The Role of Biological Processes in Base Cation Supply in Boreal Forest Podzols

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Cover: Microcosm setting of paper IV containing forest soils planted with *Pinus sylvestris* seedlings.
(Photo: Z. Fahad)

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Abstract

This thesis describes experiments designed to improve understanding of biological processes contributing to supply of base cations and other nutrients in boreal forest podzols. We used microcosms containing tree seedlings growing in natural substrates, a combination of direct measurements, modelling, stable Mg isotope analysis, and $^{13}CO_2$ pulse-labelling. Addition of ^{13}C-labelled fungal necromass to soil resulted in rapid decomposition and active incorporation of ^{13}C into RNA of *Burkholderia*, *Streptacidophilus*, *Dyella, Herminiimonas, Granulicella* and fungal species belonging mainly to the genera *Mortierella* and *Umbelopsis*. There was no evidence of ^{13}C incorporation into RNA of ectomycorrhizal fungi supporting the idea that ectomycorrhizal fungi primarily play an active role in organic matter decomposition by releasing N from recalcitrant substrates, but do not use organic matter as a source of metabolic C. Selected ectomycorrhizal and nonmycorrhizal fungi were examined for their capacity to fractionate and assimilate stable Mg isotopes *in vitro*. Ectomycorrhizal fungi mobilised and accumulated significantly higher concentrations of Mg, K and P than nonmycorrhizal fungi, when grown on granite particles. Mycorrhizal fungi were significantly depleted in heavy isotopes compared with nonmycorrhizal fungi and there was a highly significant statistical relationship between δ^{26}Mg tissue signature and mycelial concentration of Mg. *Pinus sylvestris* seedlings were grown in compartmentalised microcosms allowing their mycorrhizal mycelium, but not roots, to access different substrates, including granite particles. Root biomass and contents of Ca, K, Mg, and P in plants in granite treatments were significantly higher than in control roots. Carbon allocation by the ectomycorrhizal mycelium to soil solution was significantly and positively correlated with base cation and P content of the plants. A final experiment (using reconstructed boreal forest podzol layers) was conducted in which the relative amounts of organic and mineral substrates were manipulated to simulate different levels of intensification of the removal of organic matter. All plants were deficient in K and P but had above optimal levels of Ca and Mg. Total plant and fungal mycelial biomass was positively related to the amount of organic soil in each treatment. The δ^{26}Mg values of soil solution samples in B horizon soil increased successively with increasing plant and fungal mycelial biomass, suggesting increased uptake of Mg from the B horizon, with discrimination against the heavier isotope resulting in higher enrichment of ^{26}Mg.

Keywords: base cations, decomposition, ectomycorrhizal fungi, podzol, magnesium isotopes, nutrient mobilisation, weathering

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Dedication

To my Parents…
To Laith, Omar and Aia…

Contents

List of Publications

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

I. Fahad, Z.A., Mahmood, S., Mikusinska, A., Ekblad, A., Fransson, P., Lindahl, B., Finlay, R.D. Decomposition of fungal mycelium in podzol horizons of boreal forest – a ^{13}C-RNA stable isotope probing study (manuscript).

II. Fahad, Z.A., Bolou-Bi EB, Ekblad, A., Köhler SJ, Finlay RD, Mahmood, S. 2016. Fractionation and assimilation of Mg isotopes by fungi is species dependent. *Environmental Microbiology Reports* 8, 956-965.

III. Fahad, Z.A., Mahmood, S., Bolou-Bi, E.B., Ekblad, A., Köhler, S.J., Bishop, K., Finlay, R.D. Patterns of mycelial colonisation, base cation mobilisation, carbon allocation and Mg isotope fractionation in compartmentalised microcosms containing mineral and organic substrates (manuscript).

IV. Fahad, Z.A., Mahmood, S., Bolou-Bi, E.B., Ekblad, A., Köhler, S.J., Bishop, K., Finlay, R.D. Nutrient mobilisation from, and carbon allocation to, different soil horizons in a reconstructed boreal forest podzol – effects of organic matter depletion (manuscript).

The contribution of Zaenab Fahad to the papers included in this thesis was as follows:

I. Planned the study together with the supervisors. Maintained the phytotron materials and performed the laboratory RNA-SIP work for sequencing. Wrote the manuscript in collaboration with the supervisors.

II. Planned the study with the supervisors. Performed the laboratory work and prepared the material for chemical analyses. Analysed the data and performed statistical analyses. Wrote the manuscript with input from the co-authors and supervisors. Responsible for correspondence with the journal.

III. Planned the study with supervisors. Maintained the phytotron materials and prepared the material for chemical analyses. Analysed the data and performed statistical analyses. Wrote the manuscript with input from the co-authors and supervisors.

IV. Planned the study with supervisors. Maintained the phytotron materials and prepared the material for chemical analyses. Analysed the data and performed statistical analyses. Wrote the manuscript with input from the co-authors and supervisors.

1 Background

1.1 Boreal forests

The boreal forest biome is circumpolar in distribution, located between 50°N and 60°N and represents about one third of the total global forest area (Taggart and Cross, 2009), storing 32% of the estimated C stock of the world's forests (861 Pg C) (Pan *et al.*, 2011).

Forests present in the northern hemisphere today have been migrating, evolving, and developing since the ice retreated after the last glaciation and many of them are relatively recently established in the 18th century (Hyvönen *et al.*, 2010). In young soils, N is considered to be the primary limiting factor of production (Tamm, 1991), as N availability is very low and P is relatively abundant compared with that in old soils (Lambers *et al.*, 2008). However, the low temperature and low soil pH result in low nutrient content (Deluca and Boisvenue, 2012). The boreal forest is dominated by conifers of the family Pinaceae, including spruce (*Picea*), fir (*Abies*), pine (*Pinus*) and larch (*Larix*). Although the total number of extant species estimated in 1998 was 629 species (Lott *et al*., 2002), conifers are ecologically important since the boreal forest biome occupies a disproportionately large global area in relation to the number of tree species. *Betula and Salix* are the main deciduous components (Ostlund *et al*., 1997). The understorey components in Swedish boreal forests are ericaceous dwarf shrubs, feather mosses, and reindeer lichens.

Many boreal forests in northern Europe are intensively used for pulp, timber, and forest fuel production. Wood fuel is a renewable energy source with a high potential for exploitation (Egnell and Valinger, 2003), especially in Sweden, because two-thirds of its land area is covered with forest. Clear-felling practices in some parts of Sweden became common in the 1950s and 1960s (Lundmark *et al*., 2013) and the production of biofuels used in the Swedish energy system has steadily increased, from a little over 10% of the total energy supply in the 1980s to 20% in 2008, industrial forest residues being the

primary bioenergy source. The growing demand for renewable energy sources in Sweden has resulted in increased use of forest biomass that now includes logging residues (Egnell, 2011), and whole tree harvesting (Akselsson *et al.*, 2016). These practices render the next generation tree stands more sensitive to nutrient shortage, creating the need for compensation. Sustainable nutrient management is necessary if harvesting is high compared to the production capacity.

Boreal forest podzols

Podzols occur mainly in cool, humid climates (McKeague *et al.*, 1983) and soil stratification is the main characteristic of boreal forest podzols (**Figure 1**). Due to the slow decomposition rate of plant litter by different biota, organic matter accumulates above the mineral soil horizons. The accumulated organic horizon (O) can be subdivided into litter, fermentation, and humus- sub-horizons (Hille *et al*., 2005). During organic matter decomposition different organic acids are released into soil solution and leach down into the underlying mineral layer where another biogeochemical process, mineral weathering, takes place, leading to a weathered, ash-grey eluvial (E)-horizon. This layer contains lower concentrations of base cations, Al and Fe than the parent material and is enriched in residual Si. The further downward transport of Al and Si as inorganic colloids results in formation of an illuvial (B) horizon that is characterized by a reddish-brown colour and enriched in Al, Fe, P, and base cations (Lundström *et al.,* 2000).

The acidity of forest soil restricts the activity of burrowing soil animals such as earthworms that would otherwise cause mixing and enhances soil stratification during podzolisation (Nordström and Rundgren, 1974). These vertically separated horizons have different texture and structure and vary in nutrient content and pH; collectively these factors influence fungal community structure in each horizon (Rosling *et al*., 2003).

1.2 Microorganisms in boreal forest podzols

Microbial community composition is an important determinant of ecosystem process rates (Strickland *et al*., 2009); it is regulated by environmental factors such as nutrient availability and quality (e.g. C:N) (Swift *et al.,* 1979), pH (Alexander, 1977), soil chemistry and texture, and the presence of plant roots (McGuire, 2007).

The domain archaea was differentiated from the bacteria by Woese and Fox, (1977) that were earlier classified as anaerobic bacteria. Bomberg and Timonen (2007) showed that the presence of ectomycorrhizal Scots pine roots

in the humus layer of a boreal forest soil increased both the abundance and diversity of archaea in the phylum *Euryarchaeota*. Archaea can have an autotrophic life style, oxidizing ammonia as their sole energy source (Könneke *et al*., 2005). To date, ammonia oxidizing archaea have been found in various soils where they outnumber ammonia oxidizing bacteria (Leininger *et al*., 2006).

Boreal forest soil harbours a wide array of bacterial communities, one gram of soil is estimated to harbour 10^{10} bacterial cells (Torsvik *et al.*, 1996). Distinct bacterial communities can associate with particular soil horizons, and more than 50 phyla have been found, with 25 phyla associated with ectomycorrhizal roots (Baldrian *et al*., 2012). Generally, *Actinobacteria, Proteobacteria and Acidobacteria* are particularly abundant and can be affected by the presence of ectomycorrhizal roots in the soil (Vik *et al.*, 2013). Bacteria can decompose forest litter and degrade phenol (Persson *et al*., 1980; Manefield *et al.*, 2002), and, together with ectomycorrhizal fungi, may play a role in weathering of minerals (Uroz *et al*., 2009; Gleeson *et al*., 2006).

Fungi are heterotrophic and rely on autotrophic organisms to acquire C for their growth and metabolism. They perform different ecological functions, and occur as saprotrophs, pathogens and mutualistic symbionts. Fungi play an important role in nutrient dynamics in organic and mineral substrates. One main fungal guild in boreal forests is the saprotrophic fungi; these fungi obtain energy from dead and decaying organic matter to maintain their growth and activity. They are the main decomposers of recalcitrant woody residues and plant litter, using a wide range of extracellular, hydrolytic and oxidative enzymes. Wood decay fungi digest wood causing different types of rot based on their ability to degrade different major cell wall components. The brown rot fungi such as *Fomitopsis pinicola* and *Antrodia serialis*, white rot fungi such as *Armillaria* sp., *Bjerkandera adusta*, *Heterobasidion annosum* and *Trichaptum abietinum* are basidiomycetes, and soft rot is typically caused by ascomycetes such as *Fusarium* spp. and *Phialophora* spp. All these types can degrade cellulose but do not necessarily digest lignin. Litter decomposers in boreal forests mainly belong to two phyla, basidiomycetes, that are considered especially important because of their ability to produce ligninolytic enzymes essential for degradation of recalcitrant plant material (Osono and Takeda, 2002), and ascomycetes, that also frequently colonise plant litter but have a much lower decomposition capacity (Boberg *et al.,* 2011).

Moulds and yeasts are saprotrophic fungi, and dominate the humus layer (Deacon and Fleming, 1992). Although yeast functioning in soil is still not fully understood, they influence soil aggregation, contribute to nutrient cycles and also interact with vegetation and soil animals (Yurkov *et al*., 2012 and

references therein). Moulds and yeasts found in Swedish forests mostly belong to the genera Eurotiales, Hypocreales, Morteriellales, Mucorales, Saccharomycetales, Tremellales and Sporidiales (Sterkenburg *et al*., 2015). The other main fungal guild dominant in boreal forest is the ectomycorrhizal fungi that live in symbiosis with host plants. About 80% of present-day plant species and 92% of plant families form mycorrhizal of all types (Wang and Qiu, 2006).

Ectomycorrhizal associations are formed by more than 95% of the fine roots of coniferous trees in boreal forests (Taylor *et al.*, 2000). In Sweden, over 1100 ectomycorrhizal species have been identified and as many as 8000-10000 species may exist globally. Ectomycorrhizal roots are characterized by the presence of three structural components: 1) the Hartig net, a reticulated structure formed by hyphae that grows inwards to surround the epidermal and cortical root cells and serves as an interface between plant and fungus that adapted to the exchange of plant-derived carbohydrates for fungus-derived nutrients, 2) a mantle of fungal tissue which sheathes the fine roots close to the root tip and functions as intermediate storage for the exchangeable substances between the two symbiont partners, and 3) an extramatrical mycelium, providing a direct connection between the plant roots and the soil environment and acting as an extension to increase the surface area for nutrient exchange and water capture, and to access soil microsites that are inaccessible to the roots themselves.

Ericoid mycorrhizal fungi colonise the roots of ericaceous understorey plants, such as, *Vaccinium vitis idea* and *Vaccinium myrtillus.* The fungus penetrates the cell walls of the root and forms coiled structures within each cell without penetrating the host plasmalemma. Besides their role in N and P mobilisation, they also have the ability to metabolize toxic metals (Smith and Read, 2008). They associate with around 3400 plant species, and they are mainly Ascomycota. Arbuscular mycorrhizal (AM) fungi are the most ancient and widespread type of symbiotic fungi, colonising over 250000 plant species. They belong to the phylum Glomeromycota (Schüßler *et al*., 2001), and penetrate the cells of plant roots forming invaginations in the cell membrane and characteristic, repeatedly branched structures called “arbuscules” within the cortical cells.

Fungal functional groups and their spatial distribution

Many ecological theories treat saprotrophic and ectomycorrhizal (EMC) fungi and other soil microorganisms as a single, ubiquitously occurring, functional group. However, in stratified boreal forest podzols distinct fungal communities may occur in different soil horizons. These probably reflect differences in

trophic strategies related to the physicochemical characteristics of the different horizons (Lundström *et al.*, 2000) and competition for different nutrients because boreal forest soils are generally low in nutrient availability (Lindahl *et al.*, 2002). Litter degradation in boreal forest is largely performed by microorganisms such as fungi and bacteria (Persson *et al*., 1980). Saprotrophic fungi are the main decomposers of newly shed plant litter in the surface organic matter horizon. As free-living microorganisms, they obtain C and nutrients for growth and metabolism by degrading organic polymers using extracellular enzymes (Jennings, 1995). As the C to N ratio decreases in deeper organic horizons these saprotrophs are replaced by ectomycorrhizal fungi that receive their C directly from plant hosts and are better able to compete with saprotrophs. These fungi are able to mobilise N and P from recalcitrant organic substrates (Read and Perez-Moreno, 2003; Lindahl *et al*., 2007), and dominate the deeper mineral soils where they are able to mobilise P and base cations (Finlay *et al*., 2009; Landeweert *et al.,* 2001).

Ectomycorrhizal fungi in forest ecosystems

Boreal forests are known to play a very dynamic role in global carbon cycling (Apps *et al*., 1993). The below ground C allocation can represent 25-63% of the gross primary production on a global scale (Litton *et al*., 2007).

