fungal biomass and production, but is less important for relative differences between treatments.

Estimating fungal biomass

We used different approaches to quantify the ingrowth of EMM into the mesh-bags. The mycelial content was estimated visually with a stereo microscope (**Paper II and V**) according to a 6 graded scale (similar to Wallander et al., 2001), and the frequency of hyphae present in mesh holes was counted in vertical rows of 100 mesh holes (**Paper V**) (enabling quantitative analysis). Fungal content was then estimated chemically by analysing the fungal biomarker ergosterol (**Paper II and V**). Ergosterol is a cell membrane component specific to fungi that can be used as a quantitative indicator of fungal biomass (Nylund and Wallander, 1992). Frequency of hyphae and visual EMM estimation gave somewhat different results than ergosterol (**Paper II and V**). Even though ergosterol content usually correlate well with other methods for fungal quantification (**Paper I**), inconsistencies have been reported (Högberg, 2006; Wallander et al., 2011), and the methods likely represent different aspects of fungal biomass. The visual method may be better to estimate the proliferation and aggregation of fine hyphae while ergosterol content to a larger extent is sensitive to the abundance of rhizomorphs (thick aggregated bundles of hyphae, likely more involved in transport than in uptake and only produced by some EMF species). It is also possible that ergosterol accumulates in the mesh-bags during the incubation time, while visual estimation gives a snap shot of the current situation. Ergosterol was found to accumulate in soil during late successional stages (**Paper IV**), and it has been suggested to be less prone to degradation following N addition and reduced belowground C allocation (Zhao et al., 2005). Ergosterol content has also been shown to differ between different EMF species (Bermingham et al., 1995), and may thus be affected by differences in the environment (e.g. soil fertility) affecting the EMF community composition (Kjøller et al., 2012). Ergosterol is either "free" (unesterified) or "bound" (esterified), since ergosterol becomes bound in sterol esters during degradation (Yuan et al., 2008). We analyzed both free and total (including both free and bound) ergosterol in an attempt to separate the living fungi from the necromass, since the free part has been suggested to better resemble living fungal mycelium (**Paper I**). For the same reason, total ergosterol and free ergosterol were also analyzed in soil samples (**Paper III and IV**).

The ratio of the stable isotopes ^{15}N and ^{13}C was used to interpret the ectomycorrhizal origin of organic material in mesh-bags and in soils in the forests of the Northern

Sweden chronosequence (**Paper IV**). Plant C allocated belowground is in relation to aboveground tissue enriched in ^{13}C, and this enrichment is further enhanced during C transfer to mycorrhizal fungi (Hobbie and Werner, 2004). Mycorrhizal fungi also have higher $\delta^{15}N$ signatures than their host plants, because they supply N to their hosts that is ^{15}N-depleted relative to that retained in their own mycelium (Högberg, 1997). Thus, analyses of ^{13}C and ^{15}N can give an indication of the contribution of EMF to SOM in northern forests. Although, the results has to be interpreted with caution since other soil processes also discriminate between different isotopes. Saprotrophic fungi are generally further elevated in ^{13}C than EMF (Högberg et al., 1999). However, growth of saprotrophic fungi is typically largest in the litter layer at the surface, while EMF have been shown most active close to the border between the humus and mineral layer (Lindahl, 2007). Changes in the isotopic ratio induced by EMF activity may thus be expected occur in the deeper soil region. In regard to ^{15}N, other discriminating processes such as nitrification and denitrification are very limited in boreal forests (Schulze, 2000), and EMF activity is probably a main contributor to increased ^{15}N content with depth.

In the Tönnersjöheden fertilization project, N enriched in ^{15}N was added to EMM ingrowth mesh bags a few days before harvest. This enabled tracking and detection of ^{15}N assimilated by EMM (Clemmensen et al., 2008), while the amount of labeled N not taken up by the EMM network in the mesh-bags was collected at the bottom of the mesh-bags in ion exchange resins and used as a proxy for N leaching.

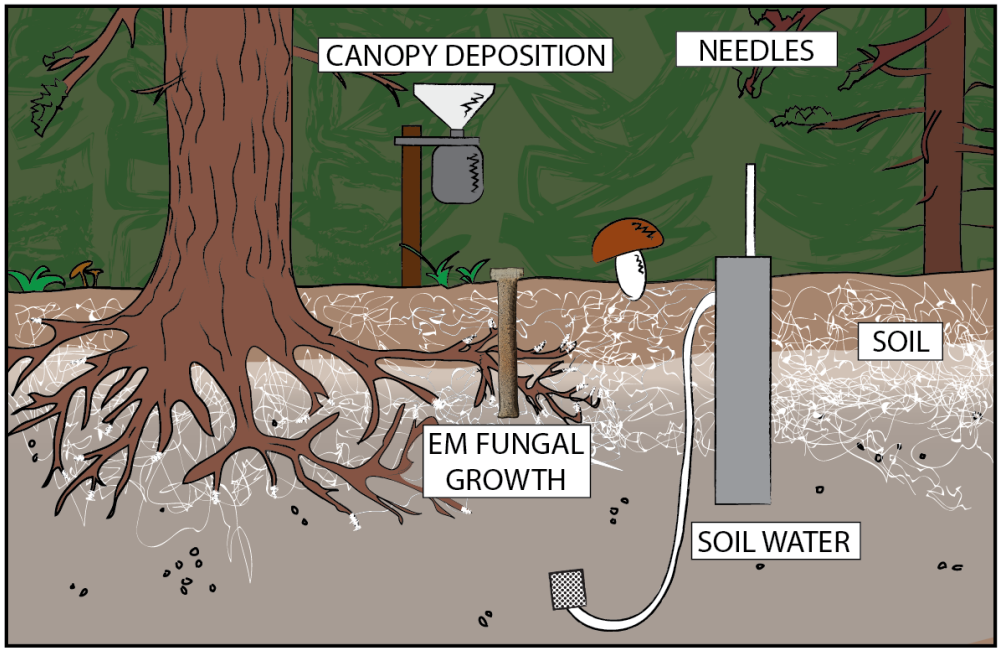

Figure 7: Overview of field sampling in this thesis. Production of ectomycorrhizal (EM) mycelium was analyzed by installing ingrowth mesh-bags in the soil. Soil samples were analyzed for chemistry, isotopic signatures of carbon and nitrogen, and the fungal specific membrane component ergosterol. Needles were analyzed for chemicals to interpret the nutrient status. Soil water was collected in suction lysimeters with ceramic filters and analyzed for pH and chemistry. Canopy deposition was sampled with funnels and analyzed for chemistry.

Soil water sampling

Ceramic cup suction lysimeters were used for soil water sampling (**Paper II and V**). The lysimeters consisted of 2 l cylindrical containers connected with plastic tubing to ceramic filters put at 50 cm depth (Fig. 7). The lysimeters were set to approximately 0.6 bar vacuum pressure and then left for two days (**Paper II**) or one month (**Paper V**). Shorter sampling periods gives a more consistent vacuum pressure in the lysimeters, but are, on the other hand, sensitive to temporary changes in soil water percolation (i.e. after heavy rainfall or during snowmelt). Thus, short sampling intervals spread out during the year may be better for instantaneous quantification of root available nutrients, while continuous long term sampling may be better to detect relative differences between treatments. Sampled soil water was quantified and analyzed for pH and N (**Paper II and V**) as well as other nutrients (**Paper II**).

ENVIRONMENTAL VARIABLES AFFECTING PRODUCTION OF ECTOMYCORRHIZAL FUNGI

Nitrogen

In the fertilization experiment at Tönnersjöheden (**Paper V**) we found repressed EMM production within four months after N fertilization (Fig. 8). Repressed EMM growth after N fertilization has been found in many earlier observations, although these measurements have usually been performed after several years of exposure to elevated levels of inorganic N (e.g. Nilsson and Wallander, 2003; Nilsson et al., 2007; Kjøller et al., 2012). The reduction in EMM growth may be due to a greater demand of photoassimilated C for above ground plant production, resulting in relatively less C being allocated belowground to support nutrient assimilation (Hobbie, 2006). In support of this Högberg et al. (2010) found a reduction in belowground

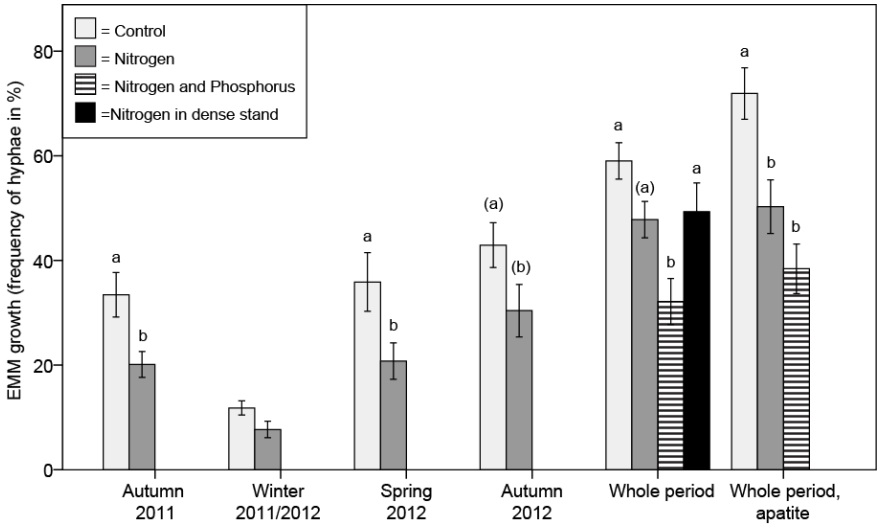

Figure 8: Tönnersjöheden fertilization experiment (**Paper V**). Growth of ectomycorrhizal extramatrical mycelium (EMM), during 4 month incubation periods as well as during the whole period of the study, according to counting of mycelium along the mesh of the ingrowth bags. Shadings and patterns illustrate fertilization regimes. Apatite refers to mesh-bags amended with the phosphorus (P) containing mineral that has been shown to enhance EMM production during P deficiency. Samples from the same period that do not share letters are significantly different (tendencies are shown within parenthesis) and the error bars represent standard error.

C allocation to soil biota of more than half after N fertilization of a pine forest in northern Sweden. In addition, the C that is allocated to EMF will, to a larger extent, be used for energy purposes and as C skeletons for amino acid production during elevated N availability, resulting in less C being available for fungal growth (Wallander, 1995). Reduced production of EMM at elevated N input may also be related to changes in the EMF community. For example, the contact exploration type (Fig. 3), described by Agerer (2001), have a very limited growth of EMM in contrast to distance exploration types (Hobbie and Agerer, 2010). Kjøller et al. (2012) found that relative abundance of contact exploration types increased with N deposition while distance exploration types decreased. Reduced growth of EMM at high N availability may thus be an effect of a shift to less C demanding exploration types.

Even though high N availability often has been observed to reduce the EMM production (e.g. Beckjord et al., 1985; Wallander and Nylund, 1992; Arnebrant, 1994; Runion et al., 1997; Nilsson and Wallander, 2003; Nilsson et al., 2007; Kjøller et al., 2012), these observations are mainly from experimental studies with rather high N addition rates (e.g. Nilsson and Wallander 2003, with an annual addition of 100 kg N ha^{-1} during 13 years). In the SWETHRO survey (**Paper II**) we show during two consecutive years that reduction of EMM production also occurs when moderate levels (< 10 kg N ha^{-1} y^{-1}) of N are added by atmospheric deposition (Fig. 9). By including a comprehensive dataset of environmental variables regarding canopy throughfall, soil water, soil chemistry and needle chemistry in a multivariate analysis (Fig. 10) we concluded that N deposition was the most important abiotic variable controlling EMM production. This suggests that the moderate deposition of N found in many boreal and boreo-nemoral regions, is not only sufficient to repress EMM production but also the main controlling abiotic variable.

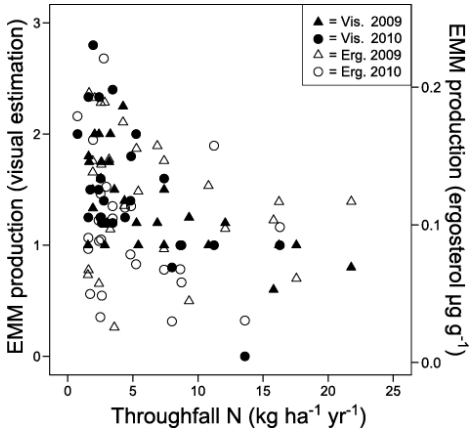

Figure 9: SWETHRO survey (**Paper II**). A negative correlation between nitrogen deposition and ectomycorrhizal extramatrical mycelium growth was found during both 2009 (r_s = -0.58, P = 0.001) and 2010 (r_s = -0.61, P < 0.001) according to visual estimation, but this could not be verified by ergosterol analyses (2009: R^2 = 0.234, P = 0.221; 2010: R^2 = 0.257, P = 0.195).

Phosphorus

Apart from N, elevated allocation to EMF has also been found in response to P deficiency in pot studies (Wallander and Nylund, 1992; Ekblad et al., 1995) and also in the field (Berner, 2013), indicating a strong role of plant P status in controlling C allocation to EMF. The importance of P provision by EMF in controlling plant C allocation to EMF was illustrated by using EMF ingrowth mesh-bags amended with the P containing mineral apatite, resulting in enhanced EMM production in P limited forests (Wallander and Thelin, 2008; Berner et al., 2012). Northern forests are generally considered N limited, and in the SWETHRO survey (**Paper II**) only one of the localities had a needle content of P below

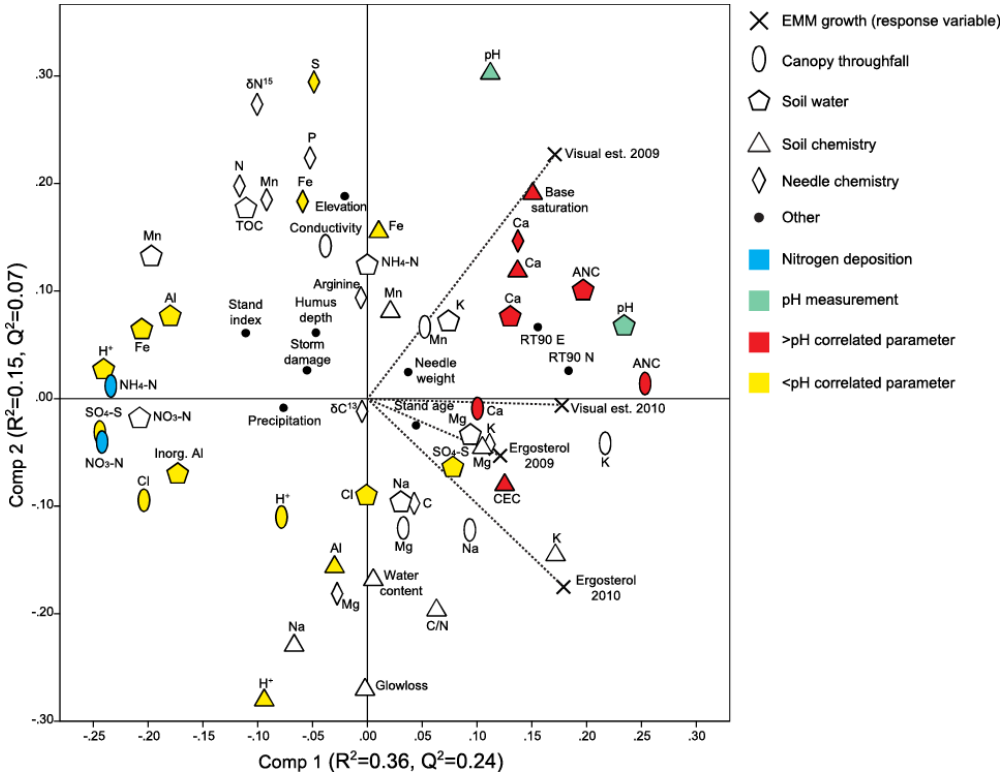

Figure 10: SWETHRO survey (**Paper II**). Loading plot from partial least square (PLS) regression using growth of ectomycorrhizal extramatrical mycelium (EMM) during 2009 and 2010, according to visual and ergosterol-based estimates, as response variables (Y-variables). Four components were significant, but only the first two are illustrated.

the deficiency level of 1.2 mg g^{-1} reported by Thelin et al. (2002). Nevertheless, a negative correlation between needle P and EMM production was found (**Paper II**), showing that P may influence EMM production, even if P is above the reported deficiency level.

Wallander and Nylund (1992) showed in pot studies that EMM production was repressed by excess N treatment when P levels was adequate, however if P was deficient no repression of EMM production was found after N addition. In the fertilization experiment at Tönnersjöheden (**Paper V**) we show that this also occurs under field conditions, since production of EMM was further repressed when N was added in combination with P (Fig. 8). This is probably an effect of sustained belowground C allocation to EMM due to enhanced P limitation in N fertilized plots. Since EMM production at control plots was elevated by apatite amendment and the P levels in the needles were below deficiency levels while N content was above (**Paper V**), it is likely that P was limiting tree growth, perhaps even more than N. Previous experiments in Sweden show that N fertilization generally results in enhanced tree growth (rev.by Nohrstedt, 2001), even though only a moderate or no increase in tree growth was reported after fertilizing forests in the same region as Tönnersjöheden with N (Persson et al., 1995). The decline in EMM growth observed after the combination of N and P addition may

however only occur when the trees are originally P limited and further studies of P addition effects on EMM growth are needed in order to test if a general effect can be expected.

pH

Although most of the variation in production of EMM in the SWETHRO survey (**Paper II**) could be explained by N availability, inclusion of needle nutrient status and pH increased the predictability further (from Q^2 = 0.27 to Q^2 = 0.36). Together, these variables seem to be the most important ones since inclusion of additional variables did not contribute to any further increase of the predictability. Strong positive correlations were found between pH and EMM production in the SWETHRO survey (**Paper II**) as well as in the study of the Central Sweden pH gradient (Fig. 11, **Paper III**).

A low pH may become stressful for fungi. Even though fungal abundance usually increase with low pH (Rousk and Bååth, 2011), Rousk et al. (2011) found reduced saprotrophic fungal growth at pH less than 4.5. Only a few field studies have analysed the effects of a reduction in pH on EMF, but it has been observed that simulated acid rain did not affect formation of mycorrhizas (Meier et al., 1989; Nowotny et al., 1998) or EMF morphotypes (Meier et al., 1989). On the other hand, liming to counteract soil acidification has a strong effect on the composition of the EMF community (Erland and Söderström, 1991; Lehto, 1994; Andersson and Söderström, 1995; Wallander et al., 1997; Bakker et al., 2000), while species richness (Kjøller and Clemmensen, 2009) and diversity (Rineau et al., 2010) seem to be rather unaffected by liming. It is often reported that species with well developed EMM increase in abundance after liming (Bakker et al., 2000; Kjøller and Clemmensen, 2009) and highly competitive ubiquitous species are more common at higher pH (Rineau et al., 2010).

Robust and long-lived plant leaves are usually associated with stressful conditions (Wright et al., 2004). Correspondingly, in fungi, long-lived mycelial structures may enable more economic resource utilization. To retain mycelium for a long time, it needs to be protected against fungivores and harsh abiotic conditions, e.g. by impregnation of cell walls with melanin and hydrophobic compounds (rev. by Ekblad et al., 2013). Fungi associated with stressful environments are often ascomycetes having traits considered adaptations to harsh conditions such as drought and acidity (Gostinčar et al., 2010). Lehto et al. (1994) found that the reduction of contact exploration type EMF was mainly due to increased pH and ionic strength. Thus the positive relation between pH and EMM production in the SWETHRO survey (**Paper II**) and the study of the Central Sweden pH gradient (**Paper III**) could be related to the composition of the EMF community.

A positive correlation between pH and EMM may, however, not imply a causal effect. Increasing the pH in acidic forest soils has been found to promote microbial activity (Bååth and Arnebrant, 1994) and, in general, to elevate nutrient availability and N mineralization. Nevertheless, the effects of liming on the N cycle in N limited, acidic soils are complex, and reduced N mineralisation after liming has also been reported (reviewed by Formánek and Vranová, 2003). The positive correlation between pH and EMM growth observed in the SWETHRO (**Paper II**) survey may be due to the acidifying effect of N deposition. Nilsson et al (2007) also found lower EMM production in areas with elevated N deposition, which also had the lowest pH

values. The narrow pH gradient and a dispersed pattern of pH-related variables (according to PLS regression, Fig. 10) in the SWETHRO survey, indicates that canopy throughfall of N was the most important variable affecting EMM growth, and probably the cause of the pH trend.

On the other hand, a positive correlation between pH and EMF biomass was also found in the study of the Central Sweden pH gradient (**Paper III**) were the forests only received natural background deposition of N. The underlying cause was most likely increased availability of nutrients at higher pH, since the most important predictor of ergosterol according to multivariate analysis as well as multiple regression was the C:N ratio of humus. We suggest that, without elevated N input through atmospheric deposition or fertilization, N availability rarely becomes sufficient to reduce the belowground allocation of C. Rather, the belowground allocation of photoassimilated C increases with fertility due to alleviation of severe nutrient limitation. Positive responses of EMF to N additions, both in terms of mycelial growth and abundance, have been observed previously in low-productive arctic heathland (Clemmensen et al., 2006). Thus, we postulate that negative effects of high N-availability on mycorrhizal fungi are primarily found at the higher inorganic N-levels associated with fertilizer addition or elevated atmospheric deposition, but that this relationship is not valid within the range of variation commonly found in undisturbed boreal forests not exposed to artificial or anthropogenic N-deposition.

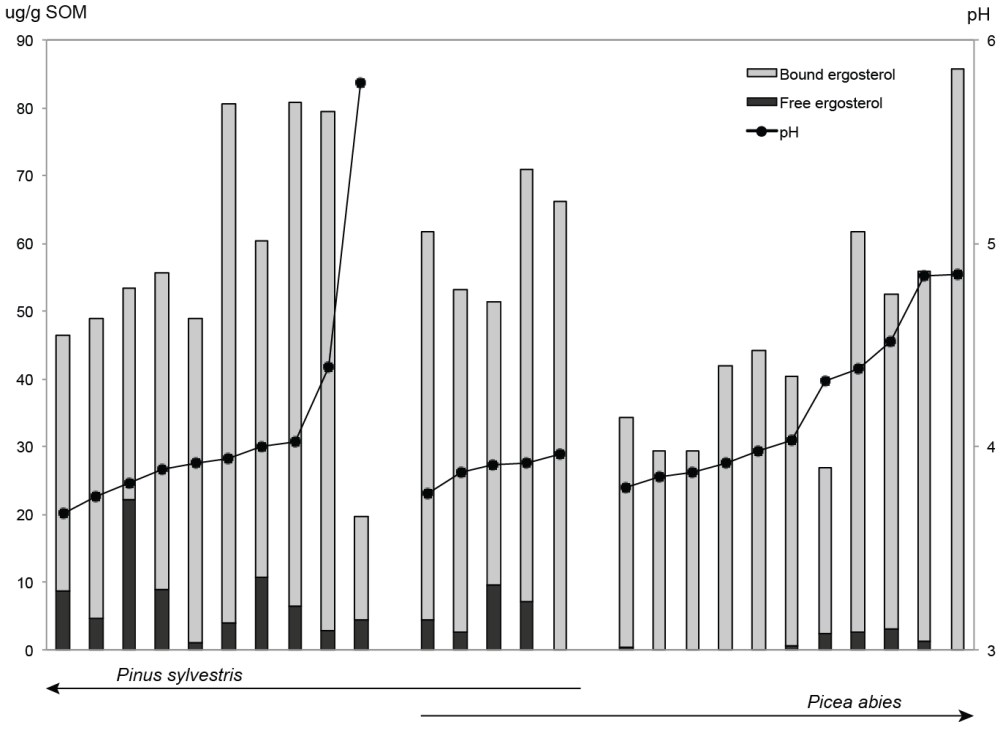

Figure 11: Bound ergosterol levels (grey part of bars) and free ergosterol levels (black part of bars) in the humus. Samples are sorted after dominant tree species of the forests, pine forests in the left part of the graph, spruce forests in the right part and codominance of the two species in the middle. For each tree species, the samples are ordered after humus pH.

NITROGEN RETENTION BY ECTOMYCORRHIZAL FUNGI

It has been hypothesised that EMM activity counteracts leaching of N (Aber et al., 1998; Nilsson et al., 2007; Högberg et al., 2011), and indications of this have been observed in a few studies (e.g. Nilsson et al., 2007). According to the SWETHRO survey (**Paper II**) elevated N losses seemed to occur at relatively low EMM production (Fig. 12). The lack of high N losses at abundant EMM production indicates a counteracting effect of EMM on N leaching. It was however not possible to draw any firm conclusions regarding the capacity of EMM to retain N in the soil, since N leaching increased with N deposition at the same time as N deposition had a negative effect on EMM production. The effects of these mechanisms on N leakage could not be separated with the approach used in the SWETHRO survey.

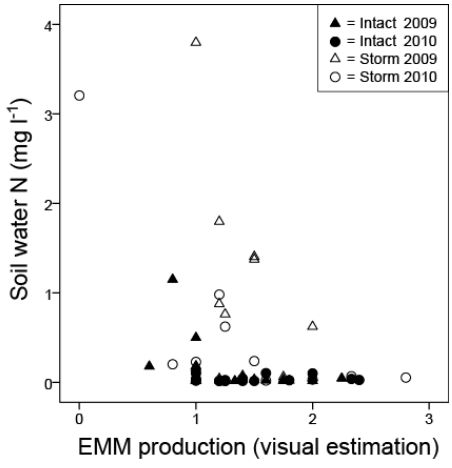

Figure 12: SWETHRO survey (**Paper II**). A tendency was found between nitrogen (N) leaching (soil water N) and visual estimation of ectomycorrhizal extramatrical mycelium (EMM) growth at intact sites (i.e. not storm damaged) during 2009 (r_s = -0.45, P = 0.073) and at storm damaged sites during 2010 (r_s = -0.34, P = 0.060). No correlations were found between N leaching and the estimates of EMM based on ergosterol (data not shown).

In order to determine the capacity of EMM to reduce N losses we applied a more intense temporal sampling strategy for both EMM production (in mesh-bags) and N leaching in the fertilization experiment at Tönnersjöheden (**Paper V**). The temporal division of sampling was made to account for the large seasonal variation in both N leaching (Stevens et al., 1993; Piirainen et al., 1998; Wright et al., 2001) and EMM growth (Wallander et al., 2001). Production of EMM, which has been suggested to increase soil N retention (Aber et al., 1998; Nilsson et al., 2007; Högberg et al., 2011), usually peaks at late summer to early fall, while it almost ceases during the winter (Wallander et al., 2001). In contrast, large temporal N flushes in the spring are typically explained by snow melting (Aber et al., 1998; Piirainen et al., 1998), intense periods of precipitation and low plant assimilation (Wright et al., 2001). In comparison to N treated plots, growth of EMM, as well as N leaching (sampled monthly in lysimeters), tended to be lower when N was combined with P application, which does not suggest that EMM had any crucial potential to reduce the N leaching. It rather seems like an alleviation of an N induced P limitation led to elevated assimilation of the tree roots directly or perhaps through EMF types not colonizing the bags. Similarly, EMM growth declined during winter but no subsequent increase in N leaching was found. However, tracking of the stable N isotope ^{15}N (that was injected into ingrowth mesh-bags two days before harvest) in EMM and ion exchange resin beads at the bottom of the mesh-bags showed that EMM take up more N and reduce leaching to a larger extent in control plots than in N fertilized plots. The ^{15}N assimilation capacity of the EMM in the mesh bags corresponded to 6 kg ha^{-1} month^{-1} during the most active EMF growth period. This is however only by the EMM produced

during the 4 month period of incubation. If a standing EMM biomass of five tons ha^{-1} is estimated (Wallander et al., 2004) an N assimilation rate by EMM of 0.31 mg g^{-1} day^{-1} would result in a monthly assimilation capacity of 46 kg ha^{-1}. Which potentially could be sufficient to retain a large part of the N solubilized from the NH_4NO_3 fertilization pellets, since previous analyses have shown that only 5 – 10 % of fertilizations with about 150 kg ha^{-1} is leached within the first years after fertilization (Nohrstedt, 2001). However, that could not be verified according to the seasonal and treatment variation in EMM production and N leaching in the present study. We show that EMF play an important role in N retention but to explain N leaching, especially after fertilization with large doses of N, many other environmental variables need to be taken into account. These may be direct assimilation by tree roots, climate variables such as precipitation and snowmelt, growth of saprophytic fungi and bacteria and chemical reactions between N and humus.

CARBON SEQUESTRATION BY ECTOMYCORRHIZAL FUNGI

EMF play an important role in the boreal forest C cycle (Smith and Read, 2008) and a reduction in EMM production may have substantial consequences for C sequestration (Godbold et al., 2006). In the fertilization experiment at Tönnersjöheden (**Paper V**), production of EMM at control plots was calculated to 583 kg ha^{-1} during four months in late summer and early autumn, and an average EMF C sequestration in mesh-bags was calculated to 320 kg ha^{-1} y^{-1} in the SWETHRO survey (**Paper II**).

The belowground contribution to SOC buildup was analyzed in northern Sweden forests at different successional stages (**Paper IV**). They were located at islands largely unaffected by anthropogenic activities, where the main disturbance has been forest fires. Due to a larger area, large islands experience lightning ignited fires more often than small islands, resulting in a restart of the succession. The mean age of succession ranged from a few hundred years at the large islands to thousands of years at the small islands (Wardle et al., 1997). In late successional stages the organic soil layer was about 1m deep, while the younger successions had a more typical boreal forest soil profile with an organic layer of about 10 cm. We analyzed organic soil profiles from 30 islands of different sizes. With a parameterized model based on ^{14}C dating (Fig. 13) we analyzed the contribution of aboveground (e.g. litter) and belowground (e.g. mycorrhiza) C inputs to the humus built up during the last 100 years. Root-derived C constituted 70 % of SOC at small islands (late succession) and 47 % at the large islands (early succession). Differences in organic matter accumulation between islands were primarily determined by processes at the interface between the fragmented litter (F) and humus (H) layers, which corresponded to the zone of highest root density. Thus, root-mediated C input to the upper part of the profile represents a major contribution to the long-term buildup of humus, especially in late successional ecosystems.

The increase in root-derived C sequestration as succession proceeds was matched by a shift in the balance between the production and decomposition of fungal mycelium. We measured the fungal-specific cell membrane lipid ergosterol as a marker for fungal biomass throughout each soil profile. Free ergosterol, characteristic of newly formed mycelia (**Paper I**), was about 20 times more abundant on large than on small islands, indicating greater mycelial production. In

contrast, bound ergosterol, the proportion of which increases during mycelial senescence (**Paper I**), was more abundant on smaller islands, indicating older mycelium with slower biomass turnover (Fig. 14). Furthermore, the fungal cell-wall polysaccharide chitin peaked in the F layer and declined in lower horizons of large islands, but remained at high concentrations at greater depths on the small islands (Fig. 14). Chitin persists longer than ergosterol in fungal tissues after death (Ekblad et al., 1998), and the high level of chitin on small islands suggests retarded decomposition of fungal cell wall residues. Thus, in spite of apparently greater mycelial production on the large islands, less mycelial necromass accumulated there than on small islands, suggesting that the large production was counterbalanced by faster decomposition of mycelial remains.

The $\delta^{13}C$ and $\delta^{15}N$ signatures in the uppermost organic layers were similar to those of leaves (Hyodo and Wardle, 2009), whereas signatures in humus layers were closer to those of mycorrhizal mycelium (Fig. 14) and sporocarps (Taylor et al., 2003). The incorporation of isotopically enriched root and fungal remains in the SOM is likely an important mechanism behind the progressive enrichment of heavier stable isotopes with soil depth in this system, indicating that mycorrhizal fungi are a large contributor to the SOC buildup. This is consistent with the observation that isotopic signatures remain relatively constant in the initial litter decomposition phase (Fig. 14) and only increase when root-associated fungi dominate C and N dynamics (**Paper IV**).

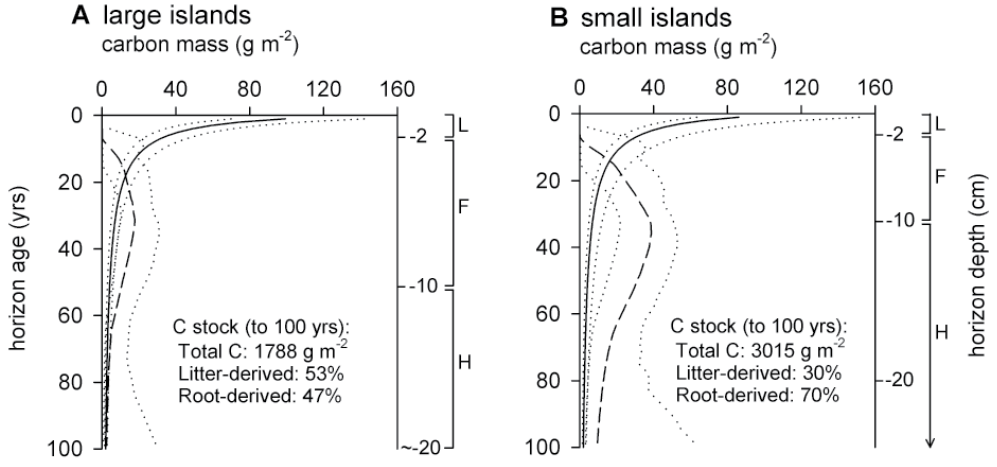

Figure 13: Northern Sweden island chronosequence (**Paper IV**). Carbon (C) dynamics in vertically stratified organic horizons of forested islands. Model estimates of C from aboveground litter (solid lines) and C introduced belowground via root transport (broken lines). Carbon mass was modeled to a horizon age of 100 years based on C mass and ^{14}C measurements in profiles from three large (A, representing early succession) and three small (B representing late succession) islands. Solid lines show means and dotted lines show the 95 % central credibility intervals. The posterior probability that the root-derived fraction is larger in small islands than in large islands is 0.97. Approximate depths are indicated for transitions between the main categories of horizons sampled; L: litter; F: fragmented litter; H: humus.

Our results elucidate the mechanisms underpinning C sequestration in boreal forests and highlight the importance of roots and root-associated fungi for ecosystem C balance and, ultimately, the global C cycle. We challenge the general view that humus accumulation is regulated primarily by saprotrophic decomposition of aboveground litter, and suggest that SOC is primarily controlled by an alternative process in which organic layers grow from below through inputs from roots and root-associated fungi.

Environmental changes, such as N fertilization and deposition, forest management, and elevated atmospheric CO_2 concentrations, are therefore likely to greatly affect soil C sequestration through their alteration of rhizosphere processes. These processes are not well described in current models of ecosystem and global C dynamics, and their more explicit inclusion is likely to improve both the mechanistic realism and future predictive power of models.

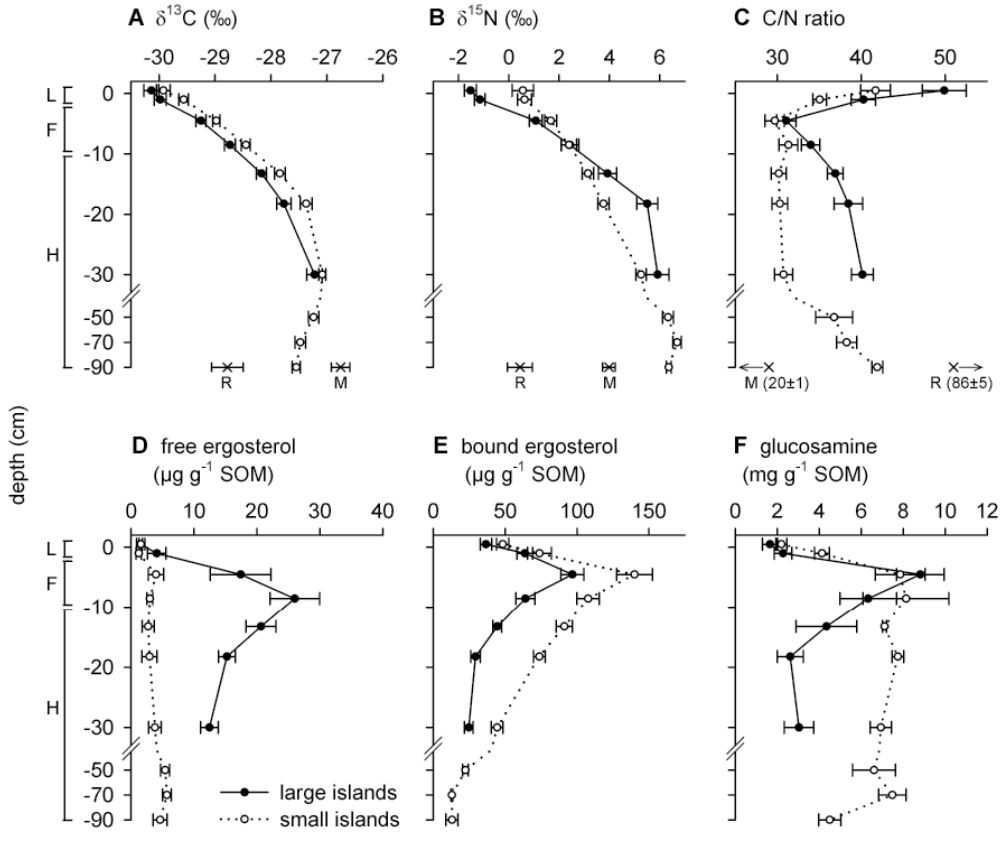

Figure 14: Northern Sweden island chronosequence (**Paper IV**). Depth profiles of $\delta^{13}C$ (A), $\delta^{15}N$ (B), C/N ratio (C) and fungal biochemical markers (D-F) in organic soil profiles of large (solid lines, representing early succession) and small (broken lines, representing late succession) forested islands. All data are means and error bars represent standard error. In the upper panel, levels measured in 1-5 mm diameter roots (R) and mycorrhizal mycelium (M) sampled at 10 cm depth are given for reference.

CONCLUSIONS

Moderate deposition of nitrogen (N) was sufficient to repress production of ectomycorrhizal extramatrical mycelium (EMM).

Elevated N leaching was detected after moderate N deposition and coincided with low EMM productivity.

Abundant EMM could not stop an immediate flush of N, to the soil water at 50 cm depth, from the added fertilizer, but it was calculated that recently produced mycelium could assimilate 6 kg N ha^{-1} $month^{-1}$, which may be sufficient to retain moderate additions of N.

The average annual C sequestration by EMM in one hectare was estimated to more than 300 kg C, and the production of EMM was up to more than 500 kg during four months.

Roots and root associated fungi were found important for C sequestration in boreal forest soils, especially in late successional stages where they contributed to 70 % of the soil organic C.

THIS THESIS IN A LARGER CONTEXT

How does inorganic nitrogen affect carbon sequestration?

The general consensus is that the C sequestration of boreal and temperate forests is enhanced by addition of N (e.g. Magnani et al., 2007; Reay et al., 2008; Sutton et al., 2008; Janssens and Luyssaert, 2009). But additions of N typically repress the production of EMM that is potentially important for C sequestration. The close connection between N and C cycles of boreal forests is obvious from simple C to N stoichiometry since the C:N ratio within various ecosystem components such as soil and microorganisms is relatively stable (Brady and Weil, 2008). Thus, to enable a major enhancement in C storage more N should be needed. The vast majority of reports point out a positive effect of N addition on C sequestration (e.g. Magnani et al., 2007; Reay et al., 2008; Sutton et al., 2008; Janssens and Luyssaert, 2009). Frequently reported underlying mechanisms include that N addition: (1) increases primary production (Magnani et al., 2007; Sutton et al., 2008) and thus litter fall (Franklin et al., 2003); (2) slows down degradation of SOM (Magnani et al., 2007; Sutton et al., 2008) due to (2a) reduced microbial production of enzymes that decay recalcitrant soil substrates (e.g. ligninases) (Janssens et al., 2010), (2b) and relatively less belowground C allocation that will prime the degradation of SOM by microorganisms (Janssens et al., 2010), and finally; (3) addition of N may result in larger C sequestration per N unit since relatively more N is allocated to tissues with higher C:N ratios (e.g. stem wood) (rev. by Janssens and Luyssaert, 2009).

On the other hand, the majority of these mechanisms relate to changes in tree/plant growth, but it is well known that most of the added N ends up in the soil (Högberg, 2012), and some is lost to ground water (Nadelhoffer et al., 1999). Leaching of N would likely be aggravated in forests that become limited by environmental variables other than inorganic N after elevated N input (rev. by Janssens and Luyssaert, 2009). Additionally, in ecosystems with severe N deficiency, addition of N may alleviate microorganism from N limitation, leading to enhanced SOM degradation (Janssens et al., 2010). Most of the studies on the effect of N on C sequestration focus on the change in aboveground plant production, litter fall and

SOM degradation processes (Hyvönen et al., 2007). Less attention has been put on the quantity and fate of the C allocated belowground by the tress, and still less to the C allocated to mycorrhizal fungi, which as showed in the work of this thesis (**Paper II, IV and V**) and other studies (Högberg and Högberg, 2002; Godbold et al., 2006; Wallander et al., 2011) can be both substantial and significant for the formation of stable SOM.

Even though it might be exaggerated (Sutton et al., 2008), Magnani et al. (2007) showed a positive correlation between N deposition and C sequestration based on the total fluxes of C in temperate and boreal forest ecosystems. Thus, it integrated all possible processes involved in the C sequestration (e.g. primary production, belowground allocation, litter fall and SOM degradation), including production and degradation of EMM. However, below a N deposition of 5 kg ha^{-1} y^{-1}, Magnani (2007) could not demonstrate any enhanced C sequestration due to N deposition, and most other studies on the effect of N on C sequestration are based on large N additions (Hyvönen et al., 2007). The majority of the boreal forests are exposed to lower N deposition rates than 5 kg ha^{-1} y^{-1}, and often they only receive the natural background deposition of less than 2 kg N ha^{-1} y^{-1} (Reay et al., 2008). Even though, in the SWETHRO survey (**Paper II**), we could not find a significant repressive effect of N deposition on EMM production below a N deposition rate of 5 kg ha^{-1} y^{-1}, the major decrease of EMM production (visual estimation) occurred below N deposition of 10 kg ha^{-1} y^{-1} (Fig. 9). Further, N additions may cause a shift towards EMF species that have less recalcitrant tissues (rev. by Ekblad et al., 2013). In the Northern Sweden forest chronosequence (**Paper IV**) we found that more than half of the SOC stock originated from belowground inputs from roots and their associated microorganisms (Fig. 13). The isotopic signal of ^{15}N and ^{13}C approached that of pure EMM and EMF sporocarps with depth, indicating that EMF was a major contributor (Fig. 14). In late successional stages the belowground contribution to SOC was further enhanced (**Paper IV**).

Nitrogen retention in northern forests

Apart from posing a threat of toxicity and eutrophication to aquatic environments, N leaching may also cause soil acidification as well as depletion of other nutrients that are leached as counter ions together with NO_3^- (rev. by Gundersen et al., 2006). Productivity in most northern forests is considered to be N limited and additional N is typically well retained. Nitrogen losses from boreal forests are small (about 0.5 – 5 kg ha^{-1} y^{-1}) (Stendahl and Hjerpe, 2007) in comparison to what is leached from agricultural lands (about 6 - 50 kg ha^{-1} y^{-1}) (Johnsson et al., 1997) in the same region. However, N leaching from boreal forests still poses a threat to the environment due to the vast areas they cover. In Sweden the total N load into the Baltic Sea that originates from forests is at level with that from agriculture land (Stendahl and Hjerpe, 2007). Most of the N leaching from forests has however, in contrast to agricultural leaching, been ascribes to natural causes. Still, previous findings (Akselsson et al., 2010) together with results from this thesis (Paper II) show that in some Swedish coniferous forests a moderate deposition is sufficient for the soil water N to exceed an annual average of 0.5 mg^{-1} l^{-1}, which has been suggested to reflect progression towards N saturation (Stoddard, 1994). If the N retention capacity of forest soils is exceeded, increased N leaching may occur (Aber et al., 1998), and the flushes of soil water N that typically follows after

storm damage (**Paper II**), fertilization (Berden et al., 1998, Paper V) and clear cutting (Akselsson et al., 2004) will likely be further enhanced. The leaching is caused as a direct effect of an increased pool of inorganic N in the soil, but it is likely further elevated as a consequence of reduced production and assimilation by EMM. I suggest based upon results in this thesis (**Paper II** and **V**) that EMM is relatively more important for the total N retention capacity in forests that receive no or only moderate anthropogenic additions of N. Given the large area of these forests, reduced EMM production, which occur already at slight elevation of N deposition, could result in increased N leaching with substantial consequences for northern forest ecosystems.

FUTURE ASPECTS

Given the large area and C stock of boreal forests, it is important to put more emphasis on resolving the missing links in the C cycle as well as the N cycle. An important question that still remains to be answered is how moderate levels of N deposition affect the sequestration of carbon in boreal areas. To answer that question and to increase the general knowledge of the role EMM play in the C sequestration of boreal forests, field surveys like the one in the analysis of the Northern Swedish chronosequence (**Paper IV**) should be repeated in a broader range of forests also including managed and fertilized forests.

While large N inputs lead to elevated aboveground inputs to SOC, belowground inputs may decrease. In order to predict future changes in the boreal C stocks it is necessary to develop models where both these two opposing processes are included, particularly given the evidence that belowground inputs can make a larger contribution to stable SOM pool than aboveground inputs.

Even though growth of EMF is typically reduced by elevated N deposition or N fertilization, severe nutrient limitation also results in less EMF production due to very limited tree growth. Further analysis is needed to identify the optimal fertility for EMM production.

Nitrogen leakage may result in depletion of other nutrients and eutrophication of aquatic systems. EMF has the potential to reduce N losses but further research is needed to estimate their N retention capacity in relation to assimilation by other microorganisms or directly by the tree roots.

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POPULÄRVETENSKAPLIG SAMMANFATTNING:

Vanliga skogssvampar är viktiga för kretsloppen av kol och kväve

Ektomykorrhiza

I alla skogar växande i ett klimat liknande Sveriges, lever nästan samtliga trädarter i ett nära samspel (symbios) med svampar. Till skillnad från nedbrytarsvampar (som kan leva enbart på dött organiskt material) är flera av de kända skogsvamparna såsom kantareller, soppar, flugsvampar, kremlor och riskor beroende av denna symbios med träd för att överleva. Symbiosen mellan träd och svamp kallas för ektomykorrhiza. Svamparna som vi ofta plockar i skogen utgör bara fruktkropparna av svampen som lever som ett nätverk av svamphyfer i marken, kallat mycel. Som liknelse kan man tänka sig att svampfruktkropparna är som äpplen på ett äppelträd. Ektomykorrhiza-svamparnas mycel ansluter till trädets rotspetsar och omsluter i regel över 90 % av dem, likt en handske. Ektomykorrhiza-svampar levererar näringsämnen och vatten ifrån marken till trädet, i utbyte mot kol som nyligen fångats in ifrån atmosfären genom trädens fotosyntes. Ektomykorrhiza-svamparna utgör en potentiellt viktig kolsänka då 10-50 % av allt det kol som binds in i trädens fotosyntes transporteras via rötterna direkt till svamparnas mycel i marken. I ett enda gram skogsjord kan det finnas upp till en kilometer svampmycel och svamparna ökar i regel trädrötternas kontaktyta med jorden över 100 gånger. Dessutom kan svamparna frigöra näringsämnen som är inbundna i organiskt material, som annars är otillgängliga för trädrötterna. Detta gör att nästan allt näringsutbyte trädet har går igenom ektomykorrhizasvampar.