Most of the nutrient uptake by trees is mediated by ectomycorrhizal roots and mycelia (Read, 1991), ectomycorrhizal fungal receive 10-30 % of photoassimilates from their hosts (Söderström, 1992; Leake, 2006). Högberg *et al*. (2001) in a large-scale girdling study of Pine forest, investigated the effect of tree girdling to xylem depth on respiratory rate of forest soil, the girdling approach was used to separate mycorrhizal root respiration from heterotrophic respiration. Their results showed a reduction in soil respiration within 1-2 months by about 54% relative to respiration on non girdled control plots, and that decreases of up to 37% were detected within 5 days. Hasselquist *et al*. (2016) examined the independent and interactive effective effects in C and N supply on the transfer of N via ectomycorrhizal fungal association with about 15 year old *Pinus sylvestris* trees. The treatments consisted of an application of a low and high N in form of $Ca^{15}NO_3$ to mor layer dominated by ectomycorrhizal fungi, and shading treatment. The authors found that reduced C uptake imposed by shading (60 % in shaded relative to non shaded control plots) resulted in lower, not higher, ^{15}N levels in foliage compared with levels in ECM root tips (expressed as the ratio ^{15}N in foliage:^{15}N in ECM roots) when a high level of N was added (150 kg N ha^{-1}) compared to when a low N level was added (20 kg N ha^{-1}), and a 30 % reduction in cumulative soil respiration compared to control plots, shading was linked to a *ca*. 25 % reduction in

ectomycorrhizal fungal biomass colonizing root tips four weeks after labeling and *ca*. 40 % reduction in cumulative ectomycorrhizal sporocarps production relative to the unshaded plots. Hasselquist *et al*. (2012) have also shown in a previous study that respiration by extramatrical mycorrhizal hyphae represents *ca.* 40% of autotrophic respiration.

In addition to nutrient and water transportation, ectomycorrhizal fungi may transfer small quantities of carbon between interconnected tree hosts (Simard *et al.*,1997), and can connect multiple plant hosts below ground across scales of cm^2 to at least tens of m^2 (Selosse *et al*., 2006). The ecological importance of ectomycorrhizal fungi in belowground C allocation was demonstrated in a study by Wallander *et al*. (2001) that estimated the total production of ectomycorrhizal fungal biomass including root tip mental in the humus layer of a Swedish forest soil to be 700-900 kg ha^{-1}. This production of fungal tissue must be of considerable importance for nutrient uptake and water translocation to plant hosts, and also represents a significant source of both C and N for saprotrophic fungi (Fernandez *et al.*, 2016). In a comparative study of C storage per unit N in different ecosystems, Averill *et al*. (2014) found that soil in systems with ectomycorrhizal plants stores 1.7 times more C than in soil colonised by arbuscular mycorrhizal hosts, highlighting the importance of the ecological role of ectomycorrhiza in C sequestration.

Ectomycorrhizal fungi, supplied with plant-derived carbohydrate molecules have the potential to extract N and P from the partially decomposed, recalcitrant litter in boreal forests (Lindahl *et al.*, 2007), and to dominate the underlying mineral horizons where P and base cations such as Mg, K and Ca mineralization and mobilization essential for the plant take place (see Landeweert *et al.,* 2001; Finlay *et al.*, 2009).

1.3 Boreal forest biogeochemical processes

Carbon dynamics in forest soil are driven by two main biogeochemical processes, while organic matter decomposition performed by saprotrophs and to some extent mycorrhizal fungi, mutualistic fungi are likely to be more important in weathering than saprotrophs because of substantial carbon costs to produce organic anions (Hoffland *et al*., 2004). The possible role of ectomycorrhizal fungi in relation to mineral weathering has been discussed in relation to the discovery of numerous tubular pores, 3-10 µm in diameter, in weatherable minerals in podzol surface soils and shallow granitic rock under European coniferous forests (van Breemen *et al.*, 2000; Jongmans *et al.*, 1997; Landeweert *et al.*, 2001).

In boreal forest, organic matter decomposition includes wood and litter degradation. Wood degraders cause either white-rot or brown-rot decays in dead wood, and decomposers have very efficient oxidative systems to degrade lignin. Most conifers are evergreens with leaves that usually have high lignin concentrations 25% (Johansson, 1995). The freshly shed litter layer can be quite substantial in volume, with the litter fall in a *P. sylvestris* forest estimated to be between 1-1.5 tonnes per hectare per year. The rate of litter decomposition in many cases is negatively correlated with lignin concentration and positively with N concentration (see Aerts, 1995). At early stages of decomposition, litter saprotrophs using mainly hydrolytic enzymes, degrade the easily degradable cellulose and other carbohydrates of dead organic matter as the principal source of metabolic C, the hydrolytic enzymes include, endocellulases, glucosidases, glucanases, cellobiohydrolases and different kinds of xylanases (Baldrian, 2008). Some conversions of organic matter may make the organic matter more resistant to further degradation (Swift *et al*., 1979) and the metabolic costs of the remaining fragmented organic matter may be higher than the gain. The partially degraded litter has a high lignin content, with organic N and P locked up in forms that are inaccessible to the plants (Northup *et al*., 1995). Lignin has a complex chemical structure consisting of phenolic residues, and to mine N that is recalcitrantly bound to the nonhydrolysable lignin, another source of C is required to perform a co-metabolic process using an enzymatic ligninolytic system based on oxidative enzymes which can be performed by ectomycorrhizal fungi using their host-derived sugars, and enzymes such as laccases, peroxidases, hydrogen peroxide producing enzyme, and peroxygenases (Bödeker, 2012).

Rineau *et al.* (2012) investigated N mobilisation from litter by *Paxillus involutus* using spectroscopic analyses and transcriptomic profiling, and observed that the mechanisms of litter decomposition involved Fenton chemistry, similar to that of saprotrophic brown rot fungi. *P. involutus* lacked many of the transcripts encoding extracellular plant cell-wall degrading glycoside hydrolases that were expressed in brown rot fungi, but retained a significant part of the oxidative decomposing machinery present in the brown rot ancestors. A large number of Class II peroxidases genes also seem to have been retained in the genome of *Cortinarius glaucopus* (Lindahl and Tunlid, 2015). The removal of N will increase the C:N ratio, indicating the activity of ectomycorrhizal fungi as decomposers acting on organic N, rather than metabolic C. Orwin *et al*. (2011) suggested that organic N uptake by ectomycorrhizal plants will limit N for free-living fungi to produce soil organic matter degrading enzymes and thus slow decomposition rate, and increase C

storage in the soil when C allocation to belowground partner is in shortage (Smith and Read, 1997).

For many decades, efforts have been made to study the interaction between ectomycorrhizal fungi and minerals in forest soil (Finlay *et al.*, 2009) and investigate the role of this group in nutrient cycling and plant growth enhancement (Wallander *et al*., 1997; Wallander, 2000; Jentschke *et al*., 2000, 2001; van Scholl *et al*., 2008). Wallander and Wickman (1999) found an enhancement of foliar K content in *P. sylvestris* seedlings colonised by the ectomycorrhizal fungus *S. variegatus* when grown with biotite, highlighting the ability of this fungus to assist plant host growth. The authors found a positive correlation between foliar K content, citric acid concentration and fungal biomass in the soil. Van Schöll *et al*. (2006) examined weathering and uptake of different base cations from mineral particles incubated in pots containing *Picea abies* seedlings colonised by the ectomycorrhizal fungi *P. involutus*, *Suillus bovinus* and *Piloderma croceum* and found that *P. involutus* was able to increase mobilisation and uptake of K from muscovite, but none of the tested fungi was able to mobilise Mg from hornblende. Adeleke *et al*. (2012) found that different roles of ectomycorrhizal fungi in mineral weathering such as nutrient absorption and translocation improve plant health and nutrient cycling in ecosystems are species specific.

Though the performance of ectomycorrhizal fungi in scavenging nutrients such as P and base cations from soil solution is well recognized (Ahonen-Jonnarth *et al.*, 2000; van Hees *et al.*, 2000; Wallander, 2000), more studies of higher resolution were performed to understand the mechanism of element dissolution, due to the difficulty to distinguish between the acid exuded by different organisms in the field, the amount of mineral dissolved, or the amount of the element taken up by the fungus due to the intimate association between plant roots and the fungal hyphae (Taylor *et al*., 2009; Landeweert *et al*., 2001; Smits *et al*., 2012).

Another study by Gazze *et al.* (2013) examined fungal exudate production in the form of extracellular polymeric substances (EPS) produced by hyphae of *P. involutus* growuing in symbiosis with *P.sylvestris* seedlings.

This study demonstrated the formation of EPS halos around hyphal tips colonising the surface of biotite flakes during fungal hyphal tip growth and discusses the possible role these halos may have in increasing the surface area of contact between fungal hyphal tip and the mineral substrates and the way in which weathering may be facilitated.

Quirk *et al*. (2012) in a field-mesh bag study compared between gymnosperms and angiosperms, associated with two groups of associated fungi (ECM and AM) in an arboretum in relation to their co-evolution on silicate

weathering intensification using silicate rocks either Ca-rich (basalt) or –poor (granite), the authors found that basalt colonization by the two groups of fungi progressively increased with advancement from arbuscular mycorrhizal to later, independently evolved ectomycorrhizal fungi, and from gymnosperm to angiosperm hosts with both fungal groups, ECM gymnosperms and angiosperms released Ca from basalt at twice the rate of AM gymnosperms.

Bonneville *et al.* (2009) studied weathering processes in a system in which *P. involutus* fungal hyphae, growing from Pine seedlings, grew on biotite flakes as a source of K. Ultramicroscopic and spectroscopic observations of these fungus-biotite interfaces revealed evidence of biomechanical forcing and altered interlayer spacing, suggesting that physical distortion of the lattice structure takes place before chemical alteration through dissolution and oxidation. A study of Saccone *et al*. (2012) using hornblende, biotite and chlorite demonstrated the ability of *P. involutus* hyphae to weather the minerals through organic acid exudation. However, there is still uncertainty about the quantitative significance of these interactions and how they are regulated (Brantley *et al.,* 2011).

1.4 Forest management and uncertainty of mineral weathering rates

Until 2013, stump harvesting was not common in Sweden (Swedish Statistical Yearbook of Forestry, 2014). Due to the increasing demand for renewable energy, Sweden has committed to achieving a share of energy from renewable sources as high as 49% of total energy consumption by 2020. Whole tree harvesting, including the stems, branches and tops, has become a common practice in Sweden. Akselsson *et al*. (2007) compared the effect of stem harvesting with whole tree harvesting in spruce and pine forests on base cations budget in Swedish forest soils and found that in spruce forests the estimated yearly net losses of Ca, Mg and K corresponded to at least 5%, 8% and 3% respectively of the exchangeable pools of base cations at 25% of 622 analysed sites. More studies need to be performed to reduce the uncertainty of weathering-rate estimates. Base cations such as Ca, Mg, and K are important nutrients for plant growth in forest ecosystems; together with Na they also determine the base saturation, and are thus critical for soil resistance to acidification (de Jong *et al*., 2011).

Increased biomass harvesting leads to an increase in base cation losses from the ecosystem, which can counteract recovery from acidification (Akselsson *et al*., 2016). Since the 1980's acid deposition has decreased dramatically and

forest harvesting is now the dominant acidification pressure (Buffam *et al*., 2008).

Seven published estimates of silicate weathering from one area using different modelling tools varied by an order of magnitude in one area (Klaminder *et al*., 2011), suggesting that there is uncertainty in these estimations. Differences in geology between different sites and inadequate understanding of the key processes such as the biological influence on mineral weathering kinetics, all complicate the production of reliable estimates.

The need for nutrient compensation through wood ash recycling or liming has been discussed in relation to possible solutions for base cation replacement (Swedish Statistical Yearbook of Forestry, 2008). The question still arising is how much is needed of any of these supplements to cover the shortfalls in base cations supplied by weathering. Many studies have been performed to estimate base cation weathering and release rates using different models based on the mass balance approach such as PROFILE in Sweden and Denmark (Sverdrup and Warfvinge, 1993; 1995) and SSMB in Finland (Warfvinge and Sverdrup, and others (see Futter, *et al.*, 2012). Sverdrup and Rosén (1998) found that weathering rates in Swedish forest soil are slow and that acidity deposition exceeds the weathering rate. The authors used PROFILE as a steady-state model based on the assumption that soil residual acidity in the percolate (H^+- and Al^{+3} -ions) will exchange with Ca, Mg and K mainly absorbed to organic matter and clays, causing the base saturation to decrease. The model was applied to compare partial and whole tree harvesting situations using data that were collected between 1983-85 from 1884 plots distributed within Sweden, and took into account soil acidification, simulating mineral weathering in one or more soil layers including different parameters (see Sverdrup and Rosén, 1998). The authors claimed if whole-tree harvesting is done without base cation return, then the negative differences between supply and removal for Ca will increase by ca. 50% and the negative differences between supply and removal for K will increase by even more.

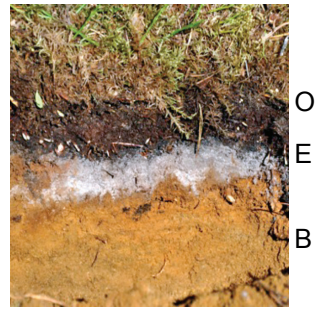
The measurements of weathering rates in these models assumed that soil-weathering rates could be given as annual mean values, and only long-term sources of acidity or alkalinity could be included in the system. Forest soil is dynamic in terms of chemical-biological properties, weathering rates in forest soil are affected by environmental factors that are not predictable in a changing climate. Studies have shown that some parameters used in these models were not valid for all sites or situations, therefore, it is suggested that changes due to seasonality, soil spatial and temporal differences, pH gradients or organic exudation, the interaction between different microorganisms in the rhizosphere in different horizons, and other biological indicators that are more sensitive to

toxic metals than the tree, such as root-microorganisms or soil fauna, should be considered in dynamic models (see Løkke *et al.*, 1996).

Al toxicity is a major factor limiting plant production on acid soil, Al solubility is enhanced by low pH (Delhaize and Ryan, 1995), mycorrhizal fungi enhance base cation availability to plants growing at elevated Al levels, and influence the ability of the plant to tolerate different anthropogenically generated stresses, they produce organic compounds allocating the C fixed by the tree and affect soil solution chemistry; such units might be a valid candidate to represent a sensitive biological indicator for weathering rate models especially root tips and the connected fungus have shorter life span than the trees.

The difficulty in measuring weathering rates in boreal forests is that different biological processes might occur simultaneously at different scales in different horizons. Both organic matter decomposition and mineral weathering are fuelled by the delivery of plant-derived C to networks of fungal symbionts that are in intimate contact with soil particles or have access to soil solution in microsites containing nutrients inaccessible to roots. Depletion of nutrients due to organic matter removal from forest floor could potentially increase nutrient mobilisation through fungal-driven weathering to compensate for nutrient shortages due to their leaching from the soil. However, it is unclear how much nutrients could be translocated to the plant, or what the C cost to the host plants would be (**Figure 2**). To understand the role of microorganisms in base cation acquisition in different soil horizons, laboratory-scale microcosms were set up to study spatial patterns of nutrient mobilisation and C allocation in different soil horizons. The overarching hypothesis behind this project is that biological weathering by symbiotic fungi and associated bacteria (and fungi) makes a significant contribution to the mineral requirements of forest trees and that this biological weathering is regulated by plant-derived C in response to changes in environmental conditions. However base cations may also be supplied through the intervention of ectomycorrhizal mycelium in decomposition and re-cycling of essential plant nutrients from organic residues.

Figure 1. A conifer forest podzol profile at Jädraås, Sweden, showing organic (O), eluvial (E) and illuvial (B) horizon soils that were used in four lab scale studies.
(Photo: Roger Finlay)



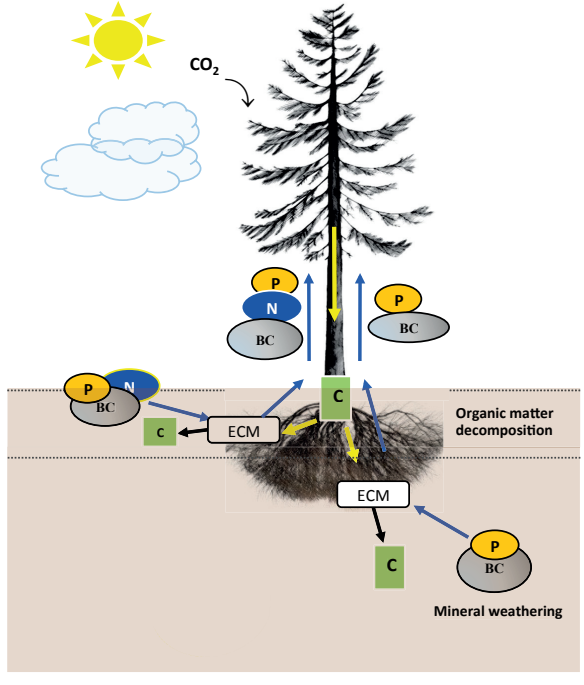


Figure 2. Schematic diagram of the proposed model of nutrient mobilisation in O, E and B horizon soils of a boreal forest podzol. The plants allocate C to roots and ectomycorrhizal fungi to fuel nutrient uptake via two different biogeochemical processes. Base cations, P and N are taken up through organic matter decomposition, while only base cations and P can be taken up through mineral weathering.

2 Project objectives

The increasing demand for renewable energy in Sweden has resulted in increased interest in whole-tree harvesting (Akselsson *et al*., 2016). The removal of this extra organic matter in the form of needles, branches, tops and roots, may create a nutrient imbalance and lead to a loss of base cations that must be compensated. How much compensatory fertilization or lime to be added to the forest is based on the estimations that early weathering models produced with some uncertainty. Although, the role of microbial symbionts in nutrient cycling in boreal forests is increasingly evident, classical models do not include the roles of microorganisms in mineral weathering. To improve weathering rate estimates, microbiological parameters should be used in dynamic weathering models. One aim of the project was to investigate the possible extent of biological weathering and how this can supply trees with base cations under different scenarios. The aim of the work in this thesis is to quantify the role of ectomycorrhizal fungi and associated bacteria in base cation release during mineral weathering and organic matter decomposition using laboratory microcosm experiments with natural forest soil. An additional field study was performed to quantify the role of the extraradical ectomycorrhizal fungal mycelium in taking up and storing base cations in different soil horizons of a boreal forest.

Paper I: Mycelial decomposition and patterns of C incorporation

Each soil horizon harbours microbial communities that are distinct in their composition and activity. To assess the activity of fungi and bacteria degrading mycelial necromass in two different podzol soils, we measured $^{13}CO_2$ respiration as a proxy for ^{13}C-labelled mycelial decomposition. The specific objectives of paper I were to:

- Identify the active bacterial and fungal communities degrading fungal necromass in organic and mineral soil horizons.

- Examine the effect of plants on the decomposition rate in different soil horizons.

Paper II: Mg fractionation and assimilation by ectomycorrhizal and nonmycorrhizal fungi

Studies of ^{13}C and ^{15}N fractionation and assimilation by ectomycorrhizal fungi have provided some knowledge about the interaction between ectomycorrhizal fungi and organic matter but information about base cation fractionation and assimilation by ectomycorrhizal fungi is scarce. We examined the relative ability of different ectomycorrhizal and non-mycorrhizal fungi to fractionate and assimilate stable isotopes of Mg, and their ability to take up Mg, Ca and P from different substrates.

The specific objectives of paper II were to:

- Determine whether different fungal species have different $\delta^{26}Mg$ signatures.
- Determine whether differences in Mg fractionation and assimilation patterns are related to the trophic status (ectomycorrhizal or saprotrophic) of the fungi.

Paper III: Base cation mobilisation, carbon allocation via ectomycorrhizal fungi to organic and mineral substrate

We examined the ability of ectomycorrhizal fungi to mobilise base cations and P from organic and inorganic matter and translocate them to their plant hosts in compartmentalized systems. The specific objectives of paper III were to:

- Determine elemental composition and content in fungal mycelium colonising organic and mineral substrates, and in soil solution associated with these two substrates.
- Determine the potential supply of nutrients from this mycelial pool to plants and its overall contribution to plant nutrient status.
- Compare ^{13}C allocation patterns by ectomycorrhizal fungi to the soil solution of organic and mineral substrates.

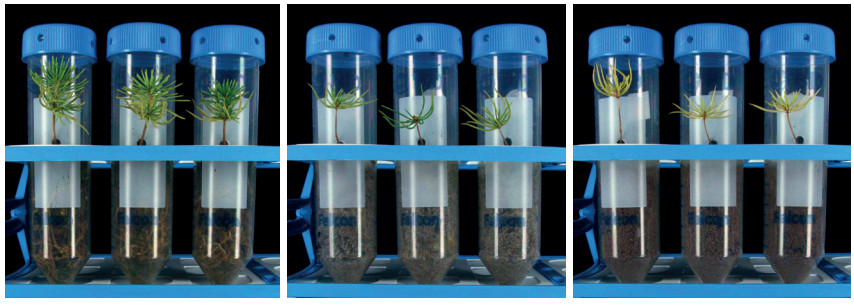
Paper IV: Effect of organic matter depletion on nutrient mobilisation and C allocation

Spatial patterns of nutrient mobilisation and C allocation were studied in stratified soils along a gradient of increasing organic matter depletion simulating different forest management regimes. The specific objectives of paper IV were to:

- Examine patterns of base cation mobilisation from organic and mineral soil horizons and how these varied with respect to different degrees of organic matter depletion.
- Estimate fungal mycelial biomass in organic and mineral soil horizons along a gradient of increasing organic matter depletion.
- Determine elemental composition of plants, fungal mycelia and soil solutions collected from different treatments representing different podzol systems varying in organic matter content.
- Examine changes in δ^{26}Mg signatures in soil solutions and how they are affected by an increased uptake of Mg from organic or mineral soils.
- Determine changes in aboveground (plant shoot) and belowground (roots, organic/mineral soils, soil solutions) C allocation patterns in relation to different degrees of organic matter depletion.

Study V: Fungal mycelial distribution and elemental composition in a forest podzol

The aim of this study was to provide complementary field data that could be used for comparison with similar measurements done in study IV. By burying mesh bags in different organic and mineral horizon soils in a boreal forest podzol at Jädraås, fungal biomass and chemical composition could be measured (with respect to podzol profile).



O horizon microcosms E horizon microcosms B horizon microcosms

Figure 3a. Microcosms consisting of modified 50 ml Falcon tubes were used, with organic (O), eluvial (E) and illuvial (B) horizon soil treatments, each replicated six times. The soil was collected from a boreal forest at Jädraås, Sweden, and planted with two-month-old *Picea abies* seedlings, allowing the shoots to protrude from hole drilled 4 cm from the top of the tube. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled *Piloderma fallax* mycelium was added one month prior to harvesting.

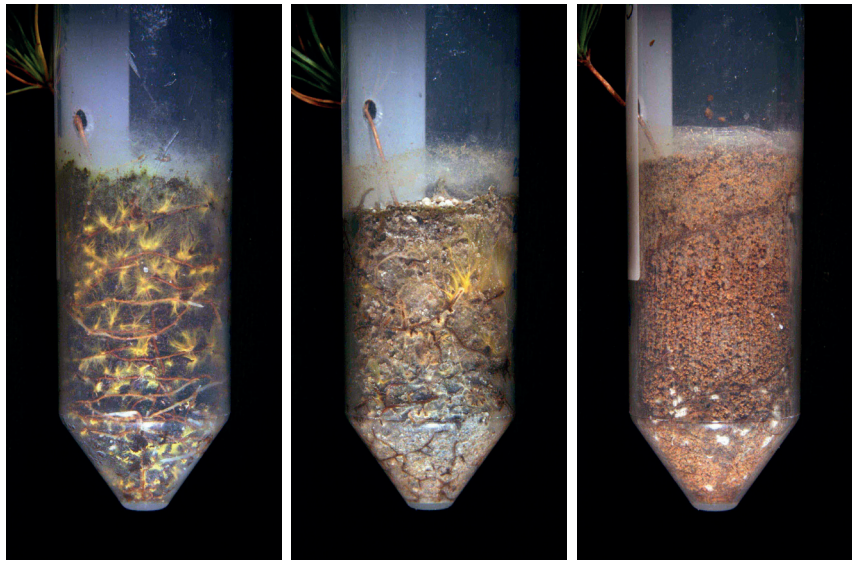


Figure 3b. A close up of three microcosms showing ectomycorrhizal colonisation of *Pinus sylvestris* roots growing in organic (O), eluvial (E) and illuvial (B) horizon soil. (Photo: Shahid Mahmood)

3 Project descriptions

3.1 Mycelial decomposition and patterns of ^{13}C incorporation (Paper I)

Soil fungal and bacterial community analysis (based on ^{13}C-RNA-SIP) and measurements of $^{13}CO_2$ evolution as a proxy for decomposition, were performed to investigate the ability of indigenous microbial communities in the organic and mineral horizons to decompose ^{13}C-labelled fungal necromass, assimilate ^{13}C-labelled decomposition products and their ability to transfer the nutrients released during decomposition to the host plant. The soil was collected from a mixed *Picea abies* (L.H. karst) and *Pinus sylvestris* (L.) forest in Jädraås, central Sweden (60°49'N, 16°30'E), in three separate locations within a 20 x 20 m area, three horizons from three locations were pooled and homogenised.

Sterile *P. abies* seedlings were transplanted into O, E or B horizon soil in modified Falcon tube microcosms (**Figure 3a & b**). Microcosms without plants were used as controls. All the microcosms were incubated in a phytotron. After eight months' incubation, when extensive formation of ectomycorrhizal roots had occurred, a slurry of homogenised ^{13}C-labelled *Piloderma fallax* (Liberta) Stalpers mycelium was injected into the centre of three replicate microcosms (either with or without seedlings) using asyringe. No mycelium was added to corresponding control microcosms.

Upon ^{13}C-mycelium addition, gas sampling was conducted to measure $^{13}CO_2$ evolution as a proxy for mycelial degradation at different time points from 0 to 28 d. Total soil RNA was extracted, ^{13}C-RNA/^{12}C-RNA fractionated, and further processed to identify active bacterial and fungal communities using 454 pyrosequencing. Plant biomass and elemental composition of shoot, root and soils were determined.

3.2 Mg isotopes fractionation and assimilation by ectomycorrhizal and nonmycorrhizal fungi (Paper II)

Fungal ^{26}Mg signatures and elemental concentrations of four species of ectomycorrhizal fungi and four species of nonmycorrhizal fungi were used to investigate the ability of different fungi to fractionate Mg isotopes, and assimilate different base cations when grown on agar containing substrates amended with granite particles. The fungi were isolated from Swedish boreal forests. The eight species were grown on either modified Melin-Norkrans (MMN) medium containing a mixture of Mg isotopes ($^{24+25+26}Mg$, supplied as $MgSO_4$) at natural abundance ratios representing the control, or mineral-free MMN medium amended with organic or granite substrates (**Figure 4**). All substrates were covered with a cellophane membrane on to which a plug of actively growing mycelium of the different selected fungi was inoculated. The resulting fungal mat was collected for isotopic values and elemental concentration measurements.

3.3 Base cation mobilisation, carbon allocation via ectomycorrhizal fungi to organic and mineral substrate (Paper III)

Pinus sylvestris (L.) seedlings were grown in O horizon soil collected from Jädraås forest (see paper I) in pots and incubated for one year in a phytotron without further addition of nutrients. The resulting partially nutrient-depleted soil (thereafter called as 'semi-depleted' soil) was collected, homogenised and transferred into the central compartment of a partitioned microcosm, and two five-week-old *P. sylvestris* seedlings were transplanted (**Figure 5a & b**). The two compartments adjacent to the central compartment, contained only glass beads, while the outer compartments contained either organic O horizon soil, or soil from the E/B interface layer (both collected from Jädraås), washed granite particles, or glass beads only (no nutrient, control). The five compartments were partitioned with a 50 µm pore size nylon mesh to allow only the actively growing mycelium to pass through the glass bead compartment and explore the substrates in the outer compartments. The glass beads in the mycelium compartment allowed harvesting of clean mycelium (without adhering mineral particles) to be used for different analyses. After 11 months' incubation in a phytotron, the microcosms were exposed to two pulses of $^{13}CO_2$ totalling 13 h to follow patterns of C allocation to different mineral and organic substrates and respective soil solutions. After a one-week chase period the microcosms were dismantled, soil solutions were extracted; subsampled for pH, elemental composition, Mg isotopic ratio and ^{13}C

enrichment analyses. The dried shoots and roots were milled and used for elemental composition and ^{13}C enrichment analyses.

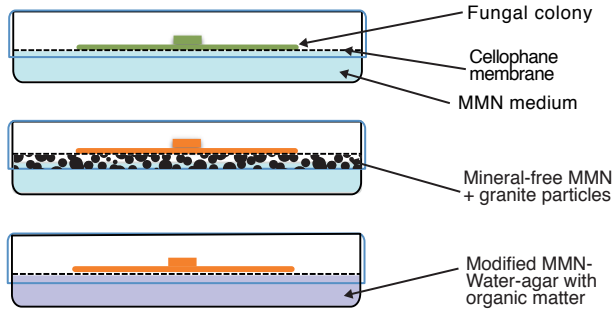


Figure 4. *In vitro* Petri dish systems to examine the ability of ectomycorrhizal and nonmycorrhizal fungi to fractionate and assimilate Mg isotopes. Fungi were grown on half-strength modified Melin-Norkrans (MMN), mineral free MMN amended with granite particles or mineral free MMN amended with solubilised organic matter. The MMN was covered with a cellophane membrane to prevent direct contact of the mycelium with growth medium. Plugs of actively growing mycelium were mounted on the cellophane and incubated at 20°C in the dark.