Kvävebegränsning i skogen

Träden i barrskogsbältet är oftast begränsade av tillgången på kväve. Om kväve tillsätts via gödsling blir det mer lättillgängligt för trädrötterna och träden behöver därför inte skicka ner lika mycket kol för att utveckla rötter eller ektomykorrhiza-svampar. Större mängder av kol binds istället in i trädets biomassa ovan mark. Mänskliga utsläpp (från bland annat trafik och industrier) har gjort att kvävet som tillsätts från atmosfären ökat från mindre än 2 kg per hektar och år till hela 50 kg per hektar och år i vissa områden i exempelvis Centraleuropa. Även om kvävenedfallet i Sverige är betydligt mindre är det ändå upp till 10 gånger högre än det naturliga nedfallet. Det har flera gånger visats att gödsling med stora mängder kväve (ofta 100 kg per hektar och år) i nordliga skogar leder till minskad tillväxt av ektomykorrhiza-mycel, men det saknas fortfarande studier på effekten av den låga kontinuerliga tillsatsen av kväve som sker via nedfall. Detta är viktigt att reda ut då det kan få stora konsekvenser gällande upplagringen av kol och kväve i skogsmarken. Resultat ifrån denna avhandling visar att kvävenedfallet i Sverige är tillräckligt för att minska tillväxten av ektomycorrhizasvampar, och att en kraftig minskning sker redan vid kvävenedfall under 10 kg per hektar och år.

Då ektomykorrhizamycel effektivt tar upp kväve i marken är det sannolikt att det bidrar till att motverka kväveläckage. Man har tidigare hittat samband med ökat kväveläckage vid låg tillväxt av ektomykorrhizasvampar efter kvävegödsling. Det har dock inte varit möjligt att särskilja betydelsen av minskat mycel från den direkta effekt som kvävegödsling har på läckaget. För att bättre reda ut svampens kapacitet att ta upp kväve satte vi upp ett gödslingsförsök i granskogar nära Simlångsdalen. Resultaten visade att ektomykorrhizasvampar har en stor kapacitet att ta upp kväve, men att detta inte var tillräckligt för att motverka det läckage som kom direkt efter gödsling.

Skogen som kolsänka

Globalt sett binder barrskogsbältet in enorma mängder kol, och det mesta av detta finns lagrat i marken. Det finns enligt den internationella klimatpanelens (IPCC) rapport, ungefär lika mycket kol lagrat i barrskogsmarken som i både mark och växtlighet i samtliga regnskogar. Detta gör det viktigt att fokusera på de processer som bidrar till kollagringen. En majoritet av de studier som gjorts hittills på effekter av kväveutsläpp och kvävegödsling visar att detta gynnar skogens kolinlagring. Dessa studier visar att kvävetillsats leder till mer kolinbindning genom en ökad tillväxt av träden och större produktion av barr samt minskade nedbrytningsprocesser i marken, men det saknas fortfarande kunskap gällande den kolinlagring som sker under markytan via rötter och ektomykorrhiza-svampar. Då kvävetillsatts minskar mängden kol som transporteras direkt ner i marken kan detta istället leda till en minskad kollagring. För att kunna utveckla en korrekt rådgivning gällande effekten av kvävetillsats på kolinbindningen är det nödvändigt att ta med båda dessa motverkande processer i beräkningarna.

Kolinlagring av ektomykorrhiza

I likhet med tidigare studier visar våra analyser av barrskogar i Svealand och Götaland att ectomycorrhiza-svamparna bildar ungefär ett halvt ton mycel per år och hektar. Detta är dock bara den årliga nyproduktionen och den stående svampbiomassan har tidigare uppskattats till ungefär 2 ton per hektar. Delar av ektomykorrhizamycelet är relativt svårnedbrytbart då det ofta är vattenavstötande och innehåller många motståndskraftiga molekyler, vilket gör att det har en potential att bidra till kolinbindningen. Något vi fann tydliga indikationer på i en undersökning av skogar med olika lång kontinuitet i Norrland. Dessa skogar var belägna på öar av olika storlek i sjöarna Uddjaure och Hornavan, och var i stort sett opåverkade av mänsklig aktivitet. Den största störningen i dessa skogar utgjordes av bränder. Då större öar oftare träffas av blixten resulterar det i mer frekventa skogsbränder som startar om successionen. Kontinuiteten av skog sträckte sig ifrån ett par hundra år (på stora öar) upp till tusentals år på små öar. Det var en enorm upplagring av organiskt material i de riktigt gamla skogarna, då humuslagret kunde vara en hel meter tjockt. I de yngre skogarna var humuslagret ungefär 10 cm tjock, likt de flesta bruksskogar. Över hälften av markkolet som bundits in i jorden de senaste 100 åren visade sig härstamma ifrån rötter och mykorrhizasvampar, och denna mängd ökade ju längre successionen hade fortgått. Tidigare har man ofta ansett att det är vad som produceras ovanför markytan som är viktigast för kolinlagringen. Resultaten i denna avhandling visar att det är viktigt att det kol som matas in i jorden via rötter och mykorrhizasvampar tas med då man beräknar och modellerar kolinbindning i skogsmark.

Slutsats

Kvävenedfall hade en negativ effekt på tillväxten av ektomykorrhizamycel, vilket kan leda till minskad kolinlagring i skogsjordarna. Detta är i direkt kontrast till många studier av andra processer som gör att kolinlagringen istället ökar efter kvävegödsling. Båda dessa motverkande system ingår i tidigare studier av det totala kolflödet mellan atmosfären och skogen, som visar på en positiv effekt av högt kvävenedfall. Men denna effekt uppstår inte förrän kvävetillsatsen når minst 5 kg per hektar och år. De flest barrskogarna är utsatta för en ökning i kvävenedfall som är mindre än detta, och våra studier visar att minskad produktion av ektomykorrhiza sker redan efter låga ökningar i kvävedeposition. Det är av stor vikt att vidare undersöka hur dessa två

motverkande system påverkas av olika mängder kväve för att bättre kunna modelera och förutsäga effekten av människoskapade kväveutsläpp. Jag visar också att ektomykorrhizasvamparna har en stor potential att motverka kväveläckage men för att ställa deras kväveupptag i relation till vad trädrötterna och övriga mikroorganismer i marken tar upp behövs fler undersökningar.

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Review

Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils – A review

H. Wallander [a,*], A. Ekblad [b], D.L. Godbold [c], D. Johnson [d], A. Bahr [a], P. Baldrian [e], R.G. Björk [f], B. Kieliszewska-Rokicka [g], R. Kjøller [h], H. Kraigher [i], C. Plassard [j], M. Rudawska [k]

[a] Department of Biology, Microbial Ecology Group, Ecology Building, Lund University, SE-223 62 Lund, Sweden
[b] School of Science & Technology, Örebro University, SE-701 82 Örebro, Sweden
[c] Institute of Forest Ecology, Universität für Bodenkultur, 1190 Vienna, Austria
[d] Institute of Biological and Environmental Sciences, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3UU, UK
[e] Laboratory of Environmental Microbiology, Institute of Microbiology ASCR, CZ-14220 Praha, Czech Republic
[f] Department of Biological and Environmental Sciences, University of Gothenburg, P.O. Box 461, SE-405 30 Gothenburg, Sweden
[g] Institute of Environmental Biology, Kazimierz Wielki University, Al. Ossolinskich 12, PL-85-093 Bydgoszcz, Poland
[h] Terrestrial Ecology, Biological Institute, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen, Denmark
[i] Slovenian Forestry Institute, Vecna pot 2, 1000 Ljubljana, Slovenia
[j] INRA, UMR Eco & Sols, 34060 Montpellier Cedex 02, France
[k] Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland

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ABSTRACT

Mycorrhizal fungi constitute a considerable sink for carbon in most ecosystems. This carbon is used for building extensive mycelial networks in the soil as well as for metabolic activity related to nutrient uptake. A number of methods have been developed recently to quantify production, standing biomass and turnover of extramatrical mycorrhizal mycelia (EMM) in the field. These methods include mini-rhizotrons, in-growth mesh bags and cores, and indirect measurements of EMM based on classification of ectomycorrhizal fungi into exploration types. Here we review the state of the art of this methodology and discuss how it can be developed and applied most effectively in the field. Furthermore, we also discuss different ways to quantify fungal biomass based on biomarkers such as chitin, ergosterol and PLFAs, as well as molecular methods, such as qPCR. The evidence thus far indicates that mycorrhizal fungi are key components of microbial biomass in many ecosystems. We highlight the need to extend the application of current methods to focus on a greater range of habitats and mycorrhizal types enabling incorporation of mycorrhizal fungal biomass and turnover into biogeochemical cycling models.

1. Introduction

A better understanding of below ground carbon (C) flux is of fundamental importance to predict how changing climate will influence the C balance of forest (and other) ecosystems (Litton and Giardina, 2008). Litton et al. (2007) reported below ground C allocation in forest ecosystems can represent 25–63% of GPP on a global scale, and this C has a large influence on the physical, chemical and biological properties of soils. The below ground allocation of C links activity in the forest canopy to the activity in the soil, and provides a flow of organic C from shoots to soil via fine roots and mycorrhizal hyphae. The pathways by which this organic C can enter soils are complex, involving both biomass turnover (Godbold et al., 2003), biomass grazing (Setälä et al., 1999) and turnover of low molecular weight exudates from roots and fungal hyphae (van Hees et al., 2005). The fate of C entering soil systems is also complex. Much of this C is lost as respiration (Janssens et al., 2001) and a small but significant fraction enters the soil organic matter (SOM) pool. Determination of the pools and fluxes of biomass inputs in isolation from fine roots and mycorrhiza provides a major scientific challenge. Some studies (e.g. Wallander et al., 2004) suggest that biomass pools and inputs from fine roots and mycorrhizal hyphae are in the same order of magnitude. However, estimates of fungal inputs rely on

* Corresponding author. MEMEG, Department of Biology, Ecology Building, Lund University, SE-223 62 Lund, Sweden. Tel.: +46 46 222 4247; fax: +46 46 222 4158.
E-mail address: hakan.wallander@biol.lu.se (H. Wallander).

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methods and conversion factors that contain a certain degree of inaccuracy that needs to be considered.

Precise measurements of production, standing biomass and turnover of extramatrical mycelium (EMM) of mycorrhizal fungi are essential in order to accurately describe the C cycle of terrestrial ecosystems. Although several techniques are available for this, they all have limitations that need to be taken into consideration.

Existing biogeochemical models often treat the uptake apparatus as a single organ, meaning that there is no distinction between roots and mycorrhizal hyphae. It is possible, and probably necessary, to amend this by allocating carbon and nutrients specifically for the fine roots and mycorrhizal hyphae respectively. This would require the development of dynamic allocation routines responsive to carbon, nutrients and water availability (Jönsson, 2006), and would allow the models to simulate nutrient uptake and carbon flux dynamically. In this review, we will discuss and compare available methods to estimate production, standing biomass and turnover of mycorrhizal mycelia (summarized in Tables 1–3). We focus on temperate and boreal forests, in which the dominant plants associate with ectomycorrhizal (ECM) fungi. From a methodological perspective, greatest progress has been made in quantification of production, biomass and turnover of ECM fungi compared to the other main mycorrhizal types (arbuscular and ericoid mycorrhizas). This progress has been driven partly by technical reasons but more importantly because of the recognition of the key roles boreal and temperate forests play in the global C cycle. However, we emphasise from the outset that greater effort must be applied to other ecosystems in which plants are primarily colonised by arbuscular and ericoid mycorrhizal fungi, which also have important roles in regulating biogeochemical cycles. We will also highlight knowledge gaps that need to be filled in order to incorporate mycorrhizal mycelia in models of biogeochemical cycles, which will enable us to better describe the C cycle in forests. Firstly methods to estimate EMM production are described and discussed, since the methodology in this field has developed rapidly over the last decades. We then discuss the advantages and disadvantages of different methods to estimate fungal biomass. Finally we discuss how we can assess the turnover of fungal hyphae. This area needs clearly to be developed in future research as it is a key process in C sequestration of forest soils. We also include aspects of sampling strategies and indirect estimates of EMM production that have great potential for the future. The mechanisms through which EMM regulate C cycling in terrestrial ecosystems have been considered recently in another review (Cairney, 2012).

2. Measurements of mycorrhizal hyphal production

A key problem in the determination of mycorrhizal hyphal production is lack of methods to distinguish growth of mycorrhizal hyphae from that of saprotrophic fungi. As ECM fungi do not form a monophyletic clade (Hibbett et al., 2000; Tedersoo et al., 2010) no single biochemical or DNA based marker can be found to quantify this group from the complex soil environment. Therefore various methods are needed to distinguish the biomass of EMM from that of other fungal mycelia. Mycelial growth can be estimated by direct observation in minirhizotrons (Treseder et al., 2005; Pritchard et al., 2008; Vargas and Allen, 2008a) and by the use of root free in-growth bags or cores, which is the most commonly applied method to measure EMM production in forests (Wallander et al., 2001; Godbold et al., 2006; Hendricks et al., 2006; Kjøller, 2006; Korkama et al., 2007; Parrent and Vilgalys, 2007; Hedh et al., 2008; Majdi et al., 2008).

2.1. Observational methods

The first observational studies used plastic sheets placed at the litter/soil interface above root clusters where individual ECM tips were observed by pulling back and replacing the litter at different times (Orlov, 1957, 1960). Lussenhop and Fogel (1999) used a method developed by Waid and Woodman (1957) to estimate hyphal production of the ECM fungus *Cenococcum geophilum* by burying nylon mesh in the soil and harvesting them at two week intervals. Rygiewicz et al. (1997) introduced the minirhizotron technique, commonly used to study fine roots, to measure temporal occurrence and lifetime of mycorrhizal root tips. However, the use

Table 1
Strengths and weaknesses of currently used methods to estimate production of ECM extramatrical mycelium (EMM).

Methods		Strengths	Weaknesses	Comments
Production of ECM mycelium	Direct minirhizotron observation	• Repeated non-destructive sampling possible. • Not dependent on conversion factors.	• Cannot differentiate between saprotophic or mycorrhizal hyphae. • High resolution needed to observe individual hyphae. • Growth might be different in observation chamber compared to soil. • Difficult to transfer to biomass per land area.	• Changes in rhizomorph production, which are easier to observe, does not automatically imply similar changes in total EMM production.
	Root free in-growth mesh-bags or cores	• Easy and relatively cheap method that can be applied in large scales. • Substrates that have no background of old mycelium, chemical markers, DNA etc. can be used. • Substrates can be 'spiked' with isotopic labelled materials, minerals etc. • Relative comparisons may be more reliable than estimates of absolute amounts.	• Growth, standing biomass and turnover may be different in mesh bags compared to soil, and this needs to be further studied. • May select for early colonizers of fungus free space. • Disturbance at installation & harvest. Interactions with soil animals are restricted. • The way the mycelial biomass is assessed may give different results.	• When bags are left in the soil over years or more, the mycelial mass is possibly a reflection of the standing biomass rather than production? • Disturbance is probably larger for larger bags or cores. • Mycelial biomass can be assessed with: dry weight, loss on ignition or with chemical markers.
	Assessment of exploration types	• Definition of exploration types is based on EMM production. • ECM communities have been studied in a number of forest ecosystems. • Possible to combine with molecular methods to indirectly non-destructively estimate EMM production.	• Estimation of EMM production is based on observations from (simplified) laboratory conditions – growth might be different in soil due to nutrient conditions and season etc.	• Only 5–10% of all ECM fungi have been characterized and are assigned into exploration types.

Table 2
Strengths and weaknesses of currently used methods to estimate the biomass of ECM extramatrical mycelium (EMM).

Methods		Strengths	Weaknesses	Comments
Biomass of ECM mycelium	Direct measurement of mycelium length in the soil	• Not dependent on chemical conversion factors.	• Difficult to separate mycelium of mycorrhizal and decomposing fungi and living biomass from necromass.	• Dependent on correct conversion factors from length to biomass.
	Root free in-growth mesh-bags or cores	• See above for mycelium production using bags.	• See above for mycelium production using bags.	• See comments on production estimates using in-growth bags above.
	Chemical markers (chitin, ergosterol, PLFAs) combined with incubation	• Highly sensitive, small amounts can be estimated.	• Dependent on conversion factors which can vary between species and growth conditions.	• Field studies on variation in conversion factors are lacking.
	Molecular DNA and RNA methods	• Possible to estimate biomass of individual species. • Targeted especially to dominant species in ECM communities. • Techniques under fast development.	• Suitable primers depend on fungal species, a number yet to be developed. • High costs of next generation sequencing.	• Techniques are under development
	Assessment of exploration types	• Data from ECM Communities on root tips can be extrapolated to EMM. • Non-destructive estimation of EMM production possible based on ECM community composition.	• EMM biomass of individual exploration types is based on a combination of previously defined estimations. • Few ECM types have been grown in cultures, therefore species-specific fungal diameter and conversion of volume into biomass needs further studies.	

of observational methods to estimate production, biomass and turnover of EMM in the field has been limited. It has mostly been used to study mycorrhizal roots tips (e.g. Rygiewicz et al., 1997; Majdi et al., 2001; Tingey et al., 2005), but few attempts have been made to estimate the length and longevity of rhizomorphs and hyphae (Treseder et al., 2005; Pritchard et al., 2008; Vargas and Allen, 2008a,b). Similar observations may also be possible using root observation windows (Stober et al., 2000). However, none of the direct techniques can distinguish between the mycelium of ECM and saprotroph mycelia. Two types of minirhizotron cameras are commonly used, which also give different image sizes; BTC 100× microvideo camera (Bartz Technologies, Santa Barbara, CA, USA) that provides image sizes of 1.9 × 1.3 cm, and a CI-600 (CID Bio-Science Inc., Camas, WA, USA) that provides a 360-degree image (21.59 × 19.56 cm). The advantage of the minirhizotron techniques, unlike other methods that rely on excavation which can disrupt extraradical hyphae, is the potential to make repeated, non-destructive observations *in situ* of the same specimen. This allows the specimen to be followed from its emergence (birth) to its disappearance (death). Although the technique has been found useful to monitor the formation and death of mycorrhizal root tips as well as rhizomorphs, several shortcomings exist. For instance, the minirhizotron technique is limited by the resolution and quality of the images (although the technology in this area is progressing rapidly, see for instance Rundel et al., 2009) and the time required for processing (which also restricts sampling intensity, depth and the number of tubes used). Since the technique cannot yet capture the production and turnover of diffuse mycelium it does not enable calculation of overall mycelium production and turnover rates. Furthermore, there is uncertainty in determining when a rhizomorph is dead, leading to the use of different criteria. For instance, Treseder et al. (2005) classified the time of death as the first visual

Table 3
Strengths and weaknesses of currently used methods to estimate the turnover of ECM extramatrical mycelium (EMM).

Methods		Strengths	Weaknesses	Comments
Turnover of ECM mycelium	Direct minirhizotron	• Birth and death of individual hyphae can be followed.	• Risk of missing the exact birth or death of the hyphae (recording frequency dependent). • May target the fast turnover pool since the length of the study period is limited.	• The problem with lag-time can possibly be solved if small vertically installed bags are used. But this needs to be evaluated.
	Direct measurements in growth mesh-bags	• In areas with rapid EMM growth and insignificant lag times for mesh bag colonization, sequential harvests at different incubation times could be a way to estimate turnover times.	• Lag-times to colonize the mesh bags may be too high for this method to give reliable results (see Fig. 1). • Turnover may be different in sand than in soil.	• The problem with lag-time can possibly be solved if small vertically installed bags are used. But this needs to be evaluated.
	Isotopic techniques	• Pulse labelling via the plant is possible. • Mesh bags amended with C_4 substrates can be used to continuously measure C input.	• Analyses of bulk mycelial materials may give false impression of a fast turnover. Analyses of isotopes in structural components would solve that problem. • The method to use C_4 materials is not very sensitive, large fluxes are needed for reliable results.	

appearance of fragmentation of the rhizomorph, whereas some authors also used the disappearance from the image for determining the death of a rhizomorph (e.g. Pritchard et al., 2008). If a rhizomorph disappears, a judgment had to be made as to whether the rhizomorph has truly died or has become obscured from view due to soil or tube movement. Both criteria are often used for estimating turnover, but may give highly variable results when compared (Børja et al., unpublished). Furthermore, it is not possible to know exactly when a rhizomorph may form or disappear from the camera's visual field between any two subsequent recording events (typically a month, but new automated minirhizotrons for recording images at multiple times per day are in progress (Rundel et al., 2009)). The long lifetime of some rhizomorphs makes it difficult to estimate turnover rate since most minirhizotron studies are conducted over a one (or two) year period. Thus, when using minirhizotrons to estimate production and turnover of rhizomorphs, it is important to consider the recording frequency and study length because both of these affect the accuracy of the estimations.

One method, which was not applied in a forest, but is worthy of mentioning is the 'root box' method of Coutts and Nicoll (1990), as it allows detailed investigation of the growth and survival of diffuse mycelium as well as of rhizomorphs over the year. These authors planted pine seedlings in peat in 2 m tall transparent acrylic tubes, placed the tubes outside and followed the growth of mycelia and rhizomorphs in detail daily from March 1987 to April 1988. This technique may be ideal for detailed studies of various ECM symbioses, for example studies of the different exploration types as defined by Agerer (see below Section 6). Although observational methods have limitations, they also have many advantages, which can substantially increase our understanding of mycelia production and turnover.

2.2. In-growth mesh bags and cores

Mesh bags (e.g. Wallander et al., 2001) are typically made from nylon mesh fine enough to prevent in-growth of roots, but large enough to allow in-growth of fungal hyphae. The fungal communities that colonize the mesh bags are usually dominated by mycorrhizal hyphae as has been verified by trenching experiments (Wallander et al., 2001) and with DNA analyses (Kjøller, 2006; Korkama et al., 2007; Parrent and Vilgalys, 2007; Hedh et al., 2008; Wallander et al., 2010). Mesh sizes between 25 and 50 μm are commonly used. In forest soils with little understorey vegetation, 50 μm prevents in-growth of tree roots, but if understorey Ericaceae or herbs are present, care should be taken so that the fine roots of these do not penetrate the mesh. For example, the fine "hair roots" of ericaceous plants can have diameters of just 20 μm (Bonfante-Fasolo and Gianinazzi-Pearson, 1979). The bags can have different forms and the sides of the nylon mesh can be sealed by sewing, heating and gluing.

The mesh bags are usually placed at the interface between mineral and organic horizons. This will maximize fungal in-growth since mycorrhizal fungi are most abundant in this region (Lindahl et al., 2007). However, when the main aim is to estimate EMM production on an area basis, tubular bags that are placed vertically to a desired soil depth have been used (e.g. Kjøller, 2006). This design also allows the comparison of adjacent soil and root samples taken with the same volume, and it is suitable for sequential harvests since the mesh bags can be replaced with minimal disturbance. In addition to bags, cores can be made of plastic tubes with windows made of mesh to allow fungal in-growth. One advantage with such cores is that they can be rotated regularly to detach fungal in-growth in order to function as controls with similar soil physical conditions but no, or little, fungal in-growth (Johnson et al., 2001, 2002a,b). This is a considerable advantage when the cores are filled with a natural substrate such as soil (see below). Keeping the volume of the in-growth bags (or cores) as small as possible is important when quantifying EMM production because this helps to ensure that soil physical and chemical conditions inside mesh bags are similar to those outside. In addition, small bags may be colonized more rapidly than larger ones.

Mesh bags are usually incubated in the soil during one growing season because this will give the net production for that year. In some cases a prolonged incubation time (two growing seasons) is necessary in order to detect EMM stimulation by specific substrates such as apatite or other minerals (e.g. Hagerberg et al., 2003; Potila et al., 2009). Berner et al. (2012) suggested that this may be an effect of early colonization by fast-growing ECM species, while species stimulated by minerals are more slowly growing. It has been shown that the stimulation of EMM by apatite was dependent on the P status of the forest (Wallander and Thelin, 2008), while other studies showed that large differences in EMM growth occurred after 5 months along a nitrogen deposition gradient (Kjøller et al., 2012) and in a nitrogen fertilized forest (Nilsson and Wallander, 2003). These findings show that effects of forest management on EMM growth can sometimes be detected with shorter incubation periods. The length of the incubation period thus depends on the purpose of the study. If the main goal is to test for differences between treatments (e.g. forest management or effects of substrates amended to the mesh bags), a longer incubation time can be used. But if the main goal is to estimate net annual production from a specific site, one growing season should be used. On the other hand, if quantifying temporal variation in fungal production is the goal, shorter incubation times than one growing season are used (e.g. Nilsson et al., 2005). Regardless of the approach, it should be noted that a lag time exists before EMM enter bags after they have been inserted into the soil. As an illustration of this, twice as much fungal biomass was found in mesh

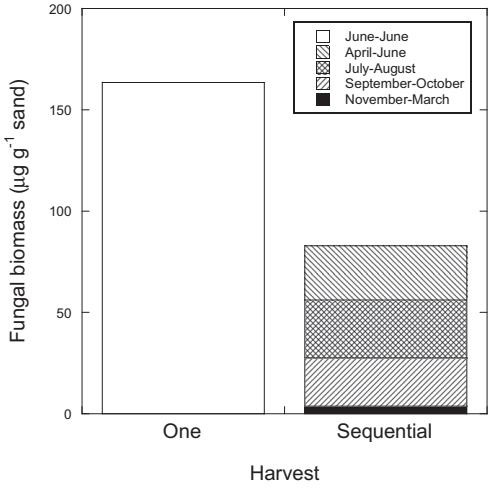

Fig. 1. Fungal in-growth into mesh bags buried in young (10–20 years) Norway spruce forests at Tönnersjöhedens experimental park. Bags were either incubated for 12 months (one harvest) or for 2 (July–August, September–October), 3 (April–June) or 5 (November–march) months periods (sequential harvests). SE for the mean EMM production after one harvest was 37.5, and the SE for the added sequential harvests was 9.3.

bags that were incubated for 12 months, compared to the added amounts in mesh bags that were incubated for 2–5 month periods in 10 young Norway spruce sites in southern Sweden (Fig. 1). Another aspect that complicates the estimate of production is the turnover of the fungal biomass in the mesh bags. A longer incubation period allows more necromass to form and decompose, which results in underestimation of the total production.

There has been concerns raised that the use of pure quartz sand in mesh bags may affect growth of EMM, which can lead to inaccuracies in production rates and biomass estimates (Hendricks et al., 2006). Hendricks et al. (2006) used 10 cm wide cores placed *in situ* for 1 month to demonstrate that mycelial in-growth was greater when natural soil was used as the in-growth substrate rather than pure sand. Whilst for many habitats the use of natural soils as a substrate is desirable, subsequent measurements can be confounded because of the large and variable amounts of background fungal biomass. If more specific methods to quantify ECM fungal biomass are developed (see below Section 3.6), natural soil could be used more reliably. Indeed, growth of arbuscular mycorrhizal fungi has been quantified in mesh bags amended with natural SOM using fatty acids (Labidi et al., 2007; Hammer et al., 2011), which are available for this mycorrhizal group (NLFA 16.1ω5, Section 3.5). Another uncertainty with the mesh bag method is that some ECM fungi appear to show preferences towards certain types of resource. In addition, some species avoid growing in mineral substrates (*Cortinarius*) probably because they are adapted to an environment where they utilize organic nutrients from SOM (Read and Perez-Moreno, 2003). Therefore, despite being abundant on root tips, species within the genera *Cortinarius* may avoid sand filled mesh bags even when they are common on the root tips while the opposite situation is the case for other species (e.g. *Xerocomus*; Kjøller, 2006; Kjøller et al., 2012). The EMM community in mesh bags may thus not represent the community that prevails in the soil, which may be a problem in some studies. An important advantage with the mesh bag method is that the fungi studied are recently formed, while fungi that we can detect in the soil can be old and inactive (see below Section 2.2).

Another important aspect that needs to be considered is that newly placed mesh bags provide a non-exploited area in the soil. Such spaces are probably rare in established forests but may be common in newly planted forests where the EMM from the previous forest can be expected to die back. In a tree age chronosequence, the EMM production was 3 times greater in young forest (10–20 y) compared to older forests (30–130 y) suggesting that young trees are investing more C to establish a mycorrhizal network, while less C is needed to sustain this network in older forests (Wallander et al., 2010). It is possible that mesh bags select for fast-growing species adapted to newly planted forests. For this reason, EMM production may be overestimated when incubating mesh bags over one growing season. As noted previously, such effects may be minimized by reducing as far as possible the volume of mesh bags and cores.

From the discussion above it seems that some factors result in overestimation while other results in underestimation of EMM production using the mesh bag method. As methods to measure fungal biomass and necromass improve (see e.g. Section 3.6), it might be possible to follow the fungal community in mesh bags over several years and quantify the yearly production after the initial empty space has been colonized. A combination of chitin and ergosterol analysis (see below) may give an indication of the ratio between biomass and necromass. Another way to quantify annual production of EMM, including necromass, is to analyse the isotopic change in $^{13}C/^{12}C$ in mesh bags that have been amended with organic material from C_4 plants, and follow this change through time (Wallander et al., 2011). A similar approach was used by Godbold et al. (2006) who filled cores with C_4 soil to estimate the contribution of fungal hyphae to new soil C over a 2.5 year period. Amendment of organic matter in the mesh bags would make the substrate more natural for growth of ECM fungi and probably produce communities more similar to those of the surrounding soil but brings with it greater abundance of saprotrophic fungi. An interesting approach to reduce in-growth by saprotrophic fungi but still use more natural soil was reported by Melanie Jones and co-workers in Canada who used an outer mesh bag with sand, which functioned as a barrier for saprotrophs, and an inner mesh bag with sterilized soil where EMM of ECM fungi proliferated (Lori Phillips and Melanie Jones, pers. comm.).

It is clear that the fungal community colonizing mesh bags may not accurately mirror the mycelial community in natural soil i.e. some species or clades may be over represented and some are underrepresented or even missing in the mesh bags. On the other hand, when working with natural soil it is also difficult to claim that only EMM are in the extracted DNA pool. One needs to be very careful in removing all roots and in reality it will be difficult to state that a soil sample is indeed completely free of ectomycorrhizal root tips or small detached pieces of ectomycorrhizal mantle. Furthermore, extraction of fungal spores in the soil may lead to false positives in the community profile. Extracting DNA or RNA from sand-filled mesh-bags at least ensures that only nucleic acid from actively (or recently active) mycelia is amplified. Another benefit is that the hyphae from the mesh bags are easily extracted from the sand and simple and cheap nucleic acid extraction methods can be applied to produce good quality templates for PCR. Whether extracting nucleic acid from sand-filled mesh bags or directly from soil, primer bias is a confounding factor preventing an accurate description of the fungal community. For each specific primer combination chosen, some groups will be over, and some groups under expressed or even completely missed (Bellemain et al., 2010). As an example of the latter, *Tulasnella* sp. are often completely missed with the standard ITS1-F and ITS4 primer combination (Taylor and McCormick, 2008). In general, careful consideration of primers combinations for the specific study system in question should be made, and the results obtained treated with sound caution.

3. Quantification of fungal biomass in mesh bags and soil

The examination of mycelia in mesh bags should start with a visual classification under a dissecting microscope. This allows a check for the presence of mycelial strands, whether or not they are hydrophilic, and gives insights in exploration types of mycorrhizal fungi (see below Section 5). The amounts of total hyphae can be estimated either by extracting fungal hyphae and converting estimates of hyphal length to biomass, or by using different chemical markers (chitin, ergosterol, phospholipid fatty acid 18:2ω6,9) as proxies for biomass. These methods are described below and the benefits and disadvantages are discussed (Tables 1–3).

3.1. Direct measurements of fungal weight and hyphal length

One approach to estimate fungal biomass that can be used in mesh bags only, is to extract the mycelium from the sand substrate and determine its weight. In this way conversion factors between biomass and a chemical marker can be avoided, but it assumes that all extractable matter is of fungal origin. This is not the case because bacteria and precipitated SOM can be present in the mesh bags, but they probably contribute very little to the weight of putative fungal material extracted. Since it is difficult to remove all sand grains, it is usually necessary to burn the extracted mycelia and use the loss on

ignition as an estimate of the biomass (Hagerberg et al., 2003; Korkama et al., 2007). The C concentration of fungal material is approximately constant (around 45%; Taylor et al., 2003) and C content can be used as a proxy for biomass in the mesh bags. When analysed on a mass spectrometer, both the content and isotopic signature of C can be obtained, which makes it possible to calculate the proportion of ECM and saprotrophic mycelia in the mesh bags because these two groups have different isotopic signatures (Wallander et al., 2001). The recovery of mycelium using this method can be tested by analysing the ergosterol content of both the sand (before and after extraction) and the extracted mycelia.

Fungal hyphae can be extracted from the mesh bags and separated from sand particles by centrifugation and collected on a filter paper for estimates of hyphal length. This approach produced similar results as direct estimates of EMM weight as described above (Wallander et al., 2004). Estimates of hyphal lengths can be converted to biomass using conversion factors from Fogel and Hunt (1979). However, a possible problem with this method is to fully account for rhizomorphs, which are multi-hyphal organs produced by many ECM fungi during growth through soil. The rhizomorphs facilitate efficient transport of carbon towards the mycelia front and mineral nutrients towards to mycorrhizal roots (Cairney, 1992). Separate counts must be carried out for rhizomorphs and hyphae, as they differ greatly in weight per unit length.

3.2. Chemical markers; chitin

Among the three possible fungal biomarkers, chitin seems the most stable parameter to assess the total fungal contribution to microbial tissue in soil (Joergensen and Wichern, 2008). Recent results (Drigo et al., 2012; Koide et al., 2011) from additions of laboratory cultivated mycelial necromass suggest rapid decomposition of chitin in soil. Indeed, fungal cell walls of all true fungi contain chitin, a structural compound with a similar role as cellulose in higher plants. In soil, other organisms may contribute to chitin contents such as microarthropods that contain chitin in their exoskeleton. However, this contribution is probably minimal as their biomass is typically below 0.5% of the fungal biomass (Beare et al., 1997; Simpson et al., 2004). An average chitin concentration of 5% of dry matter was found in a review of various species of fungi mainly grown *in vitro* and belonging mainly to Basidiomycetes, Ascomycetes and Zygomycetes (Appuhn and Joergensen, 2006). No statistically significant difference between the mean values from the three fungal orders was found, and a conversion factor from glucosamine to fungal C of 9 was proposed (Appuhn and Joergensen, 2006).

Using data from Joergensen and Wichern (2008), we estimate that ±one standard deviation around the mean gives a span of around 6–50 of the glucosamine to C conversion factor, which suggests a rather low precision in the conversion. However, it is unknown if the variation in glucosamine content is smaller or larger when in symbiosis. In the one published study known to us, extramatrical mycelium of *Paxillus involutus* in symbiosis with *Pinus sylvestris* had a glucosamine content of 4.5% (Ekblad et al., 1998). A similar value was found in mycelium extracted from mycelial ingrowth bags that were installed in the upper-most soil horizon at the tree line in a *Larix decidua* and *Pinus uncinata* stand near Davos. These two values are close to the average for the pure cultures of the Joergensen and Wichern (2008) review.

It is also possible to measure the chitin content from the pellet left after protein, lipid or DNA extraction (Kjøller and Rosendahl, 1996; Kjøller et al., 2012). This then allows measurements of enzyme activities or molecular identity in the exact same samples that are quantified for chitin. Further studies are needed on chitin concentrations in EMM of mycorrhizal fungi when in association with roots in forest soil. However, this fungal biomarker does not enable us to distinguish between saprotrophic and ECM fungi in soil samples or to separate living and dead mycelium, although this may be possible by combining ergosterol and chitin analysis (see below).

Chitin assay is easily performed using one of two steps; (i) hydrolysis either with KOH (e.g. Frey et al., 1994) that produces deacylated chitin (chitosan), or with HCl (Appuhn et al., 2004), H_2SO_4 (e.g. Zamani et al., 2008) or methanesulfonic acid (Olk et al., 2008) that produces glucosamine, and (ii) measurement of the concentrations of hydrolysis products. Chitosan and glucosamine contents can be measured with colorimetric procedures specifically assaying amino sugars (Plassard et al., 1982). Free glucosamine can also be measured with chromatographic techniques (Ekblad et al., 1998).

3.3. Chemical markers; ergosterol

The second chemical marker that has been used to estimate fungal biomass is ergosterol (22E)-Ergosta-5,7,22-trien-3β-ol ($C_{28}H_{44}O$). This compound is a membrane lipid, found almost exclusively in membranes of living fungal cells, and is the commonest sterol of Ascomycota and Basidiomycota. As ergosterol is generally not synthesized by plants and animals, and only present in low amounts in some microalgae (Grant and West, 1986; Newell et al., 1987; Weete, 1989), it has been frequently used as fungal biomarker in soils (Djajakirana et al., 1996; Möttönen et al., 1999; Bååth, 2001; Wallander et al., 2001; Hagerberg et al., 2003; Zhao et al., 2005; Högberg, 2006; Karliński et al., 2010) and correlations with other methods are usually good (Bermingham et al., 1995; Stahl and Parkin, 1996; Montgomery et al., 2000; Ruzicka et al., 2000; Högberg, 2006). Assay of ergosterol was first employed by Seitz et al. (1977) to quantify fungal infections in stored grain. In mycorrhizal fungi, the analysis of ergosterol was first applied by Salmanowicz and Nylund (1988), but has been used frequently since then (e.g. Nylund and Wallander, 1992; Ekblad et al., 1995, 1998; Laczko et al., 2004; Olsrud et al., 2007). Total ergosterol contents in mycorrhizal roots of *P. sylvestris* plants was correlated to visual estimates of root colonization (Ekblad et al., 1995) as well as to the chitin contents (Ekblad et al., 1998). In contrast, total ergosterol concentration of ericoid hair roots of dwarf shrubs from northern subarctic mires did not correlate with visual estimates of colonization but was instead positively correlated with the colonization of dark septate endophytes, which makes it questionable as a marker for ericoid mycorrhizal fungal colonization (Olsrud et al., 2007). Some studies suggest that ergosterol is a good proxy for active fungal biomass because it was found to degrade shortly after the cells death (Nylund and Wallander, 1992), and ageing mycorrhizal root tips contain low ergosterol concentrations (Ekblad et al., 1998). However, other studies suggest a slow metabolism of ergosterol under certain circumstances, such as disruption of below ground C allocation, increased N loads, addition of toxic compounds like pesticides, or existence of substantial amounts of free ergosterol in soil for considerable periods with little mineralization (Zhao et al., 2005). Soil perturbations, that may negatively influence vitality and growth of soil fungi, resulted in disruption of the proportion between soil ergosterol concentration and soil fungal biomass C (Zhao et al., 2005) and between ergosterol and phospholipid fatty acid (PLFA) 18:2ω6,9 (Högberg, 2006). These contradictory results were further criticized and discussed by Young et al. (2006) and Zhao et al. (2006). Mille-Lindblom et al. (2004) reported very slow degradation of free ergosterol in environmental samples without living mycelium when protected from sunlight and suggested that ergosterol may be stable when connected to dead fungal mycelium.

However, significant degradation of ergosterol was observed by the authors under influence of light.

Calculations of conversion factors from ergosterol to fungal biomass have been derived from various fungi and considerable variations in ergosterol concentration in fungal mycelium were reported (Lösel, 1988; Weete, 1989; Nylund and Wallander, 1992; Djajakirana et al., 1996; Montgomery et al., 2000). The average concentration of ergosterol reported thus far for different soil, aquatic and plant inhabiting fungi is 4.5 μg mg^{-1} dry mass of mycelia, and this is used to determine fungal biomass in soil. However, the ergosterol concentration in fungal mycelium extracted from mesh bags is less (1.2 μg mg^{-1}; Hagerberg et al., 2003). This may indicate that laboratory-grown mycelia contain more ergosterol than field grown mycelia, or that mycelia from mesh bags are contaminated with non-fungal material. The average relative recovery of ergosterol from soil samples was 62%, ranging from 58 to 88% (Montgomery et al., 2000), and the recovery factor value was 1.61 (1/0.62). The authors concluded that determination of fungal biomass (*FB*) on the basis of ergosterol analysis requires correcting ergosterol concentrations by the proportion of unextracted mycelial ergosterol according to the following calculation:

$$FB\left(\mu g\, g^{-1}\ \text{soil}\right) = \text{Ergosterol}\left(\mu g\, g^{-1}\ \text{soil}\right) \times f \times Rf,$$

where $f = 250$ ($1/4 \times 1000$, mg biomass μg^{-1} ergosterol), and $Rf = 1.61$ (correction factor for average percent recovery, 1/0.62) (Montgomery et al., 2000).

Separation of ergosterol into free and esterified forms might give some additional information of the vitality of the fungal mycelium. Usually total ergosterol is quantified (Nylund and Wallander, 1992), in other cases the free form is used as a biomass marker (Martin et al., 1990). Free ergosterol is a component of the cell membranes, while the esters are found in cytosolic lipid particles. A ^{14}C-labelling study of *Saccharomyces cerevisiae* indicated that the free sterols and esters are freely inter-changeable and that relatively more esters are formed when the fungus is going into a stationary phase (Taylor and Parks, 1978). Analysis of dried fungal material suggests that the free form can also be converted into the esterified form in this material and that the esterified is more stable than the free form (Yuan et al., 2008). The majority of ergosterol from in-growth bags was found in the free form (90%), while the free ergosterol was below 20% in the mineral soil, supporting the view of increasing proportion of esterified ergosterol in older SOM (Wallander et al., 2010). The relation between free and esterified ergosterol and ergosterol and chitin (Ekblad et al., 1998) could potentially be used as markers for the ratio of active and inactive fungi in soil. This possibility would be very useful but needs to be evaluated further. One problem with analysing free ergosterol in certain soils is to get the extracts clean for chromatographic analysis (Adam Bahr, pers. comm.). Ergosterol can be easily extracted from variable materials and is detectable in low concentrations. The assay comprises of extraction, purification and quantification of the molecule using high-performance liquid chromatography with a UV detector. Young (1995) developed an efficient microwave-assisted method (MAE) to extract ergosterol from a variety of matrices, which has since been applied to soil samples (Montgomery et al., 2000).

3.4. Chemical markers; PLFAs

PLFAs are essential components of cell membranes and they decompose quickly after cell death (White et al., 1979) and are commonly used as chemical markers of soil fungi. As eukaryotes and different groups of prokaryotes contain more or less specific ester-linked lipid fatty acids (Lechevalier and Lechevalier, 1988; Zelles, 1997, 1999), the analysis of PLFA composition and concentrations are useful as a tool for quantitative and qualitative examination of microbial communities in soil (fungi, bacteria, protozoa; e.g. Tunlid and White, 1992; Cavigelli et al., 1995). However, use of PLFAs for biomass estimation has recently been questioned, because the same PLFAs are stated to indicate very different groups of organism (Frostegård et al., 2011). For instance, the PLFAs cy17:0 and cy19:0, usually considered to be indicators of Gram-negative bacteria are also found in large amounts in some Gram-positive bacteria (Schoug et al., 2008). The PLFA 16:1ω5, common in arbuscular mycorrhizal fungi (Graham et al., 1995; Olsson et al., 1995), and sometimes used as a marker of Glomeromycota fungi in soil, plant roots and external mycelium (e.g. Gryndler et al., 2006), is also found in bacteria (Nichols et al., 1986). Moreover, some environmental conditions, such as temperature or toxic soil contaminants may influence the rate of PLFA degradation, independently with the turnover of soil microorganisms (Frostegård et al., 2011).

The PLFA 18:2ω6,9 is the most commonly used PLFA to estimate fungal biomass (Wassef, 1977; Lechevalier and Lechevalier, 1988; Dembitsky et al., 1992). It occurs in all eukaryotes, and is only found in low amounts in bacteria. This PLFA is a dominating fatty acid of fungal fruit bodies (e.g. Dembitsky et al., 1992; Olsson, 1999; Karliński et al., 2007) and spores (Brondz et al., 2004). A strong positive correlation was found between PLFA 18:2ω6,9 and the fungal marker ergosterol in soils from cultivated fields, gardens, grasslands and forests (Frostegård and Bååth, 1996; Kaiser et al., 2010). The PLFA 18:2ω6,9 has been used as a bioindicator of EMM in soil (Högberg et al., 2010), but it is particularly useful in experiments where other soil fungi can be eliminated or reduced, such as when using in-grow mesh bags where ECM mycelium is preferentially trapped (e.g. Wallander et al., 2001; Hagerberg and Wallander, 2002). To convert PLFA 18:2ω6,9 to microbial carbon content, Joergensen and Wichern (2008) reported a weighted conversion factor of 107 μg C nmol $PLFA^{-1}$, but values between different species grown in culture could vary 17-fold (Klamer and Bååth, 2004). Another PLFA that is common in fungi, especially Zygomycota, is 18:1ω9 (Dembitsky et al., 1992; Ruess et al., 2002; Brondz et al., 2004). The concentration of 18:1ω9 is usually closely correlated to 18:2ω6,9 (Frostegård et al., 2011). This PLFA is, however, also present in some bacteria (Schoug et al., 2008) and has not proven useful as a fungal indicator in agricultural soils (Frostegård et al., 2011).

A faster way to analyse fatty acids in soil samples is to analyse the whole cell fatty acids (WCFAs) without separation of neutral lipid fatty acids (NLFAs) and PLFAs. WCFAs reflect both microbial biomass and energy reserves of eukaryotes and are a relatively reliable method of studying fungi (Larsen et al., 2000; Thygesen et al., 2004; Karliński et al., 2007) and mycorrhiza-associated microorganisms in the field (Brondz et al., 2004; Ruess et al., 2005; Karliński et al., 2007). The analysis of WCFA composition requires 10 times less soil material than the PLFA analysis (Drenovsky et al., 2004). Since much of the WCFA is in the form of neutral lipid fatty acids (NLFAs) in triacylglycerols, a storage compound in eukaryotes, a recorded change in WCFA of NLFAs may be a result of changes in the amount of storage C rather than a change in size of the microbial population in a soil. Incorporation of glucose into fatty acids can be used to demonstrate the high microbial activity in soils. Lundberg et al. (2001) used 'solution state' ^{13}C NMR and found that the amount of ^{13}C in fatty acids peaked 3–13 days after glucose addition to a forest soil, and that it had declined by 60% 28 days after the glucose addition. A similar result was found after extraction and analyses of NLFAs and PLFAs at different time intervals after glucose additions to various soils

(Bååth, 2003). Due to the potential for large temporal variation in storage triacylglycerols, NLFAs and WCFAs are probably less suitable than PLFAs as relative measures of the microbial biomass in soils. However, the ratio of neutral lipid fatty acids (NLFAs) and PLFAs was proposed as a method to study the physiological state of the microbial population in the soil (Bååth, 2003).

The analytical procedure for PLFAs and NLFAs comprises four steps: (i) extraction of lipids, (ii) lipid fractionation, (iii) mild alkaline methanolysis, and (iv) GC analyses (White et al., 1979; Frostegård et al., 1991). Recently, the lipid fractionation was modified slightly by Dickson et al. (2009), who reported that the replacement of pure chloroform by the mixture chloroform: acetic acid (100:1, v/v) increased the effectiveness of NLFAs elution from the silica columns and eliminated an interference of NLFAs with glycolipid and phospholipid fractions. Following hydrolysis, their fatty acids (FA) are released and detected using gas chromatography (GC). PLFA analyses should be done as soon as possible after sampling since the composition may change even when stored at low temperatures (Wu et al., 2009). The best strategy is to shock-freeze the samples with liquid nitrogen and further storage at −18 °C until analysis. Homogenization of soil samples using a ball mill to a particle size less than 10 μm prior to analysis has been recommended to achieve the most reliable results (Wilkinson et al., 2002).