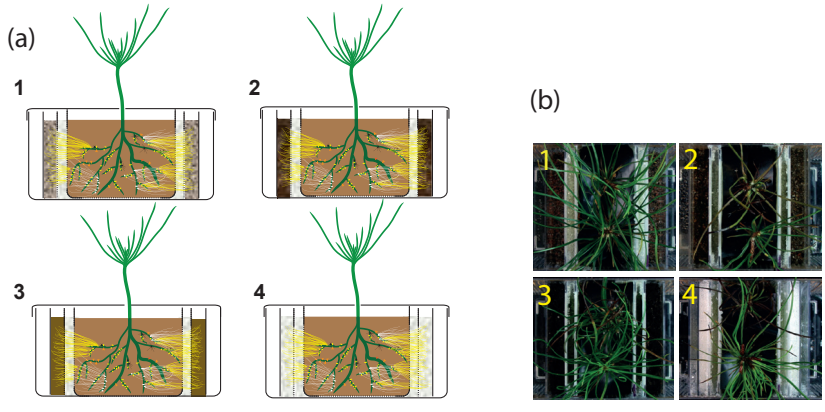


Figure 5. (a) Schematic diagram of partitioned microcosms containing 'semi-depleted' organic horizon soil in the central compartment. The partitons were constructed with 50 µm (pore size) nylon mesh that allowed only mycelium to grow through (not roots). The soil was collected from a boreal forest at Jädraås, Sweden. Two five-week-old *Pinus sylvestris* seedlings were planted in the central compartment, an inert substrate of 1 mm borosilicate glass beads was added to the buffer zone/mycelial compartment, and the outer 'nutrient' compartment contained either granite particles (1), fresh organic horizon soil (2), soil from the E/B interface layer (3) or glass beads as control (4). Fungal mycelia were able to colonise the 'nutrient' substrates in the outermost compartments by crossing the glass beads' compartment. Clean mycelium was harvested from the glass beads for elemental and δ^{26}Mg isotopic analyses. The microcosms were wrapped with thick black plastic sheet and incubated in a phytotron for 11 months. **(b)** aerial view of the actual microcosms.

3.4 Effect of organic matter depletion on nutrient mobilisation and C allocation (Paper IV)

Six *P. sylvestris* (L.) seedlings were transplanted into rectangular microcosms containing reconstructed, stratified soils consisting of different amounts of homogenized O, E, E/B interface and B horizon soils (see **Figure 6a**). Four treatments differed in the thickness of the organic (O) and mineral (B) horizons simulating different degrees of organic matter depletion associated with different intensities of harvesting, as follows: Treatment I – no organic layer, Treatment II – reduced (200 g) organic layer (Jädraås), Treatment III – normal (400 g) organic layer (Jädraås), Treatment IV – intensified (600 g) organic layer (Jädraås). The amount of E and E/B soils was fixed (**Figure 6a**). A coarse nylon mesh allowing the roots to grow through but facilitating separate collection of roots and soil from the different horizons separated the different soil layers. To simulate podzol layers mixing during whole tree/stump harvesting, a fifth treatment (V) consisted of mixed O, E, E/B, and B soils in the same proportions as treatment III (**Figure 6a**). Two nylon mesh bags, with an outer compartment containing glass beads and an inner compartment containing the same soil as the bag was located in, were placed in O, E and B soil layers to trap in-growing fungal mycelium. The microcosms were incubated in a phytotron for six months, after that soil solution was collected every month for eight months using microlysimeters installed in the O, E and B soil layers. Prior to the last soil solution collection, the microcosms were subjected to $^{13}CO_2$ pulse labelling to determine patterns of ^{13}C allocation above- and belowground, across the organic matter depletion treatments (described above). Following a one-week chase period, the microcosms were harvested destructively.

Cation exchange capacity (CEC) of the organic and mineral soils was determined. Soil solution was extracted using centrifugation, and together with the eight lysimeter-collected soil solution samples, used for analyses of elemental concentration, dissolved organic carbon (DOC), $\delta^{26}Mg$ signatures, ^{13}C enrichment and pH. The dry mass, elemental composition, and ^{13}C enrichment of shoots and roots were analysed. Fungal mycelium collected from the mesh bags in different soil horizons, was used for dry mass and elemental composition determinations.

3.5 Field study- fungal mycelial biomass distribution and elemental composition in a forest podzol (Study V)

A one-year field experiment was conducted in Jädraås forest (see paper I) in three replicate plots. Each plot was divided into four rows (treatments), the first row of each plot was assigned for control samples where both the inner and outer compartments of the mesh bags (see paper IV) contained only glass beads. The following three rows were assigned to either O, E or B horizon soils in which the buried mesh bags contained a central compartment filled with soil corresponding to the horizon the bag was buried in. The replicate samples from each plot were pooled to avoid variation due to soil heterogeneity. Biomass of fungal mycelium colonising the outer compartments of the mesh bags was determined following freeze-drying and the chemical composition of the mycelium was also determined.

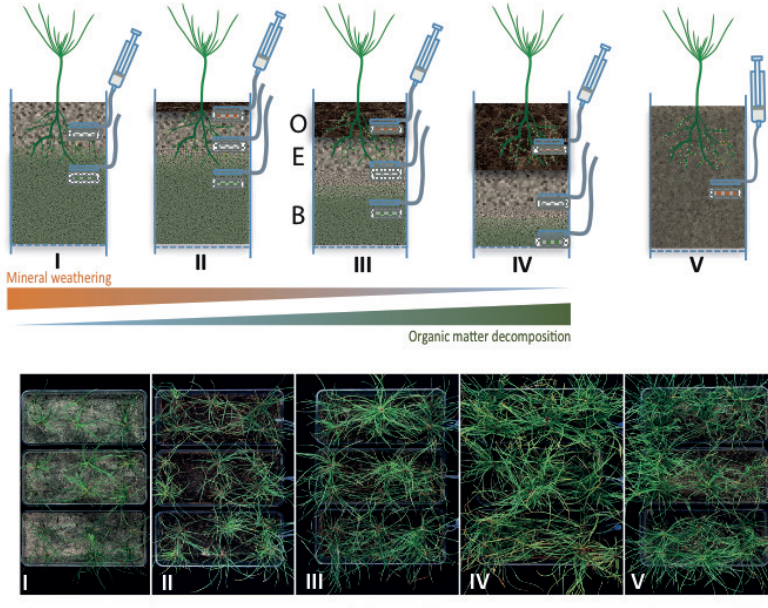


Figure 6. (a) Microcosm system consisting of five treatments representing a stratified podzol soil with different degrees of organic matter depletion: I - no organic horizon, II - reduced (½) organic horizon, III - normal organic horizon, IV - increased (x1.5) organic horizon. In treatment V the O, E and B soil horizons were completely mixed. The O, E and B layers were separated by 2 mm nylon mesh. Lysimeters were inserted in each horizon for soil solution sampling and dual compartment mesh bags were placed in each horizon. Six *Pinus sylvestris* seedlings were grown in each microcosm. The photo was taken after 14 months of growth. (Photo: Shahid Mahmood)

Figure 6. (b). Side view of microcosm showing O, E, & B soil layers and mycorrhizal colonisation of roots in treatment III
(Photo: Shahid Mahmood).

4 Materials and Methods

4.1 Study systems and growth substrates

Microcosms for different studies were designed using homogenised forest soil that was collected from Jädraås forest (described in paper I) (**Figure 1**) as the main substrate in all studies except paper II, where O horizon soil extract was added to the growth medium instead. Crushed granite rock was used as mineral substrate in paper II and III. All plant seeds used in paper I, III and IV were surface sterilized with 33% hydrogen peroxide, and propagated in vermiculite; all microcosms were covered with aluminium foil with exposed shoots and
incubated in a phytotron at a light intensity of 250 µmol m^{-2} s^{1} PAR, 18h/6h light/dark cycle. Weekly watering with deionized water was gravimetrically based, and positioning of microcosms within the phytotron was randomized to reduce environmental differences.

Mycelial decomposition and patterns of C incorporation (Paper I)

Eight-week-old *P. abies* seedlings were transplanted into (50 ml) Falcon tubes containing 25 ml of lightly compressed soil from one of three soil horizons (O 13.5 g, E and B 26 g each). Falcon tubes were modified to enable the plant shoot to protrude through a 5 mm hole (**Figure 3a**). The treatments were as follows: six replicates of O, E or B horizon soil with and without plants (O +Plant, E +Plant, B +Plant and O –Plant, E –Plant, B –plant, respectively), giving a total of 36 microcosms. The microcosms were incubated for eight months in a phytotron. After formation of ectomycorrhizal roots and mycelial networks, 1.0 ml of ^{13}C labelled *Piloderma fallax* mycelial slurry was injected (using a syringe) into the centre of soil in each microcosm. There were three replicates for each treatment and the microcosms without ^{13}C-mycelium addition were used as controls. On days 0, 1, 3, 5, 7, 14 and 28 following mycelium application, headspace gas samplings were conducted to measure

$^{13}CO_2$ evolution rate. After the last gas sampling (day 28) the microcosms were harvested destructively. Total RNA was extracted from the soils and ^{13}C-RNA/^{12}C-RNA was fractionated. Fractions with heavy and lighter densities containing ^{13}C-RNA and ^{12}C-RNA respectively, were pooled separately and RT-PCR-amplified for analysis of active bacterial and fungal communities (involved in mycelium decomposition and/or assimilating ^{13}C from decomposing mycelium), using 454 pyrosequencing. Plant biomass, and shoot and soil elemental composition, were also determined.

Base cation mobilisation, carbon allocation via ectomycorrhizal fungi to organic and mineral substrate (**paper III**)

P. sylvestris seedlings were grown in O horizon soil for one year in a phytotron to obtain a soil that was presumably lacking easily available pool of nutrients. This so called 'semi-depleted' soil was homogenised and transferred to the centre of a five-partitioned microcosm; into which two five-week-old *P. sylvestris* seedlings were transplanted (**Figure 5a & b**). The seedling compartment was located between two nylon mesh (50 µm) partitions to allow only the actively growing mycelium to pass through, and to cross an inert substrate (1 mm borosilicate beads) and to pass through another nylon mesh partition to colonise two outer substrate compartments, containing either O horizon soil, soil from the E/B interface layer, granite particles or glass beads (control). After 11 months' incubation in a phytotron, microcosms with and without plants were exposed to 99 atom % $^{13}CO_2$ for 13 h. One week after $^{13}CO_2$ labelling, the microcosms were dismantled. Soil solutions collected by centrifugation, milled plant and soil materials were stored at -20 °C.

Ectomycorrhizal biogeochemical processes (**paper IV and V**)

P. sylvestris seedlings were transplanted into microcosms containing homogenised O, E, E/B interface and B horizon soils. Four treatments differed in the thickness of the organic (O) and mineral (B) horizon soils, simulating different degrees of organic matter depletion as follows: Treatment I – no organic layer and thick B layer (1450 g), Treatment II – reduced (1/2) organic layer (200 g), 1050 g for B layer (Jädraås), Treatment III – normal organic layer (400 g), 650 g for B layer (Jädraås), Treatment IV – increased (x1.5) (600 g) organic layer, 250 g for B layer (Jädraås). The amount of E and E/B soils was fixed 570 g and 230 g respectively. The fifth treatment (V) was a mixture of O, E, E/B, and B soils. Two nylon mesh bags (50 µm) were placed in O, E and B soil layers, each bag consisted of inner compartment that contained the soil corresponding to the substrate the bag was buried in, and surrounded with an outer compartment filled with borosilicate beads to collect soil particle-free

fungal mycelium for different analyses. Plant-free microcosms were set up as controls. Soil solution was collected monthly, eight times from microlysimeters installed in the O, E and B soil layers. Before plant harvesting, the microcosms were subjected to $^{13}CO_2$ (99 atom %) pulse labelling for 8 h per day for three days. The soil, soil solution, together with the previous eight soil solution samplings, plant and mycelium were stored at -20°C.

In Jädraås forest, mesh bags (50 µm mesh size) with the same substrate composition/construction as described above for paper IV, were buried in the O, E or B podzol layers for one year. The mycelium was collected for determination of biomass and elemental composition.

In vitro-paper II

Four species of ectomycorrhizal fungi (*Amanita muscaria*, *Cenococcum geophilum*, *Suillus variegatus*, and *Piloderma fallax*), and four species of nonmycorrhizal fungi (*Heterobasidion parviporum*, *Phlebiopsis gigantea*, *Penicillium spinulosum* and *Trichoderma polysporum*) all originating from Swedish boreal forests were used. The latter two species were isolated from O horizon soil of Jädraås forest (see paper I), and were identified by fungal ITS sequencing. DNA was extracted (Griffiths *et al*., 2000), and the ITS region was amplified using ITS1F and ITS4 primers (White *et al*., 1990). Purified PCR products were subjected to Sanger sequencing. The eight species were grown in Petri dishes containing either MMN medium as control, or mineral-free MMN agar amended with granite, or organic matter as sources of Mg. The granite material was washed under running ddH_2O for about 6 h, and sonicated three times for 5 min at one hour intervals. Granite particles of 63 to 125 µm were used. The organic matter collected from Jädraås forest, was gamma irradiated (dose 25 kGy). 86 g of O soil was mixed in 500 ml dH_2O, using three mixing cycles with a blender for 3 min. The suspension was sieved using a 500 µm sieve and the filtrate was collected, these steps were repeated with the soil residuals. The filtrate was centrifuged for 30 min at 2000 g and the supernatant was filtered under a vacuum. The extract was added to MMN agar. Twenty-five ml of MMN or organic matter, and 25 ml (20 ml base layer of mineral free MMN medium and 5 ml top layer amended with granite particles) of granite substrates were poured into 100 mm Petri dishes. Upon solidification of the medium, the surface was covered with a cellophane membrane. Plugs of actively growing mycelium (5 mm diameter) were inoculated on the cellophane and incubated at 20°C in the dark for 8-12 weeks for ectomycorrhizal fungi and 2-10 weeks for nonmycorrhizal fungi.

4.2 Stable isotope analyses (SIP)

The majority of microbial species in natural environments have never been cultured (Whiteley *et al*., 2007), therefore, relating the identity of microbial communities to environmental geochemical processes driven by microorganisms in forest ecosystems is very difficult to achieve. The use of stable isotopes of elements such as ^{13}C enables better understanding of the relationship between microbial phylogeny and environmental processes. RNA is one of the oldest molecules in life, although it is inherited by daughters from the mother cell, it is also turned over independently of cellular replication, and rapidly so in periods of activity, this feature makes it an excellent biomarker for use in stable isotope probing as a target marker (Whiteley *et al*., 2007). It has been used for identifying active bacteria in ^{13}C-phenol degradation (Manefield *et al.*, 2002) and to investigate fungal activity in ^{13}C allocation to rhizosphere microbial communities (Drigo *et al*., 2010). In paper I, our approach was to use this technique to identify the microbial taxa that were able to decompose ^{13}C-labelled fungal necromass and/or assimilate ^{13}C from decomposing mycelium and incorporate into their RNA. Following total RNA extraction from soil, ^{13}C-labelled RNA (of active microbial taxa) can be separated from ^{12}C-RNA (of microbial taxa, that were active but not assimilate ^{13}C) using a density gradient ultracentrifugation method (Whiteley *et al*., 2007). In studies III and IV, plants were pulse labelled with $^{13}CO_2$ to track flow of recent photoassimilates to roots growing in different podzol horizons and to identify active bacterial and fungal communities that were assimilating plant derived ^{13}C and were involved in weathering of minerals (E or B horizons) and/or decomposition of organic matter (O soil).