3.5. Comparison of chemical markers

It is clear that each of the chemical markers described will bring different information about the fungal biomass, whether total or active. Each of them has advantages and limitations (Tables 1–3). Chitin and ergosterol assays are easier to carry-out than fatty acid (PLFAs or WCFAs) extraction, but fatty acid profiles will bring more information about microbial communities than chitin and ergosterol. On the other hand, the PLFA method is more rapid and less expensive than methods based on nucleic acids (Ramsey et al., 2006; Frostegård et al., 2011). However, none of these chemical markers will enable us to distinguish between fungal types (ECM versus non-mycorrhizal fungi) that can be present in forest soil samples. To distinguish between these "functional" types, molecular analysis (see below) should be used. Biomass estimates when using biomarkers, such as ergosterol, chitin and PLFAs, are highly dependent on the use of conversion factors. Different fungal species vary in concentrations of such biomarkers, but the biomarker to biomass ratio is probably more stable in a more complex community. The concentration of ergosterol in pure cultures of ECM fungi ranged between 1.8 and 17.6 mg g^{-1} d.wt. (Nylund and Wallander, 1992; Olsson et al., 1995) and concentration of PLFA 18:2ω6,9 ranged from 0.45 to 12 μmol g^{-1} d.wt. (Olsson et al., 2003). The content of the WCFA 18:2ω6,9 was reported as 17–75% of total WCFAs in fruit bodies and as 53–71% of total WCFAs in axenic cultures of ECM fungi (Karliński et al., 2007). Biomarker concentrations may reflect both the biomass and community composition of fungi. In addition, concentrations in a single species can change due to different environmental conditions as were reported for wood-rotting basidiomycete isolates grown in different soils (Tornberg et al., 2003) and ageing, as shown for ergosterol concentrations in the basidiomycete *Hebeloma cylindrosporum* (Plassard et al., 2000), and for ergosterol and fatty acids in pure culture of ECM fungus *Pisolithus tinctorius* (Laczko et al., 2004).

3.6. Potential of qPCR for the quantification of EMM biomass

In addition to the lipidic or polysaccharidic markers to quantify the biomass of fungi, the developments of quantitative PCR (qPCR) seem to offer a possible taxon-based alternative. The strength of DNA (or RNA) based methods is that potentially any phylogenetic level from genotypes to large groups or even total (true) fungi can be targeted (Fierer et al., 2005; Šnajdr et al., 2011). Indeed, methods to quantify general fungi or basidiomycetes have been proposed and tested (Fierer et al., 2005; Manter and Vivanco, 2007; Feinstein et al., 2009). A single species laboratory study comparing quantification of *Trametes versicolor* in wood based on chitin content, ergosterol, wood mass loss, and qPCR, showed reasonable correlations with more discrepancies occurring only with older cultures (Eikenes et al., 2005). There are currently two main limitations of the methodology: nucleic acid extraction bias and the differences in target occurrences per unit DNA or biomass. Different methods of nucleic acid extraction yield not only different quality of DNA and RNA but also different proportions of microbial taxa in the extracts (Feinstein et al., 2009). The success of qPCR rapidly decreases with fragmentation of nucleic acids, resulting in lower counts of target sequences per unit DNA. If a treatment is imposed that alters the extractability of nucleic acid or if different soil types are to be compared, this may influence the qPCR success. For the most frequently used target sequence of fungi-specific qPCR – the rDNA cassette – significant differences in copy number per genome were recorded, ranging from 10 to 200 in different species (Garber et al., 1988; Maleszka and Clark-Walker, 1990; Corradi et al., 2007; Amend et al., 2010), which adds another important source of bias. With the advance of fungal population genomics (five ECM species sequenced to date; see the website of JGI (http://genome.jgi-psf.org/) and Martin et al., 2008, 2010) in the future it may be possible to identify a universal single copy gene with adequate sequence variation for counting fungal genomes rather than rDNA copies or for delimitation of certain fungal taxa. Population genomics also brings even greater potential to test hypotheses concerning the contribution of particular genotypes to ECM fungal biomass and turnover (Johnson et al., 2012).

When qPCR specifically targets individual species of fungi, PCR-based abundance estimates represent a plausible proxy of fungal biomass content because the numbers of rDNA copies do not show high variation within a species (Amend et al., 2010). Analyses of individual fungi including *Suillus bovinus*, *P. involutus* and *Hypholoma fasciculare* in the DNA from complex samples showed that it is possible to use qPCR to specifically quantify the biomass of fungi at the species level within a community. Such data are comparable to the much more laborious or expensive approaches like cloning, pyrosequencing or DGGE approaches (Landeweert et al., 2003; Parladé et al., 2007; Šnajdr et al., 2011). Competitive PCR (a variant of qPCR) was used to demonstrate that *Hebeloma cylindrosporium* biomass in bulk soil is greatest near fruit bodies (Guidot et al., 2002). A conversion factor between qPCR-based copy number and fungal biomass and hyphal length was obtained for laboratory cultures of the ECM fungus *Piloderma croceum* showing its potential to quantify the biomass of particular species (Raidl et al., 2005). Unfortunately, due to the appearance of ECM fungi in multiple phylogenetic lineages, the finding of suitable primers to specifically amplify ECM fungal DNA and to distinguish it from non-ECM fungi is highly improbable. However, if combined with the cloning approaches or next generation sequencing, qPCR may provide estimates of ECM fungal biomass in soils. Contemporary next generation sequencing results showed that, at least in certain forest soils, fungal communities are dominated by relatively few species (Buée et al., 2009; Baldrian et al., 2012). These findings suggest that qPCR can be used to target specifically the identified dominant members of the community as an estimate of ECM fungal biomass. Recently, qPCR used for analysis of environmental samples has been expanded from the quantification of DNA towards the quantification of RNA, typically the rRNA, representing microbial ribosomes or ITS sequences in unspliced transcripts of the rDNA

operon. Although it is unknown whether the DNA or the RNA content better corresponds with the quantity of fungal biomass, it is clear that the analysis of ITS sequences in the non-spliced rDNA transcripts (indicating fungal taxa synthesizing their ribosomes) is more suitable to quantify the active part of the fungal community (Anderson and Parkin, 2007). Indeed, decomposers in spruce logs or fungi active in soil in winter with limited photosynthate allocation have been specifically identified by combining DNA and RNA analysis (Rajala et al., 2011; Baldrian et al., 2012).

4. Indirect estimation of length, space occupation and biomass of extramatrical mycelium of ectomycorrhizal fungi

Agerer (2001) proposed a classification of ECM mycelial systems into five exploration types. Accurate determination of EMM production and abundance of different exploration types within ECM communities may be used to estimate the overall production of EMM. The exploration types are described according to their pattern of differentiation, indicating their different ecology: contact type (CT), short distance (SD), medium distance (MD), long distance (LD) and pick-a-back (PB) exploration type. The exploration types have been differentiated based on about 400 different morphotypes of ectomycorrhiza, which have been identified as belonging to different fungal species on several host plant roots based on their morphological and anatomical characteristics (Agerer and Rambold, 2004–2011). The characterized ECM morphotypes represent about 5% of known fungi that can form ectomycorrhiza (Taylor and Alexander, 2005), the number of which is estimated to be 5000–6000 fungal species (Agerer, 2006). From this limited database, it appears that in many genera all known species produce only one exploration type (Agerer, 2001; Hobbie and Agerer, 2010), although some genera (i.e. *Russula* spp.) need species-based classification into an exploration type (Table 4).

An estimation of EMM of ECM fungi in natural soils could be deduced from semi-quantitative estimations of the EMM formed by SD and MD exploration types grown in rhizotrons in symbiosis with Norway spruce (Agerer and Raidl, 2004). The observations in rhizotrons have lately included other MD subtypes and LD exploration types (Weigt et al., 2011), and indices of specific space occupation, mycelial length and biomass were proposed for each exploration type. Mycelial biomass was estimated based on length measurements using calculations described in Weigt et al. (2011); the standard values for the selected exploration types are presented in Table 5. These standards for the most frequent exploration types, expressed as biomass and occupied space of EMM per unit of ECM system, are suggested as basic factors for characterizing mycelial production costs and space occupation in ecological field studies without any extraction of mycelium and for fungal communities in the soils. Since different exploration types show not only differences in distance of EMM from the root tip, space occupation, biomass and energy (C) inputs, but also in other functional relationships within the ecosystem, the ECM fungal community structure and function can be extrapolated. The differentiation into exploration types can be extrapolated from morphotype characterization based on outer morphology of ectomycorrhiza, rhizotron photographs, and fungal species identifications, using molecular based methods of fungal community composition (Grebenc and Kraigher, 2009), in which fungal species identity is linked to growth characteristics and assigned to a certain exploration type.

Indices, such as specific potential mycelial space occupation ($mm^2\ cm^{-1}$ ECM tip^{-1}), specific EMM length ($m\ cm^{-1}$ ECM tip^{-1}), specific EMM biomass ($\mu g\ cm^{-1}$ ECM tip^{-1}) can be developed for each exploration type. The specific contribution to EMM by exploration types can be achieved for cultivable and non-cultivable species, and up-scaling of cost–benefit relations is possible (Weigt et al., 2011). The method provides an estimation based on ECM fungi synthesized in experimental laboratory conditions, i.e. on prepared soil substrates, which can influence EMM growth in different exploration types. Therefore, for the calculations presented in Table 5 a number of assumptions had to be made, including i) that growth conditions concerning mycelial growth and space occupation in experimental substrates was similar to

Table 4
Representative fungal genera belonging to different exploration types (summarized from Agerer, 2001; Agerer and Rambold, 2004–2011; Agerer, 2006).

Exploration type	Morphology/anatomy	Fungal genus[a]
Contact	Smooth mantle, only few emanating hyphae, ECM tips in close contact with substrates	*Arcangeliella*, *Balsamia*, *Chroogomphus*, *Craterellus*,[b] *Lactarius*,[c] *Leucangium*, *Russula*, *Tomentella*
Short distance	Voluminous envelope of emanating hyphae, no rhizomorphs	*Acephala*, *Byssocorticium*, *Cenococcum*, *Coltricia*, *Coltriciella*, *Craterellus*,[b] *Descolea*, *Descomycetes*, *Elaphomyces*, *Genea*, *Hebeloma*, *Humaria*, *Hygrophorus*, *Inocybe*, *Pseudotomentella*, *Rhodocollybia*, *Rozites*, *Russula*, *Sebacina*, *Sphaerosporella*, *Sphaerozone*, *Tomentella*, *Tricharina*, *Tuber*, *Tylospora*
Medium distance: fringe subtype	Fans of emanating hyphae and rhizomorphs, frequent ramifications and anastomoses, rhizomorph surfaces hairy, extended contact to the soil; rhizomorphs type A,[e] exceptionally C,[e] D[e]	*Amphinema*, *Cortinarius*, *Dermocybe*, *Hydnum*, *Lyophyllum*, *Piloderma*, *Sistotrema*, *Stephanopus*, *Thaxterogaster*, *Tricholoma*
Medium distance: mat subtype	Limited range, rhizomorphs undifferentiated or slightly differentiated type A,[e] C,[e] exceptionally D[e]	*Bankera*, *Boletopsis*, *Clavariadelphus*, *Cortinarius*, *Gautieria*, *Geastrum*, *Gomphus*, *Hydnellum*, *Hysterangium*, *Phellodon*, *Ramaria*, *Sarcodon*
Medium distance: smooth subtype	Rhizomorphs internally undifferentiated, slightly differentiated or with a central core of thick hyphae. Mantles smooth with no or only a few emanating hyphae. Rhizomorphs type B,[e] C[e] and D,[e] exceptionally E[e]	*Albatrellus*, *Amanita*,[d] *Byssoporia*, *Cantharellus*, *Entoloma*, *Gomphidius*, *Hygrophorus*, *Laccaria*, *Lactarius*, *Naucoria*, *Polyporoletus*, *Pseudotomentella*, *Russula*, *Thelephora*, *Tomentella*, *Tomentellopsis*
Long distance	Smooth mantle with few but highly differentiated rhizomorphs type F.[e] ECM sparsely monopodially branched, coralloid and tuberculate.	*Alpova*, *Amanita*,[d] *Austropaxillus*, *Boletinus*, *Boletus*, *Chamonixia*, *Gyrodon*, *Gyroporus*, *Leccinum*, *Melanogaster*, *Paxillus*, *Pisolithus*, *Porphyrellus*, *Rhizopogon*, *Scleroderma*, *Suillus*, *Truncocolumella*, *Tricholoma*, *Tylopilus*, *Xerocomus*
Pick-a-back	Grow within F[e]-type rhizomorphs or mantels, can produce haustoria, can become ectendomycorrhizal. Can form contact, or smooth medium distance type.	*Gomphidiaceae* (*Gomphidius*, *Chroogomphus*) growing with *Suillus* or *Rhizopogon*; *Boletopsis leucomelaena* within unknown ECM; *Xerocomus parasiticus* within *Scleroderma citrinum*

[a] In case of controversial issues genus was categorized to exploration types according to Agerer and Rambold (2004–2011).
[b] *Craterellus tubaeformis* forms contact exploration types on *Quercus* but short distance exploration types on *Pinus*.
[c] Underlined genera have representatives in more than one exploration types.
[d] *Amanita citrina* on *Pinus* can form medium distance and long distance exploration types.
[e] The type of rhizomorphs according to Agerer (1987–1998).

Table 5
Characteristics of EMM length, space occupation and biomass for different exploration types (modified after Weigt et al., 2012a,b).

Exploration type	No. of analysed mycelia	Max. distance from root tip (cm)	Projected area per mycelial system (mm^2)	Mycelial coverage per occupied space ($mm^2\ mm^{-2}$)	Specific EMM length ($m\ cm^{-1}$ ECM tip^{-1})	Specific EMM biomass[a] ($\mu g\ cm^{-1}$ ECM tip^{-1})
Short distance	7	1.2	33 ± 9	0.39 ± 0.07	3.72 ± 1.19	3.24 ± 1.03
Medium distance	14	1.9	84 ± 5	0.58 ± 0.05	6.91 ± 0.54	6.02 ± 0.47
Long distance	3	9.6	630 ± 181	0.28 ± 0.03	55.91 ± 20.25	48.67 ± 17.62

[a] Mycelial biomass was estimated based on length measurements using calculations described in Weigt et al. (2011) combining the formula $B = r^2\pi^*L^*D^*M$ (Frankland et al., 1978), where B = fungal biomass, r = hyphal radius, L = hyphal length, D = relative hyphal density, M = % dry mass = (100 − mycelial moisture content as % of fresh weight)/100. $r^2\pi^*L$ = hyphal biovolume (assuming hyphae to be perfect cylinders) with r based on the species-specific hyphal diameter (in their study it was 2.2 μm for *Piloderma croceum*, deduced from Brand, 1991; Raidl, 1997). L was measured using WinRhizo. $D = 1.09\ g/cm^{-3}$ and $M = 21\%$ following (Bakken and Olsen, 1983) conversion of hyphal volume into biomass with $D^*M = 0.2289$ g dry mass cm^{-3}.

natural soils, ii) no competition or facilitation among mycelia of different fungi has been included, and iii) no site-related growth conditions have been addressed, and several calculation-based assumptions had to be defined (see the explanation at Table 5). However, the proposed exploration type specific standard values may provide a suitable tool for quantification of space occupation, biomass and energy trade-offs of EMM in natural soils. A combination of a further development of the database with descriptions of ECM fungi (Agerer and Rambold, 2004–2011) and functional relationships of different exploration types, grown, observed and assessed in different growth conditions, will contribute to an increasing understanding of the complex belowground mycelial interactions, cost–benefit relations and trade-offs in belowground competition or facilitation.

5. Assessment of turnover rates

Accurate estimates of the turnover of EMM are essential in order to evaluate the role of mycorrhizal fungi in the C cycle. This requires understanding of both the rate of production and decomposition of mycorrhizal mycelium. Sequential harvesting of EMM in mesh bags may be one way to estimate turnover rates, but there seems to be a lag-phase before EMM enter the mesh bags (Fig. 1). However, the lag-phase is probably dependent on the level of disturbance caused by the installation and further tests with bags of different sizes and sampling frequency are needed to evaluate the applicability of this method. Pulse labelling with ^{13}C or ^{14}C has been applied to estimate turnover in arbuscular mycorrhizal mycelium (Staddon et al., 2003; Olsson and Johnson, 2005) but not for estimates of ECM mycelium. One of the problems with isotope techniques is the risk of differences in labelling of different chemical components of the fungal biomass, some of them having a high turnover rate (respiratory substrates), while the heavy isotope will have longer residence time in structural cell materials (Dawson et al., 2002). Analyses of the turnover rate of specific components, such as chitin, may be a way to overcome this. Another issue concerns the collection of sufficient and representative amounts of ECM mycelium for isotopic analysis.

The difference in natural abundance $\delta^{13}C$ between C_3 and C_4 organic matter has been used in studies of the turnover of plant and microbial substances in soils. In these analyses a combination of pyrolysis–gas chromatography and isotope ratio mass spectrometry was used (Gleixner et al., 1999, 2002). The combination of ingrowth cores with a mesh allowing only hyphae or allowing both roots and hyphae filled with C_4 dominated soils have been used to estimate the contribution of mycelia and roots to the formation of stable SOM (Godbold et al., 2006; Wallander et al., 2011). A critical factor when using differences in natural abundance of ^{13}C is to have reliable $\delta^{13}C$ values of the end-members, e.g. mycelial and plant residues. Similar to pulse labelling techniques, differences in isotopic signature between various components within the plant and fungal materials may be a potential problem that should be considered. For example, chitin is depleted in both ^{13}C and ^{15}N compared to the total fungal biomass (Dijkstra et al., 2006). The EMM in the top soil was depleted in ^{15}N with around 5‰ compared to mycorrhizal fruit bodies in a Norway spruce site (Wallander et al., 2004), possibly reflecting lower chitin and higher protein contents in fruit bodies compared to mycelia.

We are not aware of any study that has exploited the possibility to estimate the production and turnover of mycelium in FACE-experiments (Free Air Carbon dioxide Enrichment). Sequential installation and harvest of in-growth bags in connection with the initiation or termination of CO_2 treatments should offer ideal periods to estimate the production and turnover of mycelia biomass. During these periods there are drastic shifts in the ^{13}C signal of the photosynthates (given that the CO_2 that is used to treat the plants has a different $\delta^{13}C$ than the atmosphere, which is the case if fossil C has been used to produce the CO_2). However, these experiments do not have corresponding plots that are isotopically-enriched at ambient CO_2 concentrations, and so exploiting FACE facilities would only be useful to estimate turnover under elevated CO_2 conditions.

In contrast to other types of organic inputs to soils, surprisingly little is known about the decay rate of mycorrhizal mycelium. Mesh bags of the type normally used to assess leaf litter decomposition have been used recently to demonstrate that the N concentration of hyphae explained a large part of the mass loss during the initial 4 weeks of decay (Koide and Malcolm, 2009). An alternative method is to capture and quantify CO_2 produced when hyphae are added to micro-respirometers. This approach was used to show that ECM fungal hyphae rapidly stimulated CO_2 efflux but that the effect was dependent on the species richness of the hyphae entering soil (Wilkinson et al., 2011a). Thus species richness of ECM fungi can be important both for maintaining productivity (Wilkinson et al., 2011b) and in regulating their own decomposition. These findings indicate that decomposition of ECM hyphae may be a key pathway by which C rapidly enters the saprotrophical microbial biomass in soil. The application of stable isotope probing (Radajewski et al., 2000) in which ECM fungal hyphae is enriched in ^{13}C has recently been used to demonstrate the rapidity of C incorporation into free-living soil fungi via this pathway (Drigo et al., 2012). Despite these recent advances, there is scope for considerably more research quantifying the rate of decay of different genotypes, species and morphologies of ECM fungal hyphae under a range of environmental conditions.

6. Importance of sampling design

Regardless of the effort placed in developing reliable methods to quantify production, biomass and turnover of ECM fungi, the utility of the resulting data is often dependent on the sampling design used to obtain the data in the first place. Moreover, it is often desirable to obtain similar datasets from a wide-range of different

ecosystems and habitats, particularly from a modelling perspective. This requires sampling approaches that have similar ability to quantify spatial variation in EMM abundance and biomass. Yet very few investigations employ spatially-explicit sampling strategies designed to deal with the often vast heterogeneity of EMM production in forest systems. This is in part because variation is likely to occur at a wide range of spatial scales; recent work in Douglas fir stands have demonstrated that genets of *Rhizopogon* spp. could form common mycelial networks connecting individual trees within a 30 × 30 m area (Beiler et al., 2010). In contrast, there is also clear evidence that ectomycorrhizas and their associated mycelium can form patchy clusters at scales of just a few cm (Guidot et al., 2002), perhaps due to their plasticity in responding to inputs of nutrient-rich substrates (Bending and Read, 1995). Moreover, spatial variation occurs in three dimensions. Only rarely is quantification of abundance and biomass of either ectomycorrhizal roots or EMM undertaken at multiple depths. Among surface soil horizons in a Swedish boreal forest, ECM fungi tended to be associated with slightly older partially-decomposed organic matter (Lindahl et al., 2007). In the UK, detailed analyses of the vertical distribution of 7 species of ectomycorrhizas and their EMM in a Scots pine stand showed contrasting vertical distribution patterns from 0 to 20 cm (Genney et al., 2006). The EMM of some species like *Cadophora finlandia* was distributed quite evenly with depth while the EMM of *Cortinarius* spp. was concentrated in the upper 10 cm (Genney et al., 2006). This study also demonstrated unequal distribution of the EMM of many species at 2 cm intervals. Geostatistical techniques (Legendre and Legendre, 1998) have recently been applied to provide rigorous analysis of the temporal and spatial patterns of ectomycorrhizas (Lilleskov et al., 2004; Pickles, 2007). For example, Pickles (2007) sampled 48 cores at increasing distances in a 20 × 20 m area to determine when the abundance of key common species showed spatial autocorrelation. Subsequent more intense sampling events (217 cores) in the same location exploited this information and used regular distances of either 1 or 2 m as the primary separation distance to avoid issues with spatial autocorrelation, and to provide detailed interpolated maps of species' abundance (Pickles et al., 2010). The use of geostatistical tools therefore requires an initial high investment in sampling units, but can reap benefits later once optimum sampling distances are identified. Moreover, obtaining data on spatial autocorrelation enables more meaningful inter-site comparisons and so this is an approach we advocate in future studies.

7. Conclusions

Although significant progress has been made over the last ten years in our understanding of the importance of the ECM fungal mycelium in C cycling in ecosystems, our understanding is still highly fragmented. In this paper we have summarized the state of the art in this subject as well as the strengths and weaknesses in the methods and techniques applied. Our aim is that this information will ultimately enable researchers to obtain valuable data on the production, biomass and turnover of mycorrhizal mycelium in all biomes, and modify the approaches outlined here for arbuscular and ericoid mycorrhizal systems. Such data are likely to be essential for improving process-based models of terrestrial biogeochemical cycles that currently ignore the distinct role played by mycorrhizal fungi. This may improve their potential to predict nutrient leaching and carbon sequestration. Moreover, these data could also be incorporated into spatially-explicit modelling frameworks of population dynamics.

All of the applied methods and techniques have their own sets of limitations which the users of these methods should consider before applying them (Tables 1–3). To combine several techniques in the same study, e.g. chemical markers and isotope labelling, may be a way to overcome some of these limitations. An issue that needs more attention is the turnover of EMM, especially the turnover of diffuse mycelium versus rhizomorphs. The ratio between free and total ergosterol, and the ratio between chitin and ergosterol as an indicator of the necromass/biomass ratio may be useful in such experiments and deserves further studies. Also, it could be useful to develop methods enabling us to quantify specifically the level of ^{13}C enrichment of C in glucosamine residues. Combined to environmental variation of carbon sources available to the ECM fungi (e.g. in FACE experiment using enriched or depleted $^{13}C–CO_2$ sources), such a method could fill the gap regarding the actual rate the turnover of ECM fungi in forest soils.

Indices, such as specific EMM length or specific EMM biomass, developed for different exploration types, can be used for indirect estimations of the C costs of growth and storage in ECM fungal mycelium. The utility of such indirect measures are greatest providing the ECM fungal community structure is known, that the identified species belong to different exploration types, and these show different space occupation, mycelial length and biomass.

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Growth of ectomycorrhizal fungal mycelium along a Norway spruce forest nitrogen deposition gradient and its effect on nitrogen leakage

Adam Bahr [a,*], Magnus Ellström [a], Cecilia Akselsson [b], Alf Ekblad [c], Anna Mikusinska [c], Håkan Wallander [a]

[a] Lund University, Microbial Ecology, Dept of Biology, SE-223 62 Lund, Sweden
[b] Lund University, Dept of Physical Geography & Ecosystems Science, SE-223 62 Lund, Sweden
[c] Örebro University, School of Science & Technology, SE-701 82 Örebro, Sweden

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ABSTRACT

Almost all boreal and temperate forest tree species live in symbiosis with ectomycorrhizal fungi (EMF); the trees transfer carbon (C) to the fungi in exchange for nutrients and water. Several studies have shown that experimental application of inorganic nitrogen (N) represses production of EMF extramatrical mycelia (EMM), but studies along N deposition gradients are underrepresented. Other environmental variables than N may influence EMM production and in this study we included 29 thoroughly monitored Norway spruce stands from a large geographical region in Sweden in order to evaluate the importance of N deposition on EMM growth and N leaching in a broader context. It was concluded that N deposition was the most important factor controlling EMM production and that the amounts typically deposited in boreal and boreo-nemoral regions can be sufficient to reduce EMM growth. Other factors, such as phosphorus status and pH, were also correlated with EMM production and should be considered when predicting EMM growth and N leaching. We also showed that EMM production substantially contributed to the C sequestration (320 kg ha^{-1} yr^{-1}), suggesting that it should be included in C cycle modelling. Furthermore, EMF are probably important for the N retention capacity since high N leaching coincided with low EMM growth. However, it was not possible to differentiate between the effects of EMF and the direct effect of N deposition on N leaching in the present study.

1. Introduction

Almost all boreal and northern temperate forest tree species live in symbiosis with ectomycorrhizal fungi (EMF). The EMF are dependent on photoassimilated carbon (C) allocated by the trees. In exchange, the EMF provide the trees with nutrients and water that are efficiently assimilated from the soil solution by extensive hyphal networks of EMF (Smith and Read, 2008), often referred to as extramatrical mycorrhizal mycelia (EMM). Nitrogen (N) is one of the most important nutrients for primary production and has a substantial impact on forest growth globally (LeBauer and Treseder, 2008), and is generally the limiting nutrient in temperate and boreal forests (Vitousek and Howarth, 1991). Several studies have shown that the net primary production of boreal forests was enhanced by N deposition or N fertilization (Bergh et al., 2008; Brockley, 2010; Jacobson and Pettersson, 2010), while excess N has been observed to have a negative influence on EMF biomass, growth and colonization in pot/microcosm studies (e.g. Beckjord et al., 1985; Wallander and Nylund, 1992; Arnebrant, 1994; Runion et al., 1997) as well as in field studies (e.g. Arnebrant and Söderström, 1992; Nilsson and Wallander, 2003; Nilsson et al., 2007; Högberg et al., 2011; Kjøller et al., 2012). Only a few studies have reported no effects, or even a positive effect, of N addition on EMM (reviewed by Wallenda and Kottke, 1998). Most of the field experiments on the effect of N addition on EMM production have been designed to examine the consequences of fertilization, with additions of about 50–100 kg ha^{-1} y^{-1} (e.g. Nilsson and Wallander, 2003; Wallander et al., 2011), while only a few have investigated the effect of the N added by atmospheric deposition (Nilsson et al., 2007; Kjøller et al., 2012). Even though deposition of N can reach such high levels as 50–100 kg ha^{-1} y^{-1} in some Central European areas, remote forests at higher latitudes receive considerably less (reviewed by Hyvönen et al., 2007). The mean regional deposition of inorganic N in Swedish forests during the period 2003–2007 ranged from about 2 to 16 kg ha^{-1} y^{-1} (Karlsson et al., 2012), with a declining gradient extending from the south-west to the seemingly unaffected northern part (Akselsson et al., 2010). Nilsson et al. (2007) found tendencies of reduced EMM growth due to N deposition

* Corresponding author.
E-mail address: adam.bahr@biol.lu.se (A. Bahr).

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(10–20 kg ha^{-1} y^{-1}, according to MATCH model) in oak forests in south west Sweden, but the effect was only tested in two different N deposition categories without any direct measurements of deposited N. To make accurate estimations of the effects of N deposition it is necessary with continuous direct measurements of the canopy throughfall, since it can be highly variable in both space and time in wooded ecosystems (Levia and Frost, 2006). An example of the spatial variation is seen in a recent study by Kjøller et al. (2012), who found a dramatic reduction in EMM and EMF colonised root tips with increasing N along a short distance (90 m) N deposition gradient in a Norway spruce (*Picea abies*) forest. However, the N deposition was intense (27–43 kg ha^{-1} y^{-1}) since the gradient was stretching from a forest edge facing a poultry farm and a mink farm. Moderate N deposition (0.27–2.44 kg ha^{-1} 60 d^{-1}) has been found to affect the composition of the EMF community (Lilleskov et al., 2002), but there is still a lack of studies of how moderate N deposition affects EMM production. In this study we approached that gap of knowledge by analysing production of EMM in Norway spruce forests exposed to a moderate N deposition gradient (1–25 kg ha^{-1} y^{-1}), typical for most boreal and boreo-nemoral forests regions. The study area had a large geographical distribution and included continuous sampling of the canopy throughfall at all sites. Further, the sites were thoroughly monitored for other variables than N, allowing us to test if inclusion of some of these would improve the prediction of EMF growth.

Enhanced N leaching has been observed regularly after anthropogenic disturbances (e.g. clear-cuttings, reviewed by Gundersen et al., 2006) or natural disturbances, such as storm felling (Legout et al., 2009; Akselsson et al., 2010). N mineralization rates increase due to degradation of residues and higher soil temperature, which together with reduced root assimilation leads to accumulation of NH_4^+ and increased nitrification (Fisk and Fahey, 1990; Gundersen et al., 2006). Nitrate (NO_3^-) leaching from clear-cut areas in southern Sweden has been found to be positively correlated with N deposition (Löfgren and Westling, 2002; Akselsson et al., 2004, 2010) and to remain at elevated levels for up to five years after the disturbance (Westling et al., 2004). Although most boreal forests are N limited, enhanced N losses could occur even in undisturbed stands if the N addition rate exceeds the N retention capacity of the ecosystem (Aber et al., 1998). Increased N leaching has been found to occur when the canopy N throughfall is above 10–15 kg ha^{-1} yr^{-1} (Dise and Wright, 1995; Kristensen et al., 2004; Gundersen et al., 2006). Leaching of N in forest ecosystems is most often correlated with the soil C:N ratio (Högbom et al., 2001; Nohrstedt, 2001; Kristensen et al., 2004). However, N leaching is highly variable within a broad range of soil humus C:N ratios (of about 20–30; Gundersen et al., 1998) and Högberg et al. (2011) suggested that the activity of EMF might be a better indicator of N retention in forest soil than the C:N ratio, since the growth of EMM recovered concurrently with more efficient N retention after the termination of N fertilization, while the C:N ratio did not. A reduction in EMM, resulting from a change in C allocation by the host plant, would hypothetically enhance N leaching due to a reduction in the efficiency of assimilation (Aber et al., 1998). Coincidence between abundant EMM and low N leaching has been found in an oak forest deposition gradient by Nilsson et al. (2007) but further studies are needed to interpret the specific effect and role of EMM on N leaching (Nilsson et al., 2007; Kjøller et al., 2012).

Since as much as 10–50% of the belowground allocated C can be transferred to EMF (Simard et al., 2002), it constitutes a potentially important C sink. The different isotopic composition of C4 and C3 plants have been used to quantify the carbon flux in soil (Kuzyakov and Domanski, 2000). Wallander et al. (2011) used this approach to estimate an annual C sequestration of 300–1000 kg C ha^{-1} by EMM in young Norway spruce forests. There is, however, still a lack of knowledge regarding the turnover of EMM and the role of EMF necromass in the formation of soil organic matter. Further research within this area is of importance to improve our understanding of the role of EMF in the C cycle and enable us to make better predictions of the consequences of fertilization and anthropogenic emissions in boreal forests. Belowground C allocation is dependent on many other factors than N availability, such as a deficiency of mineral nutrients closely associated with photosynthesis. Deficiencies of potassium (K), magnesium (Mg) and manganese (Mn) typically lead to a reduction in belowground C allocation, while the opposite is seen with nutrients such as N, phosphorus (P) and sulphur (S), which are more involved in the production of new plant tissue (Ericsson, 1995). Such changes in C allocation patterns will have influences on EMM production, which may also be affected by other environmental variables, such as precipitation levels (Sims et al., 2007). It is, therefore, important to include other variables apart from N when making predictions of EMM growth and its consequences for C sequestration and nitrogen leaching.

The aim of this study was to predict EMM production and N leaching in Norway spruce forests in the south part of Sweden, by including more than 50 environmental variables, as well as detailed measurements of N deposition. The hypotheses investigated were: (1) N deposition is the most important variable affecting EMM production and N leaching; and (2) improved prediction of EMM growth and N leaching will be obtained by including needle and soil chemistry in the model, in such a way that low P levels are positively related to EMM growth while low K, Mg and Mn levels are negatively related to EMM growth, and that N leaching is negatively correlated with EMM production.

2. Material and methods

2.1. Study sites

The project took place from May 2009 until October 2010 at 29 Norway spruce forest locations in southern Sweden, stretching from the county of Skåne in the south to the northernmost site in the county of Värmland (Fig. 1). The sites belong to the Swedish

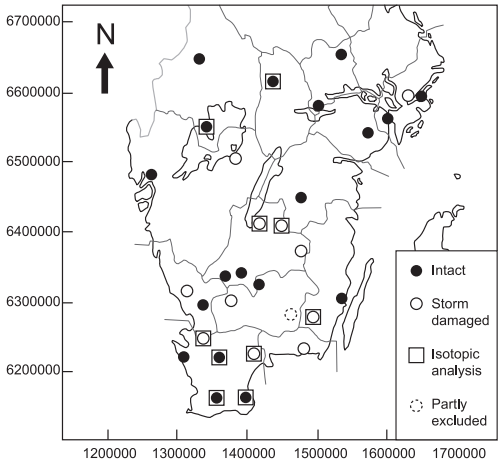

Fig. 1. Location of the field sites in the southern part of Sweden. RT90 coordinates are given.

Throughfall Monitoring Network (SWETHRO, Pihl-Karlsson et al., 2011), which is a subset of the ICP Forests (International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests), run by the IVL Swedish Environmental Research Institute and the Swedish Forest Agency. The survey covered all of the SWETHRO Norway spruce forest sites located south of the Dal River. The sites in this region represent most of the national variation in soil water N, N deposition and N accumulation (Hallgren Larsson et al., 1995; Akselsson et al., 2010). The stand age varied from 50 to 109 years (mean value = 68 years, SE = 3.1) and the soil types were sandy podzol at different stages of development, with a bedrock consisting mainly of igneous rock (Akselsson et al., 2010). Twelve of the sites had been damaged by the hurricane Gudrun that hit southern Sweden in 2005 (denoted 'Storm damaged' in Fig. 1). Eleven of those sites were classified as storm damaged by Hellsten et al. (2009) and one additional site was classified as storm damaged due to the presence of uprooted trees and cleared areas within the plot. One of the sites was excluded from soil water analyses since three of the lysimeters were positioned in an open, recently wind thrown area bordering the plot. The SWETHRO monitoring was terminated at two of the sites at the end of 2009 and thus no data were available for the 2010 analysis. Totally, in 2009, 29 sites were included in throughfall correlations and 28 in soil water correlations, and in 2010 these numbers were reduced to 27 and 26 sites (Fig. 1).

2.2. Environmental variables

At each forest site, canopy throughfall and soil water were monitored continuously (Pihl-Karlsson et al., 2011) together with analyses of soil and needle chemistry (descriptive statistics are given in Table 1). The environmental variables included in this study are listed in Table 2. Monitoring took place along two borders or the diagonals of a 30 × 30 m plot positioned within a homogeneous forest stand (Pihl-Karlsson et al., 2011). Monthly throughfall was collected in 10 aluminium foil covered polyethylene bottles with funnels (Ø = 15.5 cm) amended with a mesh filter or, during winter, in buckets (Ø = 15.5 cm). Soil water samples were collected at 50 cm depth three times a year (before, after and during the growing season) using 5 ceramic cup suction lysimeters after two days of soil water suction. The 10 throughfall samples and the 5 soil water samples from each site on each occasion were pooled before analysis. Further details regarding sampling and chemical analyses are described by Hallgren Larsson et al. (1995) and Pihl-Karlsson et al. (2011).

Table 1
Overview of the values of the explanatory environmental variables analysed. Values of canopy throughfall and soil water are based on mean annual values during the two years, while humus and needle chemistry are based on the most recent analyses.

	Variable	Range	Mean	SD
Throughfall	NO_3–N (kg ha^{-1})	0.46–11.0	3.09	0.53
	NH_4–N (kg ha^{-1})	0.50–13.5	3.11	0.60
	Tot. N (kg ha^{-1})	0.95–24.6	6.20	1.12
	SO_4–S (kg ha^{-1})	1.27–11.9	3.93	0.45
Soil water	NO_3–N (mg l^{-1})	<0.005–3.48	0.34	0.14
	NH_4–N (mg l^{-1})	<0.001–0.11	0.04	0.01
	pH_{H_2O}	3.91–5.89	4.93	0.09
Humus	C:N ratio	21.5–32.6	27.4	0.76
	K (mg g^{-1})	3.14–25.8	11.51	1.30
	pH_{H_2O}	3.35–4.80	4.04	0.10
Needles	P (mg g^{-1})	1.18–1.92	1.51	0.06
	K (mg g^{-1})	3.20–6.09	4.65	0.20
	$\delta^{15}N$	−6.93 to −2.08	−4.19	0.31

Abbreviations are given for chemical compounds.

Table 2
Environmental variables included in the multivariate analyses (PLS regressions).

Throughfall	Acid neutralizing capacity (ANC), H^+, alkalinity, SO_4–S, Cl, NO_3–N, NH_4–N, Ca, Mg, Na, K, Mn
Soil water	pH_{H_2O}, TOC[a], ANC, H^+, SO_4–S, Cl, NO_3–N, NH_4–N, Ca, Mg, Na, K, Mn, Fe, i.o. AL, o. AL
Humus	Water content, CEC[b], base saturation, org. C:N ratio, pH_{H_2O}, H^+, Na, K, Mg, Ca, Mn, Fe, Al
Needles	Weight, N, S, P, Ca, Mg, K, Fe, Mn, arginine, C, $\delta^{13}C$, $\delta^{15}N$
Other	Precipitation, stand age, stand index, elevation, humus depth, RT90 coord., storm damage

Abbreviations are given for chemical compounds.
[a] Total organic carbon.
[b] Cation exchange capacity.

Two composite humus samples of about one litre each were compiled from 15 to 20 soil cores taken at each site, and the mean values obtained from the analysis of the two composite samples were used in the calculations. The number of variables monitored differed depending on whether the site was maintained by the IVL Swedish Environmental Research Institute or the Swedish Forest Agency. There were also some regional deviations leading to fewer variables being monitored at some sites. Soil samples had been taken at 21 of the sites during the autumn of 2005, 2006 or 2007, and 10 shoots were collected from the upper canopy of at least 5 representative trees for needle analysis at 20 of the sites during January 2006. Previous year needles (<1 year old) were used for the analyses since they have been shown to generally represent the current nutrient status better than other plant tissue (reviewed by Driessche and Rieche, 1974). Composite soil and needle samples from each site were analysed to determine values of the variables listed in Table 2. Detailed descriptions of the methods of sampling and chemical analysis is given by Ladanai et al. (2010), and are thoroughly described on the web page for ICP Forests (www.icp-forests.org/manual.htm).

2.3. Growth of ectomycorrhizal fungi

Sand-filled mesh-bags were used to determine the production of EMM (Wallander et al., 2001). A square sheet of nylon mesh (7 × 7 cm) with a pore size of 50 μm were folded diagonally and then welded to give a triangular bag, which was then filled with 10 g acid-washed and burned quartz sand (0.36–2.00 mm, 99.6% SiO_2, Ahlsell AB, Sweden). The mesh-bags were buried in the soil during two separate growing seasons, the first (May 2009–October 2009) is referred to as 2009 and the second (October 2009–October 2010) referred to as 2010. To analyse EMM production, sand filled mesh-bags were buried about two metres from each of the five suction lysimeters at each site. They were placed at the interface between the organic and mineral horizons, where the production of EMM has been found to be greatest (Lindahl et al., 2007).

To investigate possible P deficiency five additional mesh-bags, amended with 0.5% of the phosphorus-containing mineral apatite and 1% maize leaf compost, were installed at each site. Enhanced EMM production in apatite-amended bags indicates P deficiency of the trees (Wallander and Thelin, 2008). It has been shown that the addition of organic material enhances EMM production (Hendricks et al., 2006; Wallander et al., 2011), and therefore compost made from maize leaves was added to the apatite amended bags and their controls to improve the detection of any possible differences in EMM growth due to P deficiency. Since it has been reported that the increase in EMM production is only observable after two growing seasons (Hagerberg et al., 2003; Potila et al., 2009), the apatite and

maize amended bags together with controls (only maize) were left in the soil during the whole study period (2009–2010). Further, additional maize compost amended bags were buried at 10 of the sites during the first period to study the contribution of EMM production to C sequestration in the bags (see Chapter 2.4). In total, 20 or 28 mesh-bags were installed at each site during the period of the study (Table 3). Directly after harvest, the mesh-bags were stored at 4 °C for up to 4 days, until they were transferred to a freezer where they were kept at −18 °C. Each of the mesh-bags was coded at harvest with a randomised identification number to avoid subjectivity in further analysis.

We used two different approaches to analyse the ingrowth of EMM into the mesh-bags, visual estimation with stereo microscope and analysis of the chemical biomarker ergosterol. Ergosterol is a cell membrane component specific to fungi that can be used as a quantitative indicator of fungal biomass (Nylund and Wallander, 1992). Each mesh-bag was wiped clean and carefully opened before visual estimation of the EMM ingrowth. The EMM ingrowth was graded according to a 6 level scale (similar to Wallander et al., 2001): (0) no mycelia, (1) few hyphae present, (2) mycelia present but no aggregation of sand particles, (3) mycelia present and some aggregation of sand particles, (4) mycelia abundant and aggregation of sand particles, and (5) mycelia abundant with aggregation of almost all sand particles. After visual determination of the fungal ingrowth, the content of each mesh-bag was transferred to a plastic zip bag and homogenised by hand before being returned to the freezer and stored at −18 °C.

The contents of the mesh-bags from each time interval were pooled site-wise and 5 g from each of the pooled samples were used for ergosterol analysis. After adding 5 ml KOH solution (10% KOH in methanol) the samples were extracted by sonication for 15 min followed by heating for 1 h in a 70 °C water bath. One ml H_2O was added to increase the polarity and 2 ml cyclohexane to serve as the lipophilic phase. After shaking the tubes for 1 min in a multivortex (Multi Reax, Heidolph, Germany), they were centrifuged for 5 min at 1000 × *g* before the lipophilic phase was separated from the methanol based phase. Phase separation was repeated once more after the addition of another 2 ml cyclohexane. The supernatants were evaporated on a 40 °C heating block under nitrogen gas flow, to prevent oxidation, and then dissolved in 200 µl methanol. The solution was filtered through a 0.45 µm filter (SUN SRi, 44504-NPC, titan syringe filter, PTFE, 0, Rockwood, TN, USA) before analysis in a reversed-phase column (Chromolith C18 column, Merck and an Elite LaChrome C18 pre-column, Hitachi, Japan) high pressure liquid chromatograph (auto sampler L2130 with UV-detector L2400 by Hitachi, Japan). The ergosterol peak was detected at 280 nm with a flow rate of 1 ml min^{-1}.

2.4. Isotopic analysis

To estimate the contribution of EMM to sequestration of C we used mesh-bags filled with sand mixed with 1% of composted maize leafs (a C4 plant) in a similar way as Wallander et al. (2011). Ten of the sites (Fig. 1), representing the total experimental range of N deposition, were selected for this study. Maize-amended mesh-bags were installed inside and outside trenched subplots (within a 30 cm deep, 16 cm diameter PVC-tube), the former acting as controls with no EMF ingrowth. This part of the experiment was restricted to the first period (2009), since longer periods can result in ingrowth of EMM into the trenching tubes from below (Wallander et al., 2011). Prior to isotopic analyses the content of the five pooled mesh-bags was freeze dried and milled with a ball mill to a fine powder. The amount of C as well as natural abundance of ^{13}C were analysed with an elemental analyser (model EuroEA3024, Eurovector, Milan, Italy) and an isotope ratio mass spectrometer (Isoprime, Manchester, UK). The ^{13}C abundance was calculated in per mille (‰) deviation from the international standard (Vienna Pee Dee Belemnite):

$$\delta^{13}C = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times 1000,$$

where R is the ratio of $^{13}C/^{12}C$. The proportion of new C was calculated according to Wallander et al. (2011) with a two component mixing model:

$$C_4\text{-carbon} = \left(\frac{\delta^{13}C_{sample} - \delta^{13}C_{new\ carbon}}{\delta^{13}C_{C_4} - \delta^{13}C_{new\ carbon}}\right),$$

where $\delta^{13}C_{new\ carbon}$ was set as −26.8‰ (Wallander et al., 2011) and δC_{C_4} values was determined from mesh-bags from trenched plots at each of the 10 sites.

2.5. Statistics

Partial least square regression (PLS) was used to interpret the multidimensional pattern of the complex dataset. PLS is an appropriate multivariate method when the response variables are few and co-linearity is apparent between *x*-variables (Wold et al., 2001). Since many ecological studies have a large array of predictor variables which often interact there is a large potential to use PLS, especially in studies with more predictors than response variables (Carrascal et al., 2009). Even though PLS modelling is typically used in other fields, such as analytical chemistry, it has been applied several times as a statistical tool in ecological publications (reviewed by Carrascal et al., 2009), including a few studies of soil microorganisms (e.g. Ekblad et al., 2005; Comstedt et al., 2011). After running a PLS on the whole dataset the number of *x*-variables was reduced, to analyse their specific contribution to the predictability of the response variables. PLS analyses were performed in Microsoft Excel (Microsoft Office Professional Plus 2010) with the Multibase 2012 plugin (Numerical Dynamics, www.numericaldynamics.com). All variables and sites with less than 20% missing values were included in the analyses and

Table 3
Overview of the mesh-bag experimental set-up. The mesh-bags were filled with 10 g acid-washed, burned quartz sand and buried in the soil to investigate growth of ectomycorrhizal mycelium. Bags amended with 1% maize leaf compost were buried with and without 0.5% apatite at all sites for the analysis of phosphorus deficiency. Additional bags containing 1% maize leaf compost were buried in trenched plots at 10 of the sites to study carbon sequestration.

Mesh-bag content	Analyses	No. Bags $site^{-1}$	No. Sites	Period	Trenched
Sand	EMM prod.	5	29	2009 May–2009 Oct.	No
Sand	EMM prod.	5	27	2009 Oct.–2010 Oct.	No
Sand + maize	P limitation	5	29	2009 May–2010 Oct.	No
Sand + maize + apatite	P limitation	5	27	2009 May–2010 Oct.	No
Sand + maize	C allocation	3	10	2009 May–2009 Oct.	Yes
Sand + maize	C allocation	5	10	2009 May–2009 Oct.	No

missing values were replaced by values estimated by the software. No transformation of data was performed prior to analysis. Each *X* and *Y* variable was standardised by auto-scaling (divided by the standard deviation and then the mean value was subtracted) prior to analysis. Cross-validation with the minimum predicted residual sum of squares (PRESS) was used to interpret significant amount of PLS components, basically R^2 values show how much the response-variables can be explained by the model while Q^2 values (from PRESS) provide a measure of the predictability of them (Eriksson et al., 2006). *T*-tests, Mann Whitney *U* tests and regression analyses were performed in SPSS (IBM SPSS Statistics, version 19.0.0), Pearson correlations (R^2) were calculated for parametric data, and Spearman rank correlations (r_s) for ordinal data (e.g. the visual estimates of EMM growth). Only two-way analyses were performed and paired tests were used for within-site differences while unpaired tests were used between sites.

3. Results

3.1. Estimation of EMM production

There was agreement between the visual estimation of ingrowth and the ergosterol content in the mesh-bags in 2010 ($r_s = 0.402$, $P = 0.019$) but not in 2009 ($r_s = 0.165$, $P = 0.197$). EMM ingrowth during 2009 and 2010 were well correlated according to the visual estimates ($r_s = 0.508$, $P = 0.008$) but this could not be verified by analysis of the ergosterol content ($R^2 = 0.097$, $P = 0.134$).

3.2. Environmental variables affecting EMM production

PLS regression using visually estimated EMM growth and ergosterol content during 2009 and 2010 as response variables to 63 environmental variables (mean values of the two years) revealed two significant components, as shown in Fig. 2 ($R^2 = 0.51$, $Q^2 = 0.31$). Performing separate PLS models for visual and chemical EMM estimation from each year resulted in $R^2 = 0.92$ and $Q^2 = 0.54$ (4 significant components) for visual growth estimation for 2009, while the corresponding values for 2010 were $R^2 = 0.57$ and $Q^2 = 0.31$ (1 significant component). Growth estimation using ergosterol gave $R^2 = 0.43$ and $Q^2 = 0.06$ (1 significant component) in year 2009, while the corresponding values for 2010 were $R^2 = 0.60$ and $Q^2 = 0.38$ (1 significant component). As the separate models gave similar results to the combined model regarding the variable importance in projection (VIP) and loadings, only the latter is presented. VIP values (Fig. 3) together with the loading plot (Fig. 2) indicated that N deposition, pH-related variables and geographical position were the most important predictors. The chemical composition of needles seemed to mostly contribute to the pattern along the second component axis. A better model was obtained by removing all environmental variables apart from throughfall N, soil water N, needle nutrient status (N, P, Mg, K and Mn), soil pH and soil water pH, resulting in a higher predictability ($Q^2 = 0.36$) than the full model ($Q^2 = 0.31$). Although N was the most important factor to explain EMM growth, predictability declined ($Q^2 = 0.27$) when throughfall N and soil water N alone were included as *x*-variables in the model. N deposition was the N

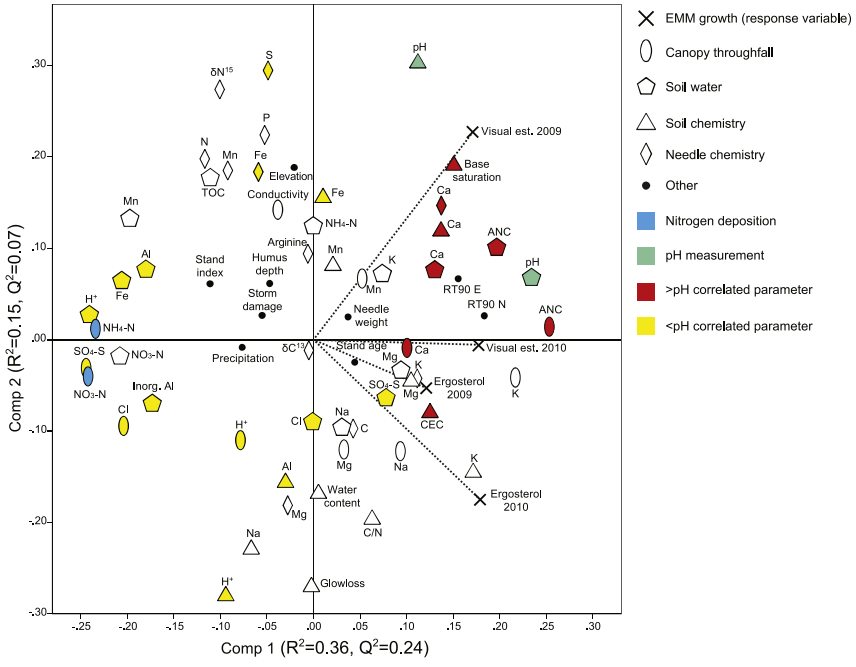

Fig. 2. Loading plot from PLS regression using growth of ectomycorrhizal extramatrical mycelium (EMM) during 2009 and 2010, according to visual and ergosterol-based estimates, as response variables (*Y*-variables). Canopy throughfall and soil water variables are based on mean annual values during the two years. Soil and needle chemistry are based on the most recent measurement (1997 respectively 2006). Cross-validation resulted in two significant components.

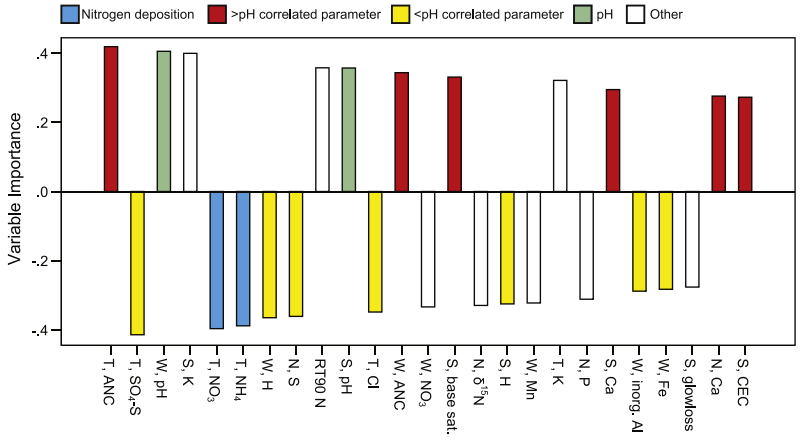

Fig. 3. Variable importance in projection (VIP) from the first PLS regression (Fig. 2), using growth of ectomycorrhizal extramatrical mycelium, according to visual estimation and ergosterol measurements, as response variables (*Y*-variables). Environmental variables are canopy throughfall (T), soil water (W), soil chemistry (S), needle chemistry (N) and geographic position (RT90). Of the 63 environmental variables used in the PLS, the 25 with highest VIP values are presented. Negative values are given for variables showing a negative trend with the response variable along the first PLS component axis.

factor most negatively correlated with EMM growth (Fig. 4) during 2009 ($r_s = -0.58$, $P = 0.001$) and 2010 ($r_s = -0.61$, $P < 0.001$) respectively according to visual estimation. This could not be verified by ergosterol analyses (2009: $R^2 = 0.234$, $P = 0.221$; 2010: $R^2 = 0.257$, $P = 0.195$). Soil water pH was positively correlated with visual EMM estimation (2009: $r_s = 0.65$, $P < 0.001$; 2010: $r_s = 0.513$, $P = 0.006$), and a trend was also observed with the ergosterol values during 2010 ($R^2 = 0.12$, $P = 0.074$). The growth of EMM increased along the north-eastern gradient (calculated as the sum of the RT90 north and east coordinates) according to visual estimates (2009: $r_s = 0.55$, $P = 0.002$; 2010: $r_s = 0.584$, $P = 0.001$), and

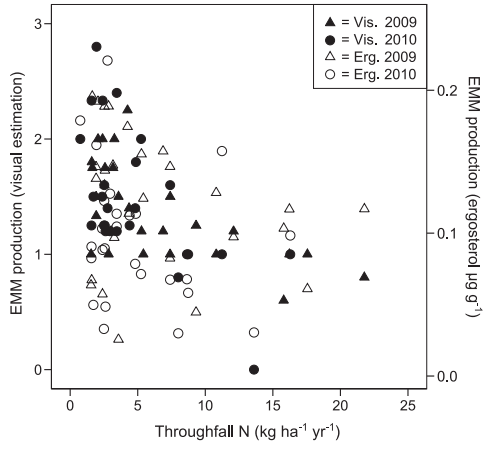

Fig. 4. A negative correlation between nitrogen deposition on ectomycorrhizal extramatrical mycelium growth (EMM) was found during both 2009 ($r_s = -0.58$, $P = 0.001$) and 2010 ($r_s = -0.61$, $P < 0.001$) according to visual estimation, but this could not be verified by ergosterol analyses (2009: $R^2 = 0.234$, $P = 0.221$; 2010: $R^2 = 0.257$, $P = 0.195$).

chemical estimation based on ergosterol showed a tendency towards the same trend (2009: $R^2 = 0.334$, $P = 0.077$; 2010: $R^2 = 0.374$, $P = 0.054$). Along the same gradient there was a decline in N deposition (2009: $R^2 = 0.674$, $P < 0.001$; 2010: $R^2 = 0.606$, $P < 0.001$). N deposition was negatively correlated to soil water pH (2009–2010: $R^2 = 0.75$, $P < 0.001$) and soil pH (2009–2010: $R^2 = 0.44$, $P = 0.051$).

Some significant correlations were observed between elemental concentration in needles and EMM production. Negative correlations were observed with concentrations of P (ergosterol 2009: $R^2 = 0.20$, $P = 0.058$; ergosterol 2010: $R^2 = 0.28$, $P = 0.022$), S (ergosterol 2009: $R^2 = 0.36$, $P = 0.008$) and Fe (ergosterol 2009: $R^2 = 0.21$, $P = 0.048$; Visual estimation 2010: $r_s = -0.602$, $P = 0.008$) while the needle Ca content increased with EMM production (ergosterol 2009: $R^2 = 0.25$, $P = 0.029$; visual estimation 2009: $r_s = 0.507$, $P = 0.027$; visual estimation 2010: $r_s = 0.482$, $P = 0.043$). Of the elements analysed in needles sampled in 2006, only N, K and P were, at least occasionally, observed at levels stated as deficient in *P. abies* foliage (Braekke and Salih, 2002) at any of the sites.

Apatite addition caused enhanced EMM production (Mann–Whitney *U* test, $P = 0.010$) at one site only. This site had the lowest needle P content of all investigated sites (1.19 mg g^{-1}), which is below the deficiency level 1.2 mg g^{-1} (Thelin et al., 2002). None of the other sites had needle P concentrations below the deficiency level.

3.3. Environmental variables affecting N leakage

When using N leaching during the two periods (the sum of soil water NO_3–N and NO_4–N) as a response variable, PLS gave two significant components ($R^2 = 0.90$, $Q^2 = 0.58$). In this model (Fig. 5) only 53 of the environmental variables were included as predictors since all the soil water variables except the response variable (N leaching) were excluded to avoid overestimation of the model due to directly correlated variables within the same medium. According to the VIP values (Fig. 6) and the PLS output (Fig. 5) the most important variables on the first component axis were pH-related variables, N deposition and growth of EMM, while storm damage

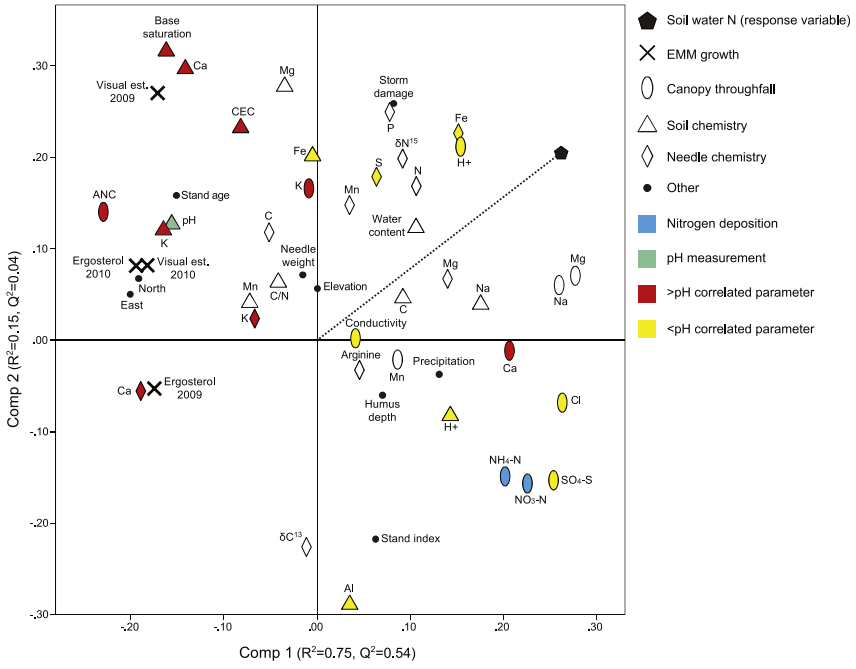

Fig. 5. Loading plot from PLS regression using the nitrogen leaching (soil water N) as response variable (*Y*-variable). Canopy throughfall and soil water variables are based on mean annual values during the two years, while soil and needle chemistry are based on the most recent measurement (1997 respectively 2006). Growth of ectomycorrhizal extramatrical mycelium (EMM) was estimated visually and chemically (ergosterol). Cross-validation resulted in two significant components.

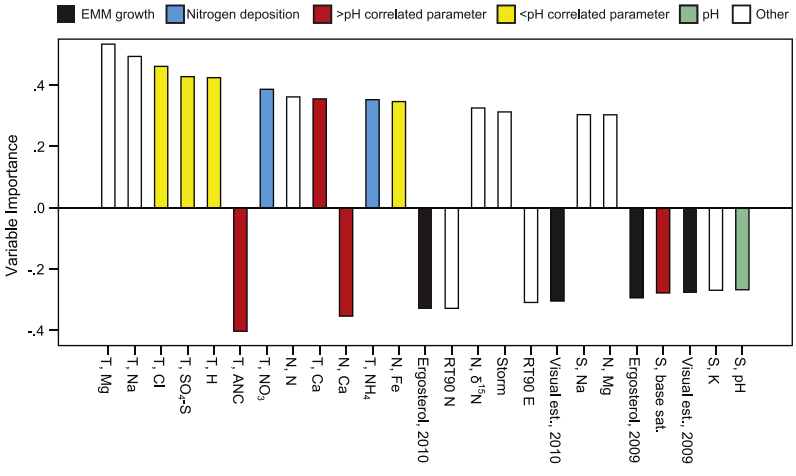

Fig. 6. Variable importance in projection (VIP) from the PLS regression, with soil water nitrogen ($NO_3^- + NH_4^+$) as response variable (*Y*-variable). Environmental variables are growth of ectomycorrhizal extramatrical mycelium (visual estimation and ergosterol), canopy throughfall (T), soil chemistry (S), needle chemistry (N), geographic position (RT90) and storm damage. Of the 51 environmental variables used in the PLS, the 25 with highest VIP values are presented. Negative values are given for variables showing a negative trend with the response variable along the first PLS component axis.

seemed to have a considerable effect on the second component. However, other variables were also important since running the PLS with pH, N deposition and growth of EMM as the only *x*-variables resulted in a substantial decline of the predictability ($Q^2 = 0.38$). Leaching of N increased with N deposition at intact sites in 2009 ($R^2 = 0.70$, $P < 0.001$) but not in 2010 (when no sites had elevated N leaching), while a positive correlation was observed at storm damaged sites in both years (2009: $R^2 = 0.69$, $P = 0.002$; 2010: $R^2 = 0.44$, $P = 0.025$). During 2009 the mean soil water N content of 0.98 mg l^{-1} (SE = 0.34) at storm damaged sites was larger ($P = 0.007$) than the values (0.15 mg l^{-1} SE = 0.069) observed at the intact sites. In 2010, intact sites (mean = 0.04 mg l^{-1}, SE = 0.01) tended ($P = 0.061$) to have lower N leaching than storm damaged sites (mean = 0.51 mg l^{-1}, SE = 0.28). Leaching of N tended to be negatively correlated with the visual estimates of EMM growth (Fig. 7) at intact sites during 2009 ($r_s = -0.45$, $P = 0.073$) and at storm damaged sites during 2010 ($r_s = -0.34$, $P = 0.060$). This trend could not be found at storm damaged sites 2009, intact sites in 2010, or at any of the above combinations according to ergosterol analysis. However, it should be borne in mind that there were no intact sites with elevated N leakage in 2010, and that observations at the two intact sites showing the greatest N leaching in 2009 was terminated at the end of 2009, and there were thus no detected N leaching to be predicted in 2010.

3.4. Isotopic analysis

The value of $\delta^{13}C$ was significantly ($P = 0.021$) higher in maize amended mesh-bags from non-trenched plots (mean = −15.3, SE = 0.29) compared to the trenched (mean = −14.4, SE = 0.19). Subtraction of the mean value from the trenched bags at each site resulted in a difference of −0.93 (SE = 0.22) regarding $\delta^{13}C$ in the non-trenched bags. Based on C_4 carbon and the total C content it was calculated that the mean C sequestration was 0.32 mg C g $sand^{-1}$ (SD: 0.35) from May 2009 until October 2009.

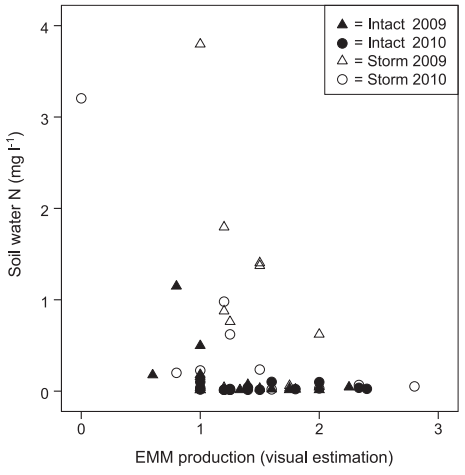

Fig. 7. A tendency was found between N leaching (soil water N) and visual estimation of ectomycorrhizal extramatrical mycelium (EMM) growth at intact sites (not storm damaged) during 2009 ($r_s = -0.45$, $P = 0.073$) and at storm damaged sites during 2010 ($r_s = -0.34$, $P = 0.060$). No correlations were found between N leaching and the estimates of EMM based on ergosterol (data not shown).

By converting this into the 10 cm top soil it was possible to estimate the annual C sequestration by EMF per ha (Wallander et al., 2011) to 320 kg (SD: 351). However, linear regression (data not shown) revealed no clear trends between C sequestration and N deposition.

4. Discussion

Our two methods to quantify fungal biomass in the mesh-bags gave somewhat different results. In separate regressions, only visual estimation revealed a negative correlation between EMM production and N deposition as well as tendencies of a negative pattern with N leakage. At the same time, negative correlations between EMM production and needle P content could only be found with biomarker estimation of the ingrowth in the mesh-bags. A reason for this could be that visual estimation likely is better to detect the proliferation of newly produced mycelia, while biomarker estimation probably reflects the total ingrowth during the whole incubation period, since the analysed chemical biomarker, ergosterol, has been shown more stable than expected during degradation of fungal biomass (Mille-Lindblom et al., 2004). Further, the ergosterol content has been shown to have both intraspecific (Yuan et al., 2008) and interspecific variation (Bermingham et al., 1995), and values may be related to the composition of the EMF community and the abundance of rhizomorphs rather than to hyphal proliferation.

The production of EMM is known to be negatively affected by high N availability (e.g. Beckjord et al., 1985; Wallander and Nylund, 1992; Arnebrant, 1994; Runion et al., 1997; Nilsson and Wallander, 2003; Nilsson et al., 2007; Kjøller et al., 2012), but this knowledge is mainly based on experimental studies with rather high N addition rates (e.g. Nilsson and Wallander, 2003, 100 kg N ha^{-1} y^{-1}). Here we show during two consecutive years that reduction of EMM production also occurs when moderate levels (<10 kg N ha^{-1} y^{-1}) of N are added by atmospheric deposition. The reduction in EMM growth may be due to a greater demand of photoassimilated C for above ground plant production, resulting in relatively less C being allocated belowground to support nutrient assimilation (Hobbie, 2006). In support of this Högberg et al. (2010) found a reduction in belowground C allocation to soil biota of more than half after N fertilization of a pine forest in northern Sweden. In addition, the C that is allocated to EMF will, to a larger extent, be used for energy purposes and as C skeletons for amino acid assimilation during elevated N availability resulting in less C being available for fungal growth (Wallander, 1995). Reduced production of EMM at elevated N input may also be related to changes in the EMF community. Agerer (2001) divided EMF into different exploration types, depending on the structure of the EMM. For example, the contact exploration type have a very limited growth of EMM and is hypothetically less C demanding than distance exploration types (Hobbie and Agerer, 2010), and the difference in biomass and distance between different exploration types can be 15-fold (Weigt et al., 2012). Reduced growth of EMM at high N availability may be an effect of a shift to less carbon demanding exploration types, in support for this Nilsson and Wallander (2003) found reduced growth of EMM in N fertilised plots where the amount of fungal biomass on roots were unchanged, and Kjøller et al. (2012) found that relative abundance of contact exploration types increased with N deposition while distance exploration types decreased.

Although most of the variation in production of EMM could be explained by N availability, inclusion of needle nutrient status and pH increased the predictability further (from $Q^2 = 0.27$ to $Q^2 = 0.36$). Together, these variables seem to be the most important ones since inclusion of additional variables did not contribute to any further increase of the predictability. According to the PLS model, needle N, P and S concentration were negatively, while

needle K concentration was positively related to EMM production. This can be explained by reductions in belowground C allocation when nutrients closely associated with photosynthesis, such as K, Mg and Mn are deficient, while the opposite is seen with nutrients such as N, P and S, which are more involved in the production of new plant tissue (Ericsson, 1995). Furthermore, enhanced production of EMM at P deficiency is commonly found in pot cultures (Wallander and Nylund, 1992; Ekblad et al., 1995) and this has also been found in the field (Berner et al., 2012). The correlation between EMM production and needle P concentration found in the present study show that it may be important to take P into account in studies of EMM production, even if P is above the deficiency level. The content of Ca in needles, which plays a fundamental role in plant biomass formation as a structural component (Marschner, 1995), was positively correlated with EMM production. In Swedish forests, Ca is however high above optimum levels in plant tissue and it has been suggested that Ca assimilation depends on availability rather than plant requirement (Knecht and Göransson, 2004). This is consistent with the results of the present study since needle Ca was correlated with Ca content in both humus ($R^2 = 0.28$, $P = 0.004$) and soil water Ca (Yr 1: $R^2 = 0.47$, $P = 0.001$), and the positive correlations between Ca and EMM production are probably due to the greater abundance of Ca at higher pH (Brady and Weil, 2008).

Strong positive correlations were found between pH and visual estimation of the amount of EMM in the present study. Only a few field studies have analysed the effects of a reduction in pH on EMF, but it has been observed that simulated acid rain did not affect formation of mycorrhizas (Meier et al., 1989; Nowotny et al., 1998) or EMF morphotypes (Meier et al., 1989). On the other hand, liming to counteract soil acidification has a strong effect on the composition of the EMF community (Erland and Söderström, 1991; Lehto, 1994; Andersson and Söderström, 1995; Wallander et al., 1997; Bakker et al., 2000), while species richness (Kjøller and Clemmensen, 2009) and diversity (Rineau et al., 2010) seem to be rather unaffected by liming. It is often reported that species with well developed EMM increases after liming (Bakker et al., 2000; Kjøller and Clemmensen, 2009) and highly competitive ubiquitous species are more common at higher pH (Rineau et al., 2010). Lehto (1994) found that the reduction of contact exploration type EMF was mainly due to increased pH and ionic strength. Thus the positive relation between pH and EMM production in our study could be related to the composition of the EMF community. Increasing the pH in acidic forest soils has been found to promote microbial activity (Bååth and Arnebrant, 1994) and, in general, to elevate nutrient availability. Nevertheless, the effects of liming on the N cycle in N limited, acidic soils are complex, and reduced N mineralisation after liming have also been reported (reviewed by Formánek and Vranová, 2003). However, the positive correlation between pH and EMM growth observed in the present study may also be due to the acidifying effect of N deposition. Nilsson et al. (2007) found lower EMM production in areas with elevated N deposition, but these also had the lowest pH values. Acidification is caused by the atmospheric formation of HNO_3 from NO_x and Swedish air concentrations of NO_2 were about ten times higher than NH_3 around the turn of the millennium (Pihl-Karlsson et al., 2011). The amount of deposited NO_3–N in the present study was similar to deposited NH_4–N (NO_3–N $= -0.28 + 1.10 \times NH_4$–N, $R^2 = 0.93$, $P < 0.001$). Kjøller et al. (2012) found no change of pH in an N deposition gradient where the pollution consisted mainly of NH_3. The acidifying effect of HNO_3 deposition together with a narrow pH gradient and a dispersed pattern of pH-related variables (according to PLS regression, Fig. 2) in the present study, indicates that canopy throughfall of N was the most important variable affecting EMM growth, and probably the cause of the pH trend.

4.1. C sequestration by EMF

EMF play an important role in the boreal forest C cycle (Smith and Read, 2008) and a reduction in EMM production may have substantial consequences for C sequestration (Godbold et al., 2006). In this study, an average C sequestration of 320 kg ha^{-1} y^{-1} was calculated by extrapolating the amount of fungal C accumulating in the mesh-bags to an areal basis. However, this data has low precision since it is based on small differences in $\delta^{13}C$ (0.93, SE = 0.34). From the ten sites included in this part of the study, we could not find any relation between N deposition and C sequestration.

4.2. Environmental variables affecting N leakage

Since the surface area for plant nutrient assimilation can be increased in the order of two magnitudes through formation of ectomycorrhiza (Smith and Read, 2008), it is likely that high abundance of EMM increase the N retention capacity of the soil. It has been hypothesised that EMM growth counteracts leaching of N (Aber et al., 1998; Nilsson et al., 2007; Högberg et al., 2011), and indications of this has been observed in a few studies (e.g. Nilsson et al., 2007). The effect of EMM on nitrogen retention may be affected by the composition of the EMF community and the abundance of specific exploration types. Kjøller et al. (2012) found that the relative abundance of contact exploration types (e.g. *Lactarius* spp.), with potentially less capacity to retain N, increased in areas with higher N leaching. According to the visual estimation of EMM production (Fig. 7) elevated N losses seemed to occur at relatively low EMM production, while low N leaching was detected at all levels of EMM production. The lack of high N losses at abundant EMM production indicates a counteracting effect of EMM on N leaching. It was however not possible to draw any firm conclusions regarding the capacity of EMM to retain N in the soil, since N leaching increased with N deposition at the same time as N deposition had a negative effect on EMM production. The effects of these mechanisms on N leakage couldn't be separated with the approach used in the present study. In order to determine the capacity of EMM to reduce N losses it might be better to apply more intense temporal sampling strategies for both EMM production and N leaching. EMM production in the boreal and boreo-nemoral regions generally peaks during the autumn while N leaching vary more depending on the climate: northern and central areas of Sweden tend to have increased N leaching during snowmelt in spring, while further south N losses usually occur in late autumn due to high precipitation (Gustafson, 1983). Another approach to detect the N retention capacity of the soil could be to add isotopically labelled nutrients, which would enable the detection of assimilation (Harrison et al., 1995). In an ongoing experiment, we are exposing plots differing in EMM proliferation to ^{15}N-labelled NO_3^- to investigate the N retention capacity of EMM.

4.3. Conclusions

By including a comprehensive dataset of environmental variables regarding canopy throughfall, soil water, soil chemistry and needle chemistry we here conclude that N deposition is the most important abiotic variable controlling EMM production in the surveyed forest type, and that the N deposition of 2–20 kg ha^{-1} y^{-1}, typical for the boreal and boreo-nemoral region, is sufficient to repress EMM production. Other important abiotic factors found to be negatively related to EMM growth were low pH and high needle P concentration. Further, we also conclude that the most important variables explaining the N leakage were N deposition, low pH, storm damage and low EMM production, but a more experimental approach to investigate mechanisms, and more intense seasonal

sampling efforts are needed to improve our understanding of the N retention capacity of EMM.

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III

Shift in composition and functioning of the fungal community along a natural boreal forest nutrient gradient

Erica Sterkenburg[a], Adam Bahr[b], Mikael Brandström-Durling[a], Karina E. Clemmensen[a] and Björn D. Lindahl[a]
[a] *Swedish University of Agricultural Sciences, Dept. of Forest Mycology and Plant Pathology, Uppsala BioCenter, Box 7026, SE-750 07 Uppsala, Sweden.*
[b] *Lund University, Microbial Ecology, Dept. of Biology, SE-223 62 Lund, Sweden*

Abstract
In the boreal forest ecosystem, fungi are the principal decomposers of organic matter and pivotal symbionts of trees. Yet, compared to plants and animals, large knowledge gaps remain concerning fungal niche differentiation. Here we investigate if fungal communities reflect patterns of ecosystem productivity, aiming for a better understanding of the functional ecology of fungi and their interplay with the plant community and environmental factors.

We used high throughput 454-sequencing of ITS2 amplicons to analyse the fungal community in humus and needle litter in 26 pristine boreal forests in central Sweden. Ergosterol was used as a fungal biomass-marker. The forests were selected to represent a gradient in nitrogen availability and pH as well as different vegetation types with respect to dominant tree species (*Pinus sylvestris, Picea abies*) and understory vegetation.

We found a significant relationship between the fungal community composition and soil acidity as well as C:N-ratio. Moving from the low-productive to the high-productive end of the gradient, there was a shift from a community dominated by ascomycetes to a community with increased contribution of basidiomycetes, both in humus and litter. The levels of ergosterol decreased at low pH, indicating environmental stress, but superimposed on this general pattern, the presence of *Pinus sylvestris* had a positive influence on fungal biomass in the humus layer. Against our expectations, the relative abundance of ectomycorrhizal fungi in the humus layer did not decline in the most fertile forests, but remained at 50-70% of the amplicons.

With this study, we show that it is possible to make statistical predictions about the composition and biomass of fungal communities within the range of forest types that we covered. This implies that it is possible to establish general relationships that are of potential use in theoretical and quantitative ecosystem modelling.

Introduction
Fungi are the principal decomposers of organic matter in forest ecosystems and pivotal symbionts of trees (Smith and Read, 2008). Yet, compared to plants and animals, large knowledge gaps remain concerning fungal diversity and ecology. Like plants, fungi are immobile and, thus, bound to their habitats, potentially forming habitat-specific communities. Variation of plant communities along habitat gradients, in e.g. productivity, mineralogy and hydrology (e.g. Lahti and Väisänen, 1987), is well described. In contrast, niche differentiation of fungal species and prevalence of functional groups along such gradients are yet not well described and understood. Until recently, microbial soil ecology has been driven by methods with no or low phylogenetic

resolution, such as microscopic observation, total biomass/activity measurements or phospholipid analysis. Since closely related taxa may have very different ecology (Hibbett et al., 2000), it is necessary to analyse the fungal community at levels of orders, genera or even species, to separate and quantify functional groups. Only recently, methodological advances in molecular biology, for example high-throughput sequencing of molecular markers (Lindahl et al., 2013), enable detailed and at least semi-quantitative analysis of fungal communities, and subsequent testing for correlation with stand properties and potential environmental drivers.

Boreal forests are, in general, nitrogen (N) limited (Vitousek and Howarth, 1991), and dominated by coniferous trees producing recalcitrant litter with a high content of lignin and phenolic compounds (Aerts, 1995). In these forests both mycorrhizal and saprotrophic fungi play a fundamental role in the cycling of nutrients and carbon (Lindahl et al., 2007). Saprotrophic fungi are the main decomposers of litter, woody debris and soil organic matter (Rayner and Boddy, 1998; Baldrian, 2009; Fontaine et al., 2011), recycling carbon (C) to the atmosphere. Furthermore, symbiotic mycorrhizal fungi provide their host plants with soil-derived N and other nutrients in return for recently photosynthesised C (Smith and Read, 2008; Högberg et al., 2010), which they route into the soil, making a major contribution to long term C sequestration (Clemmensen et al., 2013). In the boreal forest, nutrient availability, hydrology and soil acidity are the principal, and usually co-varying environmental determinants of plant communities. Since mycorrhizal fungi depend on allocation of recently photoassimilated C and saprotrophic fungi are dependent on plant litter production, it seems likely that fungal communities vary along similar gradients.

In the most low-productive habitats, low N-availability may directly constrain fungal growth of both saprotrophs (Boberg et al., 2008) and mycorrhizal fungi (Clemmensen et al., 2008). In addition, low-productive plant communities provide less C to fungi, in terms of litter to saprotrophs and, in absolute terms, also with respect to root sugars to mycorrhizal fungi. Furthermore, plants produce more recalcitrant litter (Aerts, 1995). Acidic soils, typical for boreal forests (Brady and Weil, 2008), may also constitute a stressful environment for fungi. Even though, in a broader perspective, fungal abundance usually increase with low pH while bacterial abundance decrease (Rousk and Bååth, 2011), reduced fungal growth was observed at pH less than 4.5 (Rousk et al., 2011). Furthermore, in a large-scale boreal forest field survey (Bahr et al., 2013) it was found that the production of mycorrhizal fungi increased with increasing pH (although acidifying N-deposition is a confounding factor here). Among bacteria, resource availability is the principal determinant of community composition, with oligotrophic (as opposed to copiotrophic) groups being characterized by low resource utilization rates (Fierer et al., 2007). Similarly, in fungi, scarce resource availability and stressful conditions are likely to favour species with slow mycelial growth (Cooke and Rayner, 1984). In plant leaves, low productivity is generally associated with robust and long-lived tissues (Wright et al., 2004), traits being favoured under stressful conditions (Grime, 1977). Correspondingly, in fungi, long-lived mycelial structures may enable more economic resource utilization. To retain mycelium for a long time, it needs to be protected against fungivores and harsh abiotic conditions, e.g. by impregnation of cell walls with melanin and hydrophobic compounds (rev. by Ekblad et al., 2013). Fungi associated with stressful environments are often ascomycetes adapted to e.g. drought and acidity (Gostinčar et al., 2010).

Alleviation of severe environmental stress leads to elevated plant productivity, with more C potentially becoming available to fungi via increased plant litter production and root-mediated fluxes, concomitant with increased N availability and decomposability of organic matter (Peltzer et al., 2010). Improved conditions probably enable higher mycelial production and open up for colonisation by fungal species without prominent stress tolerant traits, and thus results in enhanced competition. Assuming a trade-off between stress tolerance and combative strength, the incidence of species with high capacity of interference and competitive antagonism should increase with decreasing environmental stress (Cooke and Rayner, 1984), and basidiomycetes are thought to be particularly successful in this respect (Boddy, 1999, 2000). Many basidiomycetes have highly differentiated mycelia, with rapidly advancing mycelial fronts, which degrades and regress into a sparse network of aggregated cords, as the mycelium senesce. This strategy has been proposed to provide an advantage with respect to colonisation of heterogeneously distributed resources, as well as combative interactions (Boddy, 1999, 2000). By redistribution of acquired nutrients, cord-forming basidiomycetes may optimize the efficiency by which they exploit colonised resources. Many basidiomycetes are also known for their ability to produce a potent repertoire of degrading enzymes (Rayner et al., 1987; Floudas et al., 2012). Combining high combative strength with efficient substrate degradation, translocation capacity and, for some groups, efficient ectomycorrhizal symbiosis, cord-forming basidiomycetes maximize the efficiency by which they exploit available resources in stable environments (Cooke and Rayner, 1984). They display many traits that are associated with K-selection, resulting in a competitive advantage at high mycelial densities with strong competition for space.

As abiotic stress is replaced by biotic stress (antagonism) along the gradient of increasing productivity, competition does not only intensify between fungal species, but also between entire functional groups. Interactions between mycorrhizal fungi and saprotrophic fungi in soil microcosm (Lindahl et al., 2001), as well as field experiments (Gadgil and Gadgil, 1971), have indicated antagonism and competition for nutrients between the two functional groups. The relative competitive strength of the two different groups has been hypothesised to change along a gradient in plant productivity, due to changed allocation of photosynthesis products (Högberg et al., 2003). At low nutrient availability, mycorrhizal fungi may exchange plant C for soil nutrients, and any surplus of photosynthetic sugars should preferably be allocated to them. Mycorrhizal fungi have, thus, been proposed to out-compete saprotrophs from the humus layers in low productive boreal forests, restricting their niche to the uppermost litter layer (Lindahl et al., 2007; Clemmensen et al., 2013). Sufficient availability of nutrients shifts tree production above ground (Janssens et al., 2010), resulting in less relative allocation of carbon to roots (Högberg et al., 2010). For example, anthropogenic N addition usually results in repressed mycorrhizal production (Nilsson et al., 2007; Kjøller et al., 2012; Bahr et al., 2013). This altered resource partitioning may, supposedly, shift the dominance from mycorrhizal fungi to saprotrophic fungi as fertility increases and relax the strict vertical partitioning of these functional groups that occurs in low-productivity forests.

At high fertility, trees and undergrowth species composition shifts to a higher inclusion of deciduous species that shed litter seasonally, resulting in flushes of easily available resources into the soil. With higher nutrient availability and better quality of litter, mineralization results in a

mobile, inorganic N-pool, whereby competition for nutrients decreases, reducing the benefit of differentiated mycelia with cords. Further, fertile soils usually constitutes a better environment for many soil animals , which may disturb the soil by mixing and grazing, preventing establishment of large and long-lived mycelial networks (Crowther et al., 2012). Thus, in such disturbed and fluctuating environments, r-selected fungi such as yeasts and moulds should be favoured, since they grow rapidly, are short-lived and reproduce fast, predominantly by asexual conidia or budding.

By using DNA extractions from soil and organic matter, the entire fungal community in the sample can be characterized at species level by using sequencing of the ITS region of the ribosomal RNA genes. Identification of species subsequently enables assembling of taxa into functional guilds. We can, thus, now begin to elucidate niche differentiation and specific ecosystem functions of fungal community components, in order to formulate hypotheses about the prevalence of different functional guilds in contrasting environments (Moorhead and Sinsabaugh, 2006; Koide et al., 2011). Such information is pivotal, since different functional guilds transform organic matter in strikingly different, often opposite ways, and play contrasting roles in C- and N-cycling (Lindahl et al., 2007; Clemmensen et al., 2013).

We analysed fungal communities in litter and humus layers from 26 pristine boreal forests in central Sweden by 454-pyrosequencing of PCR amplified molecular markers (Ihrmark et al., 2012; Lindahl et al., 2013). The fungal specific membrane lipid ergosterol was used as a marker of fungal biomass (Nylund and Wallander, 1992). Both free and total ergosterol was analysed; the free form has been shown to decline during senescence of fungal tissues (Yuan et al., 2008) and may be used to indicate the contribution of freshly formed mycelium (rev. by Wallander et al., 2013). Fungal biomass and community composition was related to the plant community and soil parameters. The forests were selected to represent a gradient of increasing fertility, as indicated by increasing soil NH_4 concentration and pH and decreasing C:N-ratio of litter and soil organic matter.

We hypothesize that:

1. In the most low-productive habitats, fungal biomass is low and stress tolerant ascomycetes dominate
2. In habitats with intermediate productivity, fungal biomass is high and cord-forming basidiomycetes dominate
3. In the most high-productive habitats, fungal biomass is low, and opportunistic moulds and yeasts dominate
4. Mycorrhizal fungi are progressively replaced by saprotrophs with increasing productivity and that the strict vertical separation of these functional groups between litter and humus is relaxed at high nutrient availability

Materials and Methods

Site description and sampling procedure

Twenty-six forests, located in central Sweden were selected to represent a gradient in fertility with contrasting levels of pH and N availability of soils, in addition to the relative composition of Norway spruce (*Picea abies* (L.) H.Karst.) and Scots pine (*Pinus sylvestris* L.) as well as understory plants (supplementary material). To reduce variation due to anthropogenic disturbance, areas outside the range of elevated N deposition were chosen based on national inventories of forests with high conservation values. The forest stands are all old (>100 years) and have never been subjected to intense forestry. Dominant type species of trees and ground vegetation were noted.

In September of 2008, ten soil cores (Ø = 2 cm) were randomly collected at an approximate minimum distance of 10 m between samples from each forest. The humus part from each soil core was pooled within sites, resulting in one humus sample per forest. In addition, 10 needles were picked from the ground at each spot where a soil core was collected, and then pooled within sites. When both dominant tree species were co-occurring, needles from each species were collected and kept as separate samples. The samples were stored in a cold container and frozen within 6h after collection at -20 °C.

Soil chemical analyses

The samples were thawed and homogenized before further analyses. A part of each homogenized sample was freeze dried and analysed for C:N ratio using an elemental analyser. Humus samples were analysed for the following parameters: Extractable NH_4 was determined by shaking 5 g of soil in 25 ml 0.5 M K_2SO_4 for one hour. The extracts were filtered (Munktell OOH S1-80-40) and measured with a Flow Injection Analyser (FIAstar ™, Foss Tecator, Höganäs, Sweden). Water content was determined by oven drying subsamples at 60 °C for 12h. Organic matter content was analysed by burning subsamples at 550 °C for 6h. Soil pH was measured in deionized water in a 1:3, soil:water ratio (744 pH meter, Metrohm, Herisau, Switzerland).

Ergosterol analysis

Free and total ergosterol content was analyzed according to Clemmensen *et al.* (2013) with minor adjustments (free ergosterol was analysed in humus samples only). Total ergosterol in 75 mg humus or 150 mg mineral soil was extracted with 5 ml 10 % KOH in methanol, while free ergosterol in 200 mg humus or 400 mg mineral soil was extracted with 5 ml methanol. After 15 min sonication and 60 min 70°C water bath, 1 ml H_2O was added to increase the polarity of the methanol phase. After adding 2 ml cyclohexane the tubes were shaken 1 min on a multivortex (Multi Reax, Heidolph, Germany) followed by 5 min centrifugation at 1 000 g, and the non-polar phase was then transferred into new tubes. The phase separation was repeated once more after addition of another 2 ml cyclohexane to the original tubes. The cyclohexane was evaporated on a 40°C heating block under N_2 gas flow. The ergosterol was then extracted in 200 µl methanol in a 40°C water bath. The samples were transferred into 2 ml microcentrifuge tubes and centrifuged at 13 000 g before the supernatants were filtrated (0.45 µm titan syringe filter, 44504-NPC by Sun sri, USA) into high-performance liquid chromatograph (HPLC) vials with inserts. The samples were analyzed in a reversed-phase column (C18 column: Chromolith model by Merck, C18 pre-column: Elite LaChrome model by Hitachi, Japan) HPLC (auto sampler L2130 with UV-detector L2400 by Hitachi, Japan) with a flow rate of 1 ml min^{-1}, and the ergosterol peak was detected at 280 nm.

DNA analysis

Prior to DNA analysis, the freeze-dried soil was cleared from roots larger than 2 mm in diameter and carefully homogenised in a mortar. Freeze-dried needles were homogenised in a ball mill (Retsch GmBH, Haan, Germany). Approximately 50 mg of humus or needles or 400 mg of mineral soil were used for DNA extraction with 1 ml extraction buffer (3 % cetyl trimethylammonium bromide (CTAB), 2mM EDTA, 150 mM tris-HCl and 2.6 M NaCl, pH8) at 65 °C for 1 h. After extraction with chloroform, DNA was precipitated with 1.5 volumes of isopropanol. Following centrifugation, the pellet was cleaned with ethanol and re-suspended in 50 µl water. The DNA extracts were further purified with the Wizard purification systems kit (Promega, Madison, USA). DNA samples were subjected to PCR, using the two primer combinations ITS4-gITS7 and ITS4-fITS9 (Ihrmark et al., 2012). In both combinations, the ITS4 primer was elongated by a unique sample tag of eight bases, designed using the BARCRAWL software (Frank, 2009). An annealing temperature of 56 °C and 55 °C was used respectively. By using qPCR, the number of cycles in the PCR was optimized for each sample and ranged between 25 and 35 cycles. Three PCR reactions from each sample and primer pare were pooled and purified with the AMPure kit (Beckman Coulter Inc., Brea, CA, USA). The concentration of the purified PCR products was established with a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA) and equal amounts of DNA from each sample were pooled to provide one sample per primer combination for the entire study. Adaptor legation and sequencing were performed by LGC Genomics, Berlin, Germany, on a 454-Genomic Sequencer (Roche, Basel, Switzerland), using titanium chemistry.

Sequence analysis

Sequences were analysed using the SCATA pipeline (scata.mykopat.slu.se). Sequences were filtered for quality, removing sequences with an average quality score below 20 or with score below 10 at any position, using the high quality region (HQR) extraction option. Sequences were then screened for primers and sample identifying tag and complimentary reversed when necessary. For sequences obtained with the ITS4-fITS9 primer combination, 44 bp of the 5.8 region were removed, resulting in equal lengths of sequences from both primer combinations. The sequences were then compared for similarity, using BLAST as the search engine, requiring a minimum match length of 85 %. Pairwise alignments were scored using a scoring function with 1 in penalty for mismatch, 0 for gap open and 1 for gap extension. Homopolymers were collapsed to 2 bp before clustering. Sequences were clustered into into operational taxonomic units (OTU) by single linkage clustering, with the maximum distance allowed for sequences to enter clusters set to 1.5 %.

The two datasets resulting from different primer combinations were merged. If a cluster differed more than three fold in relative abundance between the datasets, the highest relative abundance was used. Otherwise, the average relative abundance was recorded. Relative abundances were then adjusted to sum up to one.

Taxonomic and functional identification

From each sample, OTU:s were taxonomically identified in decreasing order of abundance until at least 80 % of all sequences were identified in each sample. The most abundant sequence in each operational taxonomic unit (OTU) was used as a representative. For each OTU, the most

closely related reference sequences were retrieved from the UNITE database or GenBank (NCBI) by BLASTn massblaster in PlutoF (Abarenkov et al., 2010). OTU representatives were aligned together with reference sequences in MegAlign (DNAStar) and taxonomic identities were assigned based on neighbour joining trees. Plant sequences were removed from the dataset and relative abundances were adjusted accordingly.

When an OTU, according to the neighbour joining tree was closely related to a reference sequence from an identified species with a known ecological function, it was assigned to that species and that specific function. However, when an OTU was closely related to a reference sequence from unidentified material (*i.e.* from community sequencing) with unknown function, it was assigned to a putative function depending on the substrate from which closely related reference sequences were derived.
When possible, the identified fungal taxa were ordered into groups according to their ecological function. Six groups were defined:

- *ECM*; fungi known to be ectomycorrhizal, both basidiomycetes and ascomycetes. This group was further divided into two subgroups.
    - *ECM with differentiated mycelium*; ectomycorrhizal fungi with medium distance fringe exploration type and long distance exploration type (Agerer, 2001)
    - *ECM with undifferentiated mycelium*; ectomycorrhizal fungi with contact, short-distance, medium distance (mat and smooth subtype) exploration types (Agerer, 2001).

- *Root associated ascomycetes*; ascomycetes associated with living plant roots, but not known ECM (e.g. ericoid mycorrhiza). The following two groups were used.
    - *Known root associated ascomycetes*; OTUs within the Ascomycota known to be associated with roots.
    - *Known and putative root associated ascomycetes*; OTUs within the Ascomycota known to be associated with roots and OTUs within the Ascomycota with close match to reference sequences from living root material.

- *Yeasts and molds*; Species in the orders Eurotiales, Hypocreales, Morteriellales, Mucorales, Saccharomycetales, Tremellales and Sporidiales.

- *Litter associated ascomycetes*; ascomycetes related to litter, saprotrophic fungi as well as endophytic. The following two groups were used.
    - *Known litter associated ascomycetes*; OTUs known to be associated with litter.
    - *Known and putative litter associated ascomycetes*; OTUs known to be associated with litter and OTUs with close match to reference sequences from intact, living or dead leaves or needles.

- *Litter associated basidiomycetes*; basidiomyctes related to litter, mainly saprotrophic. The following two groups were used.
    - *Known litter associated basidiomycetes;* OTUs known to be associated with litter.
    - *Known and putative litter associated basidiomycetes*; OTUs known to be associated with litter and OTUs with close match to reference sequences from intact, living or dead leaves or needles.

- *Unknown function*; fungi with unknown ecological role or fungi unclassified down to a sufficient taxonomical level.