4.3 Molecular and chemical analyses

Molecular biological techniques and sequencing analyses

In forest soil, fungi are most probably functionally different in foraging for different nutrients for their host plants; active members should be identified at species level because different species of the same family may have different functions (Matheny *et al.*, 2006). Fungal identification using culture-independent techniques based on the ITS region of ribosomal encoding gene became common with the development of fungal specific primers ITS1 and ITS4 (White *et al*., 1990) that covers ITS1, 5.8S, ITS2 of fungal ITS region. In general, ITS spacer regions evolve more rapidly than coding regions (Suh *et al*., 1993) as it has high degree of variation even between closely related species, and highly conserved regions, in addition to accessible sites by the

universal ITS1 and ITS4 primers. Some species might have insertions in the ITS region generating long PCR fragments (Kåren *et al.*, 1997) for these species, by using ITS1F and ITS4 primers, the varied length might cause biased PCR amplification against the long fragments and distort the composition of the microbial communities in the downstream analysis, instead, a combination of fITS7 and RITS4 primers will amplify shorter regions and increase amplification efficiency since fewer PCR cycles are required (Ihrmark *et al*., 2012).

In paper II, DNA was extracted from two soil-isolated cultures (Griffiths *et al.*, 2000), the fungal ITS region was amplified using the combination of ITS1F and ITS4 primers. Purified PCR products were subjected to Sanger sequencing (Sanger *et al.*, 1977) and species were identified based on sequence similarity to the GenBank database (Altschul *et al.*, 1990).

For soil microbial community studies, 454-pyrosequencing is commonly used for both fungi and bacteria (Rothberg and Leamon, 2008). It enables rapid characterization of microbial communities using high numbers (>1 000 000) of sequences in parallel to be synthesised in one run and it differs fundamentally from Sanger's sequencing method in the order of nucleotide incorporation (Ramon *et al*., 2003). Four enzymes involved in the process where DNA polymerase incorporates one of four dispensed nucleotides into a nascent DNA template leading to inorganic pyrophosphate (PPi) to generate that in a quantity equimolar to the amount of the incorporated nucleotide. ATP sulfurylase converts PPi to adenosine triphosphate (ATP); the latter is used by luciferase to convert luciferin to oxyluciferin emitting light that corresponds to the amount of ATP, and displayed as a peak in a pyrogram. In each cycle only one nucleotide is incorporated and the others, together with the surplus of ATP, will be degraded by Apyrase. The detecting light allows parallel sequencing of templates in a mixture.

In paper I, 0.5 g of O horizon, and 2.0 g of each mineral horizon soil were extracted using RNA PowerSoil® total RNA isolation kit. The extracts were Residual DNA was removed using RTS DNase™ Kit. For studying metabolically active microbial community, ^{13}C-RNA was reversed to cDNA using iScript ™ cDNA Kit. To test the reproducibility of RNA samples, we amplified fungal ITS region with ITS1F and ITS2 primers and screened them using DGGE analysis. Due to the similarity of the resulting banding profiles, triplicate soil RNA samples of each treatment were pooled prior to further analyses. To separate ^{13}C-RNA from ^{12}C-RNA of soil-RNA pooled samples were subjected to isopycnic density gradient ultracentrifugation at 140 000 *g*, at 20^{o}C for 48 h, using caesium trifluoroacetate (CsTFA) gradients (Manefield *et al.*, 2002).

Following fractionation, densities of all fractions were determined by measuring refractive index using a refractometer. ^{13}C-RNA (heavy) fractions and ^{12}C-RNA (light) fractions were pooled separately before complimentary cDNA synthesis. Reverse transcription of pooled RNA fractions to cDNA was conducted using iScript ^{TM} cDNA Kit. Three technical replicates of heavy and light RNA pooled fractions were used for fungal-ITS region amplification. ITS4 (with 8-base pairs long unique sample-identifying tags) and fITS7 primers ((White *et al*., 1990; Ihrmark *et al*., 2012) were used. Bacterial-16S rRNA gene amplification was preformed using 515F and 806R primers (Caporaso *et al.*, 2011; Bates *et al.*, 2011), the later consisted of (12-base barcode, unique for each sample). PCR products were 454-pyrosequenced using 2 x 1/8th of a GS FLX Titanium Pico Titer Plate (Macrogen, South Korea). Bacterial sequences were analysed using the RDP pipeline (Ribosomal Database Project version 11.1) (Cole *et al*., 2014). Fungal sequences were analysed using SCATA pipeline (Sequence Clustering and Analysis of Tagged Amplicons; www.scata.mykopat.slu.se).

Elemental and isotopic analyses

An indicative parameter that reflects the role of ectomycorrhizal fungi in nutrient translocation essential for the host plant is the chemical composition of the plant. Chemical analyses were performed for plants and soil (paper I, III, and IV), soil solution (paper III and IV), and fungal mycelium (paper II, III, IV and field study V) using an inductively coupled plasma mass spectrometer (ICP-AES). Other soil solution properties measured were: dissolved organic carbon (DOC), cation exchange capacity (CEC) and pH.

Dissolved organic carbon DOC measurement was performed for a pooled sample that was diluted with milliQ H_2O up to 20 g, sealed with Parafilm and stored at 4°C overnight. DOC was measured with a Shimadsu TOC-5050 Total Organic Carbon Analyzer using high temperature, catalytic oxidation. Carbonates were removed by sparging the acidified (pH 2) soil solutions with 1.5% HCl.

For CEC, three replicates of O, E and B horizon and OEB mixed soils were pooled equivalently and air-dried at 40°C overnight. 1.0 and 2.5 g of air-dried organic soil, and mineral and mixed soils respectively, were extracted with 10 ml of 1M NH_4OAc solution. The air-dried samples, were further dried at 100°C for absolute dryness to calculate the moisture correction factor (mcf). The concentration of Ca, Mg, NA and K was analysed by ICP-OES.

$^{13}CO_2$ gas and ^{13}C in plant, soil and soil solution materials were measured using isotope ratio mass spectrometry (IRMS).

Multicollector-Inductively Coupled Plasma Mass Spectrometer (MC-ICP MS) was used to for ^{26}Mg isotope measurement.

5 Results and discussion

5.1 Paper I

Mycelial decomposition kinetics

Addition of dead mycelium resulted in a rapid release of $^{13}CO_2$ to the gaseous phase, irrespective of soil type (**Figure 7a & b**). The initial losses (day 0) of ^{13}C from systems containing E horizon soil with plants were significantly higher (P<0.0001) than those from equivalent systems without plants suggesting that the presence of plants might have primed higher, initial levels of degradative activity in this horizon. Kuzyakov (2010) demonstrated that even after a substrate is exhausted, some microorganisms remain active and extracellular enzymes produced during a period of high activity remain in the soil and can contribute to soil organic matter decomposition. This high initial activity might also be caused by different microorganisms that were able to produce degrading enzymes in E horizon soil but absent from, or inactive in, organic and deeper mineral soil.

There was a significant increase (P=0.0006) in $^{13}CO_2$ release after 24 h (day 1) in O horizon soil with and without plants, and in E horizon soil with no plants. This could be explained both by induction of microbial activity and the action of an existing pool of enzymes. From day 1 onwards there was a steady decline in $^{13}CO_2$ release to day 14, reflecting respiratory losses during decomposition and, presumably, incorporation of ^{13}C into microbial biomass. From day 14 until day 28 $^{13}CO_2$ release was stable, reflecting incorporation of the labile pool into microbial biomass and steady-state respiratory loss.

Similar results were obtained by Wilkinson *et al.* (2011) who found a rapid decomposition of macerated ectomycorrhizal fungal necromass incubated in soil under laboratory conditions that peaked at 14 d and that addition of mycelium stimulated CO_2 efflux from free-living soil microbes. Schweigert *et al.* (2016) incubated forest arenosol with ^{13}C-labelled *Laccaria bicolor* in a

closed weathering reactor. They found that the isotopic composition of the mineralised CO_2 decreased steeply over the first 30 days. A DNA-SIP laboratory study by Drigo *et al*. (2012) using ^{13}C-labelled *Pisolithus microcarpus* fungal mycelium, which was applied to forest soil to trace ^{13}C incorporation into active microbial biomass and ^{13}C in the soil over 28 days, demonstrated a 48% reduction in soil ^{13}C from day 0 to day 28 with the largest reduction (33%) occurring within the first 7 days after incorporation of the ^{13}C necromass.

Bacterial communities

In systems containing O horizon soil **(Figure 8a**), *Betaproteobacteria* were active in assimilating ^{13}C from the dead mycelium but they were much less active in the absence of added mycelium. Incorporation of ^{13}C was higher in the absence of plants. *Gammaproteobacteria* were also active in assimilating ^{13}C from the dead mycelium but only the presence of plants, *Sphingobacteria* and *Subdivision3* were also active in assimilating ^{13}C from the dead mycelium, but only in the absence of plants. *Actinobacteria*, *Alphaproteobacteria, Planctomycetia* and *Acidobacteria* Gp1 and Gp3 were active but incorporated less ^{13}C. In systems containing E horizon soil, (**Figure 8b**) *Betaproteobacteria* were even more active in assimilating ^{13}C from the dead mycelium, but this was not affected by the presence of plants. The same effect was observed for *Sphingobacteria* although this group was less active. In the presence of plants, *Gammaproteobacteria* followed the same pattern as in the O horizon. *Actinobacteria*, *Alphaproteobacteria, Planctomycetia, Deltaproteobacteria and Acidobacteria* Gp1, Gp2 and Gp3 were all active.

Input of easily available organic C compounds via plant root exudation (Paterson *et al.*, 2007), or by direct or indirect supply from mycorrhizal mycelium could prime microbial activity (Zhang *et al.*, 2016) but there was limited evidence for these priming effects in our study. Only the activity of *Gammaproteobacteria* appeared to be stimulated by the presence of plants.

Brabcová *et al*. (2016) studied the decomposition of *Tylopilus felleus* fruit bodies in forest litter and organic horizon soil, and found DNA-based evidence that *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were initially dominant in the organic horizon soil. Following insertion of mesh bags containing cut fragments of fruit bodies, the bacterial communities became largely dominated by *Gammaproteobacteria* within 1 to 3 weeks and then by *Bacteroidetes* and *Alphaproteobacteria* up to 21 weeks. Similar bacterial groups were found to be active after four weeks in our study, with the exception of *Bacteroidetes,* but direct comparison between the two studies is difficult since one is DNA-based and the other is RNA-based.

Separate analyses of RNA from bacterial genera that responded to mycelium addition are displayed in (**Paper 1, Figure 3a & b**). *Burkholderia*, was the most active genus, mainly in E horizon soil, and there was clear evidence of ^{13}C incorporation in the presence of plants. *Streptoacidophilus*, *Granulicella*, *Rhodanobacter*, *Flavisolibacter* and *Mucilaginibacter* were also active (in decreasing order) in O horizon soil to which mycelium had been added (**Paper 1, Figure 3a**). In E horizon soil *Streptoacidophilus*, *Granulicella*, *Herminiimonas, Dyella, Pedobacter* and *Mucilaginibacter* were all active (in decreasing order) (**Paper 1, Figure 3b**).

Burkholderia belong to the phylum *Betaproteobacteria* and are commonly associated with ectomycorrhizal mycelium (Uroz *et al.*, 2010; Nguyen and Bruns, 2015; Marupakula *et al.*, 2016), although there was clear activity in the absence of plants in our study. Some bacteria of the genus *Burkholderia* have the ability to solubilise inorganic phosphate (Kim *et al*., 2005) and to mobilise iron using siderophores (Vial *et al*., 2007) and it has been suggested (Uroz *et al.*, 2010) that they may also be involved in weathering of minerals. *Dyella*, and *Sphingomonas,* (belonging to the *Gamma-* and *Alphaproteobacteria,* respectively) and *Bacillus* have been shown to have mineral weathering, P solubilisation and Fe-chelation capability in a study by Uroz *et al.* (2011). Actinomycetes decompose Ca-oxalate crystals, and recycle Ca permitting formation of Ca bicarbonates (Cromack *et al.,* 1977).

Fungal communities

In systems containing O horizon soil, the active fungi decomposing the dead mycelium were mainly nonmycorrhizal, saprotrophic fungi, even in the presence of plants (**Figure 9a**). The activity of *Umbelopsis* sp*., Umbelopsis angularis, Mortierella humilius, Penicillium spinulosum, Sugiyamaelia paludigena, Trichoderma viride* and *Mucor silvaticus* increased greatly following the addition of dead mycelium, but the activity of *Ascomycota* sp., *Entyloma chrysosplenii, Pulchromyces fimicola, Rhodotorula lamellibrachiae* and *Entyloma microsporum* decreased. Ectomycorrhizal fungi including *Cenococcum geophilum, Piloderma olivaceum,* in the O horizon (**Figure 9a**) and *Piloderma sphaerosporum*, in the E horizon (**Figure 9b**), were active in systems containing plants, but their activity was greatly reduced in systems to which dead mycelium was added. *Umbelopsis dimorpha,* and an unidentified fungal species (SH234809.06FU) in the E horizon, and *Sugiyamaelia paludigena, Mucor silvaticus* and *Trichoderma viride* in the O horizon, were active in assimilating ^{13}C from the dead mycelium.

Brabcová *et al*. (2016) studied the decomposition of *Tylopilus felleus* fruit bodies in forest litter and organic horizon soil, and observed rapid initial

decomposition of dead fungal mycelium, with a loss of 48% of the dry mass within the first 3 weeks. The authors also found DNA-based evidence that the fungal communities in the early stages of mycelial decomposition were almost exclusively composed of saprotrophs, such as *Mortierella*, *Aspergillus*, *Cladosporium*, *Mucor*, and *Kappamyces* in the soil. The genera *Penicillium*, *Cladosporium, Aspergillus* and *Mortierella* were highly abundant, often in tens of per cent and up to 87%, whereas the parasitic and ectomycorrhizal fungi increased later.

Drigo *et al*. (2012) added ^{13}C-labelled *Pisolithus microcarpus* to forest soil to study mycelial decomposition rates and used ^{13}C-DNA-based DGGE approach to study soil-fungal community structure and incorporation of ^{13}C. The authors found that ectomycorrhizal mycelial degradation was rapid during the first 7 days and that ^{13}C was incorporated primarily into Basidiomycete fungi, including both saprotrophic and ectomycorrhizal taxa. Koide and Malcolm (2009) in a study on the relationship between N concentration in ectomycorrhizal fungal tissue and decomposition rate using different strains varying in their C:N ratio grown on culture medium, found that 20-80% of the mass of fungal mycelium grown axenically on growth medium was lost within one month of addition to soil. N concentration was positively correlated with fungal necromass decomposition, suggesting that fungal C:N ratio is an important factor controlling mycelial decomposition in forest ecosystems. Lindahl *et al*. (2010) in a study based on DNA analysis demonstrated that opportunistic, saprotrophic fungi proliferate in response to inputs of fungal necromass produced by disruption of ectomycorrhizal mycelium caused by soil coring.