Statistics

Ordination analyses were performed in Canoco for Windows v. 4.55 (Microcomputer Power, Ithaca, NY, USA). A detrended correspondence analysis (DCA) was carried out in order to obtain a graphical representation of fungal community similarity in the different forests and substrates. Correlation between environmental parameters and fungal community composition was established by canonical correspondence analysis (CCA), both on species level and on the level of genera and orders. Genera were used when possible, but in orders with many less clearly defined genera (e.g *Helotiales*) the order was used instead. The CCA:s were followed by Monte Carlo permutation test with forward selection of explaining variables, in order to establish the level of significance for the tested variables.

To test how the different functional groups of fungi were related to the environmental parameters, a multiple regression analyses were performed using General Regression Model, Multiple regression, Forward stepwise selection, in the Statistica software (Statsoft inc., Tulsa, OK, USA). Multiple regression was also used to test how the levels of ergosterol in the samples were related to environmental parameters as well as the functional groups of fungi.

Results

Correlation between environmental parameters

In the litter, litter C:N-ratio, pine litter, lingonberry and heather were significantly and positively correlated, while all of these were negatively correlated with humus NH_4, spruce litter, blueberry and herbs.

In the humus, pH, NH_4 and mineral content were significantly and positively correlated, while all of these were negatively correlated with C:N, together forming a set of fertility indicators. Scots pine was negatively correlated with these fertility indicators whereas Norway spruce was predominantly found in forests with low C:N-ratio. Lingonberry and heather were predominantly found in forests with low NH_4-levels, while herbs were more common in forests with high NH_4-levels.

Ergosterol

In the litter, there was a significant negative correlation ($p=0.0001$) between total ergosterol and litter C:N-ratio (Table 1; Fig. 1a). With C:N ratio in the model, the plant species origin of the litter did not significantly contribute additional explanatory power, and the interaction term between C/N-ratio and litter species was not significant. Total ergosterol levels in the humus were

significantly correlated with, pH, presence of Scots pine, mineral content, and relative abundance of yeasts and moulds, in order of decreasing additional explanatory power (Table 1). Ergosterol levels were higher in the presence of Scots pine, and within tree species the levels increased with higher pH. In combination with the fact that Scots pine forests commonly grow on more acidic soils, these additive effects result in a complex overall relationship between soil acidity and fungal biomass across the entire range of pH variation (Fig. 1b). Higher abundance of yeasts and moulds correlated with lower amounts of total ergosterol. There were no significant between-effects between tree species or any of the other significant parameters. The levels of free ergosterol in the humus were significantly and positively correlated with C:N-ratio and the presence of herbs (Table 1).

Community composition

In the litter, the DCA showed that a large portion of the variation in fungal species composition could be explained by host tree species (Fig. 2). In a CCA analysis with fungal species, the first axis explained a significant part of the variation in fungal community composition (F=4.1 and p=0.0001), and was positively correlated with C:N, pine litter, heather and lingonberry but negatively correlated with spruce litter and blueberry. Humus NH_4 and herbs correlated with the second CCA axis. Forward selection picked out litter type (p=0.0001), humus NH_4 (p=0.0008), presence of blueberry (p=0.034) and litter C:N (p=0.034) in order of decreasing additional explanatory power, as the best explanatory variables. The additional explanatory power contributed by other variables was not significant. The CCA axes (representing variation explained by environmental variables) together accounted for 39 % of inertia in the data. In a CCA analysis with species merged into genera or orders, the first CCA axis was positively correlated with C:N-ratio and pine litter but negatively correlated with spruce litter, and explained a significant part of the variation in fungal community composition (F=7.2 and P=0.0001)(Fig. 4). Forward selection picked out C:N-ratio (p=0.0001), presence of herbs (p=0.004) and litter type (p=0.03) in order of decreasing additional explanatory power, were the best explanatory variables for the fungal community composition in the litter. The additional explanatory power contributed by other variables was not significant. The CCA axes together accounted for 44 % of inertia in the data.

Multiple regression of the relative abundances of functional groups in relation to environmental parameters showed that the relative abundance of litter-associated ascomycetes was positively and significantly correlated with C:N-ratio, but negatively with the presence of herbs. When only taxonomically identified species were included in the analysis, the relative abundance of litter ascomycetes correlated positively with pine litter. Litter-associated basidiomycetes decreased with higher C:N-ratio. Ectomycorrhizal fungi in the litter decreased in the presence of *Calluna*, while root-associated ascomycetes were not affected by any of the environmental parameters. Root associated fungi together never exceeded 12 % of the amplicons in the litter. The relative abundance of yeasts and moulds was positively correlated with pine litter, but never exceeded 7 % of the amplicons (Fig. 6).

As shown by the DCA, the fungal species composition in the humus could, to a large extent, be predicted by the environmental parameters (Fig. 3). In a CCA analysis with fungal species, the first axis was positively correlated with pH, NH_4, mineral content, Norway spruce, blueberry and

herbs but negatively correlated with C:N, Scots pine, heather and lingonberry, and explained a significant part of the variation in fungal community composition (F=1.3 and p=0.025). Forward selection indicated that pH (p=0.0001), mineral content (p=0.003) and *Calluna* (p=0.003) in order of decreasing additional explanatory power, were the best explanatory variables. The additional explanatory power contributed by other variables was not significant. The CCA axis accounted for 48 % of inertia in the data. In a CCA analysis with species merged into genera or orders, the first CCA axis was positively correlated with pH, NH_4, herbs, mineral content and Norway spruce but negatively correlated with C:N-ratio, Scots pine and heather and explained a significant part of the variation in fungal community composition (F=3.056 and p=0.007)(Fig. 5). Forward selection indicated that pH (p=0.0001), C:N-ratio (p=0.02) and presence of *Calluna* (p=0.04) in order of decreasing additional explanatory power, were the best explanatory variables. The additional explanatory power contributed by other variables was not significant. The CCA axis accounted for 53 % of inertia in the data.

Multiple regression analysis showed that, in the humus, the relative abundance of root associated ascomycetes was positively and significantly correlated with C:N-ratio and with the presence of *Calluna*. In contrast, the total relative abundance of ectomycorrhizal fungi as well as ectomycorrhizal fungi with undifferentiated mycelium was significantly lower in forests where *Calluna* was present. When all forests were included in the analysis, yeasts and moulds did not correlate significantly with any environmental parameters. However, when the analysis was separated into mono-specific Norway spruce and Scots pine forests, yeasts and moulds correlated negatively with C:N-ratio in the spruce forests. The relative abundance of litter-associated fungi in the humus was higher in forests with higher N-availability, but never exceeded 5 % of the amplicons (Fig. 6).

Discussion

The fungal community composition varied strongly along the studied gradient. In each set of pooled subsamples from each site, there were a few highly frequent species that dominated the fungal community, and forests with similar characteristics shared dominant fungal species. Just as plant communities vary in predictive ways along gradients (Lahti and Väisänen, 1987), variation in the dominant components of the fungal communities could, to a large extent, be explained by pH, C:N and NH_4 content in the humus. In the litter, the fungal community depended strongly on the host tree species (Fig. 1 and 2), whereas in the more degraded humus layer the fungal community rather correlated with the understory plant community and less so with tree species (Fig. 3). Ergosterol concentrations also varied with environmental parameters in a predictable way. Thus, we here show that it is possible, with some statistical certainty, to predict the composition and biomass of fungal communities from environmental variables, within the range of forest types that we covered. Predictions are valid at the level of species, but also on higher taxonomic levels, as well as broad functional groups. This implies that it is possible to generate general relationships, which are useful in theoretical and quantitative ecosystem modelling.

There was a clear shift from a community dominated by ascomycetes in the low-productive end of the gradient to a community with increased contribution of basidiomycetes in the rich end. This observation was consistent both in the litter samples and the humus samples, although ascomycetes generally were more abundant in the litter than in the humus (Fig. 6). The dominant ascomycetes in the poor forests could largely be assigned to species in the

Leotiomycetes and Chaetothyriales. This is consistent with our hypothesis (hypothesis 1) that there would be an increase in more stress tolerant ascomycetes in poor environments, since fungi in Leotiomycetes and Chaetothyriales commonly display traits associated with stress tolerance, such as melanised cell walls (Gostinčar et al., 2010).

After manipulating soil acidity in a field experiment in arable soils, Rousk *et al.* (2010) also observed increasing dominance of Leotiomycete 18S markers at a pH below 4.5 (in a range from pH 8.3 to pH 4.1). Furthermore, within both Scots pine and Norway spruce forests, there was a negative correlation between ergosterol and acidity in humus, and for litter there was a negative correlation between ergosterol and C:N ratio (Fig. 1). This lends further support to the negative influence of environmental stress on the ascomycetes found in acidic forests with low N-availability. We hypothesise that stress tolerance with respect to acidity and low nutrient availability has been a major driver during early evolution of these sections of Ascomycota, underlying their generally high abundance in low-productive, acidic ecosystems globally. Within these sections, functional differentiation into saprotrophic litter fungi, endophytes and root-associated symbionts seem to have evolved much later in smaller clades roughly corresponding to the genus level.

The identified OTUs with the highest abundance, among the root associated ascomycetes, in the humus were *Rhizoscyphus ericae, Meliniomyces variabilis* and *Oidiodendron pilocola.* These Leotiomycetes are all mycorrhizal on ericaceous plants, and it could be argued that their distribution is regulated by the presence of their plant hosts. However, from a more 'mycocentric' perspective, the success of these plants under stressful conditions could just as well depend on their capacity to form symbiosis with fungi that facilitate survival in a stressful environment. A more balanced view would be that the two partners have co-evolved into a symbiosis that is highly successive in harsh environments (Read and Perez-Moreno, 2003).

In high C:N ratio litter, *Lophodermium pinastri* and *Lophodermium picea* dominated. These fungi are endophytes/pathogens and their persistence in litter after abscission seems to be favoured by high C:N ratios.

At intermediate N-availability and pH, the need for traits associated with stress-tolerance should be smaller, and fungi with competitive traits would increase. Moving towards more high-productive forests, we found an increase in the abundance of basidiomycetes relative to ascomycetes, both in litter and humus (in agreement with hypothesis 2). The higher combative strength of the basidiomycetes seems to have evolved on the expense of acidity/nutrient tolerance. Among the root-associated fungi in the humus, the apparent competitive balance between stress-tolerant ascomycetes and combative basidiomycetes also implied a shifting proportion of ericoid mycorrhizal fungi (mainly ascomycetes) and ectomycorrhizal symbionts (mainly basidiomycetes; Fig. 5), which most likely paralleled the relative abundance of their host plants (Read and Perez-Moreno, 2003).

In parallel with the increase in relative abundance of basidiomycetous fungi, the level of total ergosterol in low C:N needle litter was three times higher than in high C:N litter (Fig. 1). Boberg et al (2008) showed that NH_4 addition resulted in increased mycelial production of saprotrophic basidiomycetous fungi (in the genus *Mycena*), elevated respiration rates and increased needle mass loss in single species laboratory systems. In our study a similar increased fungal biomass in

response to higher N-content of the litter was observed in a range of forests and within complex fungal communities.

We further hypothesised that with increasing N-availability the mycorrhizal community would be replaced by saprotrophs, primarily opportunistic yeasts and moulds (hypothesis 3 & 4). The established view is that when nutrient availability increases, plant C allocation to roots would decline with negative effect on the presumably C-limited mycorrhizal community, leaving saprotrophic fungi with a competitive advantage (Högberg et al., 2003; Högberg et al., 2010). Additionally, a higher disturbance of mixing soil fauna (Butenschoen et al., 2007) in combination with temporal fluctuations in substrate availability should favour opportunistic saprotrophs. Previous studies have shown a decline in fungal biomass (relative to prokaryotes) with increasing nitrogen availability by quantifying PLFA's (Pennanen et al., 1999; Nilsson and Wallander, 2003).

In contrast to our hypothesis, the relative abundance of ectomycorrhizal fungi did not decline even under the most nutrient rich conditions, but remained high at 50-70 % of the amplicons (Fig. 6). Neither did the total fungal biomass decrease (Hypothesis 3), as shown by the high ergosterol levels in the more fertile forests with higher pH (Fig. 1). Positive responses of ectomycorrhizal fungi to N-additions, both in terms of mycelial growth and abundance, have been observed previously in low-productive arctic heathland (Clemmensen et al., 2008). We postulate that negative effects of high N-availability on mycorrhizal fungi are primarily found at the higher inorganic N-levels associated with fertilizer addition or atmospheric deposition, but that this relationship is not valid within the range of variation commonly found in undisturbed boreal forests not exposed to artificial or anthropogenic N-deposition. In fact, a single Scots pine forest, with much higher pH and inorganic N levels than commonly found in Scandinavian boreal forests, had the lowest ergosterol content of the entire study (and was therefore excluded from data analysis). This single observation may be due to a decrease in fungal biomass further along the fertility gradient, but such conditions are found only rarely in boreal forest not exposed to deposition or fertilization of N.

Dividing ectomycorrhizal fungi into subgroups based on the level of mycelial differentiation, we found that species with undifferentiated mycelium (e.g. *Tylospora*) were relatively more common towards the richer end of the gradient, whereas cord-forming species with differentiated mycelium (e.g. *Suillus* and *Cortinarius*) were more common in the low-productive forests, at least in relation to other ectomycorrhizal taxa (Fig. 5). This finding is in agreement with previous studies of colonised root tips (Lilleskov et al., 2002; Toljander et al., 2006; Cox et al., 2010; Kjøller et al., 2012), and here we corroborate this relationship based on total soil DNA, across wide geographical distances, and within a natural range in N-availability.

The shift in ectomycorrhizal morphology may imply that a larger proportion of the fungal biomass was found in ectomycorrhizal mantle tissues, closely associated with the roots at higher levels of nutrient availability. Thereby, removal of roots prior to analysis (e.g. by sieving) could contribute to previous observations of declining fungal biomass with increasing ecosystem productivity, explaining discrepancies between studies.

Even though litter-associated basidiomycetes increased in the humus layer in the most fertile forests, they still contributed a minor part (<5 %) of the amplicons only (Fig. 6). This small

increase may be explained by mixing of litter components into the more decomposed humus by soil fauna (in line with findings of larger mineral content mixed into the humus), possibly in combination with decreased competition from the ectomycorrhizal fungal community. However, we did not find significant amounts of DNA from terricolous, non-litter saprotrophs, such as members of the family Agaricaceae. A small increase in the abundance of yeasts and moulds with increasing N-availability was observed, but only in Norway spruce forests where Scots pine was absent. Here some groups (tremellales) increased in abundance with decreasing C:N-ratio (Fig. 5), and there were also some indications of a small increase in free ergosterol in these herb rich spruce forests (Fig. 1). However, including Scots pine forests in the analysis, the general pattern was completely opposite to our expectations; yeasts within Saccharomycetes, as well as moulds in the genera *Mortierella* and *Penicillium* were most abundant in acidic, high C:N ratio pine forests (Fig. 5), and free ergosterol was also positively correlated with the C:N ratio (Table 1). Thus, the expected major shift to a community dominated by saprotrophs, yeasts and moulds towards the richest part of the gradient was not observed. It is possible that the saprotrophic niche in humus is occupied primarily by bacteria, as proposed by (Högberg et al., 2003), but among fungi the vertical separation of a litter-associated, saprotrophic community above and a humus-associated mycorrhizal community below (Lindahl et al., 2007; Clemmensen et al., 2013) was maintained across the full range of ecosystem fertility.

The Scots pine forests deviated from our hypotheses with high levels of both free and total ergosterol in spite of their stressful growth conditions with low pH and severe nutrient limitation (Fig. 1). This is partly contradictory to our hypothesis (hypothesis 1) that fungal abundance generally would increase towards intermediate fertility. In addition, the abundance of free ergosterol was higher in the low-productive end of the gradient (Fig. 1), suggesting a higher proportion of recently formed fungal biomass (rev. by. Wallander et al., 2013) and a higher turn-over rate of the mycelium. Presumably, by allocating a higher proportion of photoassimilates to their mycorrhizal symbionts, Scots pines are better able than Norway spruces to support a high mycelial biomass of competitive ectomycorrhizal fungi, in spite of high levels of environmental stress. We postulate that Scots pines, by investing more C in cord-forming ectomycorrhizal symbionts, such as Suillus and Cortinarius species, with a high potential to forage recalcitrant organic pools for nutrients (Bending and Read, 1995; Bödeker et al., 2009; Hobbie and Agerer, 2010; Hobbie et al., 2013), are able to efficiently exchange C for scarcely available nutrients and compete successfully with ericoid plants and their associated stress-tolerant ascomycetes (Collier and Bidartondo, 2009).

The capacity of mycorrhizal fungi to support their host with nutrients is largely regulated by their propensity to immobilise nutrients in their own mycelia, particularly under nutrient limiting conditions (Alberton et al., 2007; Corrêa et al., 2012). Näsholm et al. (2013) recently showed that the more C the plants allocate to their mycorrhizal symbionts, the larger fraction of tracer ^{15}N was immobilised in mycelial biomass. The dilemma of the ectomycorrhizal symbiosis is that efficient foraging for nutrients from organic matter is conditional on high mycelial production, but efficient transfer of nutrients to the host plants requires a low degree of N-immobilisation in mycelial biomass. We postulate that under strongly nutrient limiting conditions, some ectomycorrhizal fungi combine high biomass production with rapid mycelial turn-over (here evident as high levels of free ergosterol), thereby enabling efficient substrate colonisation while

still maintaining a high proportional transfer of acquired nutrients to their hosts. Such properties are well described from cord-forming saprotrophs, which take advantage of their highly differentiated mycelia to efficiently explore heterogeneous environments and redistribute nutrients between resource units of contrasting qualities (Boddy, 1999). Similar strategies with rapidly advancing mycelial fronts, which degrades and regress into a sparse network of aggregated cords as the mycelium senesce, have also been described in ectomycorrhizal fungi, particularly in the genus *Suillus* (Finlay and Read, 1986). We further postulate that this self-induced fast mycelial turn-over support a community of opportunistic saprotrophs. After killing mycorrhizal mycelium by severing root connections, Lindahl et al. (2010) found increased abundance of yeasts and molds (again *Mortierella*, Saccharomycetes and *Penicillium*). It, thus, seems as if pines support a fungal community that combines functional groups of stress-tolerant, competitive as well as ruderal fungi, but to a high cost in terms of root derived carbon. Furthermore, the significant negative relationship between yeast and mould abundance and total ergosterol levels in the humus (Table 1) support that that these opportunists take part in rapid degradation of recently dead ectomycorrhizal mycelium. A correlation between high C allocation to roots, rapid production and turn-over of mycorrhizal mycelium, high levels of free ergosterol, and efficient N recycling was recently demonstrated by Clemmensen et al. (2013) in low-productive old-growth boreal forest ecosystems. Similar to the present study, these features were associated with the presence of Scots pine, and primarily connected to ectomycorrhizal fungi in the genera *Suillus* and *Cortinarius* as well as opportunistic molds and yeasts (Clemmensen et al., 2013).

In conclusion, we found a significant relationship between the fungal community composition and soil acidity as well as C:N-ratio. Moving from the low-productive to the high-productive end of the gradient, there was a shift from a community dominated by ascomycetes to a community with increased contribution of basidiomycetes, both in humus and litter. The levels of ergosterol decreased at low pH, indicating environmental stress, but superimposed on this general pattern, the presence of Scots pine had a positive influence on fungal biomass in the humus layer. Against our expectations, the relative abundance of ectomycorrhizal fungi in the humus layer did not decline in the most fertile forests, and we propose that further enhanced N addition by deposition or fertilization, above the levels usually encountered in non-disturbed boreal forests, is needed for a negative effect to occur. With this study, we show that it is possible to make statistical predictions about the composition and biomass of fungal communities within the range of forest types that we covered, which is potentially useful in theoretical and quantitative ecosystem modelling.

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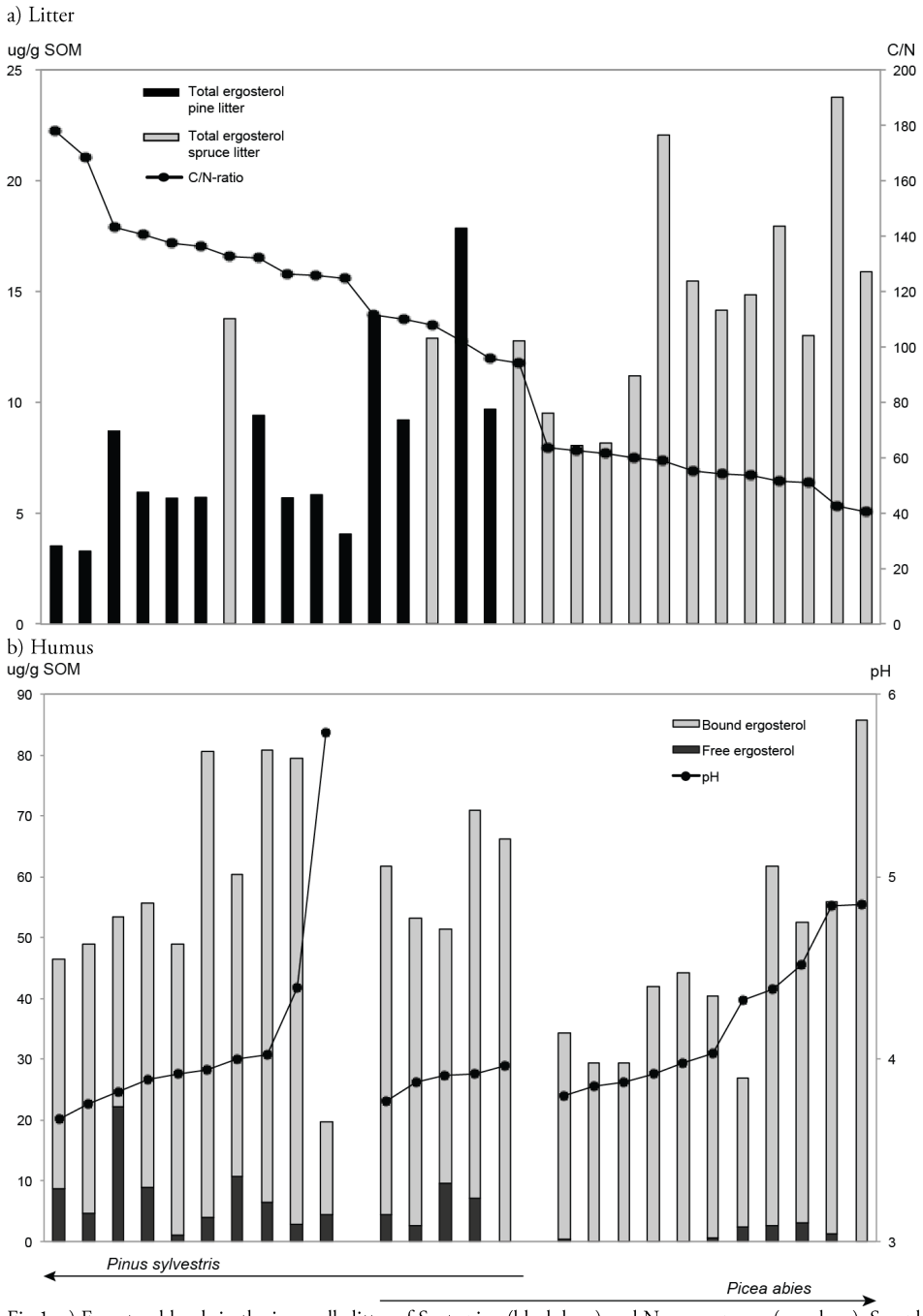


Fig 1. a) Ergosterol levels in the in needle litter of Scots pine (black bars) and Norway spruce (grey bars). Samples are sorted after litter C:N-ratio.
b) Bound ergosterol levels (grey part of bars) and free ergosterol levels (black part of bars) in the humus. Samples are sorted after dominant tree species of the forests, pine forests in the left part of the graph, spruce forests in the right part and codominance of the two species in the middle. For each tree species, the samples are ordered after humus pH.

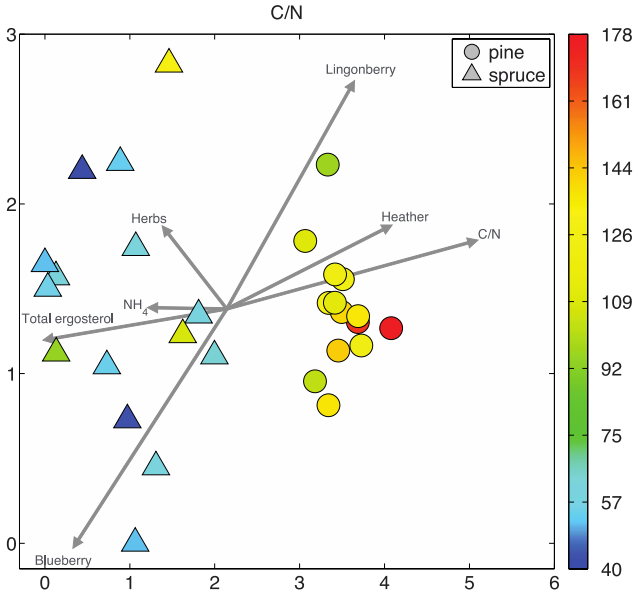
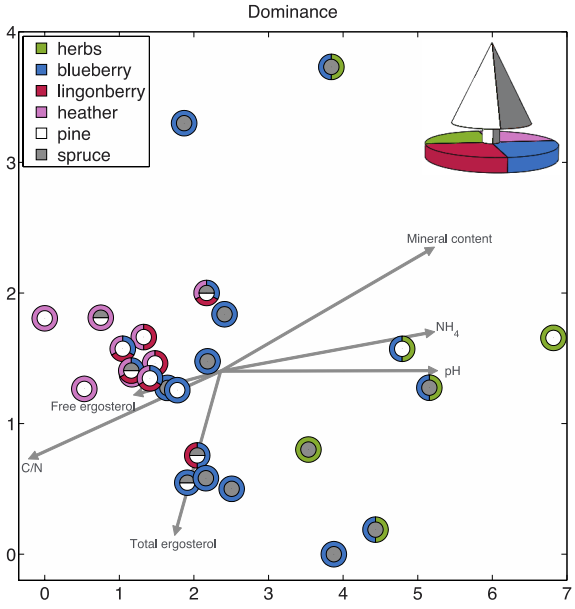


Fig 2. Joint biplot derived from a detrended correspondence analysis (DCA) of fungal communities in the litter layer of 26 Swedish boreal forest. Circles represent fungal communities in pine litter and triangles represents the fungal communities in spruce litter, both colour coded after litter C:N ratio. Vectors indicate the direction and degree (arrow length) of the correlation between quantitative soil parameters and the two first DCA axes.

a)



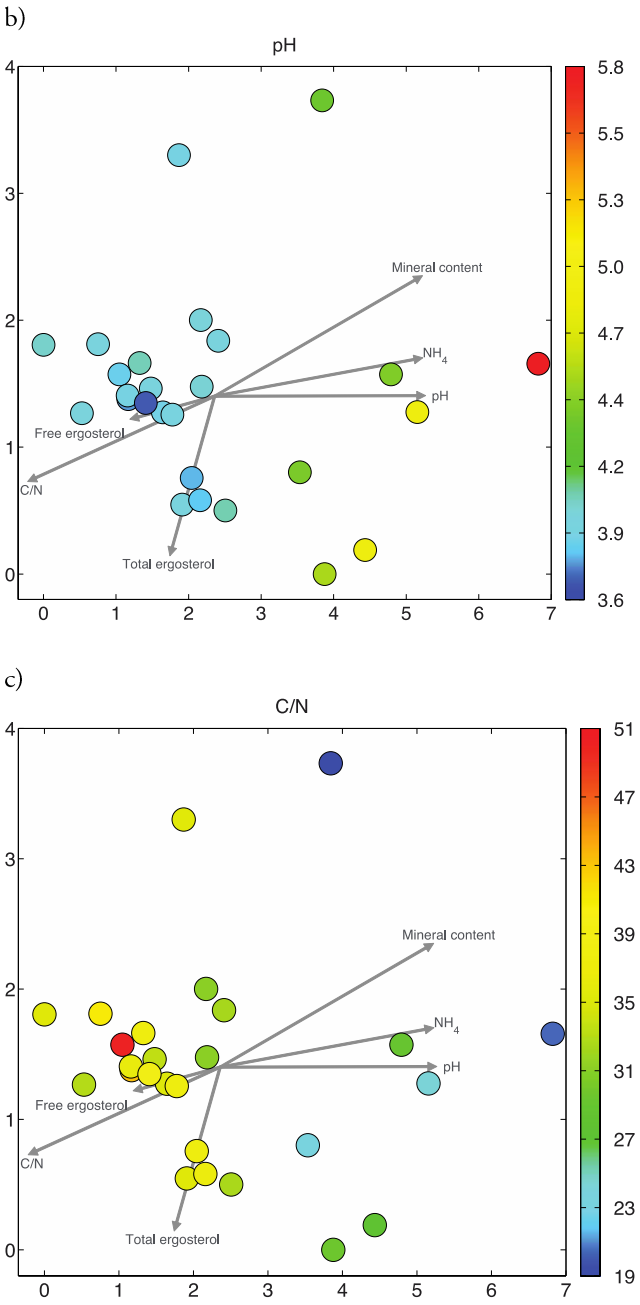


Fig. 3. Joint biplot derived from a detrended correspondence analysis (DCA) of data from the humus fungal communities in humus of 26 Swedish boreal forests. Each circle represents the fungal community in one forest where a) the outer part is colour-coded after understory vegetation and the inner part represents dominant tree species of the forest b) circles are colour-coded after humus pH and c) circles are colour-coded after humus C:N-ratio. Vectors indicate the direction and degree (arrow length) of the correlation between quantitative soil parameters and the two first DCA axes.

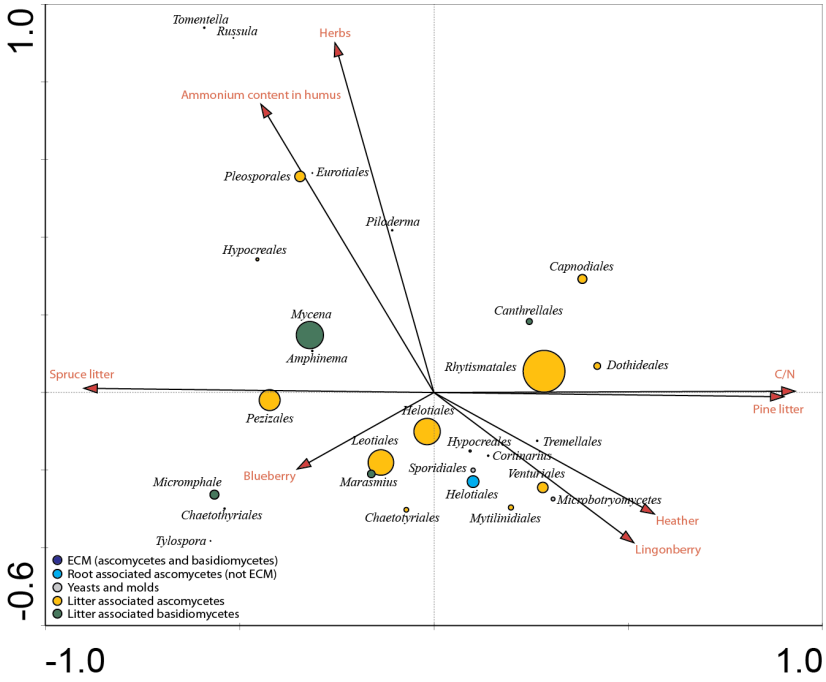


Fig 4. Joint biplot derived from a canonical correspondence analysis (CCA) of fungal communities in litter layers of 26 Swedish boreal forests, where species have been merged into genera or orders. Circles are colour coded depending on function of the genus or order. Vectors represent the constraining environmental variables in the CCA.

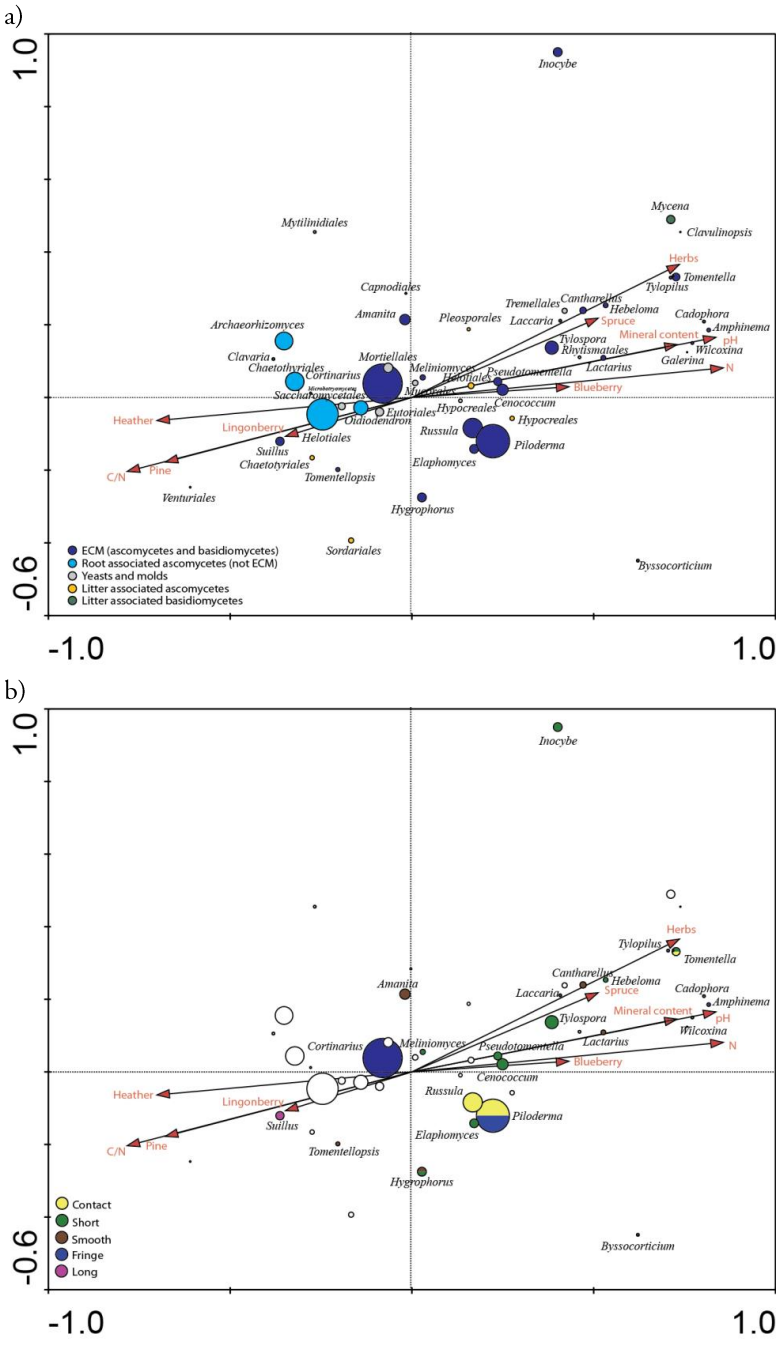


Fig 5. Joint biplot derived from a canonical correspondence analysis (CCA) of fungal communities in humus of 26 Swedish boreal forests, where species have been merged into genera or orders a) Circles are colour coded depending on function of the genus or order and b) Ectomycorrhizal genera are colour coded depending on exploration type of the genus according to Agerer (2001). Vectors represent the constraining environmental variables in the CCA.

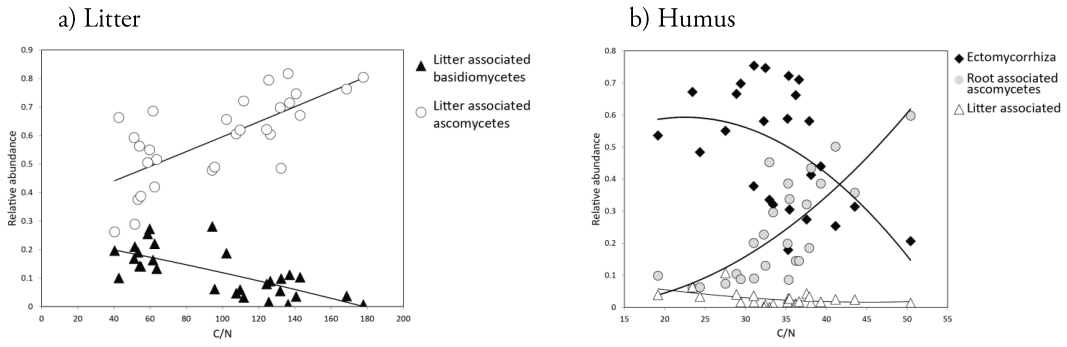


Fig 6 . Relative abundance of the ITS2 amplicons categorized as species being ectomycorrhizal (diamonds), root associated ascomycetes (dots) or litter associated (triangles), with increasing soil C:N-ratio in the a) litter and b) humus.

Table 1. Result from a general regression model, multiple regression of how free and total ergosterol levels in humus and litter was related to environmental parameters. P-value specified when significant, and direction of coefficient between brackets.

	Humus Total ergosterol	Humus Free ergosterol	Litter Total ergosterol
pH	p<0.0001 (+)		
NH_4 in humus			
C:N-ratio		p=0.0001 (+)	p<0.0001 (-)
Mineral content	p=0.004 (-)		
Pine	p<0.0001 (+)		
Spruce			
Herbs		p=0.038 (+)	
Blueberry			
Lingonberry			
Heather			
ECM diff			
ECM undiff			
Root ass asco			
Yeasts and moulds	p=0.01 (-)		
Litter ass asco			
Litter ass basidio			
Whole model	p<0.0001, R^2=0.47	p=0.0003, R^2=0.47	p<0.0001, R^2=0.46

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IV

We have found major changes in a plant-pollinator network over the past 120 years. This is partly explained by the nonrandom extirpation of bee species that are expected to be the most vulnerable to land-use and climate change, such as rare and specialized species, species occupying higher trophic levels, and cavity-nesting species. We found large changes in phenology of both forbs and pollinators and the potential for interaction mismatches, and these phenological changes can explain some of the species and interaction losses observed in this system. Our more optimistic finding was that plant-pollinator interaction networks were quite flexible in the face of strong phenological change and bee species extirpations, with many extant species gaining interactions through time. However, the redundancy in network structure has been reduced, interaction strengths have weakened, and the quantity and quality of pollinator service has declined through time. Further interaction mismatches and reductions in population sizes are likely to have substantial negative consequences for this crucial ecosystem service.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S10
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Roots and Associated Fungi Drive Long-Term Carbon Sequestration in Boreal Forest

K. E. Clemmensen,[1]* A. Bahr,[2] O. Ovaskainen,[3] A. Dahlberg,[1,4] A. Ekblad,[5] H. Wallander,[2] J. Stenlid,[1] R. D. Finlay,[1] D. A. Wardle,[6] B. D. Lindahl[1]

Boreal forest soils function as a terrestrial net sink in the global carbon cycle. The prevailing dogma has focused on aboveground plant litter as a principal source of soil organic matter. Using ^{14}C bomb-carbon modeling, we show that 50 to 70% of stored carbon in a chronosequence of boreal forested islands derives from roots and root-associated microorganisms. Fungal biomarkers indicate impaired degradation and preservation of fungal residues in late successional forests. Furthermore, 454 pyrosequencing of molecular barcodes, in conjunction with stable isotope analyses, highlights root-associated fungi as important regulators of ecosystem carbon dynamics. Our results suggest an alternative mechanism for the accumulation of organic matter in boreal forests during succession in the long-term absence of disturbance.

Globally, the boreal forest biome covers 11% of the land surface (*1*) and contains 16% of the carbon (C) stock sequestered in soils (*2*). Aboveground plant litter quality and decomposition rates have been proposed as the fundamental determinants of long-term soil organic matter accumulation (*3*–*6*). However, a large proportion of photosynthetically fixed C is directed belowground to roots and associated microorganisms (*7*, *8*), potentially affecting C sequestration either positively or negatively (*9*–*12*). A better mechanistic understanding of how the belowground allocation of C affects long-term sequestration rates is crucial for predictions of how the currently large C stock in boreal forest soils may respond to altered forest management practices, climate change, elevated CO_2 levels, and other environmental shifts.

Here we present evidence from a fire-driven boreal forest chronosequence that enables the study of soil C sequestration over time scales of centuries to millennia. The system consists of forested islands in two adjacent lakes, Lake Hornavan and Lake Uddjaure (65°55′ to 66°09′N; 17°43′ to 17°55′E), in northern Sweden. The islands in these lakes were formed after the most recent glaciation and have since been subjected to similar extrinsic factors. Larger islands, however, burn more frequently because they have a larger area to intercept lightning strikes (*6*, *13*); several large islands have burned in the past century, whereas some small islands have not burned in the past 5000 years. It has previously been shown that as the time since fire increases, soil and total ecosystem C accumulates unabated and linearly (*6*, *14*), leading to humus layers that can exceed 1 m in depth on the smallest islands. This has been attributed to a decline in the quality of aboveground litter inputs and impaired litter decomposition as the chronosequence proceeds (*6*, *14*, *15*). We studied organic soil profiles on 30 islands representing three size classes with increasing belowground C stocks (*14*): 10 large islands (>1.0 ha; on average, 6.2 kg of C m^{-2} accumulated belowground; mean time since fire 585 years), 10 medium islands (0.1 to 1.0 ha, 11.2 kg of C m^{-2}, 2180 years), and 10 small islands (<0.1 ha, 22.5 kg of C m^{-2}, 3250 years).

[1]Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, Box 7026, SE-75007 Uppsala, Sweden. [2]Department of Biology, Microbial Ecology Group, Lund University, Box 117, SE-221 00 Lund, Sweden. [3]Department of Biosciences, University of Helsinki, Box 65, FI-00014 University of Helsinki, Finland. [4]Swedish Species Information Centre, Swedish University of Agricultural Sciences, Box 7007, SE-750 07 Uppsala, Sweden [5]School of Science and Technology, Örebro University, SE-701 82 Örebro, Sweden. [6]Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden.

*Corresponding author. E-mail: karina.clemmensen@slu.se

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We explored C dynamics across the chronosequence by analyzing bomb ^{14}C (*16*) to determine the age since fixation of soil C, and then fitted a mathematical model to measurements of C mass and age distribution across vertical organic matter profiles for six representative islands; three large and three small (Fig. 1) (*17*). The model assumes two sources of C inputs: (i) a series of consecutively deposited cohorts of aboveground plant litter with negligible vertical mixing, and (ii) belowground inputs through root transport and rhizosphere processes. The dynamics of both C sources were estimated by a Bayesian parameterization of the model. The observed distribution of C mass and age was adequately predicted only when root C import was accounted for (Table 1 and figs. S1 and S2). The parameterized model estimates that the proportion of root-derived C accumulated over the past 100 years is larger on small islands (70%) than on large islands (47%), and the larger total C sequestration on small islands during this period can be explained entirely by root-derived inputs (Fig. 1). Differences in organic matter accumulation between islands were primarily determined by processes at the interface between the fragmented litter (F) and humus (H) layers, which corresponded to the zone of highest root density (Fig. 2B) and where the aboveground litter was 10 to 60 years old. The model was run for 100 years, covering almost the entire humus profile of the large islands, but on small islands a major proportion of C is stored in deeper horizons that are older than this. However, the model indicates that below 20 cm depth, root-derived C inputs are low and the C remaining from the horizons above decomposes slowly, as is also supported by ^{14}C depletion in the deeper layers of small islands (Table 1). Thus, root-mediated C input to the upper part of the profile represents a major contribution to the long-term buildup of humus, especially in late successional ecosystems.

Fungi play central roles in boreal forest ecosystems, both as decomposers of organic matter and as root-associated mediators of belowground C transport and respiration. We profiled the relative abundance of major functional groups of fungi through the depth profile of each island by DNA barcoding based on 454 pyro sequencing of the ITS2 region of ribosomal RNA genes (*17*, *18*). These analyses suggest that fungal communities in the uppermost litter layers were dominated by free-living saprotrophs, whereas mycorrhizal and other root-associated fungi dominated at greater depth (Fig. 2A). Thus, root-associated fungi dominate the part of the soil profile where the model indicates the largest difference in C sequestration between the island size classes. At this depth, free-living saprotrophs (mainly molds and yeasts) make a much reduced contribution, suggesting a correspondingly greater role of root-associated fungi in the regulation of organic matter dynamics.

The increase in root-derived C sequestration as the chronosequence proceeds is matched by a shift in the balance between the production and decomposition of fungal mycelium in the F-H transition zone of the soil profile. We measured the fungal-specific cell membrane lipid ergosterol as a marker for fungal biomass throughout each soil profile. Even though standing fungal biomass, as indicated by total (free plus bound) ergosterol (Fig. 3, A and B) and ITS copy numbers (table S1), was roughly similar on all islands, free ergosterol (characteristic of newly formed mycelia) (*19*–*21*) was about 20 times more abundant on large than on small islands, indicating a larger proportion of freshly produced mycelium and thus greater mycelial production. In contrast, bound ergosterol (the proportion of which increases during mycelial senescence) (*19*, *20*) was more abundant on smaller islands, indicating older mycelium with slower biomass turnover. Furthermore, the fungal cell-wall polysaccharide chitin (Fig. 3C) peaked in the F layer and declined in lower horizons of large islands, but remained at high concentrations at greater depths on the small islands. Chitin persists longer than ergosterol in fungal tissues after death (*21*), and the high level of chitin on small islands suggests retarded decomposition of fungal cell wall residues. Thus, in spite of supposedly greater mycelial production on the large islands, less mycelial necromass accumulated there than on small islands, suggesting that the large production was counterbalanced by faster decomposition of mycelial remains. Correspondingly, the ^{14}C model indicated faster decomposition of root-derived C on large islands, despite inputs being conservatively constrained to be equal across all islands. Taken together, our results point to impaired

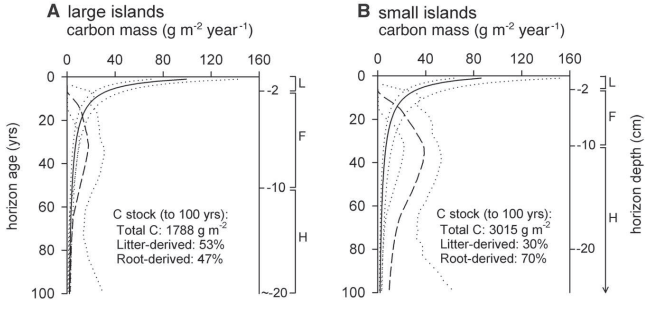

Fig. 1. Carbon dynamics in vertically stratified organic horizons of forested islands. Model estimates of C from aboveground litter (solid lines) and C introduced belowground via root transport (broken lines) are shown. C mass was modeled to a horizon age of 100 years, based on C mass and ^{14}C measurements in profiles from three large (**A**) and three small (**B**) islands. Dotted lines show the 95% central credibility intervals around posterior means. The posterior probability that the root-derived fraction is larger on small islands than on large islands is 0.97. Approximate depths are indicated for transitions between the main categories of horizons sampled; L, litter; F, fragmented litter; H, humus.

Table 1. C mass, ^{14}C abundance, and estimated C mean age of sampled organic layers on large and small islands. Means ± SE, $n = 3$ ($n = 2$ for the deepest layer in both size classes; both values are given).

Layer	Large islands			Small islands		
	C mass (g m^{-2})	$\Delta^{14}C$ (‰)	C age (years)	C mass (g m^{-2})	$\Delta^{14}C$ (‰)	C age (years)
Litter, on surface	103 ± 3	70 ± 3	7 ± 0	95 ± 7	62 ± 1	6 ± 0
Litter, 0 to 2 cm	185 ± 17	81 ± 17	9 ± 3	180 ± 11	84 ± 11	9 ± 2
Fragmented litter, 2 to 7 cm	454 ± 91	118 ± 12	15 ± 2	539 ± 63	101 ± 14	12 ± 2
Fragmented litter, 7 to 10 cm	493 ± 76	165 ± 19	20–39*	494 ± 77	136 ± 7	18 ± 1
Humus, 10 to 16 cm	1170 ± 90	150 ± 31	42–51*	1150 ± 165	191 ± 14	23–33*
Humus, 16 to 20 cm	1620 ± 230	21 ± 16	53 ± 1	910 ± 105	195 ± 17	34–50*
Humus, 20 to 40 cm	3410, 1846	9.4, -14.3	53, 57	6440 ± 815	14 ± 15	59 ± 7
Humus, 40 to 60 cm				7660 ± 700	−99 ± 14	780 ± 120
Humus, 60 to 80 cm				7380 ± 1760	−194 ± 9	1670 ± 90
Humus, 80 to 100 cm				5620, 3650	−285, −267	2628, 2432

*The mean age is within the given interval in samples that include the 1960s peak in bomb ^{14}C.