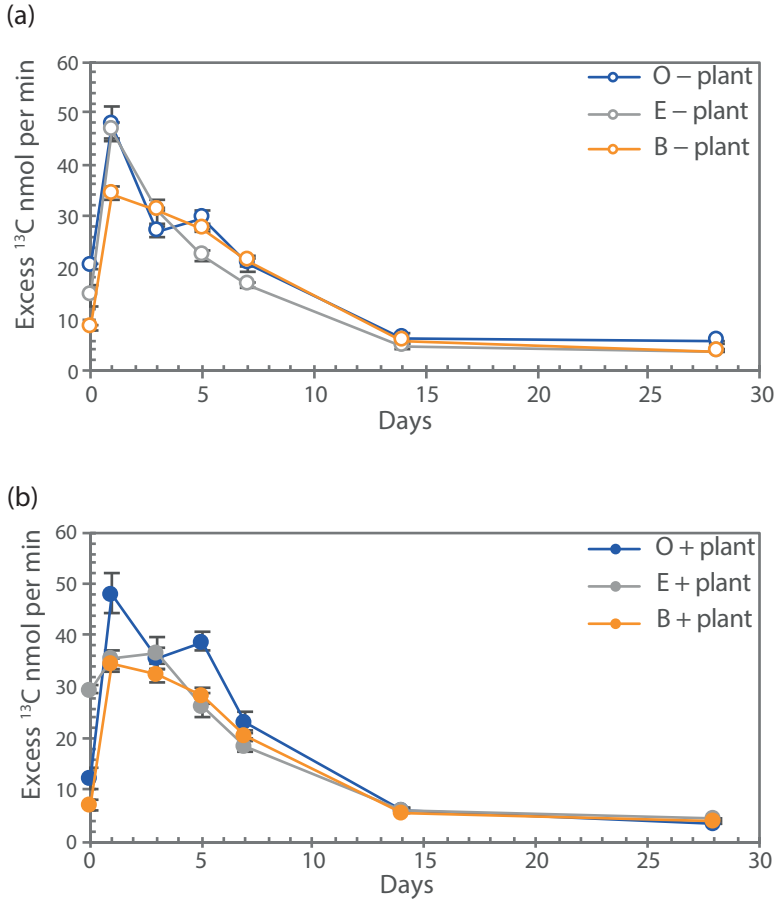


Figure 7. $^{13}CO_2$ evolution rate (^{13}C excess nmol min^{-1}) over 28 days as a proxy for decomposition of ^{13}C-labelled *Piloderma fallax* mycelium added to O, E and B horizon soils collected from a boreal forest at Jädraås, Sweden. The soil microcosms were either without plants **(a)** or planted with *Picea abies* seedlings **(b)**. The microcosms were incubated in a phytotron for eight months. Data points represent the mean ± SE (n = 3). Error bars are not shown when smaller than the symbols.

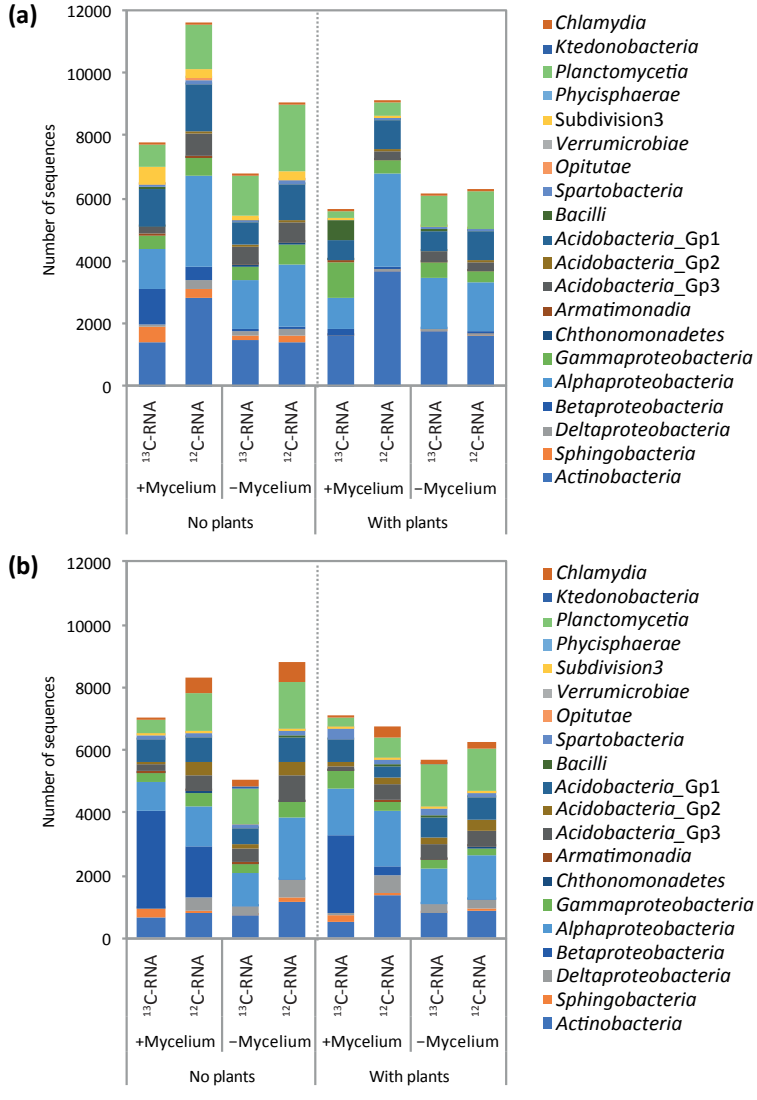


Figure 8. Abundance of the 20 most active bacterial classes including the ones that were able to incorporate ^{13}C in their RNA following addition of ^{13}C-labelled *Piloderma fallax* mycelial necromass into O horizon **(a)** and E horizon **(b)** soil microcosms that were either without plants or planted with *Picea abies* seedlings. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled mycelium was added one month prior to harvesting. RNA was extracted from three biological replicates and pooled for ^{13}C-RNA stable isotope probing and 454 pyrosequencing analyses.

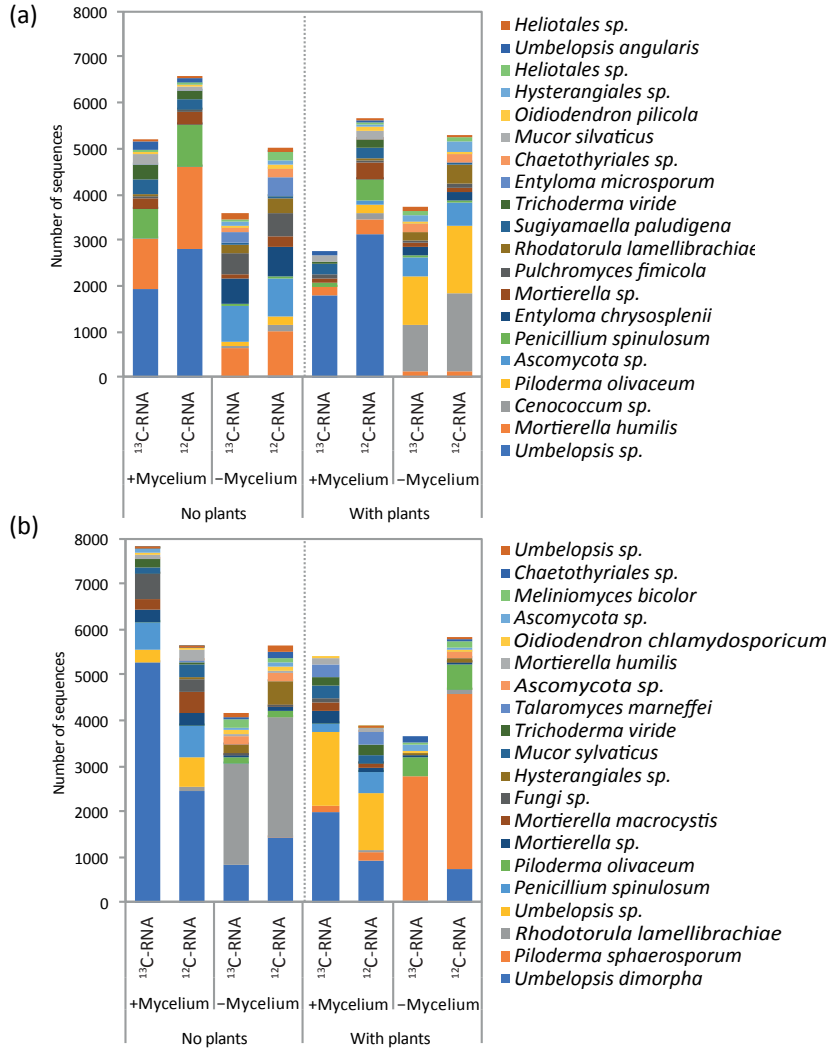


Figure 9. Abundance of the 20 most active fungal taxa including the ones that were able to incorporate ^{13}C in their RNA following addition of ^{13}C-labelled *Piloderma fallax* mycelial necromass into O horizon **(a)** and E horizon **(b)** soil microcosms that were either without plants or planted with *Picea abies* seedlings. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled mycelium was added one month prior to harvesting. RNA was extracted from three biological replicates and pooled for ^{13}C-RNA stable isotope probing and 454 pyrosequencing analyses.

5.2 Paper II

Stable isotope studies based on the fractionation and distribution of ^{13}C and ^{15}N have provided much information about the interactions of ectomycorrhizal fungi with organic matter. Mycorrhizal ectomycorrhizal fungal sporocarps have consistently higher $\delta^{15}N$ values than saprotrophic fungi (Hobbie *et al.*, 1999) and foliar in $\delta^{15}N$ values are consistently more depleted than in mycorrhizal fungi, suggesting that discrimination against the heavy isotope takes place during mycorrhizal uptake. However, there are no equivalent studies of mycorrhizal interactions with minerals. We set up an axenic system to test three main questions: a) Do ectomycorrhizal fungi fractionate Mg isotopes? b) Do they have different ^{26}Mg signatures from nonmycorrhizal fungi when grown on mineral substrates? c) Do they assimilate base cations and phosphorus in higher amounts than nonmycorrhizal fungi?

Mg isotope fractionation

The $\Delta^{26}Mg$ values in **Figure 10a** represent the difference between the tissue and substrate $\delta^{26}Mg$ signatures. The ectomycorrhizal fungi were generally more depleted in the heavy isotope ^{26}Mg compared with substrate, particularly on the granite medium.

Three of the four nonmycorrhizal fungi had significantly less depleted signatures than three of the four ectomycorrhizal fungi. The signature for *Cenococcum geophilum* was less depleted than those of the other ectomycorrhizal fungi. Conversely *Heterobasidion parviporum* had a more depleted signature than the other nonmycorrhizal fungi. Similar differences were found for the fungi grown on the organic matter substrate and MMN, although the values were much less depleted and most of the nonmycorrhizal fungi were enriched in the heavy isotope. The general conclusion for the selected mycorrhizal group is that they were able to discriminate against the heavy Mg isotope, confirming observations of $\delta^{26}Mg$ signatures in other microorganisms such as cyanobacteria (Black *et al.*, 2006) and foraminifera (Chang *et al.*, 2004). Variation in these parameters may occur due to different factors such as availability and type of nutrients (Emmerton *et al.*, 2001a, 2001b). Alteration in all fungal isotopic signatures relative to the Mg source suggests that both groups of fungi were able to fractionate Mg, but with pronounced differences between the groups. On granite substrate *S. variegatus* exhibited the most depleted $\delta^{26}Mg$ value in both groups. In natural ecosystems isotope fractionation may be influenced by resource availability or differences in the overall uptake capacity of different fungi. Hobbie and Agerer (2010) found that long distance exploration type fungi such as *Suillus* spp. with high biomass were more enriched in ^{15}N than medium or short distance exploration

type fungi with lower biomass (such as *Amanita*). However, in our system the fungi produced similar amounts of biomass and differences in isotope fractionation are unlikely to have been caused by differences in the physical extent of colonisation.

Bivariate plots of δ^{26}Mg (‰) versus Mg concentration (mg g^{-1}) in mycelia of ectomycorrhizal and nonmycorrhizal fungi grown on the three different substrates (**Paper 2, Figure 2**) revealed a negative relationship between Mg concentration and the δ^{26}Mg signature in the mycelium. The values for the two groups of fungi differed significantly when cultured on the granite substrate, showing strong statistical separation (p=0.0042) but the statistical separation of the two groups of fungi was not significant when they were grown on the organic substrate or MMN medium.

Fungal nutrient uptake

The higher overall depletion in δ^{26}Mg in ectomycorrhizal fungi, when grown on granite substrate, may be explained by a higher capacity to mobilise Mg from the mineral substrate and this is supported by their significantly higher mycelial concentrations of Mg, K and P (**Figure 10b**). Uptake of P from apatite by the ectomycorrhizal fungus *P. involutus* has been demonstrated by Smits *et al*. (2012) and it is well known that ectomycorrhizal hyphae are able to allocate plant derived carbon selectively to minerals such as apatite (Smits *et al*., 2012) and potassium feldspar (Rosling *et al*., 2004). Wallander and Wickman (1999) demonstrated increased foliar content of K in *P. sylvestris* seedlings colonised by the ectomycorrhizal fungus *S. variegatus* when grown with biotite. They found a positive correlation between foliar K content, citric acid concentration and fungal biomass in the soil. Other experiments have shown production of citric acid (Fransson and Johansson, 2010), oxalic acid (van Hees *et al*., 2006) and siderophores (Haselwandter *et al*., 2011) by ectomycorrhizal fungi.

Although the present study revealed a significant difference in mycelial concentrations of Mg, K and P in ectomycorrhizal fungi compared with nonmycorrhizal fungi grown on granite, Vaario *et al.* (2015) did not detect any significant increase in these elements in mycelia of *Tricholma matsutake* or *P. fallax* in systems incubated with mineral fragments collected from the field. The concentration of these elements in the latter study was between 9 and 48 times lower than in our study and this difference is probably explained by the fact that the authors used fewer, larger (5-10 mm diameter) mineral fragments with a much smaller total surface area than the minerals (63-125 μm in diameter) used in our study. Data available from field studies of Mg in

mycorrhizal fungal fruitbodies (Falandysz *et al.*, 2012; Kojta *et al.*, 2012) are comparable to the values reported in our study (0.4-1.3 mg g^{-1}).

The higher capacity to mobilise Mg from the mineral substrate might explain the higher overall depletion in δ^{26}Mg in ectomycorrhizal fungi, when grown on granite substrate. Generally, most physical, chemical and biochemical processes favour initial incorporation of the lighter isotope in products mobilised from substrates enriched in heavy isotopes (Hobbie and Högberg, 2012; Shearer and Kohl, 1986)

Ectomycorrhizal fungi are thought to have evolved repeatedly and independently from saprotrophic precursors with multiple reversals to the free-living trophic habit (Hibbett *et al.*, 2000). The extant fungi forming symbiotic associations with forest trees may therefore exhibit different degrees of mutualism that may explain the variation within each trophic group found in our study.

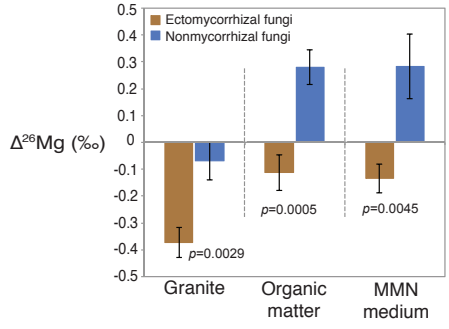


Figure 10a. Mean values of Δ26Mg (‰) for four ectomycorrhizal and four nonmycorrhizal fungi grown either on a mineral free Modified Melin-Norkrans (MMN) medium amended with granite particles, mineral free MMN medium amended with solublised organic matter from the organic layer of a boreal podzol or MMN medium. Vertical bars represent ± SE (n = 4). The fungi were grown on cellophane membranes covering the growth substrates in Petri dish microcosms.

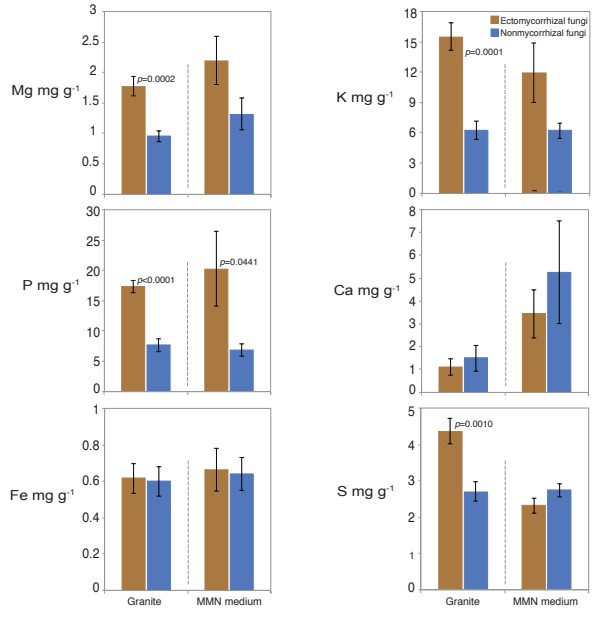


Figure 10b. Mean values of Mg, K, P, Ca, Fe, S concentration (mg g^{-1}) in ectomycorrhizal and nonmycorrhizal fungal mycelia grown on mineral free Modified Melin-Norkrans (MMN) medium amended with granite particles or MMN medium. Vertical bars represent ± SE (n = 4). The fungi were grown on cellophane membranes covering the growth substrates in Petri dish microcosms.

5.3 Paper III

Plant growth, mycelial growth and elemental content

Shoot biomass was statistically higher in systems containing fresh O horizon soil than in systems containing E/B soil ($P<0.05$). However, none of the other treatment differences were statistically significant. Root biomass in systems containing granite and O horizon soil was statistically higher than root biomass in systems with E/B horizon soil and the control ($P<0.0001$) (**Figure 11a**).

Mean shoot content of Ca was statistically higher in systems containing O soil than in systems containing E/B soil ($P<0.05$), but not in systems containing granite or the control (**Figure 11b)**. Mean shoot content of K and P was statistically higher in systems containing O soil than in the other treatments ($P<0.0001$). Mean root content of Ca was statistically higher in systems containing granite than in systems containing E/B soil and the control ($P<0.001$) but did not differ statistically from the equivalent value in systems containing O soil (**Figure 11b**). Mean root content of K and P was statistically higher in granite containing systems than in control ($P=0.032$ for K, $P= 0.017$ for P) but did not differ statistically from O and E/B soil treatments. Mean root content of Mg was statistically higher in granite and O soil containing systems than in E/B soil and the control ($P<0.0001$) (**Figure 11b**). Concentrations of Mg, K and P in soil solutions were higher in the O soil compartment than in the other treatments ($P<0.01$ for Mg, $P<0.0001$ for K and P) (**Figure 11b**). Ca concentrations in systems containing granite substrate were significantly higher than those in systems containing other substrates ($P<0.0001$). The pH of soil solutions ranged from 3.1 in O soil to 6.1 in granite soil solution and was statistically different between the substrates (data not shown).

Mycelial biomass in the outer compartments did not differ significantly between the different substrates (data not shown). Elemental contents (Ca, Mg, Na and K) of the fungal mycelium were measured but the mycelial material needed to be pooled prior to analysis of the elemental contents, so it was not possible to carry out statistical analysis of the data. However, these data establish that the mobilised base cations were taken up and transported by the ectomycorrhizal fungi (data not shown).

Soil solution elemental composition and C allocation

Figure 11c shows a bivariate plot of the allocation of ^{13}C in soil solution in the outer substrate compartments in relation to the total plant content of base cations and P. The regression is statistically significant ($P<0.001$) and shows that more ^{13}C is exuded into soil solution per unit base cations assimilated by plants in systems containing O soil than in systems containing granite or E/B

interface substrate. The form in which this C was exuded was not examined but it is known that ectomycorrhizal fungi use different mechanisms while mobilising nutrients and can produce low molecular weight organic acids (Wallander *et al*., 2002; Ahonen-Jonnarth *et al*., 2000; van Hees *et al*., 2002; 2006; Fransson and Johansson, 2010), and siderophores (Holmström *et al*., 2004; Haselwandter *et al*., 2011) to promote mineral weathering and extracellular enzymes to promote organic matter decomposition (Lindahl and Tunlid, 2015; Schimel and Bennett, 2004). Differential allocation of C by ectomycorrhizal mycelia to different minerals has been shown by Rosling *et al.* (2004) for feldspar and quartz, by Smits *et al.* (2012) for apatite and quartz. In the latter study *Pinus sylvestris* seedlings allocated more ^{14}C to *P. involutus* mycelium colonising apatite when P was limiting. The fungus allocated 17 times more ^{14}C to apatite grains, compared to quartz and fungal colonisation of the apatite increased P mobilisation by a factor of three. Other studies by Schmalenberger *et al.* (2015) have also demonstrated mineral-specific patterns of oxalate secretion by the ectomycorrhizal fungus *P. involutus*. Wallander and Wickman (1999) demonstrated increased foliar content of K in *P. sylvestris* seedlings colonised by the ectomycorrhizal fungus *S. variegatus* when grown with biotite. They found a positive correlation between foliar K content, citric acid concentration and fungal biomass in the soil. Recently Quirk *et al*. (2014) in a weathering reactor system study found that ^{14}C allocation by ectomycorrhizal fungi into mesh bags containing basalt was correlated with the extent of Ca dissolution from basalt.

The importance of recently fixed C in biological processes was reviewed by Högberg and Read (2006) and it is likely that the high rates of ^{13}C allocated to the soil solution associated with O soil (**Figure 11c**), reflect plant-derived C exuded by mycorrhizal hyphae. The role of ectomycorrhizal fungi in decomposing organic substrates to mobilise N (and possibly also base cations and P) from organic residues using hydrolytic or oxidative enzymes has been demonstrated (Shah *et al.*, 2016; Rineau *et al.*, 2012; Bödeker *et al.*, 2014; Lindahl and Tunlid, 2015) and is obviously dependent upon a supply of C (Rineau *et al*., 2013).

The low concentrations of Mg, K and P in soil solution in granite and E/B soil containing systems (**Figure 11b**) have two possible explanations: 1) that the rate of element dissolution in these two substrates is low, 2) that the elements mobilised and dissolved by ectomycorrhizal fungi are taken up by the fungi but translocated to plant hosts that act as active sinks. In the study by Fahad *et al.* (2016), higher concentrations of base cations (1.7, 15, 18 mg g^{-1} of Mg, K and P respectively) were found in the mycelium of ectomycorrhizal fungi exposed to granite particles, however these *in vitro* systems did not

include plant hosts that might have acted as sinks and lowered the base cation or P concentration. Wallander *et al*. (2002) used PIXE (Particle-induced X-ray emission) to analyse *Rhizopogon* sp. mycelium connected to *Pinus muricata* and associated with a mixture of apatite and biotite in laboratory systems; and apatite or biotite in mesh bags buried in a *Picea abies* forest soil and colonised by unidentified fungal species. The authors found that fungal rhizomorphs in laboratory systems contained 12-31 mg g^{-1} Ca when connected to apatite. Ectomycorrhizal fungal rhizomorphs collected from the field contained up to 11 mg g^{-1} K (mean value, 3.3 mg g^{-1}), 11 mg g^{-1} Ca and 1.0 mg kg^{-1} P.

Organic P and K accumulated in the soil solution of O soil and were significantly higher than in all other treatments. The amounts of these two elements in plant shoots were also significantly higher than in the other treatments, however both P and K were deficient in these seedlings (**Figure 11b**)

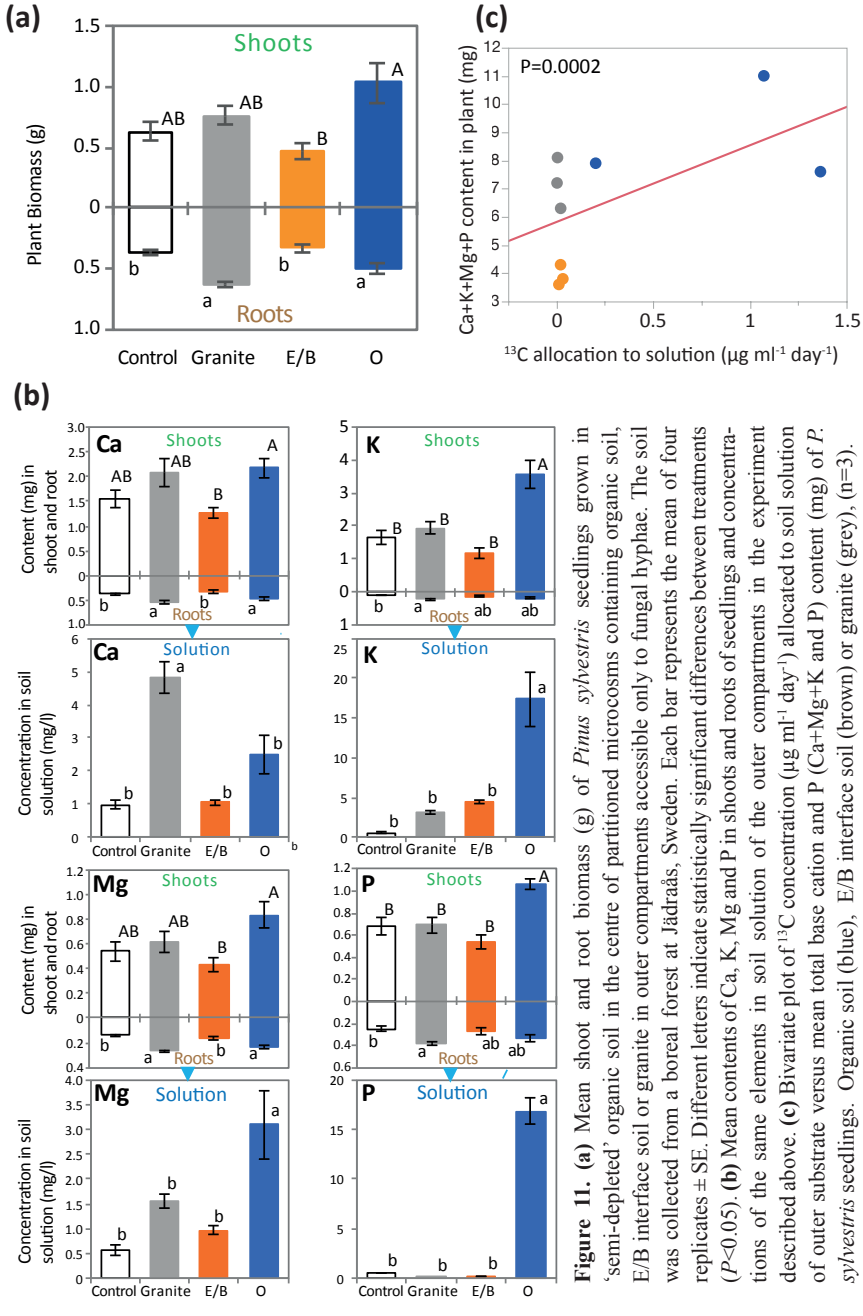


Figure 11. **(a)** Mean shoot and root biomass (g) of *Pinus sylvestris* seedlings grown in 'semi-depleted' organic soil in the centre of partitioned microcosms containing organic soil, E/B interface soil or granite in outer compartments accessible only to fungal hyphae. The soil was collected from a boreal forest at Jädraås, Sweden. Each bar represents the mean of four replicates ± SE. Different letters indicate statistically significant differences between treatments ($P<0.05$). **(b)** Mean contents of Ca, K, Mg and P in shoots and roots of seedlings and concentrations of the same elements in soil solution of the outer compartments in the experiment described above. **(c)** Bivariate plot of ^{13}C concentration (µg ml^{-1} day^{-1}) allocated to soil solution of outer substrate versus mean total base cation and P (Ca+Mg+K and P) content (mg) of *P. sylvestris* seedlings. Organic soil (blue), E/B interface soil (brown) or granite (grey), (n=3).

5.4 Paper IV

Plant growth and elemental composition

The total plant biomass in treatment IV (1.5x natural amount O horizon soil) was significantly higher than in all other treatments ($p<0.0001$) (**Figure 12)**, declining successively and significantly with each reduction in the amount of O horizon soil. The shoot biomass in the mixed horizon treatment V was intermediate between treatments III and IV, and the root biomass did not differ significantly from that of treatment II. Shoot N content was highest in treatments III, IV and V, declining significantly with successive reductions in the amount of O horizon material in treatments II and I (**Paper 4, Figure 2a**). Shoot N concentration in treatment III was optimal but strongly deficient in treatment 4 (**Paper 4, Figure 2b**).

Shoot Ca, K and P contents were also related to the organic layer volume, and significantly higher in treatments IV and V for K and Ca and treatment IV for Mg (**Paper 4, Figure 2a**). Plants in all treatments were deficient in K and strongly deficient or deficient in P, whereas concentrations of Ca and Mg were above optimal levels in all treatments (**Paper 4, Figure 2b**).

Canonical variates analysis of root concentration of Ca, Mg, K, Fe, Mn, P, S and N revealed distinct chemical composition of roots growing in the O, E, B and mixed soil horizons **(Figure 13).**

Plant growth in boreal forests is regulated by N, P and K - the main elements generally limiting plant productivity (Näsholm *et al*., 1998; Chapin, 1980). Our systems were designed to imitate increasing intensities of harvesting in Swedish forests by using a gradient of organic matter depletion. Our main aim was not to examine the direct impact of contrasting harvest intensities *per se* but to quantify the potential nutrient inputs from organic soil through decomposition and from mineral soil through weathering to gain a better understanding of the biological processes contributing to supply of N, P and base cations in a forest podzol. Our results showed that organic layer thickness had a significant impact on nutrient uptake and consequent plant growth (**Figure 12)**. The shoots, and the roots in B horizon soil were both affected by organic layer thickness indicating the importance of the organic layer in regulating the shoot growth and belowground root and mycelium development.