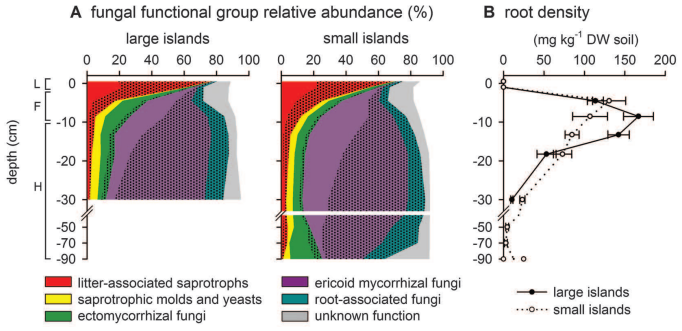

Fig. 2. Depth profiles of the relative abundance of fungal functional groups (percent of amplified ITS sequences) (**A**) and root (1 to 5 mm in diameter) density (**B**) on large and small forested islands. The profile corresponds to the organic layers L, F, and H. Functional groups comprise identified species with known function (unshaded) and species putatively assigned to a function (shaded) (*17*). The data set contains 650,000 sequence reads, and the globally most abundant 583 clusters are analyzed, covering 82 to 95% of the reads in individual sample types. The total ITS copy number was not affected by island size but decreased with depth (with 3×10^9, 4×10^8, and 2×10^7 copies g^{-1} of organic matter in L, F, and H layers, respectively) (table S1). The abundances of different functional groups should be compared with caution because of possible differences in ITS copy numbers per unit of biomass. All values are based on means of n =10 islands (except that n = 2, 5, and 7 for the lowest horizons) (*17*).

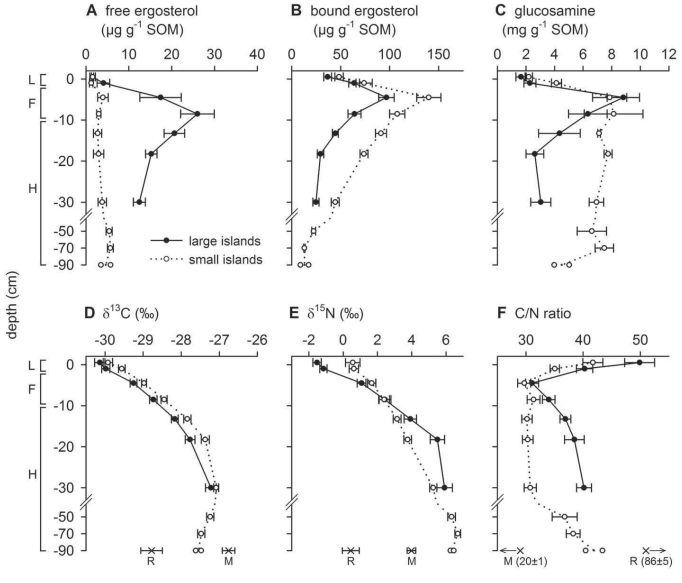

Fig. 3. Depth profiles of fungal biochemical markers (**A** to **C**), $\delta^{13}C$ (**D**), $\delta^{15}N$ (**E**), and the C/N ratio (**F**) in organic soil profiles of large (solid lines) and small (broken lines) forested islands. All data are means ± SE, n =10 (except that n = 3 for glucosamine and n = 2, 5, and 7 for the lowest horizons) (*17*). Medium-sized islands are not shown but are included in the statistical analyses presented in table S1. In the lower panel, levels measured in roots (R) 1 to 5 mm in diameter and mycorrhizal mycelium (M) sampled at 10 cm depth are given for reference.

decomposition of fungal residues as an important regulator of C accumulation as the chronosequence proceeds.

The often observed increases in ^{13}C and ^{15}N abundances with soil depth have been interpreted as evidence of increasing contributions of enriched microbial components to residual soil organic matter (*22*–*24*). In our system, where the dominant plant species form ecto- or ericoid mycorrhizal associations, the $\delta^{13}C$ and $\delta^{15}N$ signatures in the uppermost organic layers were similar to those of leaves (*25*), whereas signatures in humus layers were closer to those of rhizosphere mycelium (Fig. 3, D and E, and table S1) and mycorrhizal fungal sporocarps (*26*). Plant C allocated belowground is relatively enriched in ^{13}C, and this enrichment is further accentuated during C transfer to mycorrhizal fungi (*27*). However, historic changes in atmospheric $\delta^{13}C$ (*28*) may also contribute to the depth gradient (*22*, *24*). Mycorrhizal fungi also have higher $\delta^{15}N$ signatures than their host plants, because they supply N to their hosts that is ^{15}N-depleted relative to that retained in their own mycelium (*29*). Thus, the incorporation of isotopically enriched root and fungal remains is likely to be an important mechanism behind the increasing stable isotope signatures with soil depth in this system. This is consistent with the observation that isotopic signatures remain relatively constant in the initial litter decomposition phase and only increase when root-associated fungi dominate C and N dynamics (Figs. 2 and 3) (*30*, *31*).

Previous studies in this (*6*, *14*) and other (*3*, *4*) systems have pointed to the input and quality of aboveground litter as important regulators of C and N sequestration during long-term ecosystem development and succession. Our results show that aboveground plant litter dynamics on its own cannot explain the increasing rate of organic matter accumulation with time since wildfire, and that the dynamics of roots and associated fungi is an important additional factor explaining C accumulation in boreal forests. Although we observe less C accumulation on large islands, it is reasonable to assume that C allocation to roots and associated mycelium is greatest on those islands, especially given their higher root densities (Fig. 2B), free ergosterol levels (Fig. 3A), and net primary productivity (*6*). This apparent contradiction corroborates recent results (*32*, *33*) showing that increased C input to roots in response to CO_2 enrichment accelerates the turnover of soil organic matter, counteracting C accumulation and enhancing N cycling through the microbial pools. In our system, a similar stimulation of N recycling by large C inputs is supported by the steeper ^{15}N gradient (*31*) and higher C:N-ratio in the humus of large islands (Fig. 3, E and F) (*30*). In contrast, the less steep ^{15}N gradient and lower C:N-ratio on smaller islands suggest impaired mycorrhizal N mobilization (*31*) and accumulation of N in biochemically stabilized fungal remains, consistent with the high levels of bound ergosterol and

chitin on those islands. The consequential reduced N availability to plants leads to progressive nutrient limitation and compositional changes in the vegetation with increasing time since a major disturbance (*14*, *34*). Changes in plant productivity and community composition may, in turn, influence total belowground C allocation and distribution to fungal associates. Together, these feedbacks result in continuing C and N accumulation in the humus layer and decreasing plant production, and this process is only reset by major disturbances, such as wildfire.

Our results elucidate the mechanisms underpinning C sequestration in boreal forests and highlight the importance of root-associated fungi for ecosystem C balance and, ultimately, the global C cycle. We challenge the previous dogma that humus accumulation is regulated primarily by saprotrophic decomposition of aboveground litter, and envisage an alternative process in which organic layers grow from below through the continuous addition of recently fixed C to the organic matter profile in the form of remains from roots and associated mycelium. Environmental changes, such as N fertilization and deposition, forest management, and elevated atmospheric CO_2 concentrations, are therefore likely to greatly affect soil C sequestration through their alteration of rhizosphere processes. These processes are not well described in current models of ecosystem and global C dynamics, and their more explicit inclusion is likely to improve both the mechanistic realism and future predictive power of models.

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Supplementary Materials

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The Biological Underpinnings of Namib Desert Fairy Circles

Norbert Juergens

The sand termite *Psammotermes allocerus* generates local ecosystems, so-called fairy circles, through removal of short-lived vegetation that appears after rain, leaving circular barren patches. Because of rapid percolation and lack of evapotranspiration, water is retained within the circles. This process results in the formation of rings of perennial vegetation that facilitate termite survival and locally increase biodiversity. This termite-generated ecosystem persists through prolonged droughts lasting many decades.

Fairy circles (FCs) are large, conspicuous, circular patches devoid of vegetation in the center but with perennial grasses at the margin. These patches occur in large numbers in the desert margin grasslands of southern Africa (Fig. 1, A and B). Early observers considered poisonous plants, ants, or termites as causal factors; however, most of these early hypotheses were systematically tested and rejected (*1*, *2*). It has also been proposed that an unknown semivolatile substance in the soil might be responsible for the absence of grass within the FCs (*2*, *3*). In fact, a wide range of volatile organic compounds are found in FCs (*4*). Measurements of carbon monoxide and hydrocarbons in the soil led to the proposal of a geochemical origin of FCs (*5*). Carnivorous ants (*6*) and "self-organizing vegetation dynamics" (*7*) have also been considered as causes for FCs. Despite the many hypotheses, the origin and the ecosystem function of FCs are still a much-debated mystery. I used a long-term data set describing the environmental and biogeographical characteristics and dynamics of FCs to identify the most likely cause of these unique formations. Additionally, I analyze the function of FCs in terms of water management, biodiversity, and adaptation to arid conditions.

FCs occur along a narrow belt at the eastern margin of the Namib Desert, running from mid-Angola to northwestern South Africa. The area of distribution is closely associated with the isohyet of 100-mm mean annual precipitation (MAP) (Fig. 1B). The disjunct occurrence of FCs is caused by their pronounced restriction to sandy soils.

High soil humidity within FCs has been observed previously (*1*, *2*). To confirm and quantify this potentially adaptive function, I measured volumetric soil water content ($m^3/m^3 \times 100$) from 2006 to 2012 within and around FCs. At sites with a MAP of 100 mm, more than 53 mm of water were stored in the upper 100 cm of soil, even during the driest time of the year (table S1). At a depth >40 cm, a soil humidity of more than 5% volumetric water content was recorded over 4 years.

Higher temporal resolution of water flux was gained by automatic measurements recorded every hour within the bare patch and the grass matrix at 10-, 30-, 60-, and 90-cm depths using FDR sensors. During the observation period of 4 years, the humidity at 60-cm depth within the FC was either at or well above 5% volumetric water content (Fig. 2A). In the typical sand texture of FC soils with dominant grain sizes around

Biocenter Klein Flottbek, University of Hamburg, Ohnhorststrasse 18, 22609 Hamburg, Germany. E-mail: norbert.juergens@t-online.de

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Supplementary Materials for

Roots and Associated Fungi Drive Long-Term Carbon Sequestration in Boreal Forest

K. E. Clemmensen,* A. Bahr, O. Ovaskainen, A. Dahlberg, A. Ekblad, H. Wallander, J. Stenlid, R. D. Finlay, D. A. Wardle, B. D. Lindahl

*Corresponding author. E-mail: karina.clemmensen@slu.se

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This PDF file includes:

Supplementary materials:

Materials and Methods

Field sampling

The study was conducted in a boreal forest chronosequence situated on a series of forested islands in the two adjacent Swedish lakes, Lake Hornavan and Lake Uddjaure (65°55′ N to 66°09′ N; 17°43′ E to 17°55′ E) in N Sweden (*6, 13*). We studied 30 islands that represent three size classes: 10 large islands (>1.0 ha; on average 6.2 kg C m^{-2} accumulated belowground; mean time since fire 585 years), 10 medium islands (0.1 to 1.0 ha; 11.2 kg C m^{-2}; 2180 years), and 10 small islands (<0.1 ha, 22.5 kg C m^{-2}; 3250 years).

In August 2009, ten 3-cm diameter soil cores at least 10 m apart were sampled from each island using a PN425 JMC Sub-Soil Probe PLUS (JMC Soil Samplers, Newton, USA). The full humus depth of up to 1.2 m was sampled directly into separate plastic tubes and the cores were frozen within a few hours after sampling and kept intact until further processing. In the laboratory, the ten cores from each island were sorted according to organic layer thickness represented by the depth of the coring hole measured in the field (mineral parts subtracted if present). The shortest and longest cores out of the ten were discarded. The remaining eight cores were split into 20-cm horizons, accounting for their compression in the sampling tubes, as estimated by sample length in tube divided by depth of coring hole. The uppermost 20 cm was further split into two litter (L) horizons (intact litter on the surface and more degraded intact litter in the upper 0-2 cm of the core), two fragmented litter (F) horizons (2-7 and 7-10 cm depth) and two humus (H) horizons

(10-16 and 16-20 cm depth), representing sequentially more decomposed organic material by depth. Material from the same horizon was pooled within each island; living roots and rhizomes with a diameter of >1 mm were removed, and remaining organic matter homogenized, freeze-dried, weighed and milled to a fine powder before sub-sampling for further analyses. The island system consisted of a continuum of humus depths. From small islands we sampled up to 10 horizons, with the 60-80 cm horizon present on seven islands (i.e. n=7 for this depth), and the 80-100 cm horizon present on n=2 islands. For medium islands, the three lowest horizons (20-40, 40-60, 60-80 cm) were present on n=9, n=5 and n=2 replicate islands. For large islands, the lowest horizon (20-40 cm) was present on n=7 replicate islands. All other horizons were represented by n=10 islands. In total the data set consisted of 232 soil samples.

To obtain reference data from potential sources of soil organic matter (apart from roots collected from soil cores), fungal mycelium was sampled with sand-filled (20 g) 50 µm mesh fungal ingrowth bags (*35*). The bags (2 per island) were incubated at 10 cm depth from August 2005 to August 2006. The sand was extracted in water and mycelium floating in the water phase collected and freeze-dried. Due to small sample sizes mycelium was pooled within island size classes, yielding 3 composite samples per island size class.

Chemical analyses

All soil samples, roots and mycelium were analyzed for total C and N, as well as ^{13}C:^{12}C and ^{15}N:^{14}N ratios on an Isoprime isotope ratio mass spectrometer (Micromass-GV Instruments, Manchester, UK) coupled to an Eurovector CN elemental analyzer (model EuroEA3024; Eurovector, Milan, Italy) using continuous flow. Natural abundance of isotopes is expressed in

the δ-notation relative to international standards, Vienna Pee Dee Belemnite for C and atmospheric N_2 for N; δX_{sample} (‰) = 1000 × [(R_{sample}:$R_{standard}$)-1] where R is the molar ratio of $^{heavy}X$:$^{light}X$ isotope. The standard deviations of isotopic measurements of the working standards used were ± 0.1‰ for $\delta^{15}N$ and ± 0.05‰ for $\delta^{13}C$.

The ^{14}C content was analyzed on profiles from a representative subset of three small and three large islands, totaling 49 samples. The ^{14}C:^{12}C ratio in the dried and milled bulk organic material was analyzed by accelerator mass spectrometry at the Tandem Laboratory (Uppsala University, Sweden). Radiocarbon data are expressed as $\Delta^{14}C$, defined as the difference between the ^{14}C:^{12}C ratio in the sample and that of a universal standard (oxalic acid I, decay-corrected to 1950). For calculation of $\Delta^{14}C$, sample ^{14}C activity was corrected for isotopic discrimination after C fixation by correcting sample ^{13}C content to that of the atmosphere.

Ergosterol was analyzed in freeze dried and milled soil from each of the pooled samples using the method described by Nylund and Wallander (*36*) with some adjustments. To analyze free ergosterol, 5 ml methanol was added to 100 mg soil while 5ml 10% KOH in methanol was added to 50 mg soil for analysis of total ergosterol. Apart from those differences both free and total ergosterol was analyzed using the same protocol. The samples were extracted with a 15 min sonication and 1 h incubation at 70°C in a water bath. Distilled water (1 ml) was added to increase the polarity and 2 ml cyclohexane to serve as a lipophilic phase. After shaking for 1 min on a multivortex, samples were centrifuged for 5 min at 1000 g before collection of the lipophilic supernatant. The phase separation was then repeated once more after addition of another 2 ml of cyclohexane. The supernatant samples were evaporated on a 40°C heating block under nitrogen

gas flow (to prevent oxidation) and then extracted in 200 µl methanol and filtered through a 0.45 µm titan syringe filter (44504-NPC by Sun sri, USA) before they were analysed in a reversed-phase column (C18 column: Chromolith model by Merck, C18 pre-column: Elite LaChrome model by Hitachi, Japan) high-performance liquid chromatograph (auto sampler L2130 with UV-detector L2400 by Hitachi, Japan) with a flow rate of 1 ml min^{-1}. The ergosterol peak was detected at 280 nm.

The chitin content was measured using HPLC as described by Ekblad and Näsholm (*37*). In short, 10 mg of freeze dried and milled material was treated with 0.2 N NaOH to remove protein and amino acids. Glucosamine residues were released by acid hydrolysis with 6 N HCl and converted to fluorescent derivatives by treatment with 9-flourenylmethylchloroformate. Separation and detection of FMOC-derivatives was carried out on a Waters liquid chromatograph system consisting of a 600E pump and a WISP 700 autoinjector. Reversed-phase separation was carried out at a flow rate of 1 ml min^{-1} on a 250 mm x 5 mm (I.D.) ODS-hypersil (5 µm) column. The column effluent was directed to a Waters 740 fluorescence detector (excitation 260 nm, emission 330 nm). Chitin content was corrected for a recovery rate of 81%.

Fungal communities

Freeze-dried organic matter (50 mg) was extracted in 1.5 ml CTAB buffer (3% cetyltrimethylammonium bromide, 2 mM EDTA, 150 mM Tris-HCl and 2.5 M NaCl, pH 8) at 65°C for 1½ h interrupted twice for homogenization (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France). After centrifugation, the supernatant was extracted once with one volume of chloroform. The DNA was then precipitated from the supernatant with 1.5

volumes of 2-propanol at -20°C overnight. After centrifugation, the pellet was washed once with 70% cold ethanol and re-suspended in water. The resulting 50 µl DNA extracts were cleaned with the Wizard DNA clean-up system using a vacuum manifold (Promega, Madison,USA) and stored at -20°C.

The content of extractable fungal DNA was estimated using the ITS region of the ribosome encoding genes as a marker. The forward primer fITS9 (*18*) and the reverse primer ITS4 (*38*) were used to target the fungal ITS2 region, using a SYBR green mix (Maxima SYBR Green/Fluorescein qPCR Master Mix, Fermentas, Sweden) in a quantitative PCR cycler (Bio-Rad iQ5, Life Technologies, Carlsbad, CA, USA). The DNA extracts were diluted 40-4000 times in 20 µl PCR reactions, with 1 µM of the fITS9 primer and 0.3 µM of ITS4 primer. The PCR was run under the following conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C, 20 s at 55°C, 45 s at 60°C and SYBR green was quantified during the 60°C elongation step. A dilution series of plasmids containing an ITS insert from *Pilidium concavum* (Desm)Hoehn was used as a standard. Samples and standards were run in triplicates, and the analysis was repeated with a known quantity of standard plasmid added to each sample, in order to correct for PCR inhibition.

For production of fungal ITS2 amplicons for 454-sequencing, the two forward primers fITS9 and gITS7 (*18*) were used in separate reactions in combination with the ITS4 primer in order to maximize coverage of the fungal community. The ITS4 primer was extended with a sample-specific 8bp long identification tag, which was generated using the Barcrawl software (*39*). Analysis by PCR was conducted with an initial denaturation phase of 5 min at 95°C and 24 - 35 cycles of 30 s at 95°C, 30 s at 55°C (fITS9) or 56°C (gITS7) and 30 s at 72°C, and a final

elongation phase of 7 min at 72°C. The PCR mix consisted of 0.2 mM dNTPs, 0.75 mM $MgCl_2$, 0.3 µM of ITS4 primer, 1 µM of fITS9 or 0.5 µM of gITS7 primer and 0.5 U of DNA polymerase (Dream Taq, Fermentas) in 50 µl reactions. The number of cycles (22-35) was optimized to reach the exponential phase of the PCR reaction for each sample as determined by qPCR results. Samples were run in technical triplicates that were inspected on a 1% agarose gel and pooled. Amplicons were then purified with the AMPure PCR purification kit (Agencourt Bioscience Corporation, Beverly, MA, USA) and DNA concentrations were measured by spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA). Aliquots of each sample containing equal amounts of PCR product were pooled, concentrated by freeze-drying, re-suspended and further purified by the GeneJET PCR purification columns (Fermentas). Adaptor ligation and 454-sequencing were performed by LGC Genomics GmbH (Berlin, Germany) on a GL FLX Titanium system (Roche, Basel, Switzerland).

Sequences were processed using the bioinformatic pipeline SCATA (http://scata.mykopat.slu.se/). Sequences with an average quality score below 20 or below 10 at any single position were discarded, using the high quality region (HQR) extraction option. Sequences with missing primer sequences or identification tags were also discarded, allowing for one mismatch in each of the primer sequences. Out of the total of 442994 and 456918 sequences obtained for the fITS9 and gITS7 datasets respectively, 316211 and 351122 sequences passed this quality control. After removing primer and tag sequences, an additional 44 bp were removed from sequences obtained with the fITS9 forward primer to equalize sequence lengths in the two data sets. This left 41 bp of the conserved 5.8S region, 105-317 bp (on average 185 bp) of the ITS2 region and 38 bp of the LSU, on which the clustering was based. Sequences were compared

for similarity using BLAST as a search engine, with the minimum length of pairwise alignments set to 90% of the longest sequence. Pairwise alignments were scored using a scoring function with 1 in penalty for mismatch, 0 for gap opening and 1 for gap extension. Homopolymers were collapsed to 3bp before clustering. Sequences were assembled into clusters by single-linkage clustering with a 98.5% sequence similarity required to enter clusters.

For species identifications, the entire UNITE database (*40*) and a curated selection of sequences from the NCBI nr database were included in the clustering procedure in SCATA providing some species identifications based on the same criteria as the clustering. For identification of clusters with no in-cluster reference, the most abundant genotype was compared to databases using the BLASTn algorithm. Based on this, all clusters were split into major phylogenetic groups of fungi (Basal lineages, Leotiomycetes, Eurotiomycetes, other Ascomycota, Basidiomycota) and most abundant genotype aligned together with selected reference sequences using ClustalW (*41*) in MegAlign (DNASTAR Lasergene 9). Relatedness of the aligned sequences was investigated by neighbor-joining (NJ) analysis with total character difference using PAUP* 4.0b10 (AltiVec; *42*). Fungal clusters were then assigned to known or putative functional groups based on their placement in relation to reference sequences in the NJ trees (Fig. S3A-E); i.e. we employed a phylogenetically and taxonomically flexible delimitation level of functional groups guided by external reference sequences downloaded from public databases. Fungi identified to species or genera with a well-known function or life-form (based on published literature) were categorized into the functional groups: “Litter-associated saprotrophs”, “Ectomycorrhizal fungi”, “Ericoid mycorrhizal fungi” or “Molds and yeasts”. Fungi included in supported clades representing either a higher phylogenetic level with common function (*i.e.* Archaeorhizomycetes) or a group

of reference sequences derived from a particular substrate (*i.e.* surface-sterilized roots of ectomycorrhizal- or ericoid hosts or litter) were categorized into putative functional groups: "Putative litter-associated saprotrophs", "Putative ectomycorrhizal fungi", "Putative ericoid mycorrhizal fungi" or "Putative root-associated fungi". "Molds and yeasts" are free-living saprotrophs and this group contained clusters included in the major clades of Mucorales, Mortierellales, Saccharomycetales, Microbotryomycetes, Tremellales as well as *Penicillium* and *Trichoderma* spp.. "Putative litter-associated saprotrophs" included litter degraders, such as unidentified *Mycena* spp., and endophytes and pathogens isolated from litter materials (persisting as saprotrophs in dead litter). "Putative root-associated fungi" included species matching reference sequences isolated from roots or rhizoids (other than ericoid or ectomycorrhizal), such as Archaeorhizomycetes, as well as fungi with uncertain mycorrhizal status. Clusters of low abundance identified as lichens or pathogens on soil animals were left in the group of species with unknown function.

For establishment of relative abundance of fungal sequence clusters, all non-fungal sequence reads (on average 2% and 15% of the sequences obtained with primers fITS9 and gITS7, respectively) were removed from the data. Thereafter, each of the 233 samples were represented by on average 1329 (fITS9) plus 1275 (gITS7) reads. Potential differences in specificity of the two forward primers were tested by comparing the relative abundance across all samples of each cluster in amplicons obtained with the two primers. When one of the primers clearly disfavored a cluster (<30% of the other primer), data from the highest yielding primer was used, and the relative abundance of the rest of the clusters in each sample was adjusted accordingly (<0.5% of clusters affected). For all other clusters, relative cluster sizes were calculated as the average

between the amplicons obtained with the two different forward primers. In order to limit the identification work load, only the globally most abundant 583 clusters were included in analyses, covering 90% of all reads in the data set and with the smallest clusters represented by 70 reads.

Carbon sequestration model

A mathematical model was designed to estimate aboveground plant litter age in different horizons, as well as the amount of non-decomposed C of above- and belowground origin. We used an elaboration of the approach described by Franklin *et al.* (*43*). The model input data include mass and ^{14}C abundance of sampled soil horizons, yearly deposition of aboveground litter C (hereafter referred to as "litter C"), and previously acquired short term (2 years) decomposition rates of litter C (*6, 44*). In contrast to other ^{14}C based soil C models (e.g. *45*) an assumption here is that the soil consists of a series of consecutively deposited cohorts of litter C with insignificant vertical mixing, which makes the current model applicable to this soil type that contains little activity of burrowing animals. Litter C is assumed to decompose over time according to the equation of Bosatta and Ågren (*46*). Additionally, recently photosynthesized C is deposited via roots and associated microorganisms at all depths in the humus layer (hereafter referred to as "root C").

Model description

We define the layer of soil that contains the litter C that was deposited on the ground t years ago as the cohort t. We denote by $l(t)$ the mass of litter C (per square meter) in cohort $t = 0,1,2,\ldots$ The age of the C that is deposited on the ground may not be zero and is thus given a parameter

a_0 to be estimated. The degradation of litter C is described according to the equation of Bosatta and Ågren (*46*):

$$l(t) = l(0)(1 + c_l t)^{-\gamma_l},$$

where $c_l > 0$ and $\gamma_l > 0$ are parameters. The above equation assumes stationarity, *i.e.* that the annual deposition of litter $l(0)$ has remained constant over the years.

We denote by $r(t, \Delta t)$ the mass of root C in cohort t that was deposited Δt years ago and is thus currently of age Δt. We denote by $s(t)$ the mass of root C that is added annually to cohort $t = 1,2,\ldots$ Here we also assume stationarity, *i.e.* that the amount of root C added to the system (and its distribution over the soil layers) has remained the same over the years. Thus, using the same decomposition model as for litter C, the root C follows the equation:

$$r(t, \Delta t) = s(t - \Delta t)(1 + c_r \Delta t)^{-\gamma_r},$$

where $c_r > 0$ and $\gamma_r > 0$ are parameters. The total amount of C in cohort t is $c(t) = l(t) + \sum_{\Delta t=0,\ldots,t-1} r(t, \Delta t)$.

The average age of the C in cohort t is

$$a(t) = \frac{l(t)(t + a_0) + \sum_{\Delta t=0,\ldots,t-1} r(t, \Delta t)\Delta t}{c(t)}.$$

The data are not directly on the average age of the C, but on the average ΔC_{14} value. We describe the relationship between ΔC_{14} and C age as $\Delta C_{14} = F(\mathrm{a})$, where the function F was parameterized according to records of stratospheric ^{14}C content of the Northern hemisphere during the last 50 years (*47*).

The average ΔC_{14} of C in cohort t is

$$\Delta C_{14}(t) = \frac{l(t)(F(t + a_0)) + \sum_{\Delta t=0,\ldots,t-1} r(t, \Delta t) F(\Delta t)}{c(t)}.$$

Data

There are three kinds of data used to parameterize the model.

Soil samples: A soil sample consists of n_h horizons for which the total mass and the average ΔC_{14} age of the C have been determined. Each horizon h consists of an unknown number of sequential cohorts, e.g. first horizon of cohort 1, second of cohorts 2-4, third of cohorts 5-20, etc. We denote the duration (the number of years each cohort stays in the horizon) of horizon h by d_h. We denote the model-predicted amount of C in horizon h by c_h and the model-predicted average ΔC_{14} age content of C in the horizon by z_h (these can be computed from the cohort-based predictions). We denote the measurements of c_h and z_h by C_h and Z_h. We assume that the measurements relate to the model-predicted values through a proportional error, modeled as $C_h = c_h \exp(e_c)$, $Z_h = z_h \exp(e_z)$, where the error terms are distributed as $e_c \sim N(0, {\sigma_c}^2)$, $e_z \sim N(0, {\sigma_z}^2)$.

Litter C deposition: The yearly amount of C in the litter deposited on the soil surface, i.e. $l(0)$, has been measured as combined tree litter fall (2000-2008 average), dwarf shrub production (2000-2008 average) and moss production (2010) (*6, 44*). We denote the measurement by $L(0)$ and assume that $L(0) = l(0)\exp(e_l)$ with $e_l \sim N(0, {\sigma_l}^2)$.

Litter C decomposition bags: Bags of fresh litter ($t = 0$) were left to decompose on the soil surface and measured for their C content at times $t = 0$ and $t = 2$ (*6*). We denote these measurements as $B(0)$ and $B(2)$. As these measurements are likely to be more accurate (in relative terms) than the other measurements described above, we simplify the model parameterization by ignoring the measurement error, thus assuming that

$$B(2) = B(0)(1 + c_l t)^{-\gamma_l},$$

i.e. that

$$c_l = \frac{\left(\frac{B(2)}{B(0)}\right)^{-1/\gamma_l} - 1}{2}.$$

Parameterization

Root C input $s(t)$ is unknown for all values of t. To allow suitable flexibility but avoid over-parameterization, we assume that $s(t)$ is piecewise linear in the $\log t$ -scale, with values determined at $t = 1, 2, 4, 8, 16, 32, 64, 128$. For $t > 128$, we assume that $s(t) = s(128)$. This discretization roughly matches the resolution of the sampled horizons. In Model 1 we assume that $s(t) = 0$ for all t, *i.e.* that all stored C has its origin in aboveground plant litter. In Model 2 we assume that $s(t) = 0$ for $t = 1, 2, 3, 4$, but otherwise $s(t)$ is a free function. The assumption that no root C enters the uppermost horizons is based on the absence of roots and DNA from

root-associated fungi in these layers (Fig. 2). We fixed the parameters $\gamma_l = \gamma_r = 1.19$ based on literature (*46*).

The parameters to be estimated are the following:

- Same for all islands: $s(t), \sigma_c, \sigma_z, \sigma_l$
- Specific to each island: $l(0)$, a_0, ,c_r and d_h, where $h = 1,2,$ ... is an index for the cohort

We set to log-transformed standard deviation parameters $\sigma_c, \sigma_z, \sigma_l$ a N(0,1^2) prior, i.e. normal distribution with mean zero and standard deviation 1. For a_0 we assume a uniform prior in the range $3 \leq a_0 \leq 5$. For the remaining parameters, i.e. for $s(t)$ with $t = 1, 2,4, 8,16, 32,64,128,$ for $l(0)$ for each island, for the d_i parameters for each island, and for c_r, we set the essentially uninformative N(0,10^2) prior. Additionally, the horizon durations d_i were constrained to total at most 100 years.

We fitted the models to data using a slightly adapted version of the MCMC scheme of Ovaskainen *et al.* (*48*). Thus, the proposal distributions needed in the Metropolis-Hastings algorithm were adjusted adaptively during the burn-in (with 10,000 iterations) to obtain an optimal acceptance ratio, after which the posterior was sampled with 100,000 iterations thinned to 1,000 samples. The estimation was performed with Mathematica 8.0.

Statistical analyses

Differences in element, biochemical marker and root (1-5 mm) concentrations in organic profiles were analyzed by generalized linear mixed models, using the GLIMMIX procedure in the SAS 9.3 package (Statistical Analysis System Institute, 2002-2010). To quantify overall effects of

island size and sampling layer (upper six horizons included, n=180), island size class (df = 2; except df = 1 for chitin) and layer (df = 5) and their interaction (df = 10; df = 5 for chitin) were defined as fixed factors. To account for possible dependency between measures at different depths within individual islands, residual covariance structures were specified through RANDOM statements. Layer was treated as a repeated measure with island as the subject and a first-order autocorrelation structure. Satterthwaite-type of degrees of freedom based on the Kenward-Roger adjustment were used to calculate Wald F test statistics of the fixed factors and results were evaluated using Tukey's adjustment for multiple comparisons with $\alpha = 0.05$. Residuals were tested for normality with the Shapiro-Wilk test. A lognormal distribution type was specified for all data.

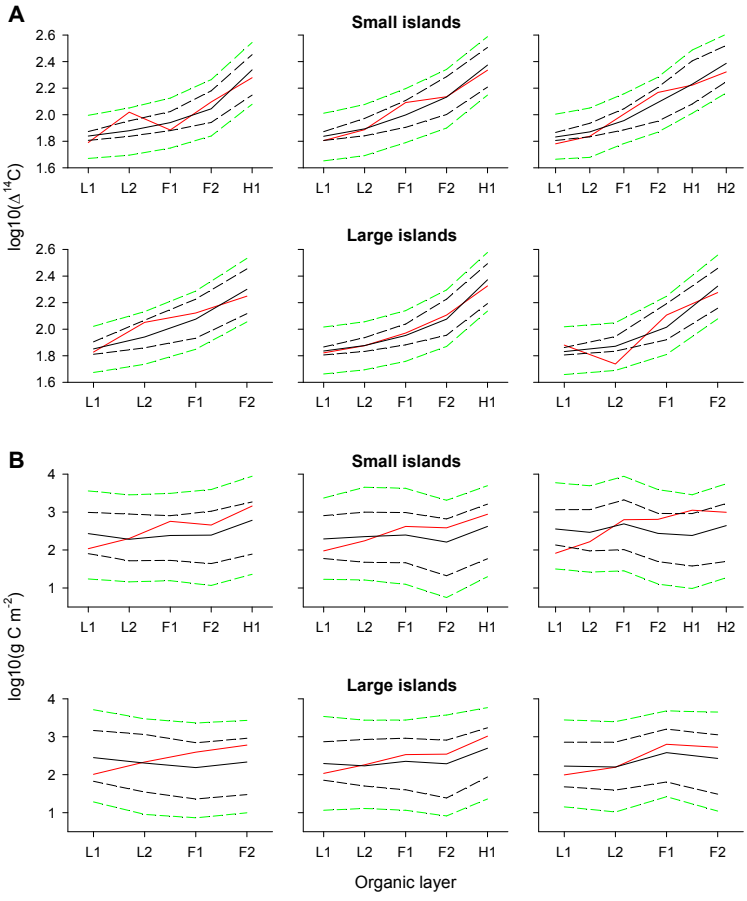


Fig. S1. Predictions of $\Delta^{14}C$ (A) and C mass (B) in sampled horizons by Model 1 allowing C input from aboveground litter only. Red lines show measured data and black lines show model posterior means. Broken lines represent the 95% central credibility intervals for the mean model predictions (black) and individual measurements (green).

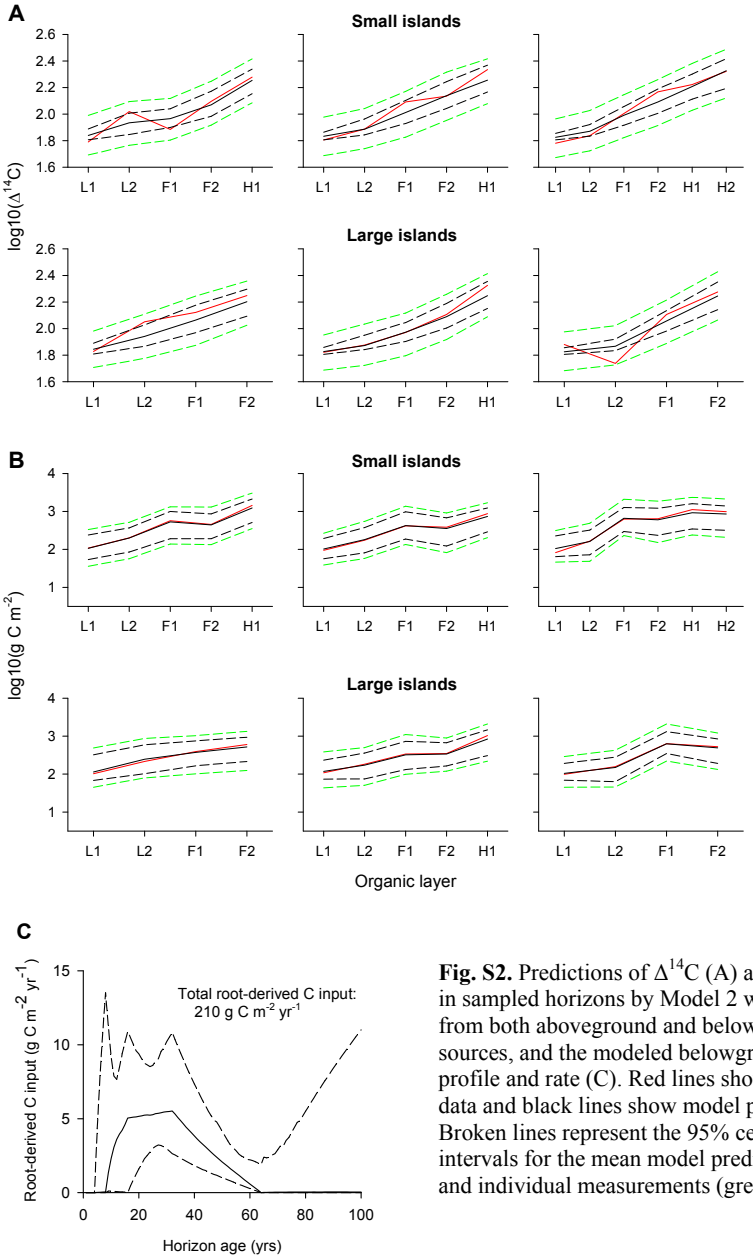


Fig. S2. Predictions of $\Delta^{14}C$ (A) and C mass (B) in sampled horizons by Model 2 with C input from both aboveground and belowground sources, and the modeled belowground C input profile and rate (C). Red lines show measured data and black lines show model posterior means. Broken lines represent the 95% central credibility intervals for the mean model predictions (black) and individual measurements (green).

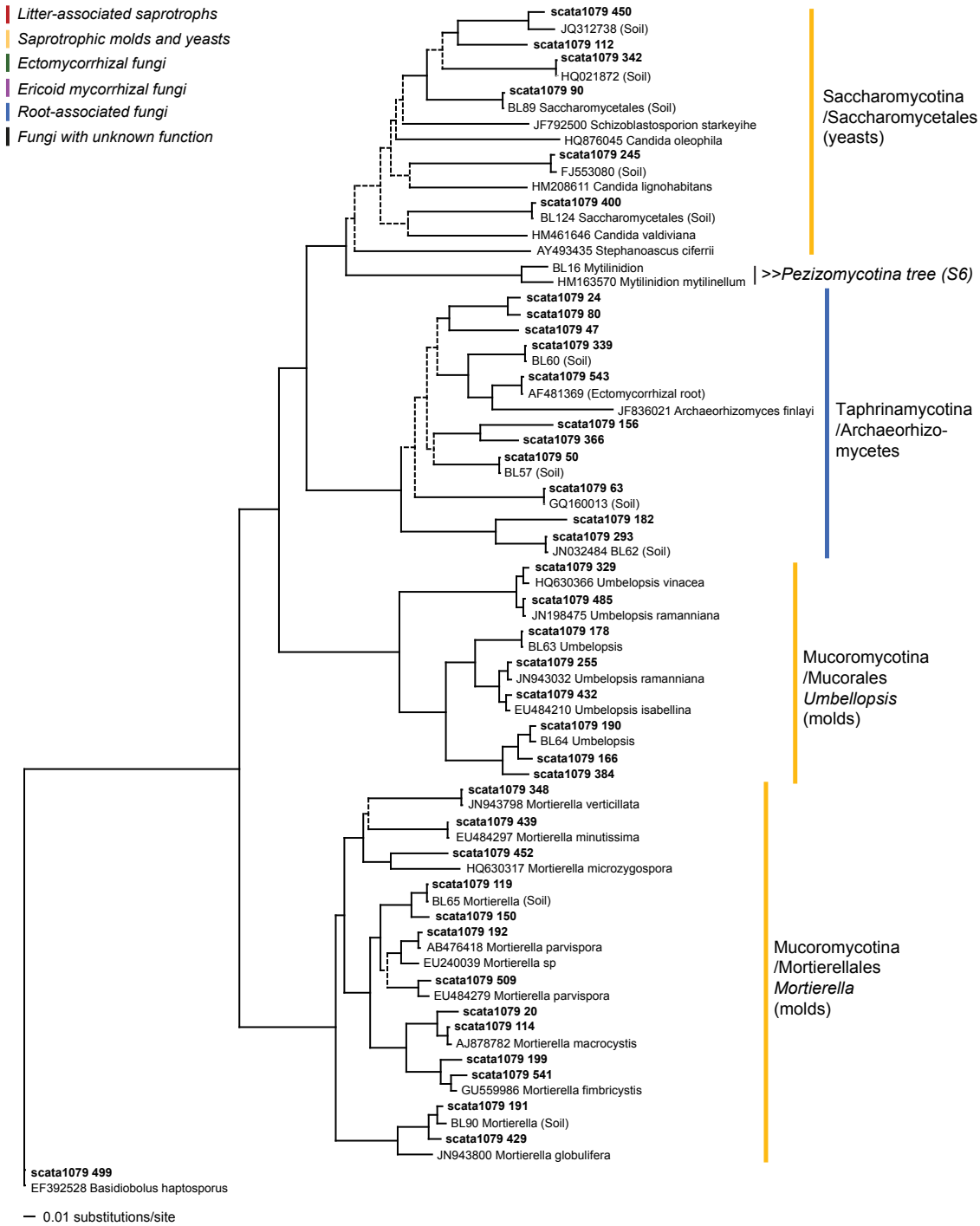


Fig. S3A. Neighbor-joining tree of representative sequences from obtained clusters (name in bold) and reference sequences belonging to Basal fungal lineages and basal clades of Ascomycota. Putative functional assignments are shown by color-coded bars. All branch nodes with boot-strap values of >70 have full lines; broken lines indicate boot-strap values of <70. More abundant clusters have lower identification number.

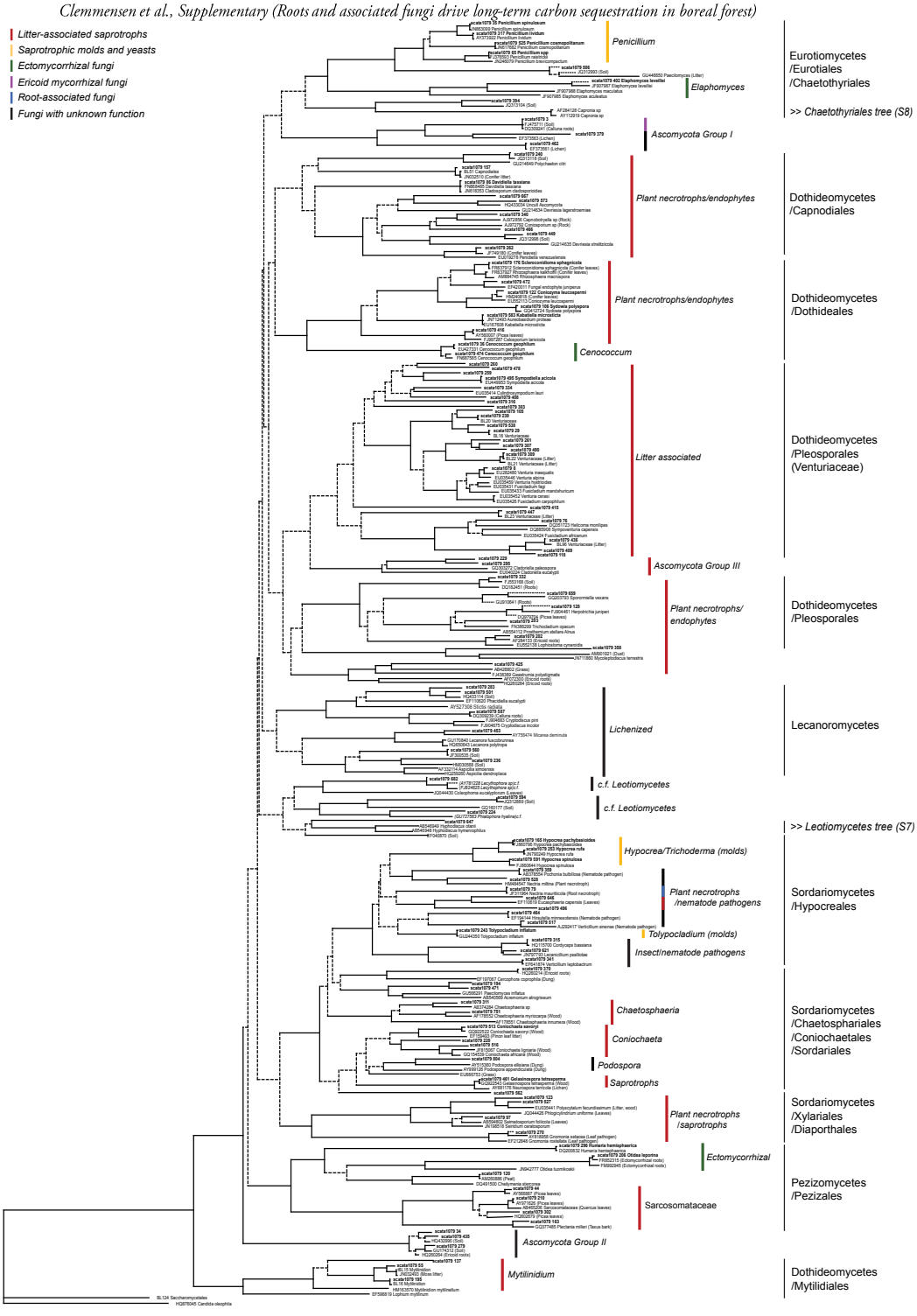


Fig. S3B. Neighbor-joining tree of representative sequences from obtained clusters (name in bold) and reference sequences belonging to Pezizomycotina (Ascomycota). Putative functional assignments are shown by color-coded bars; only identified clusters (with name indicated after sample ID number) are included in known functional groups. The environmental source material of reference sequences is shown in parentheses. Branch nodes with boot-strap values of >70 have solid lines; broken lines indicate boot-strap values of <70. More abundant clusters have lower identification number.

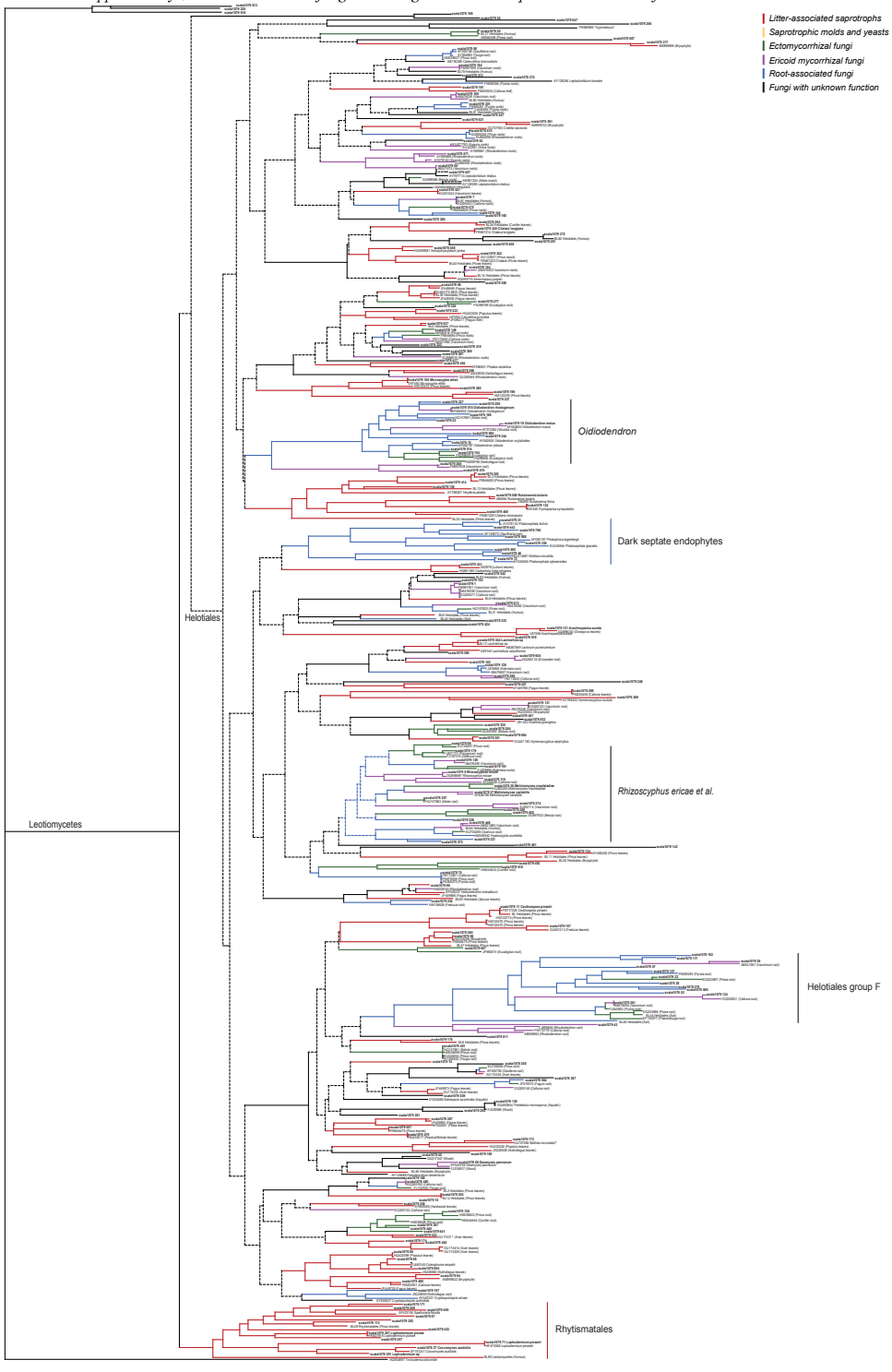


Fig. S3C. Neighbor-joining tree of representative sequences from obtained clusters (name in bold) and reference sequences belonging to Leotiomycetes (Pezizomycotina, Ascomycota). Putative functional assignments are shown by color-coded branches or bars; only identified clusters (with name indicated after sample ID number) are included in known functional groups. The environmental source material of reference sequences is shown in parentheses. Branch nodes with boot-strap values of >70 have solid lines; broken lines indicate boot-strap values of <70. More abundant clusters have lower identification number.

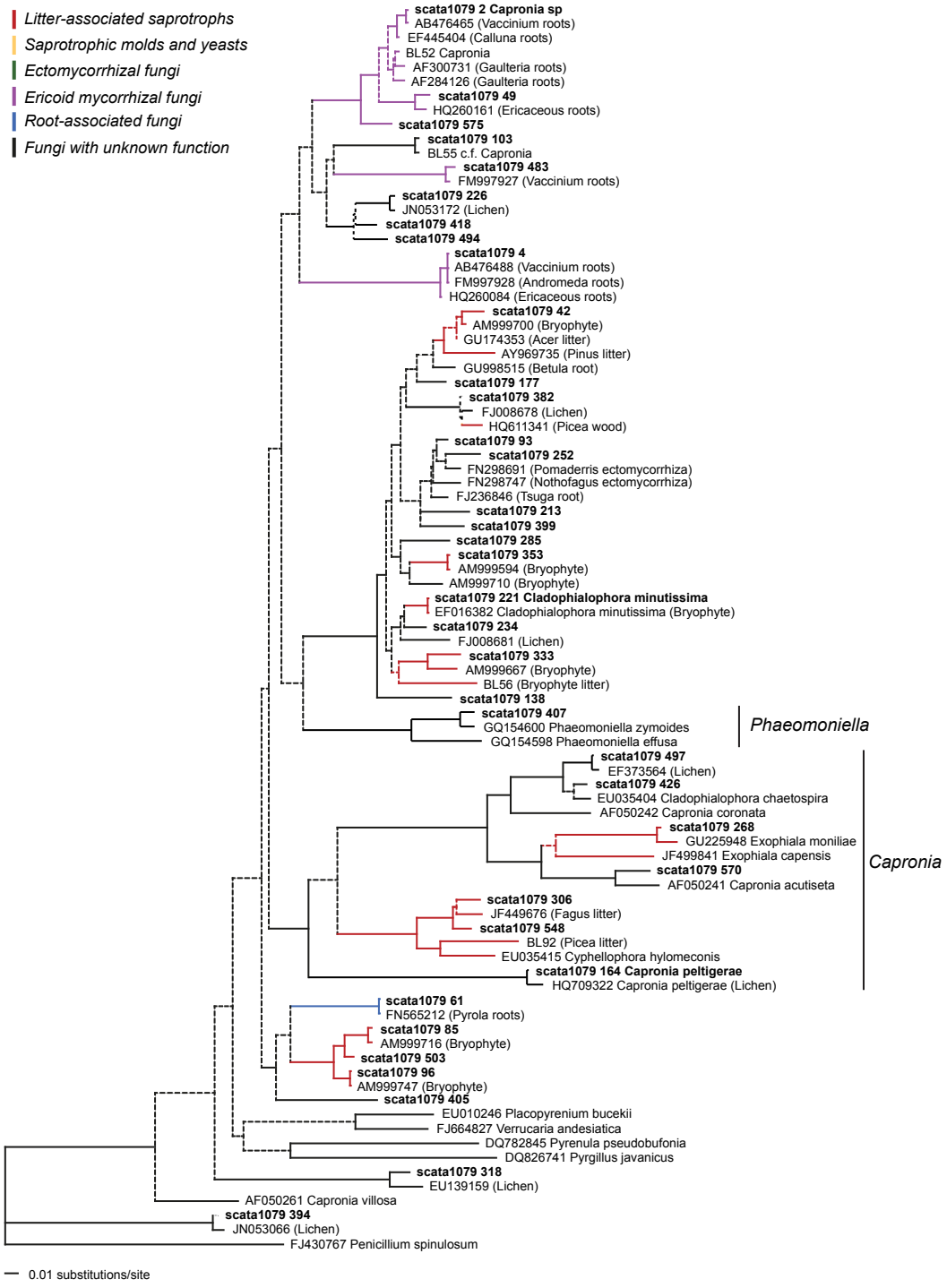


Fig. S3D. Neighbor-joining tree of representative sequences from obtained clusters (name in bold) and reference sequences belonging to Chaetothyriales (Eurotiomycetes, Pezizomycotina, Ascomycota). Putative functional assignments are shown by color-coded branches; only identified clusters (with name indicated after sample ID number) are included in known functional groups. The environmental source material of reference sequences is shown in parentheses. All branch nodes with boot-strap values of >70 have full lines; broken lines indicate boot-strap values of <70. More abundant clusters have lower identification number.

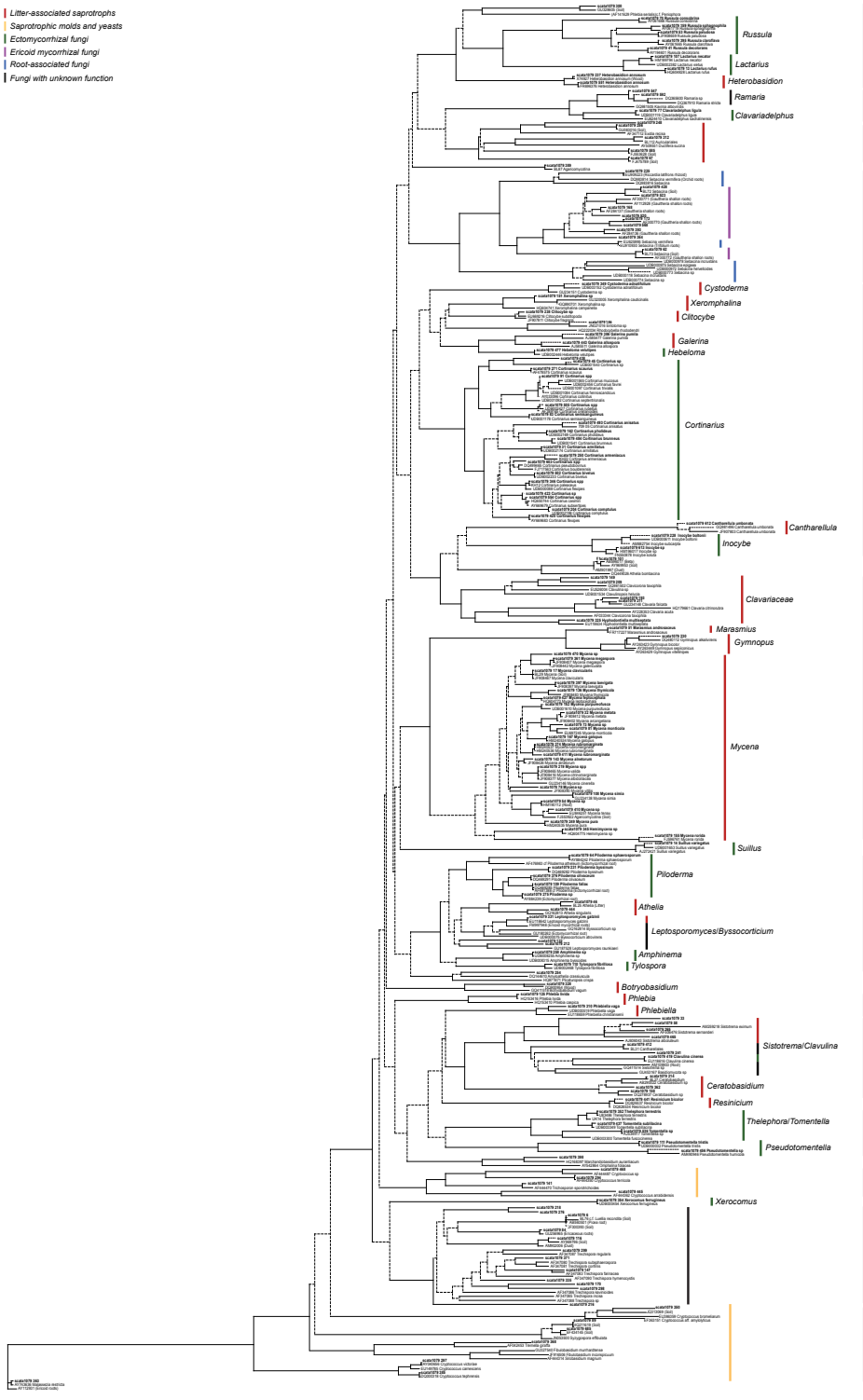


Fig. S3E. Neighbor-joining tree of representative sequences from obtained clusters (name in bold) and reference sequences belonging to Basidiomycota; sequences representing 7 clusters belonging to Microbotryomycetes (putative yeasts) were not included in the tree due to too large divergence. Putative functional assignments are shown by color-coded bars; identified clusters (with name indicated after cluster identification number) are included in known functional groups. Branch nodes with boot-strap values of >70 have solid lines; broken lines indicate boot-strap values of <70. More abundant clusters have lower identification number.

Table S1. *F* and *P* values derived from analyses of variance of results presented in Figs. 2 and 3. Island size class (three levels; two for glucosamine), organic layer (six levels; four for root density) and their interaction were included as fixed factors and layer treated as a repeated measure within each island (30 islands in total) in the random term of a generalized linear mixed model. Degrees of freedom (df) for *F* values (effect, error) were estimated using the Kenward-Roger adjustment.

variable	island size		layer		size x layer interaction	
	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*
C/N ratio	$9.64_{2,37}$	**0.0004**	$44.33_{5,130}$	**<0.0001**	$1.05_{10,133}$	0.4041
$\delta^{13}C$ (‰)	$5.69_{2,39}$	**0.0069**	$312.65_{5,131}$	**<0.0001**	$0.41_{10,135}$	0.9378
$\delta^{15}N$ (‰)	$1.80_{2,38}$	0.1798	$260.32_{5,135}$	**<0.0001**	$11.6_{10,137}$	**<0.0001**
Total ergosterol (mg g^{-1} SOM)	$10.58_{2,47}$	**0.0002**	$46.72_{5,129}$	**<0.0001**	$1.40_{10,134}$	0.1863
Free ergosterol (µg g^{-1} SOM)	$16.32_{2,33}$	**<0.0001**	$17.40_{5,126}$	**<0.0001**	$4.04_{10,130}$	**<0.0001**
Bound ergosterol (µg g^{-1} SOM)	$20.74_{2,44}$	**<0.0001**	$35.56_{5,130}$	**<0.0001**	$2.68_{10,134}$	**0.0051**
Glucosamine (mg g^{-1} SOM)	$6.10_{1,6}$	**0.0466**	$13.56_{5,19}$	**<0.0001**	$3.35_{5,19}$	**0.0239**
ITS copy numbers (g^{-1} SOM)	$0.65_{2,42}$	0.5265	$58.19_{5,127}$	**<0.0001**	$0.20_{10,132}$	0.9958
Root density (mg kg^{-1} soil)	$0.65_{2,30}$	0.5351	$17.01_{3,74}$	**<0.0001**	$2.62_{6,77}$	**0.0228**

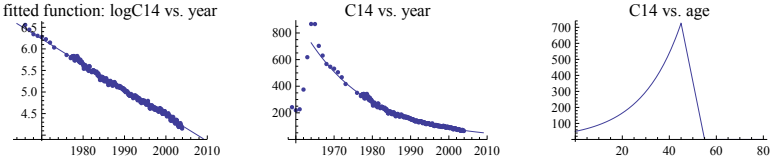
Model code S1. (16 following pages)
The Mathematica 8.0 source code used to estimate the C sequestration model.

```
Needs["ErrorBarPlots`"];

sfunction[t_, st_, ss_] := Module[{res, ns, smaller},
   ns = Length[st];
   res = -1;
   If[t ≤ Min[st], res = ss[[1]]];
   If[t ≥ Max[st], res = ss[[ns]]];
   If[Min[st] < t < Max[st],
    smaller = Position[Sort[Append[st, t]], t][[1, 1]] - 1;
    res = ss[[smaller]] + (ss[[smaller + 1]] - ss[[smaller]]) *
        (Log[t] - Log[st[[smaller]]]) / (Log[st[[smaller + 1]]] - Log[st[[smaller]]]);
   ];
   N[res]];

SplitPars[pars_] := Module[{pars2, res, dh},
   pars2 = pars;
   res = {Take[pars2, 3], Take[pars2, {4, 3 + nt}], Take[pars2,
      {4 + nt, 4 + nt + nsites - 1}], Take[pars2, {4 + nt + nsites, 4 + nt + 2 nsites - 1}],
     Take[pars2, {4 + nt + 2 nsites, 4 + nt + 3 nsites - 1}]};
   pars2 = Drop[pars2, 4 + nt + 3 nsites - 1];
   dh = {};
   Do[
    dh = Join[dh, {Take[pars2, nh[[i]]]}];
    pars2 = Drop[pars2, nh[[i]]];
    , {i, nsites}];
   res = Join[res, {dh}];
   res];

CarbonModel[pars_, site_, AdditionalOutput_] :=
  Module[{cl, sigmac, sigmaz, sigmal, ss, a0, l0, cm, dh, ah, l, dm, linage, dmt,
    dmC14, s, m, tm, c, ma, mC14, tma, tmC14, a, C14x, mass, age, C14, res},
   cl = lcl[[site]];
   {{sigmac, sigmaz, sigmal}, ss, a0, l0, cm, dh} = SplitPars[pars];
   ss = Exp[ss];
   ss = Join[{0, 0, 0}, ss];
   If[! source, ss = Table[0, {i, 3 + nt}]];
   a0 = Exp[a0[[site]]];
   l0 = Exp[l0[[site]]];
   cm = Exp[cm[[site]]];
   dh = Round[Exp[dh[[site]]]];
   ah = Table[Total[Take[dh, h]], {h, nh[[site]]}];
   If[Max[ah] + a0 ≤ MAXT && Min[dh] ≥ 1,
    l = Table[l0 (1 + cl t)^(-gammal), {t, MAXT}];
    dm = Table[(1 + cm dt)^(-gammam), {dt, 0, MAXT - 1}];
    linage = N[Table[t, {t, MAXT}]];
    dmt = dm * (linage - 1);
    dmC14 = dm * Map[F, linage - 1];
    s = Table[sfunction[t, st, ss], {t, MAXT}];
    m = Table[Reverse[Take[s, t]] * Take[dm, t], {t, MAXT}];
    tm = Map[Total, m];
    c = l + tm;
    ma = Table[Reverse[Take[s, t]] * Take[dmt, t], {t, MAXT}];
    mC14 = Table[Reverse[Take[s, t]] * Take[dmC14, t], {t, MAXT}];
    tma = Map[Total, ma];
    tmC14 = Map[Total, mC14];
```

```
    a = (l * (linage + a0) + tma) / c;
    C14x = (l * (Map[F, linage + a0]) + tmC14) / c;
    mass =
     Table[Total[Take[c, {If[h == 1, 1, ah[[h - 1]] + 1], ah[[h]]}]], {h, nh[[site]]}];
    age = Table[Total[Take[c * a, {If[h == 1, 1, ah[[h - 1]] + 1], ah[[h]]}]],
       {h, nh[[site]]}] / mass;
    C14 = Table[Total[Take[c * C14x, {If[h == 1, 1, ah[[h - 1]] + 1], ah[[h]]}]],
       {h, nh[[site]]}] / mass;
    If[AdditionalOutput, res = {mass, C14, age, l, tm}, res = {mass, C14, age}],
    res = {-1, -1, -1}];
   res
  ];

pri[pars_] := Module[{sigmac, sigmaz, sigmal, res, ss, a0, l0, cm, dh},
   {{sigmac, sigmaz, sigmal}, ss, a0, l0, cm, dh} = SplitPars[pars];
   res = Log[PDF[NormalDistribution[0., 1], sigmac]] +
     Log[PDF[NormalDistribution[0., 1], sigmaz]] +
     Log[PDF[NormalDistribution[0., 1], sigmal]] +
     Total[Log[PDF[NormalDistribution[0., 10], ss]]] +
     Sum[If[3 ≤ Exp[a0[[site]]] ≤ 5, 0., -Infinity] +
       Log[PDF[NormalDistribution[0., 10], l0[[site]]]] +
       Log[PDF[NormalDistribution[0., 1], cm[[site]]]] +
       Total[Log[PDF[NormalDistribution[0., 10], dh[[site]]]]], {site, nsites}];
   res
  ];


like[pars_] := Module[{sigmac, sigmaz, sigmal,
    res, ss, a0, l0, cm, dh, mass, C14, age, L0, B2, Dmass, DC14},
   {{sigmac, sigmaz, sigmal}, ss, a0, l0, cm, dh} = SplitPars[pars];
   {sigmac, sigmaz, sigmal} = Exp[{sigmac, sigmaz, sigmal}];
   l0 = Exp[l0];
   res = 0;
   Do[
    {L0, B2, Dmass, DC14} = data[[site]];
    {mass, C14, age} = CarbonModel[pars, site, False];
    If[Length[mass] == 0,
     res = -Infinity,
     res =
       res + Total[Log[PDF[NormalDistribution[0, sigmac], Log[Dmass] - Log[mass]]]] +
        Total[Log[PDF[NormalDistribution[0, sigmaz], Log[DC14] - Log[C14]]]] +
        Log[PDF[NormalDistribution[0, sigmal], Log[l0[[site]]] - Log[L0]]];
    ];
    , {site, nsites}];
   res];

post[pars_] := pri[pars] + like[pars];

trunc[x_] := Min[Max[x, 10.^(-5)], 10.^(5)];

(* update parameter (combination) i *)
update[i_] := Module[{new, nli},
   ac[[i]] += {1, 0};
   new = pars;
   new = new + Re[Random[NormalDistribution[0, kk[[i]] Sqrt[la[[i]]]]] vect[[i]]];
```

```
    nli = post[new];
    If[Random[] < Exp[nli - li],
     pars = new;
     li = nli;
     ac[[i]] += {0, 1};
    ];
   ];

updatelavect := Module[{cov, met},
    cov = ((s2 - Transpose[{s1}].{s1} / ns1s2) / (ns1s2 - 1));
    met = cov + 10^(-5.) IdentityMatrix[npars];
    {la, vect} = Eigensystem[met];
    la = Abs[la];
   ];

Iterate[repl_] := Module[{q, w, rpars},
    Do[
      If[adapt,
       q = 1. + Exp[-Length[ta] / 500.];
       w = 1. - 0.1 Exp[-Length[ta] / 500.],
       q = w = 1];
      Do[update[j], {j, npars}];
      AppendTo[ta, pars];
      AppendTo[tali, li];
      s1 += w * pars;
      s2 += w * Transpose[{pars}].{pars};
      ns1s2 += w;
      If[adapt,
       If[Length[ta] > 50, updatelavect];
       rpars = ac[[All, 2]] / ac[[All, 1]];
       kk = Map[trunc, kk * q^(rpars - 0.44)];
      ];
      ac *= w;
      , {i, repl}];
   ];
dat = Import["model input data_4.xlsx"];
f1 = ListPlot[dat[[5]], PlotRange → All];
dax = Drop[dat[[5]], 5];
dax[[All, 2]] = Log[dax[[All, 2]]];
ff[x_] = Fit[dax, {1, x}, x];
f[x_] = Exp[ff[x]];
DataYear = 2009;
MAXT = 100;
f2 = Plot[f[x], {x, 1964, DataYear}];
F[age_] := If[DataYear - age < 1964, If[DataYear - age < 1954, 0,
     f[1964] - (1964 - (DataYear - age)) f[1964] / (10)], f[DataYear - age]];
Print["C14 vs. age:\n", GraphicsGrid[
    {{Show[{Plot[ff[x], {x, 1964, DataYear}], ListPlot[dax]},
       PlotLabel → "fitted function: logC14 vs. year"],
      Show[f1, f2, PlotLabel → "C14 vs. year"],
      Plot[F[age], {age, 0, 80}, PlotLabel → "C14 vs. age", AxesOrigin → {0, 0}]}},
    ImageSize → {Automatic, 120}]];
```

C14 vs. age:




```
sites = Drop[dat[[2, All, 1]], 1];
Print["sites = ", sites];
nsites = Length[sites];
data = Table[{}, {i, nsites}];
nh = Table[0, {i, nsites}];
Print["Red: measurement of C14, Black: value of C14 inferred from age"];
Do[
  da = dat[[1]];
  fsite = sites[[i]];
  take = Flatten[Position[da[[All, 1]], fsite]];
  {site, horizon, Dmass, Dage, DC14} = Transpose[da[[take]]];
  nh[[i]] = Length[horizon];
  da = dat[[2]];
  take = Flatten[Position[da[[All, 1]], fsite]][[1]];
  L0 = da[[take, 2]];
  da = dat[[3]];
  take = Flatten[Position[da[[All, 1]], fsite]][[1]];
  B2 = 1 - da[[take, 2]] / 100;
  data[[i]] = {L0, B2, Dmass, DC14};
  Print["data for site ", i, " = ", fsite];
  Print["L0=", L0];
  Print["B2=", B2]; Print[GraphicsGrid[
    {{ListPlot[Dmass, Joined → True, PlotLabel → "mass", PlotRange → All],
      ListPlot[Dage, Joined → True, PlotLabel → "age", PlotRange → All],
      ListPlot[{DC14, Map[F, Dage]}, Joined → True, PlotStyle → {Red, Black},
       PlotLabel → "C14", PlotRange → All]}}, ImageSize → {Automatic, 120}]];
  ,
  {i,
   nsites}];
```

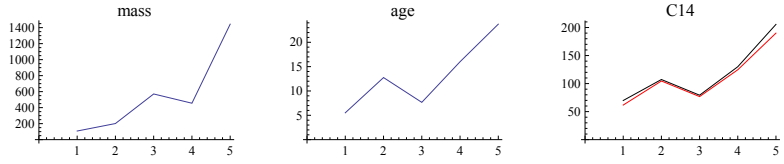
```
sites = {U105, U33, U37, H32, H12, U58}
```

```
Red: measurement of C14, Black: value of C14 inferred from age
```

```
data for site 1 = U105
```

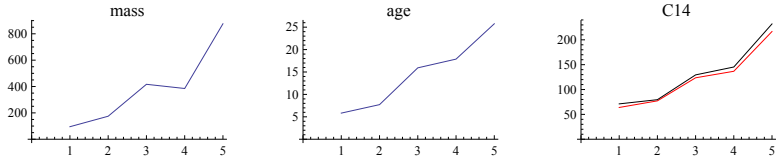
```
L0=79.45
```

```
B2=0.5578
```

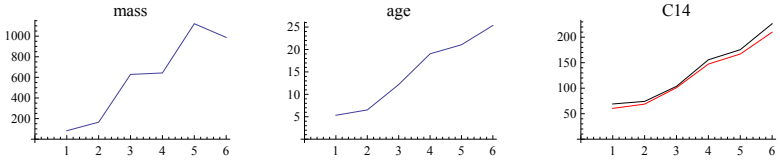



```
data for site 2 = U33
```

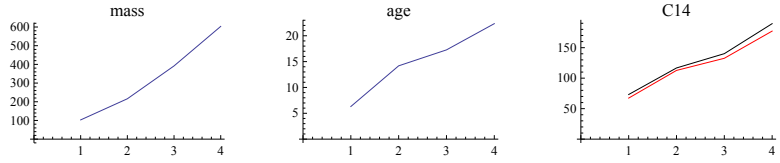
```
L0=79.4

B2=0.6146
```



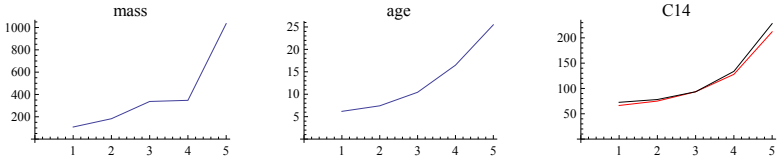

```
data for site 3 = U37

L0=139.25

B2=0.524667
```



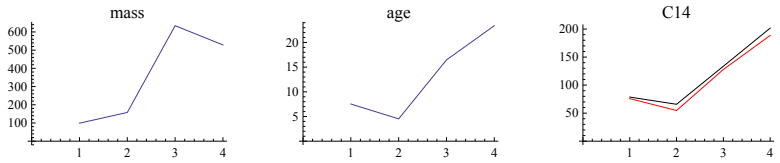

```
data for site 4 = H32

L0=112.55

B2=0.456833
```




```
data for site 5 = H12

L0=102.8

B2=0.559
```

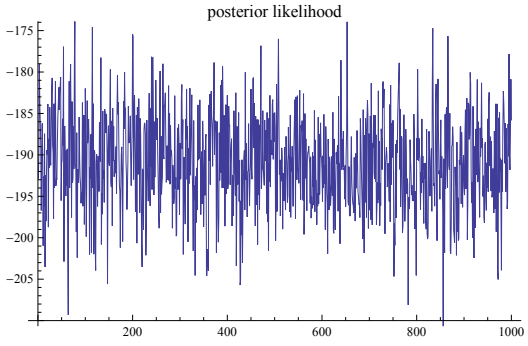



```
data for site 6 = U58

L0=119.65

B2=0.508
```




```
gammal = gammam = 1.19;
lcl = ((data[[All, 2]]^(-1 / gammal)) - 1) / 2;
source = ! True;
st = {1, 2, 4, 8, 16, 32, 64, 128};
nt = Length[st] - 3; (* values of s for t=8,16,32,64, 128 *)
inpars = Table[3 + nh[[i]], {i, nsites}];
(* island specific parameters to be estimated: a0, l0, cm, and all dh *)
npars = 3 + nt + Total[inpars];
(* global parameters to be estimated: sigmac, sigmaz, sigmal,
and all st *)
estimate = ! True; (* true if to do estimation and scae results,
false if loading estimates from file *)
replicate = 1;
```

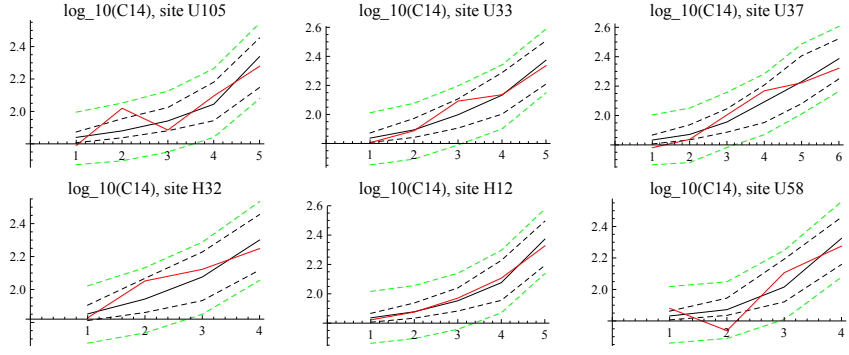
```
If[estimate,
  trans = 10 000;
  iter = 100 000;
  pars = Table[0, {i, npars}];
  Do[pars[[i]] = Log[4.], {i, 4 + nt, 4 + nt + nsites - 1}];
  Print["initial likelihood = ", li = post[pars]];

  {la, vect} = N[Eigensystem[IdentityMatrix[npars]]];
  ns1s2 = 0;
  s1 = Table[0, {i, npars}];
  s2 = Table[0, {i, npars}, {j, npars}];
  kk = Table[1., {i, npars}];
  ac = Table[{0, 0}, {i, npars}];

  Print["INITIAL ITERATION STARTS, NEEDED TO
     GET RID OF THE TRANSIENT AND TO ADJUST THE PROPOSALS"];
  ta = tali = {};
  adapt = True;
  Print[AbsoluteTiming[Iterate[trans]]];
  Print[ListPlot[ac[[All, 2]] / ac[[All, 1]],
    PlotRange → {0, 1}, PlotLabel → "accept ratios"]];
  Print[ListPlot[tali, Joined → True, PlotLabel → "posterior likelihood"]];
  Print["MAIN ITERATION STARTS"];
  ac = Table[{0, 0}, {i, npars}];
  ta = tali = {};
  adapt = False;
  Print[AbsoluteTiming[Iterate[iter]]];
  Print[ListPlot[ac[[All, 2]] / ac[[All, 1]],
    PlotRange → {0, 1}, PlotLabel → "accept ratios"]];
  Print[ListPlot[tali, Joined → True, PlotLabel → "posterior likelihood"]];
 ];
file =
  StringJoin["C:\\HY-data\\OVASKAIN\\all stuff\\manuscripts\\InPreparation\\Björn
     Lindahl\\otsos work\\results\\source=",
   ToString[source], " replicate=", ToString[replicate], "_thinned"];
If[estimate,
 Quiet[DeleteFile[file]];
 Save[file, {ta, tali, trans, iter}],
 Get[file];
 Print[ListPlot[tali, PlotRange → {All, {Min[tali], Max[tali]}},
   Joined → True, PlotLabel → "posterior likelihood"]];
];
```




```
tl0 = tc14 = tmass = tl0e = tc14e = tmasse = ts = tcm = ta0 = tah = tal = tatm = {};
Do[
  pa = RandomChoice[ta];
  {{sigmac, sigmaz, sigmal}, ss, a0, l0, cm, dh} = SplitPars[pa];
  tl0 = Append[tl0, Exp[l0]];
  tl0e = Append[tl0e, Exp[l0] * Exp[Random[NormalDistribution[0, Exp[sigmal]]]]];
  prediction = Table[CarbonModel[pa, site, True], {site, nsites}];
  tc14 = Append[tc14, prediction[[All, 2]]];
  tc14e = Append[tc14e, Table[
     prediction[[site, 2]] * Table[Exp[Random[NormalDistribution[0, Exp[sigmaz]]]],
       {j, nh[[site]]}], {site, nsites}]];
  tmass = Append[tmass, prediction[[All, 1]]];
  tmasse = Append[tmasse, Table[
     prediction[[site, 1]] * Table[Exp[Random[NormalDistribution[0, Exp[sigmac]]]],
       {j, nh[[site]]}], {site, nsites}]];
  tal = Append[tal, prediction[[All, 4]]];
  tatm = Append[tatm, prediction[[All, 5]]];
  ss = Exp[ss];
  ss = Join[{0, 0, 0}, ss];
  If[! source, ss = Table[0, {i, 3 + nt}]];
  s = Table[sfunction[t, st, ss], {t, MAXT}];
  ts = Append[ts, s];
  tcm = Append[tcm, Exp[cm]];
  ta0 = Append[ta0, Exp[a0]];
  ltah = {};
  Do[
   dh2 = Round[Exp[dh[[site]]]];
   ah = Table[Total[Take[dh2, h]], {h, nh[[site]]}];
   ltah = Append[ltah, ah];
   , {site, nsites}];
  tah = Append[tah, ltah];
  , {repl, 1000}];
```

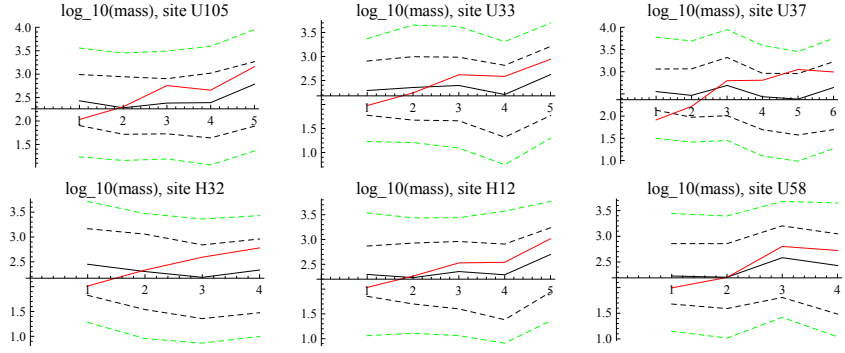
```
Tme = Tlow = Thigh = Tlow2 = Thigh2 = {};
Do[
  me = Median[Log[10, tc14[[All, site]]]];
  low = Quantile[Log[10, tc14[[All, site]]], 0.025];
  high = Quantile[Log[10, tc14[[All, site]]], 0.975];
  low2 = Quantile[Log[10, tc14e[[All, site]]], 0.025];
  high2 = Quantile[Log[10, tc14e[[All, site]]], 0.975];
  AppendTo[Tme, me];
  AppendTo[Tlow, low];
  AppendTo[Thigh, high];
  AppendTo[Tlow2, low2];
  AppendTo[Thigh2, high2];
  f1 = ListPlot[me, PlotStyle → Black, Joined → True];
  f2 = ListPlot[low, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  f3 = ListPlot[high, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  f2b = ListPlot[low2, Joined → True, PlotStyle → {Green, Dashing[{0.02, 0.02}]}];
  f3b = ListPlot[high2, Joined → True, PlotStyle → {Green, Dashing[{0.02, 0.02}]}];
  f4 = ListPlot[Log[10, data[[site, 4]]], Joined → True, PlotStyle → Red];
  figu[site] = Show[f1, f2, f3, f2b, f3b, f4, PlotRange → All,
    PlotLabel → StringJoin["log_10(C14), site ", sites[[site]]]];
  , {site, nsites}];
Print["red color = real data; black color = model prediction
    for truth; green color = model prediction for measurement"];
GraphicsGrid[{{figu[1], figu[2], figu[3]}, {figu[4], figu[5], figu[6]}},
 ImageSize → {Automatic, 240}]
```

```
red color = real data; black color = model prediction
  for truth; green color = model prediction for measurement
```



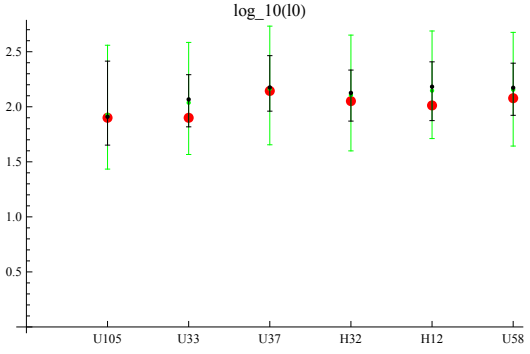
```
Tme = Tlow = Thigh = Tlow2 = Thigh2 = {};
Do[
  me = Median[Log[10, tmass[[All, site]]]];
  low = Quantile[Log[10, tmass[[All, site]]], 0.025];
  high = Quantile[Log[10, tmass[[All, site]]], 0.975];
  low2 = Quantile[Log[10, tmasse[[All, site]]], 0.025];
  high2 = Quantile[Log[10, tmasse[[All, site]]], 0.975];
  AppendTo[Tme, me];
  AppendTo[Tlow, low];
  AppendTo[Thigh, high];
  AppendTo[Tlow2, low2];
  AppendTo[Thigh2, high2];
  f1 = ListPlot[me, PlotStyle → Black, Joined → True];
  f2 = ListPlot[low, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  f3 = ListPlot[high, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  f2b = ListPlot[low2, Joined → True, PlotStyle → {Green, Dashing[{0.02, 0.02}]}];
  f3b = ListPlot[high2, Joined → True, PlotStyle → {Green, Dashing[{0.02, 0.02}]}];
  f4 = ListPlot[Log[10, data[[site, 3]]], Joined → True, PlotStyle → Red];
  figu[site] = Show[f1, f2, f3, f2b, f3b, f4, PlotRange → All,
    PlotLabel → StringJoin["log_10(mass), site ", sites[[site]]]];
  , {site, nsites}];
Print["red color = real data; black color = model prediction
    for truth; green color = model prediction for measurement"];
GraphicsGrid[{{figu[1], figu[2], figu[3]}, {figu[4], figu[5], figu[6]}},
 ImageSize → {Automatic, 240}]
```

```
red color = real data; black color = model prediction
  for truth; green color = model prediction for measurement
```



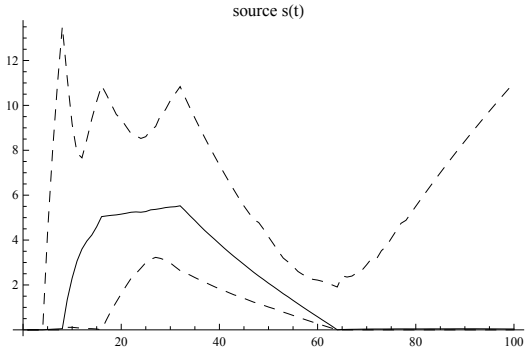
```
tx = Log[10, tl0]; f1 = ErrorListPlot[Table[{{site, Median[tx[[All, site]]]},
     ErrorBar[{-Median[tx[[All, site]]] + Quantile[tx[[All, site]], 0.025],
       Quantile[tx[[All, site]], 0.975] - Median[tx[[All, site]]]}]},
    {site, nsites}], PlotStyle → Black, PlotRange → All];
tx = Log[10, tl0e]; f1b = ErrorListPlot[Table[{{site, Median[tx[[All, site]]]},
     ErrorBar[{-Median[tx[[All, site]]] + Quantile[tx[[All, site]], 0.025],
       Quantile[tx[[All, site]], 0.975] - Median[tx[[All, site]]]}]},
    {site, nsites}], PlotStyle -> Green, PlotRange → All];
f2 = ListPlot[Log[10, data[[All, 1]]], PlotStyle → {Red, PointSize[Large]}];
Print["red color = real data; black color = model prediction
     for truth; green color = model prediction for measurement"];
Show[f2, f1b, f1, PlotRange → All, AxesOrigin → {0, 0}, PlotLabel → "log_10(l0)",
 Ticks → {Table[{site, sites[[site]]}, {site, nsites}], Automatic}]
```

```
red color = real data; black color = model prediction
  for truth; green color = model prediction for measurement
```

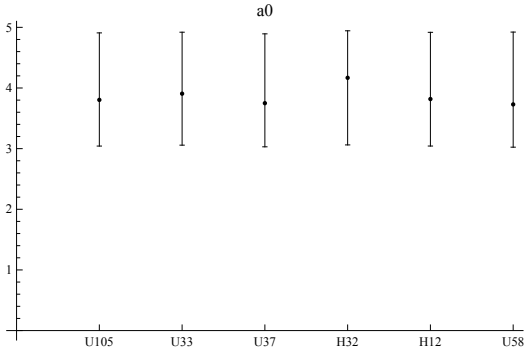


```
me = Median[ts];
low = Quantile[ts, 0.025];
high = Quantile[ts, 0.975];
f1 = ListPlot[me, PlotStyle → Black, Joined → True];
f2 = ListPlot[low, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
f3 = ListPlot[high, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
sts = Map[Total, ts];
Print["total source (sum over cohorts) {0.025,0.5,0.975} quantiles: ",
  Quantile[sts, {0.025, 0.5, 0.975}]];
Show[f1, f2, f3, PlotLabel → "source s(t)", PlotRange → {All, All}]
```

```
total source (sum over cohorts) {0.025,0.5,0.975} quantiles:
 {133.813, 209.099, 460.594}
```

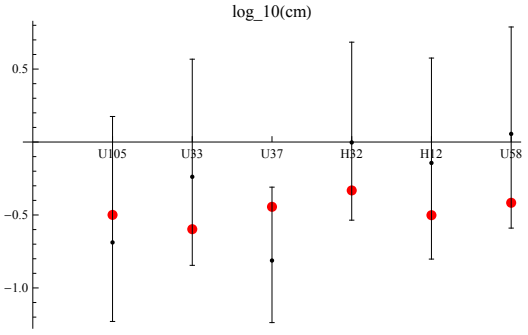



```
tx = ta0; f1 = ErrorListPlot[Table[{{site, Median[tx[[All, site]]]},
    ErrorBar[{-Median[tx[[All, site]]] + Quantile[tx[[All, site]], 0.025],
      Quantile[tx[[All, site]], 0.975] - Median[tx[[All, site]]]}]},
   {site, nsites}], PlotStyle → Black, PlotRange → All];
Show[f1, PlotRange → All, AxesOrigin → {0, 0}, PlotLabel → "a0",
 Ticks → {Table[{site, sites[[site]]}, {site, nsites}], Automatic}]
```

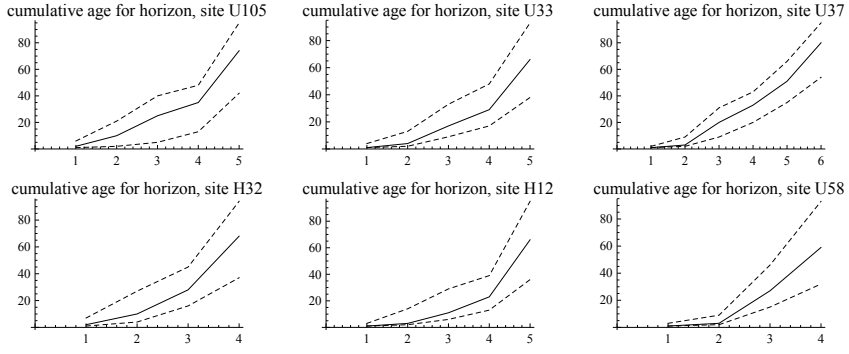


```
tx = Log[10, tcm];
f1 = ErrorListPlot[Table[{{site, Median[tx[[All, site]]]},
      ErrorBar[{-Median[tx[[All, site]]] + Quantile[tx[[All, site]], 0.025],
        Quantile[tx[[All, site]], 0.975] - Median[tx[[All, site]]]}]},
    {site, nsites}], PlotStyle → Black, PlotRange → All];
f2 = ListPlot[Log[10, lcl], PlotStyle → {Red, PointSize[Large]}];
Print[
  "Probability(average cm for small islands < average cm for large islands) = ",
  N[Count[Sign[Sum[tcm[[All, i]], {i, 3}] / 3 - Sum[tcm[[All, i]] / 3, {i, 4, 6}]], -1] /
     Length[tcm]] ];
Print["red color = cl; blue color = model prediction"];
Show[f2, f1, PlotRange → All, AxesOrigin → {0, 0}, PlotLabel → "log_10(cm)",
 Ticks → {Table[{site, sites[[site]]}, {site, nsites}], Automatic}]
```

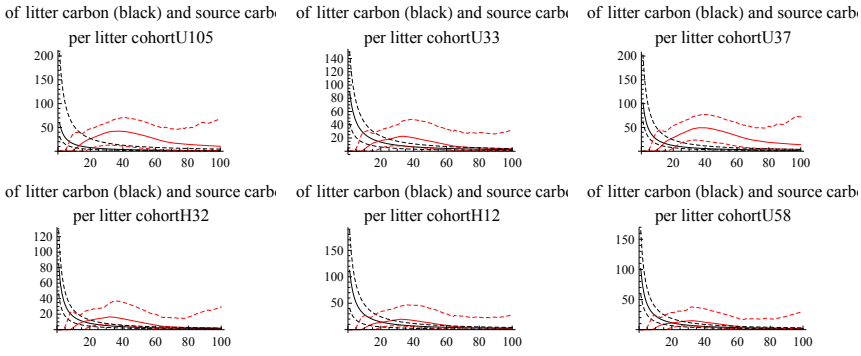
```
Probability(average cm for small islands < average cm for large islands) = 0.931
red color = cl; blue color = model prediction
```



```
Do[
  me = Median[tah[[All, site]]];
  low = Quantile[tah[[All, site]], 0.025];
  high = Quantile[tah[[All, site]], 0.975];
  f1 = ListPlot[me, PlotStyle → Black, Joined → True];
  f2 = ListPlot[low, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  f3 = ListPlot[high, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]}];
  figu[site] = Show[f1, f2, f3, PlotRange → All,
    PlotLabel → StringJoin["cumulative age for horizon, site ", sites[[site]]]];
  , {site, nsites}];
GraphicsGrid[{{figu[1], figu[2], figu[3]}, {figu[4], figu[5], figu[6]}},
 ImageSize → {Automatic, 240}]
```



```
Do[
 me = Table[Median[tal[[All, site, t]]], {t, MAXT}];
 low = Table[Quantile[tal[[All, site, t]], 0.025], {t, MAXT}];
 high = Table[Quantile[tal[[All, site, t]], 0.975], {t, MAXT}];
 f1a = ListPlot[me, Joined → True, PlotStyle → Black, PlotRange → All];
 f2a = ListPlot[low, Joined → True,
   PlotStyle → {Black, Dashing[{0.02, 0.02}]}, PlotRange → All];
 f3a = ListPlot[high, Joined → True, PlotStyle → {Black, Dashing[{0.02, 0.02}]},
   PlotRange → All];
 me = Table[Median[tatm[[All, site, t]]], {t, MAXT}];
 low = Table[Quantile[tatm[[All, site, t]], 0.025], {t, MAXT}];
 high = Table[Quantile[tatm[[All, site, t]], 0.975], {t, MAXT}];
 f1b = ListPlot[me, Joined → True, PlotStyle → Red, PlotRange → All];
 f2b = ListPlot[low, Joined → True,
   PlotStyle → {Red, Dashing[{0.02, 0.02}]}, PlotRange → All];
 f3b = ListPlot[high, Joined → True, PlotStyle → {Red, Dashing[{0.02, 0.02}]},
   PlotRange → All];
 figu[site] = Show[f1a, f1b, f2a, f2b, f3a, f3b, PlotRange → {All, All},
   PlotLabel → StringJoin["amount of litter carbon (black) and
        source carbon (red)\n per litter cohort", sites[[site]]]];
 , {site, nsites}]
GraphicsGrid[{{figu[1], figu[2], figu[3]}, {figu[4], figu[5], figu[6]}},
 ImageSize → {Automatic, 240}]
```



```
lsmall = Sum[Sum[tal[[All, site, t]], {t, MAXT}], {site, 3}] / 3;
llarge = Sum[Sum[tal[[All, site, t]], {t, MAXT}], {site, 4, 6}] / 3;
msmall = Sum[Sum[tatm[[All, site, t]], {t, MAXT}], {site, 3}] / 3;
mlarge = Sum[Sum[tatm[[All, site, t]], {t, MAXT}], {site, 4, 6}] / 3;
Print["Total amount of litter carbon included in cohorts from
    1 to MAXT {0.025 quantile, 0.5 quantile, 0.975 quantile}:"];
Print["average for small islands: ", Quantile[lsmall, {0.025, 0.5, 0.975}]];
Print["average for large islands: ", Quantile[llarge, {0.025, 0.5, 0.975}]];
Print["Total amount of mycelial carbon included in cohorts from
    1 to MAXT {0.025 quantile, 0.5 quantile, 0.975 quantile}:"];
Print["average for small islands: ", Quantile[msmall, {0.025, 0.5, 0.975}]];
Print["average for large islands: ", Quantile[mlarge, {0.025, 0.5, 0.975}]];
Print[
  "Out of all carbon included in cohorts from 1 to MAXT, the fraction F of source
    carbon is {0.025 quantile, 0.5 quantile, 0.975 quantile}:"];
Print["small islands: ", Quantile[msmall / (msmall + lsmall), {0.025, 0.5, 0.975}]];
Print["large islands: ", Quantile[mlarge / (mlarge + llarge), {0.025, 0.5, 0.975}]];
Print["Probability that F(large)>F(small): ",
 N[Count[Sign[msmall / (msmall + lsmall) - mlarge / (mlarge + llarge)], -1] /
   Length[msmall]]]
```

```
Total amount of litter carbon included in cohorts
  from 1 to MAXT {0.025 quantile, 0.5 quantile, 0.975 quantile}:
average for small islands: {674.209, 923.207, 1627.66}
average for large islands: {659.599, 955.153, 1377.48}
Total amount of mycelial carbon included in cohorts
  from 1 to MAXT {0.025 quantile, 0.5 quantile, 0.975 quantile}:
average for small islands: {1047.8, 2128.44, 3268.19}
average for large islands: {452.199, 836.207, 1722.44}
Out of all carbon included in cohorts from 1 to MAXT, the fraction F
  of source carbon is {0.025 quantile, 0.5 quantile, 0.975 quantile}:
small islands: {0.411271, 0.692914, 0.807554}
large islands: {0.294549, 0.470859, 0.682351}
Probability that F(large)>F(small): 0.055
```

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V

Evaluation of N retention capacity by ectomycorrhizal fungi in a Norway spruce forest fertilized with nitrogen and phosphorus

Adam Bahr[a], Magnus Ellström[a], Johan Bergh[b] and Håkan Wallander[a]
[a] *Lund University, Microbial Ecology, Dept of Biology, SE-223 62 Lund, Sweden*
[b] *Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre, SE-230 53, Alnarp, Sweden*

Abstract
Mycelium of ectomycorrhizal fungi (EMF) encloses almost all fine roots of trees growing in boreal and boreo-nemoral forests. The EMF are dependent on belowground allocation of photoassimilated C by the tree, in return they deliver nutrients and water. The EMF mycelium increase the absorptive surface area of tree roots by two orders of magnitude and within on single gram of humus hundreds of meters of EMF mycelium can be found. This makes the EMF mycelium potentially important for the nitrogen (N) retention capacity of boreal forest soils. Previous studies have found coincidences between elevated N leakage and low EMF mycelium production, but it has still not been possible to separate the specific role of EMF from other N retaining processes (e.g assimilation by tree roots and heterotrophic microbes). We approached this gap of knowledge in a fertilization experiment in a Norway spruce forest in south Sweden by: 1) Relating seasonal variations in N leakage to EMF production; 2) Analyzing if N leaching after fertilization was affected by combined addition of phosphorus (P). Nitrogen combined with P should hypothetically reduce production of EMF while it should promote tree N assimilation due to alleviation of N induced P limitation; 3) Addition of isotopically labeled N to EMF ingrowth mesh-bags to enable tracing of N assimilation as well as N leached through the EMF mycelium. We found a large N assimilation capacity of EMF mycelium (0.31 mg ^{15}N g^{-1} EMF mycelium day^{-1}, *SE* = 0.03), but the assimilation of EMF mycelium was not sufficient to hamper large immediate losses of N after fertilization. Further, tendencies of reduced N leaching and reduced EMF mycelium production after combined addition of N and P indicated that other processes have to be taken into account.

Introduction

When nutrients such as nitrogen (N) and phosphorus (P), that are important for plant biomass synthesis, become deficient, trees typically enhance belowground carbon (C) allocation to roots and root associated fungi to sustain the assimilation of these elements (Ericsson, 1995). In boreal and boreo-nemoral forests, that usually are N limited (Vitousek and Howarth, 1991), ecto-mycorrhizal fungi (EMF) can receive a large portion of the photoassimilated C (Simard et al., 2002). The ectomycorrhizal extra-matrical mycelium (EMM) extends through the soil and assimilates N from both organic and inorganic sources (Smith and Read, 2008). This is generally thought of as a symbiosis in which the tree gains nutrients in exchange for the allocated C (Smith and Read, 2008). The relationship may however be more or less mutual (Karst et al., 2008), and it has recently been suggested that C allocation to EMF under N poor conditions could cause increased N retention within the EMM, resulting in aggravated N deficiency in the trees and further increased C allocation to the associated fungi in a positive feedback loop (Näsholm et al., 2013).

Coincidences of elevated N leaching and impaired EMM production found after N

deposition or fertilization has led to suggestions that active growth of EMF prevents N losses (Nilsson et al., 2007; Nilsson et al., 2012; Bahr et al., 2013). However, the capacity of EMF to reduce N leaching still remains uncertain since the effect of impaired EMM production, typically seen after N addition (Arnebrant and Söderström, 1992; Nylund and Wallander, 1992; Nilsson and Wallander, 2003; Nilsson et al., 2007; Högberg et al., 2011; Kjøller et al., 2012; Nilsson et al., 2012; Bahr et al., 2013), hasn't been separated from the direct effect of an increased N pool in the soil as well as other environmental variables. Apart from repressing the belowground C allocation and thus the EMF growth, enhanced availability of N may also alter the capacity of the present EMM network to take up N (Clemmensen et al., 2008).

Even though N is generally considered well retained in the N poor boreal and boreo-nemoral forest soils (Gundersen and Rasmussen, 1995), leaching of N may occur if the N retention capacity is exceeded due to extensive N input (Aber et al., 1998; Gundersen et al., 2006; Akselsson et al., 2010). Elevated N leaching has been recorded in boreo-nemoral forests as a result of N deposition (Nilsson et al., 2007; Akselsson et al., 2010; Bahr et al., 2013), N fertilization (Berden et al., 1998) and clear-cutting (Akselsson et al., 2004). Further, deposition or fertilization with N may shift the limitation to other nutrients and resources (Tamm, 1991), and there have been findings of P limitation caused by addition of N in coniferous forests in the boreal region (Quesnel and Côté, 2009), boreo-nemoral region (Clarholm and Rosengren-Brinck, 1995) and nemoral region (Mohren et al., 1986; Valentine and Allen, 1990; Houdijk and Roelofs, 1993; Braun et al., 2010). Alleviation of N induced P limitation by fertilization of P, has been shown to increase the autotrophic N retention capacity in coniferous forest soils (Stevens et al., 1993; Blanes et al., 2012), but the role of EMF were not tested in these studies.

Wallander and Nylund (1992) showed in laboratory experiments that EMM production was repressed by excess N treatment when P levels was adequate, however if P was deficient no repression of EMM production was found after N addition. The importance to take P into account when analyzing EMM production was also highlighted in a recent large scale field study in boreo-nemoral forests, where EMM production was negatively correlated with needle P content (Bahr et al., 2013). Even though the combined effect of N and P levels on EMM production has been analyzed in laboratory experiments (Wallander and Nylund, 1992) this, to our knowledge, still remains to be tested in the field.

In the present study we simultaneously analyzed N leakage and growth of EMM during four-month periods over one year in a Norway spruce (*Picea abies*) forest fertilized with N or N and P in combination. The temporal division of sampling was made to account for the large seasonal variation in both N leaching (Stevens et al., 1993; Piirainen et al., 1998; Wright et al., 2001) and EMM growth (Wallander et al., 2001). Production of EMM, which has been suggested to increase soil N retention (Aber et al., 1998; Nilsson et al., 2007; Högberg et al., 2011), usually peaks at late summer to early fall, while it almost ceases during the winter (Wallander et al., 2001). In contrast, large temporal soil water N flushes in the spring are typically explained by snow

melting (Aber et al., 1998; Piirainen et al., 1998), intense periods of precipitation and low plant assimilation (Wright et al., 2001).

Production of EMM was estimated with sand filled ingrowth mesh-bags. The response of EMM to apatite amendment was tested in some of the mesh-bags, and used as a proxy for P status since it has earlier been found that addition of the P rich mineral apatite stimulate EMM growth in P deficient soil (Wallander and Thelin, 2008). Isotopically labeled N was added to some of the ingrowth mesh-bags to quantify N assimilation by EMM (Clemmensen et al., 2008). The amount of labeled N passing through the EMM network was collected in ion exchange resins and used as a proxy for N leaching. To maximize the prerequisite for N leaching we also included N treated plots in denser (unthinned) forest stands, where the potential to assimilate N would be reduced since the photosynthetically active radiation can become limiting at a sufficient supply of water and nutrients (Aber et al., 1998).

Our aim was to analyze the N retention capacity of EMM under different nutrient conditions and seasons. It was hypothesized that (1) N addition would cause both enhanced N leaching and reduced EMM production. (2) EMM production should be further repressed when N addition was combined with P addition (if P becomes limiting for primary production in N treated plots). (3) N leaching should mainly occur in plots where apatite amendment stimulates EMM growth since this indicates P limitation and thus excess N. (4) Largest N losses were expected to co-occur with low EMM production in early spring, while elevated EMM production in autumn should counteract N leaching. (5) N fertilization was expected to cause reduced N assimilation by EMM and increased N leaching in mesh-bags amended with ^{15}N.

Material and methods

Field site

The experiment was conducted in South Sweden at Tönnersjöheden research park (Lat. 56° 41′-42′ Long. 13° 5′-7′), in the county of Halland, which is managed by the Swedish University of Agricultural Sciences. The experimental site consisted of 32 year old (at the initiation of the experiment) Norway spruce (*Picea abies*) forest, which was thinned during the winter 2011. The analysis was conducted at 24 plots (30-40 m x 25 m) with 4 different treatments (Table 1). 200 kg ha^{-1} of N was added once (July 2011) as ammonium nitrate (Skog-CAN, Yara AB, Helsingborg, Sweden) to 18 of the 24 analyzed plots. Six of the N treated plots were also treated twice (July 2011 and July 2012) with 200 kg ha^{-1} of P (in total 400 kg ha^{-1}) added as superphosphate (P20, Yara AB, Helsingborg, Sweden). Another 6 of the N treated plots were not thinned prior to the treatments and were thus forming denser stands (1500 stems ha^{-1}, SE = 40) than the thinned plots (940 stems ha^{-1}, SE = 70). The remaining 6 plots served as controls. The N plots were split into 5 m wide areas, and the fertilizers where distributed by hand to one area at a time to improve the homogenization of the distribution. Half of the plots, 3 of each treatment, were located on a blocky moraine soil while the other where located at sandy alluvial deposits with potentially larger percolation capacity. The soils where podzols with a humus depth ranging from 3 to 16 cm with a mean of 9.8 cm (*SD* = 3.1). Treatment at different plots was selected randomly.

Production of extramatrical ectomycorrhizal mycelium

Ingrowth mesh-bags were used to determine the production of EMM, a fine mesh (pore

size: 50 µm) allowed for EMM ingrowth while it prevented tree roots to penetrate into the bag (Wallander et al., 2001). The mesh-bags were made cylindrical (length: 16 cm, diameter: 2 cm) to cover most of the active EMM region. They were filled with 70 g acid washed quarts sand (0.36-2.00 mm, 99.6% SiO_2, Ahlsell AB, Sweden) to reduce ingrowth of saprotrophic fungi due to lack of C. Some of the mesh-bags were amended with 0.5% of the P containing mineral apatite, since this can give an indication of P deficiency (Wallander and Thelin, 2008). An overview of the mesh-bags is given in Table 1. Parallel to the longest sides in each plot (at least 2 m from the boarders) two transects were placed, and the mesh-bags were positioned systematically along these. Six sets of mesh-bags were installed at each plot. One set of mesh-bags, including non-amended and apatite amended bags (apatite bags were not installed at unthinned plots) were installed in July 2011 and left during the whole study period, until November 2012. To analyze seasonal variation in EMM production, non-amended mesh-bags where installed during consecutive four month periods (15 July 2011 – 15 November 2011, 15 November 2011 – 15 March 2012, 15 March 2012 – 15 July 2012 and 15 July 2012 – 15 November 2012). A hole, for each mesh-bag, was made with a soil corer (diameter: 2.0 cm) and the humus depth was noted. After harvest the mesh-bags were stored in a fridge (+4°C) for less than 3 days and then transferred to a freezer (-18°C).

Before analyses the humus depth was marked on the side of the mesh-bags, the bags were then opened by cutting a hole in one end and then gently pulling of a string of mesh along the whole mesh-bag. Along that string the frequency of hyphae was counted in vertical rows of 100 mesh holes. This was done at three different depths; at the border between humus and mineral soil as well as in the middle of these two layers. The abundance of EMM in the mesh-bags was also estimated visually in a stereo microscope according to Wallander et al. (2001), with the use of a six graded scale. The sand from the mesh-bags were then split up into the humus part and the mineral part and pooled for each plot and period. Fungal content in these samples was further estimated by analyzing the ergosterol content. Ergosterol is a fungal specific membrane lipid that can be used as a fungal biomarker (Wallander et al., 2001). During degradation of fungal hyphae ergosterol becomes bound in sterol esters (Yuan et al., 2008) and the amount of free ergosterol has been suggested to better resemble living fungal mycelium (reviewed by Wallander et al., 2013). Free and total ergosterol was analyzed in 5 g sand from each of the pooled samples. Ergosterol was extracted in 5 ml methanol with 15 min sonication and 1 h 70°C water bath. 2.5 ml of the solution was then transferred into new 10 ml glass tubes together with 2.5 ml methanol for analyses of free ergosterol. To enable detection of total ergosterol (including the bound partition), 2.5 ml KOH solution (20% KOH in methanol) was added to the original tubes and they were shaken on a multivortex (Multi Reax, Heidolph, Germany) for 3 minutes. The ergosterol analyses were then continued according to Bahr et al. (2013). In brief: After extraction of ergosterol into the methanol based solvent, 1 ml H_2O was added to increase the polarity. After adding 2 ml cyclohexane, the samples were shaken on a multivortex to transfer ergosterol into the nonpolar phase and then centrifuged to enable phase separation into new tubes, another 2 ml cyclohexane was added to the original tubes and the phase separation was repeated. After

Table 1: Overview of treatments as well as number of lysimeters, mesh-bags (MB) and nitrogen labeling (^{15}N) samples included in the study. In the fertilized plots, 200 kg nitrogen (N) ha^{-1} was added as ammonium-nitrate, while 400 kg phosphorus (P) ha^{-1} was added as superphosphate.

		Control	N	NP	N, dense
Number of plots	Moraine soil	3	3	3	3
	Alluvial deposit soil	3	3	3	3
	Total	6	6	6	6
Lysimeters $plot^{-1}$	Alluvial deposit soil	4	4	4	4
Sand mesh-bags $plot^{-1}$	Whole period	6	6	6	6
	4 month periods	6	6		
Apatite mesh-bags $plot^{-1}$	Whole period	6	6	6	
^{15}N labeled mesh-bags $plot^{-1}$	July 2012 - Nov 2012	6	6		

evaporating the cyclohexane the ergosterol was dissolved in 400 µl methanol before ergosterol detection in a reversed-phase column (Chromolith C18 column, Merck and an Elite LaChrome C18 pre-column, Hitachi, Japan) high pressure liquid chromatograph (auto sampler L2130 with UV-detector L2400 by Hitachi, Japan). The ergosterol peak was detected at 280 nm with a flow rate of 1 ml min^{-1}.

Labeled nitrogen – trace experiment

The labeling experiment was implemented during the last study period (July 2012 – November 2012) at the N treated plots as well as the controls (Table 1). Labeled N was added as $^{15}NH_4Cl$ according to Clemmensen et al. (2008). Ammonium has been found preferentially assimilated by EMM, in contrast to NO_3^- (Finlay et al., 1992; Clemmensen et al., 2008), and there is larger interspecific variation in NO_3^- assimilation (Finlay et al., 1992). Further, NH_4^+ is by far the most common form of inorganic N found in Norway spruce forests soils (Tamm, 1991). To enable detection of leached N, 2 g ion-exchange resin beads (Amberlite® MB6113 mixed resin, BDH laboratory supplies, Poole, England) were put in the bottom of each mesh-bag. Labeling with ^{15}N was performed 2 days before harvest since Clemmensen et al. (2008) found this sufficient for EMM assimilation in mesh-bags. We injected 5 ml $^{15}NH_4Cl$ (14.5 mM), corresponding to 17 kg N ha^{-1}, in the mesh-bags with a syringe inserted to a depth of 2 cm. Before harvest the mesh-bags were flushed twice with 10 ml H_2O. After harvest these mesh-bags were treated as the others, but after visual estimation of EMM a pooled sample of totally 10 g sand was combined from the mesh-bags of each plot. The pooled samples were put into 10 ml glass tubes together with 7 ml H_2O. The tubes were shaken for one minute in a multivortex (Muli Reax, Heidolph, Germany). Separately, each tube was then shaken on a single vortex (Vortex-genie 2 model: G560E, Scientific industries inc., Bohemia, USA) for ten seconds followed by immediate decantation of the liquid to a 50 ml polyethylene Saerstedt tubes. This process was repeated three more times after addition of another 5 ml H_2O to the original glass tubes. For further separation from mineral particles the 50 ml tubes were then vortexed for ten seconds before immediate decantation into new 50 ml tubes. After centrifuging the 50 ml tubes for three minutes at 1000 *g* the supernatant was removed and the mycelial pellet left to dry overnight at 40°C. The dried mycelium was weighed and put in tin capsules (SC0009, SerCon ltd., Crewe, United Kingdom) for further analysis (see below).

Ammonium was extracted from resin beads with the use of acid traps, according to IAEA (2001), in brief: The collected resin beads from all mesh-bags at each plot were pooled and split into two technical replicates per plot. Ammonium was extracted from the ion-exchange resin beads in 50 ml 1M KCl in plastic jars. After addition of 0.2 g MgO and an acid trap (a quartz filter treated with 10 µl $KHSO_4$, sealed between two Teflon tapes), the plastic containers where immediately sealed. Liberated gaseous ammonia was slowly collected in the acid traps during three days in darkness at 100 RPM shaking. The quartz filters were then put into tin capsules and left to dry in a desiccator. Mycelial and resin bead samples were analyzed for the ratios of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ as well as total N and C content at the stable isotope facility at the Department of Biology, Lund University. Samples were flash-combusted in a Flash 2000 elemental analyzer, and the isotopic ratios determined by a Delta V Plus isotope-ratio mass spectrometer (IRMS) connected to the elemental analyzer via the ConFlow IV interface (Thermo Scientific Inc., Bremen Germany)

Soil water sampling

Four suction lysimeters were installed at each of the 12 plots located at sandy alluvial deposit soil with potentially larger percolation capacity, where sufficient soil water percolation was expected. The lysimeters consisted of 2000 ml cylindrical containers connected with plastic tubing to ceramic filters put at 50 cm depth. Each month the lysimeters were set to approximately 0.6 bar vacuum pressure and then left for one month before collection of the samples. The total amount of soil water in each lysimeter was noted and 50 ml water samples were transferred to plastic tubes and stored in a fridge (+4°C) overnight before pH measurements. The samples were then stored in a freezer (-18°C) until further analyses. Prior to N analyses water samples were pooled plot wise, in relation to the total sampled amount, for four month periods corresponding to the mesh-bag periods. To pinpoint possible N flushes due to snowmelt, winter and spring samples (November 2011 – April 2012) were pooled for each month separately. No water samples were collected during February 2011 due to frozen ground.

Needle chemistry

Previous year's needles were collected at three out of six plots (two from sandy alluvial deposit plots and one from moraine plots) of each treatment (in total needle samples were collected from 12 plots), during January 2013 to analyze the effect of fertilization on tree nutrient status. They were analyzed for N and P content at the Swedish university of agricultural sciences (department of soil and environment, Uppsala, Sweden) with an inductively coupled plasma optical emission spectrometry (ICP Optima 7300 DV, PerkinElmer Inc., Waltham, USA).

Statistics

The data was statistically analyzed with IBM SPSS statistics 21. Differences in parametric data (frequency of hyphae, ergosterol, needle chemistry, soil water chemistry and IRMS results) were tested with T-tests for 2 groups, ANOVA and Tukey's post hoc tests for 3 or more groups, while regressions were analyzed with Pearson correlations (R^2). The homogeneity of variances was verified with Levene's test. Differences in nominal data (visual estimation of EMM production) were tested with Mann-Whitney U for 2 groups, Kruskal-Wallis ANOVA for 3 or more groups, while regressions were analyzed with Spearman rank correlations (r_s). Ergosterol

content and soil water N values were log transformed before statistical analysis, apart from that no other transformations were applied. Only two-way T-tests were performed and independent tests were used to analyse differences between fertilization treatments while dependent tests were used for differences within a fertilization treatment (such as the effect of season or apatite amendment).

Results

No significant differences in EMM abundance were found between humus and mineral soil and the data was combined for each mesh-bag. Similarly, the two different soil types were combined due to lack of differences in EMM production. Due to good correspondence between visual estimation of EMM production and frequency of hyphae, only the latter (allowing for quantitative analysis) is shown.

Fertilization generally resulted in reduced EMM production according to frequency of hyphae (Fig. 1) and visual estimation (data not shown), while this could only be statistically verified (T-test, P = 0.020) according to ergosterol estimation during spring 2012 and a tendency (T-test, P = 0.057) of that during autumn 2012 (Fig. 2). Ingrowth in mesh-bags filled with pure sand was most repressed by combined N and P addition according to frequency of hyphae (ANOVA with Tukey post hoc, P = 0.001) and visual estimation (Kruskal-Wallis ANOVA, P = 0.007, data not shown), while no difference could be seen in ergosterol content (Fig 2.). Compared to unamended mesh-bags, ingrowth of EMM was larger in apatite amended bags in control plots according to frequency of hyphae (T-test, P = 0.049) and ergosterol content (T-test, P = 0.045). Ergosterol content was almost doubled in apatite amended mesh-bags at N fertilized plots compared to unamendended bags (T-test, P = 0.035), but that difference could not be detected according to frequency of hyphae or visual estimation of EMM production. At plots fertilized with both N and P, apatite amendment had no effect on EMM production according to any of the EMM estimations. Compared to control plots, ingrowth (based on frequency of hyphae) of EMM in apatite amended mesh-bags was lower (P = 0.019) in N treated plots while the ergosterol content tended to be opposite (Fig. 2), however not significant (P = 0.438).

Free ergosterol constituted an average of 29.7% (SE = 2.5) of the total ergosterol in mesh-bags at the different treatments and seasons. The proportion of free ergosterol was reduced during the winter at both control plots (ANOVA with Tukey, $P < 0.001$) and N fertilized plots (ANOVA with Tukey, $P < 0.001$), apart from that no other clear patterns could be revealed in regard to different treatments or seasons (Fig. 2). However, there was a large variation within treatments and seasons in the free partition of ergosterol, typically ranging from less than 10% to about 70%.

EMM biomass production during autumn 2012 was calculated to be 583 kg C ha^{-1} (SE = 48) in control plots and 361 kg (SE = 17) C ha^{-1} in N fertilized plots. These data are based on the C content (IRMS analysis) in the mesh-bags (which was 1.18 mg C g $sand^{-1}$ in control plots and 0.73 mg C g $sand^{-1}$ in the N fertilized plots). Furthermore, we assumed that all C in the mesh-bags were of EMF origin, the EMM biomass contained 45% C, and that any transport of dissolved organic C into the mesh-bags was neglectable. The EMM biomass was significantly higher in control plots compared to N treated plots (T-test, P = 0.001).

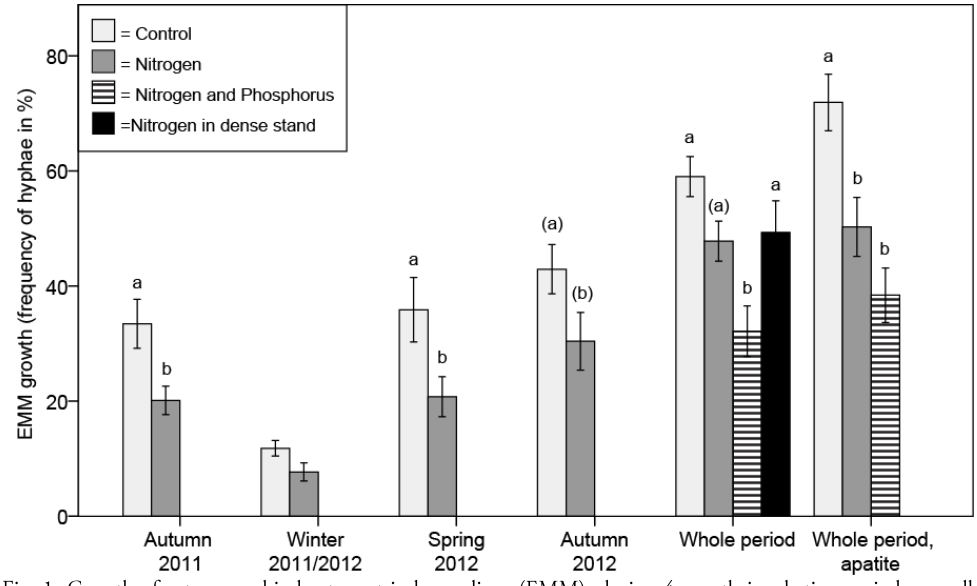

Fig. 1. Growth of ectomycorrhizal extramatrical mycelium (EMM), during 4 month incubation periods as well as during the whole period of the study (15 July 2011 until 15 November 2012), according to counting of mycelium along the mesh of the ingrowth bags. Shadings and patterns illustrate fertilization regimes. Samples from the same period that do not share letters are significantly different ($P < 0.05$) according to T-test for the four month periods and according to ANOVA with Tukey's post hoc test for the whole period. Error bars represent SE.

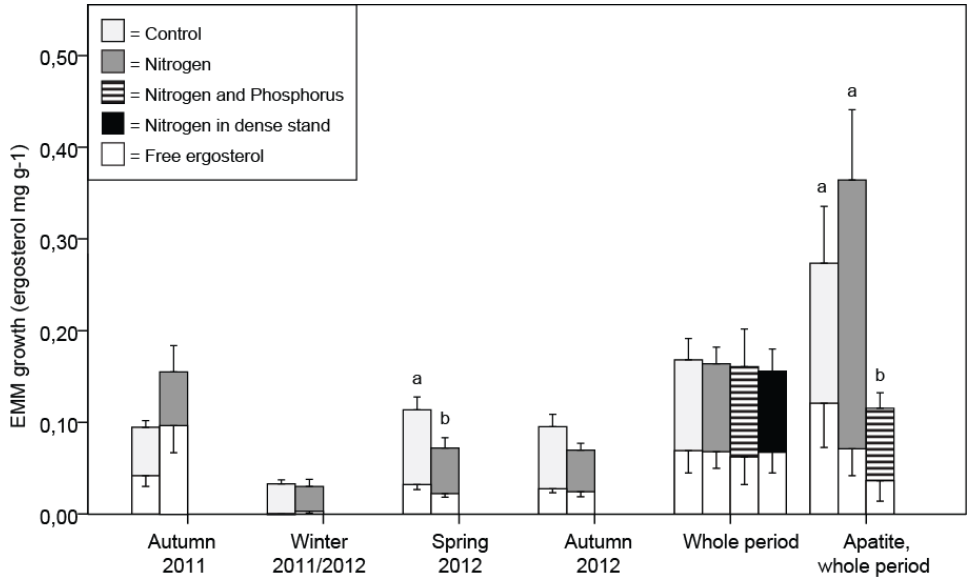

Fig. 2. Growth of ectomycorrhizal extramatrical mycelium (EMM), during 4 month incubation periods as well as during the whole period of the study (15 July 2011 until 15 November 2012), according to analysis of free and total ergosterol in sand from the ingrowth mesh-bags. Shadings and patterns illustrate fertilization regimes. Samples from the same period that do not share letters are significantly different ($P < 0.05$) in total ergosterol content according to T-test for the four month periods and according to ANOVA with Tukey's post hoc test for the whole period. Error bars represent SE.

Delta ^{13}C ratio of the EMM was -27.6 ‰ (*SE* = 0.09) and didn't differ between treatments. The average ergosterol concentration in the EMM biomass (calculated from C content according to IRMS, by estimating 45% C content of EMM biomass) was 0.40 µg g^{-1} (*SE* = 0.10), and it didn't differ between treatments.

Only low levels of N leaching, on average 0.18 mg l^{-1} (*SE* = 0.02), was detected during the pretreatment analysis of soil water in March 2011 (Fig. 3), consisting of 57% NO_3^- and 43% NH_4^+. During the first period after fertilization, the levels of inorganic N in soil water at fertilized plots were elevated (ANOVA with Tukey, *P* = 0.001) to 34.5 mg l^{-1} (*SE* = 12.2). Leaching of N then declined during the research period but elevated (ANOVA with Tukey, *P* = 0.015) levels due to fertilization were still detected one year after the application (mean = 3.00 mg l^{-1}, SE = 0.68), between July and November 2012. After the second winter, in February 2013, no enhanced N leaching could be statistically verified in N treated plots, but that sampling period did take place during low soil water percolation.

At control plots, needle concentration of P was below the deficiency level reported by Thelin et al. (2002) while N content seemed to be sufficient. Elevated levels of both N (ANOVA, mean = 18.4, *SE* = 0.5, *P* < 0.001) and P (ANOVA, mean = 3.19, *SE* = 0.20, *P* < 0.001) in needles was detected at plots fertilized with those nutrients, but there were no differences in needle N content between different treatments that included N fertilization (Table 2).

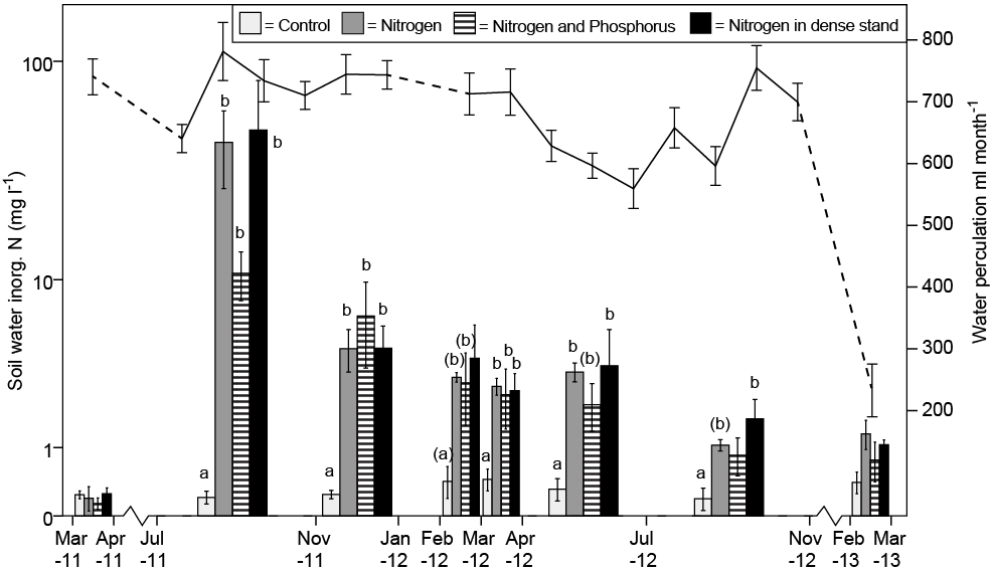

Fig. 3. Inorganic N concentration and volume of soil water collected in ceramic filter suction lysimeters. Shadings and patterns illustrate the fertilization regimes (that were initiated in July 2011). Samples from the same period that do not share letters are significantly different (P < 0.05) according to ANOVA with Tukey's post hoc test. Error bars represent SE .

Table 2: Content of nitrogen (N) and phosphorus (P) in previous year's needles, collected at half of the plots during January 2013 (one and a half year after fertilization). Average content at different fertilization treatments are given together with standard error (SE). Deficiency levels suggested by Thelin et al. (2002) are given.

Treatment	N (%)	SE	P (mg g-1)	SE
Control	16.1	0.4	1.1	0.1
N	17.8	0.6	1.2	0.1
NP	20.2	0.3	3.2	0.2
N dense	17.1	0.1	1.3	0.1
Deficiency	12		1.3	

The assimilation of the ^{15}N (injected into the mesh-bags) in EMM (Fig. 4) was larger (P = 0.013) at control plots (1.13% of added ^{15}N, SE = 0.17%) than in N fertilized plots (0.58%, SE = 0.07%). Based on that result it was estimated that the EMM had a potential to assimilate 217 g inorganic N ha^{-1} day^{-1} (SE = 32) at control plots and 113 g ha^{-1} day^{-1} (SE = 13) at fertilized plots. In contrast more ^{15}N was leached (P = 0.05) to the resin placed at the bottom of the bags in N fertilized plots (mean = 4.8%, SE = 0.98%) than in the controls (mean = 1.9%, SE = 0.84%). However, no significant difference was found in ^{15}N content per unit EMM biomass (0.31 mg ^{15}N g^{-1} day^{-1}, SE = 0.03, based on C content) collected from control and N treated plots. The growth of EMM during the autumn of 2012, when the labeling experiment was implemented, was positively correlated with recovery of ^{15}N (Fig. 5), both according to frequency of hyphae (R^2 = 0.57, P = 0.004) and ergosterol analysis (R^2 = 0.57, P = 0.004).

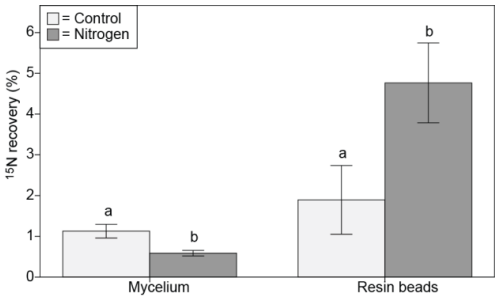

Fig. 4. Recovery of 15N added to the mesh-bags, in mycelium extracted from the ingrowth mesh-bags and in ion-exchange resin beads from the bottom of the mesh-bags. Shadings illustrate fertilization regimes. Samples of the same medium that do not share letters are significantly differed (P < 0.05) in total ergosterol according to T-test. Error bars represent SE.

Discussion

In the present study we found repressed EMM production starting within four months after N fertilization. Repressed EMM growth after N fertilization has been found in many earlier observations, although these measurements have usually been performed after several years of exposure to elevated levels of inorganic N (e.g. Nilsson and Wallander, 2003; Nilsson et al., 2007; Kjøller et al., 2012; Bahr et al., 2013).

However, even though N fertilization consistently repressed EMM growth according to frequency of hyphae and visual EMM estimation, ergosterol content didn't reveal that pattern as clear. Even though ergosterol content usually correlate well with other methods for fungal quantification (rev. by Wallander et al., 2013), inconsistencies have been reported (e.g. Högberg, 2006; Bahr et al., 2013), and the methods likely represent different aspects of fungal biomass. The visual method may be better to estimate the proliferation and aggregation of fine hyphae while ergosterol content, to a larger extent is sensitive to the abundance of rhizomorphs. It is also possible that ergosterol accumulates in the mesh-bags during the incubation time, while visual estimation gives a snap shoot of the current situation. Ergosterol has been found to accumulate in soil during late successional stages (Clemmensen et al., 2013), and it has been suggested as being less prone to degradation during N addition and reduced belowground C allocation (Zhao et al., 2005).

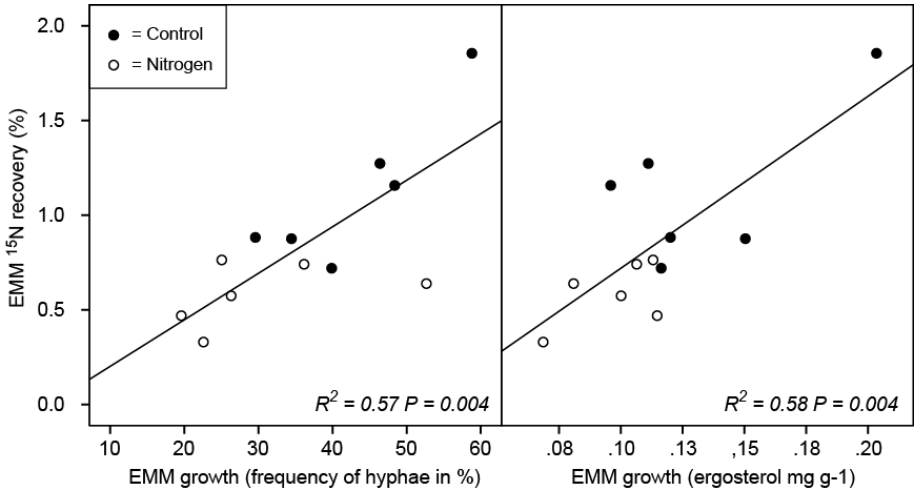

Fig. 5. Recovery of 15N added to the mesh-bags, in mycelium extracted from the ingrowth mesh-bags in relation to production of ectomycorrhizal extramatrical mycelium (EMM). EMM was estimated by counting of mycelium along the mesh of the ingrowth bags (A) and analysis of free and total ergosterol in sand from the ingrowth mesh-bags (B). Filled circles illustrate nitrogen fertilized plots.

In the long term, elevated soil fertility has been found to change the EMF community composition (Kjøller et al., 2012). This may affect the ergosterol content which has been shown to differ between, as well as within, different EMF species (Bermingham et al., 1995). It is however not likely that the short time period of the present study was sufficient for considerable changes in the EMF community. Delta ^{13}C ratio of the EMM was -27.6 ‰ (*SE* = 0.09) and didn't differ between treatments. Further, the average ergosterol concentration in the EMM biomass was low (0.40 µg g^{-1}) in comparison to previous findings of 1.00 µg g^{-1} by Hagerberg et al. (2003), although, they found large variation between different years (0.63-1.30 µg g^{-1}).

It was expected that analysis of free ergosterol would result in better correlations with the frequency of hyphae and visual estimations, since the free partition of ergosterol has been suggested as an indicator of living fungi (rev. by Wallander et al., 2013). However, there was a large variation in free ergosterol, and no clear differences could be detected in regard to fertilization treatment or incubation time. However during the winter, there was almost no free ergosterol which could be caused by a stagnated production of new EMM. But, these results must be interpreted with caution since the ergosterol levels were very low in the winter and the level of free ergosterol was close to the detection limit. In comparison with previous analyses of ergosterol in ingrowth mesh-bags (Wallander et al., 2010), in which 90% consisted of the free form, relatively low amounts (30%) of free ergosterol was detected in the present study. This discrepancy might be related to the extraction method used for free ergosterol, which is more crude and contains compounds that interfere with the ergosterol signal and make quantification of free ergosterol less reliable. Further research regarding ergosterol degradation during different environmental conditions is needed to improve our understanding and interpretation of the ergosterol analysis. The interpretation of free ergosterol could be

improved by manipulative experiments examining how it is affected by fungal degradation during e.g. soil incubation with fungicide addition to prevent production of saprotrophic fungi, or with freeze-thaw treatments. Further, development of the method for analysis of free ergosterol might result in more accurate HPLC chromatograms and improved detectability.

Enhanced repression of EMM growth when N is combined with P fertilization has earlier been found in microcosms (Wallander and Nylund, 1992). Our results indicate that this also occurs under field conditions, since EMM production tended to be further repressed by combined addition of N and P. This is probably an effect of sustained belowground C allocation to EMM due to enhanced P limitation in N fertilized plots.

Since EMM production in control plots was elevated by apatite amendment, and the P levels in the needles were below deficiency levels while N content was above (Table 2), it is likely that P was limiting growth in control plots. This, together with previous observations of P affecting EMM production in unfertilized boreo-nemoral forest (Hagerberg et al., 2003; Berner et al., 2012; Bahr et al., 2013) show that, apart from N, it may be important to take P into account in studies of environmental variables affecting EMF. Future estimates of growth responses will reveal to what extent tree growth is limited by N, P or both nutrients.

The limitation of P probably became more severe after N addition and could have caused the enhanced ergosterol levels in the apatite mesh-bags in N treated plots (Fig. 2). The lack of , decline in the needle content of P after N fertilization may suggest that tree growth did not respond to N addition, or that the time period for a growth response (about one year) is too short. The combination of N and P fertilization completely eradicated the positive effect of apatite on EMM growth and P levels in the needles rose far above deficiency levels indicating that any P limitation was relieved. Further studies are needed to state if the negative effect of P addition on EMM production (Fig. 1 and 2) is general, or if it only occurs when trees are initially P limited.

In relation to previous observations of annual EMM production of about 160 kg ha-1 (rev. by Ekblad et al., 2013), the production of EMM at control plots was large, calculated to 583 kg ha^{-1} during four months in late summer and early autumn. This can be compared to an aboveground NPP of 11.6 t ha^{-1} y^{-1} in a Norway spruce forest in Skogaby (located about 20 km from the present study) of similar age and fertility (Schulze, 2000). In Skogaby the production of belowground plant tissue was 40 % (7.4 t ha^{-1} y^{-1}) of the total NPP. Thus, the estimated four month EMM production of 583 kg ha^{-1} (the EMM production in the mesh-bags is similar to the soil environment) of the present study indicate that a substantial part of the C is allocated to EMF.

The abundant EMM was apparently not sufficient to retain all the added N, since an immediate flush of soil water N occurred after fertilization with up to about 100 mg l^{-1}. Elevated N losses were recorded at all fertilization treatments and not only where P was limiting. There was, however, a tendency of reduced N losses after fertilization at the plots fertilized with both N and P, which could be due to alleviation of the P limitation that was present in plots receiving N only (Stevens et al., 1993). Thus, N leakage after fertilization may be reduced by combined addition of P, but further studies are needed to verify this effect.

After the first autumn, soil water levels of N decreased to less than 10 mg l^{-1} and one year after the application, during the second autumn, to about 1 mg l^{-1}. Average N levels was however still elevated at N fertilized plots, but only significantly so in unthinned forest were limited photosynthetically active radiation might restrain belowground C allocation (Aber et al., 1998) and thus also N assimilation. The rapid decline in N leaching after application is in accordance with previous findings of reduced soil water NO_3^- within a few years after interrupted N treatment (reviewed by: Högberg et al., 2011).

Contrary to our expectations, no enhanced N leaching was found during the spring when no active EMM growth was detected, indicating that other processes than EMM production are also important for N retention. The rapid reduction in N leaching may have overshadowed any seasonal effect on N leaching making it difficult to relate it to EMF growth. However, EMM production as well as N leaching was reduced in the NP treatment, which does not suggest that reduced EMM production necessarily enhance the risk of N leaching. For a better evaluation of the role of EMF to counteract N leaching, estimates of N assimilation is necessary, and by tracking ^{15}N in the mesh-bags we found that EMM had a potential to retain 6 kg N ha^{-1} $month^{-1}$ in the control plots while this declined to half in the fertilized plots due to less abundant EMM growth. This suggest a significant role of EMM in N retention, although only a small portion of the initial 200 kg N application can be assimilated during the first growing season. In contrast, EMF uptake may be sufficient to retain moderate levels of inorganic N several years after fertilization or due to elevated N deposition. This data is based on the EMM produced in the mesh-bags during the experimental period, but if the total standing EMM biomass is assumed to have a similar assimilation potential, a different pattern will emerge. Wallander et al. (2004) estimated a standing biomass of EMM in a Norway spruce forest in southern Sweden to 5000 kg ha^{-1}. This will correspond to a monthly assimilation capacity of 46 kg ha^{-1}, meaning that 4 months would be enough to take up the whole application of 200 kg N ha^{-1}. It should be noted that the potential to assimilate N by EMM is probably underestimated since a portion of the N taken up will be translocated to the host tree during the two day incubation period. Näsholm et al. (2013) recently found that N fertilization resulted in enhanced allocation of $^{15}NO_3^-$ to the host tree, which suggest that the N assimilation potential may be even more underestimated in the N fertilized plots. On the other hand, enhanced N leaching in the mesh-bags of N fertilized plots suggest lower N retention of the EMM in this treatment. In addition, the Näsholm et al. (2013) study was performed in a severely N limited forest while our study was done in a forest with sufficient N, which would guarantee efficient allocation of N to the host trees also in the control plots. However, a true estimate of the N assimilation potential by EMM requires that the amount of N allocated to the host trees is also quantified.

In the present study we show that EMF play an important role in the N retention but to explain N leaching, especially after fertilization with large doses of N, many other environmental variables has to be taken into account. These may be direct assimilation by tree roots, climate variables such as precipitation and snowmelt, growth of saprophytic fungi and chemical reactions between N and humus. A large initial flush

of N after the fertilization show that some of the added N cannot be retained. Usually this correspond to around 5% of the added amount after a standard fertilizer application (Nohrstedt, 2001). It is possible that N leaching could be prevented by more moderate application of the N fertilizer (Bergh et al., 2008), and similar to the work by Stevens et al. (1993), our results suggest that N leakage can also be reduced by combining N with a P fertilizer. However, this may only be relevant in P deficient soils and further studies are needed to evaluate this treatment in different types of forests.

Our findings of reduced ^{15}N retention by EMM after N fertilization supports earlier suggestions that EMM prevents N leaching which are based on correlations between elevated N losses and low EMM production (Nilsson et al., 2007; Kjøller et al., 2012; Bahr et al., 2013). Högberg et al. (2013) found that the fungal to bacterial ratio in the soil was the variable most correlated to N losses. It was however not clear to what extent saprophytic vs. mycorrhizal fungi contributed to the N retention (Högberg et al., 2013). In the present study, elevated percolation of isotopically labeled NH_4 in the ingrowth mesh-bags at the N fertilized plots is likely due to repressed EMM production. Still, saprophytic fungi maybe important for N retention in the natural soil environment. Reduced N assimilation by EMM in N treated plots may however also been affected by changed specific N assimilation capacity per mycelial biomass after N fertilization (Finlay et al., 1992; Clemmensen et al., 2008), but it has been reported that interspecific differences regarding NH_4^+ assimilation are small (Finlay et al., 1992).

Conclusions

Elevated N losses coincided with reduced EMM production after N fertilization, but seasonal variation of EMM production was not correlated to seasonal variation in N leaching. It was thus not possible to draw any firm conclusions of the role of EMM in the total ecosystem N retention capacity. However, by tracing stable N isotopes added to ingrowth mesh-bags we were able to demonstrate enhanced assimilation and reduced leaching of labeled N in control compared to fertilized plots. EMM thus have a significant role in reducing N leaching but other factors are also important. We calculated a potential N retention capacity by EMM to more than 6 kg ha^{-1} $month^{-1}$ at control plots, while this value was reduced to half in N fertilized plots.

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