Although shoot N content was the highest in plants grown in treatment IV, shoot N concentrations were deficient according to the definition of Brække and Salih (2002) based on one-year-old needles of Norway Spruce and Scots pine since they were <18 mg g^{-1}. N concentration can decrease with time due to the dilution effect of greater biomass. Irrespective of organic layer thickness,

shoots were either deficient in K or strongly deficient in P, suggesting that the supply of these elements (either through decomposition of organic matter or weathering of mineral) was not adequate to match with seedlings demand, though the shoots in treatments III-V had significantly higher contents of K and P. Any deficiency in macronutrients such as K and P may result in poor growth and could lead to reduction in overall productivity of the system. Ca and Mg concentrations were above optimal level in all treatments and the soil solution concentrations of these elements (data not shown) also reflect the fact that the release of these elements (Mg in particular) from O, E and B layers has been sufficient and steady during this short-term experiment. However, for reliable estimates of nutrient concentrations long-term studies should be conducted with multiple sampling points. Several studies of needle nutrient composition in Sweden have been conducted over longer time periods, for example Olsson *et al*. (2000) and Akselsson *et al*. (2007).

Nutrient translocation belowground to the plant occurs through two mechanisms in the soil, directly by the roots from soil solution, or indirectly by ectomycorrhizal fungi and associated bacteria. However, the relative extent to which these elements are taken up by roots or by ectomycorrhizal fungi is difficult to distinguish due to the ubiquitous nature of the fungal symbionts in the soil, unless stable isotope tracers were used to trace the elements isotopic signatures in roots and mycelium directly colonising the substrate. Canonical discriminant analysis of root concentrations of Ca, Mg, K, Fe, Mn, P, S and N revealed that roots growing in the O, E, B and mixed soil horizons had a distinct chemical composition and the vectors suggest that P and Mn uptake might be mainly associated with mineral weathering in B horizon soil and that organic matter might mainly supply Mg and Ca and that E horizon provides Fe (**Figure 13**). The mixed soil (treatment IV) appears to behave like the organic matter in supplying Mg, Ca and Mn predominantly through decomposition. Bilodeau-Gauthier *et al.* (2013) studied the effect of mounding of hybrid poplars-podzol soil on N mineralisation, by burying the organic layer under a quantity of mineral soil to modify the vertical arrangement of soil layers. The buried organic layer was extensively explored by the proximal roots and led to faster proximal root development. Mounding also developed a condition to favour N dynamics; exploring a large soil volume in the mounding treatment resulted in more abundance of the roots compared to the control. The authors concluded that mounding enhances the above ground growth of the planted tree, greater soil N mineralisation and greater production and vertical distribution of proximal roots. Heiskanen and Rikala (2006) have reported that soil mounding and mixing practices enhance root and shoot growth, N and nutrient uptake, shoot N concentration of 1- and 2-year-old *Picea abies*

seedlings. Similarly, in our study, pine seedlings that were grown in mixed O, E and B horizon soil (treatment 5) attained significantly higher shoot biomass compared to treatment 3 that had stratified O, E and B horizon layers. These seedlings had significantly higher contents of N, Ca, K and P than seedlings grown in treatments 1-3 microcosms (**Paper 4, Figure 2a**).

Olsson *et al*. (1996) found that exchangeable pools of Ca and Mg in the soil were lower after whole tree harvesting than in treatments with needles or all logging residues left on site. However, in our short-term experiment, we did not see any major differences in exchangeable Ca and Mg pools across the treatments both in organic and mineral soils due to successive reduction in O horizon thickness (data not shown).

Fungal standing mass and elemental composition

The estimated mean mycelial dry mass per microcosm **(Figure 12)** was significantly greater in treatment V compared to treatment I ($p<0.02$), but did not differ in a statistically significant manner from treatments II, III or IV. However, in the stratified systems (treatments I to IV) mycelial density in the B horizon soil was statistically significantly greater in treatment IV than any B horizon soil of other treatments. The dry mass of mycelium in treatment V represented 32% of the total belowground biomass (including roots) in the mixed soil (**Figure 12**).

We needed to pool the mycelial samples to obtain sufficient material for analysis of elemental concentrations but the composition of different elements in the pooled samples from treatments I, II, III and IV appeared to be very similar (**Paper 4**, **Figure 4**). The total elemental content of mycelium in the mixed soil (treatment V) was higher, reflecting the increased amount of mycelium described above but the elemental composition was similar to the other treatments with large pools of Al, Fe, and Ca.

It is challenging to estimate ectomycorrhizal mycelial biomass in soil due to the difficulty in achieving soil-particle-free biomass. Methods to estimate fungal biomass based on mesh bags containing either sand or soil, buried in the upper 10 cm in different forest soils, have advantages and disadvantages (see Wallander *et al.*, 2013). Different estimates have been produced, based on ergosterol content (390 kg ha^{-1}), phospholipid fatty acid 18:2ω6,9 in sandy soil (320 kg ha^{-1}) (Wallander *et al.*, 2001), loss of ignition in podzol (390 kg ha^{-1}) (Hagerberg *et al.*, 2003), elemental carbon analysis of extracted mycelium in sandy soil (215 kg ha^{-1}) (Boström *et al*., 2007). One of the problems with PLFA and ergosterol is that ericoid mycorrhiza and dark septate endophytes contain two fungal marker molecules (Jumpponen and Trappe, 1998), which might affect the interpretation of the estimated biomass. Our estimation in

different soil horizons was based on direct mycelial dry mass measurements. We scaled up the total fungal biomass in treatment III microcosms that were growing for 15 months and found an estimated biomass around 489 kg ha^{-1}. Treatment III represents a natural forest podzol at Jädraås (Sweden) in terms of O and E horizon thickness though B horizon thickness was greatly reduced in our reconstructed podzol system. However, mycelium standing biomass in our laboratory microcosms followed a similar trend as reported in natural forest soils (see above). During sample preparation, strict washing steps were performed to allow fungal elemental and isotopic composition analyses. This resulted in losses of small hyphae, conidia, spores and sclerotia at every washing step. Despite that, the fungal dry mass based estimates in our study have given relatively higher mycelial biomass in treatment IV and V than the above mentioned methods because of the composition of soil in the treatment IV that was extremely high in organic matter thickness which might have accelerated both plant and fungal growth, while in treatment 5 though the composition is based on the same layers as in treatment 3 the spatial and physical structure are different (we mixed organic and mineral soils to mimic the disturbance after tree harvesting in forest soil) and this might create a soil environment with homogeneously distributed organic and mineral nutrient sources and probably also facilitating colonisation of the root system by ectomycorrhizal mycelia. On the other hand, this perturbation might influence the biogeochemical processes and nutrient pools availability which in turn may also change the microbial community composition and activity. We are currently analysing ^{13}C-RNA-SIP based metabolically active bacterial and fungal communities in these podzol microcosm systems.

Base cation composition of the mycelium growing in organic or mineral soil layers did not differ markedly, however the observation that mycelial biomass was consistently higher in mineral soils, suggests that the total contents/pools of base cations (and P) in mineral horizon mycelium could be significantly bigger than in organic soil layer.

^{13}C allocation

^{13}C-labelling revealed that the total amount of ^{13}C allocation per day was highest into roots growing in treatment III and that total allocation (per day) into mineral horizon roots was higher than that into organic horizon roots for treatments II and III (**Paper 4, Figure 5a**). The proportion of total plant C allocated belowground was highest in treatments II and III (~31%), followed by treatments I and V (~22%). Shoot ^{13}C allocation did not differ significantly between treatment III, IV and V, but was significantly lower in treatments I and II (P<0.05). Mineral horizon soils received 10-20 folds more ^{13}C per day

than the organic soil and there was a successive increase in the amount of ^{13}C allocated per day in soils from treatments II-IV (**Paper 4, Figure 5b**). The overall ^{13}C recovered in the soil solution pool appeared to be a small fraction of the C that plants allocated belowground (**Paper 4, Figure 5c**). However, there was a clear trend of successive increase in ^{13}C allocation rate from treatment I (lowest), to treatment IV (highest).

Competition between the fungus and the roots for photosynthates is the main factor responsible for the typical larger shoot:root dry weight ratio in mycorrhizal plants (Berta *et al.*, 1990). While C allocation aboveground promotes competition for light, plants simultaneously allocate C belowground to produce roots and root exudates, support mycorrhizal associations, and compete for soil N and P (Litton *et al.*, 2007). In this study shoot ^{13}C allocation depended on size of the shoot and belowground C allocation to roots also followed a similar trend, except for treatment IV that had significantly higher ^{13}C in shoot but allocated relatively little to the roots and ^{13}C content values were comparable with roots of treatment II (that had significantly smaller shoots) (**Paper 4, Figure 5a**). One possible explanation for this discrepancy could be that plants in this treatment were probably allocating more C to mycelium that was growing in soil or investing C in root or mycelial exudates. ^{13}C allocation patterns to organic and mineral soils and soil solutions, also support our speculation (**Paper 4, Figure 5b & c**). The overall content of ^{13}C that plants allocated to mineral soils was several folds greater than allocated to organic soils also provides the evidence that biogeochemical weathering of minerals probably costs more recently fixed photosynthetic C than recycling of nutrients through decomposition of organic matter. The overall ^{13}C recovered in the soil solution pool appeared to be a small fraction of the ^{13}C that plants allocated belowground (**Paper 4, Figure 5c).** However, there was a clear trend of successive increase in ^{13}C allocation to O horizon soil in treatment II (lowest) to treatment IV (highest). Photosynthates are mainly incorporated into plant biomass, and plants allocate estimated 35-80% of the fixed C belowground for root production and respiration, and also to support a diverse soil microbial community either directly, via mycorrhizal fungi, or via root exudates/rhizodeposits (Ryan *et al*., 2004; Nehls *et al.*, 2007; Jones *et al.*, 2010).

Soil solution chemistry

In the presence of plants and associated ectomycorrhizal fungal mycelium, the $\delta^{26}Mg$ signatures of pooled soil solution samples in B horizon soil increased successively from –0.29 to 0.24 with increasing plant and fungal biomass in

treatments I-IV (**Figure 14a, b, c**), suggesting discrimination against the heavy ^{26}Mg by ectomycorrhizal fungi (and roots) in B soil, coupled with increased mycorrhizal uptake in response to increasing thickness of the organic soil layer. Fahad *et al.* (2016) studied Mg isotope fractionation and assimilation by ectomycorrhizal fungi grown on medium containing granite particles as the main source of Mg in an axenic system, and found that ectomycorrhizal fungi had significantly depleted δ^{26}Mg signatures and higher concentrations of total Mg compared with nonmycorrhizal fungi and accumulated significantly higher concentrations of Mg, K and P than the nonmycorrhizal fungi. In the treatments without plants there was no corresponding gradient. In treatment V the δ^{26}Mg values were more depleted (–0.50) and there was no clear difference between systems with and without plants.

Soil solution pH in systems containing plants was lower than the controls except for B soil, which showed a slight increase in pH (**Paper 4, Figure 7**).

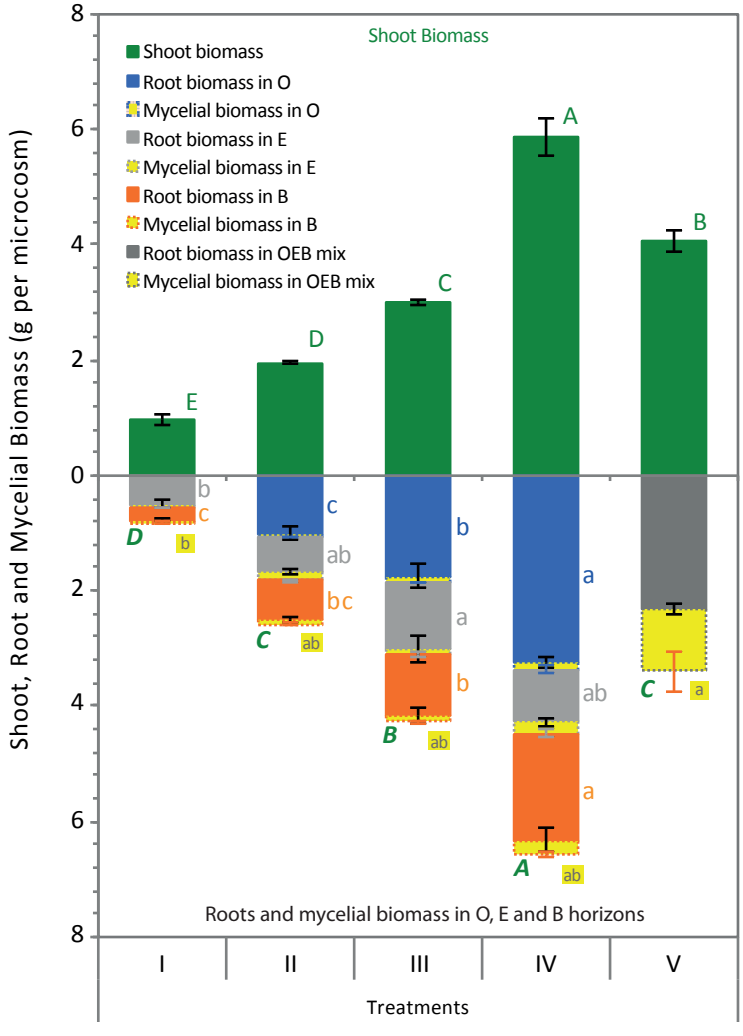


Figure 12. Mean shoot, root and ectomycorrhizal fungal biomass in microcosms with a gradient of organic matter depletion. **I** - no organic horizon, **II** - reduced (½) organic horizon, **III** - normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the O, E and B soil horizons were completely mixed. Vertical bars = ± 1 SE (n = 4). Six *Pinus sylvestris* seedlings were grown in each microcosm. O, E and B horizon soils were from a boreal forest podzol at Jadraås, Sweden.

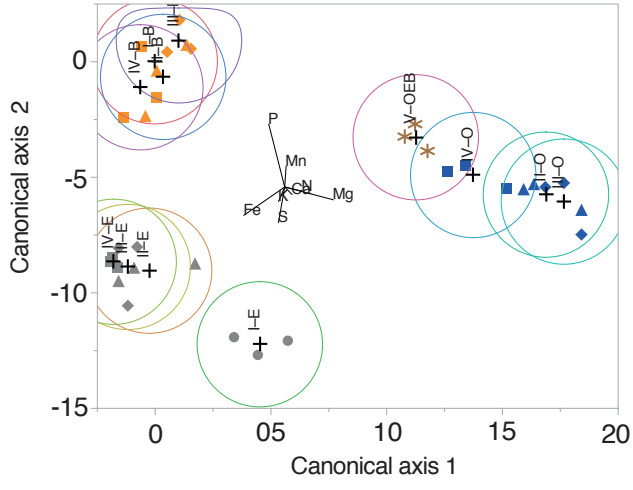


Figure 13. Canonical variate analysis of root concentrations of Ca, Mg, K, Fe, Mn, P, S and N in *Pinus sylvestris* seedlings growing in different podzol horizon soils in microcosms with gradient of organic matter depletion. **I** - no organic horizon, **II** - reduced (½) organic horizon, **III** - normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the O, E and B soil horizons were completely mixed.

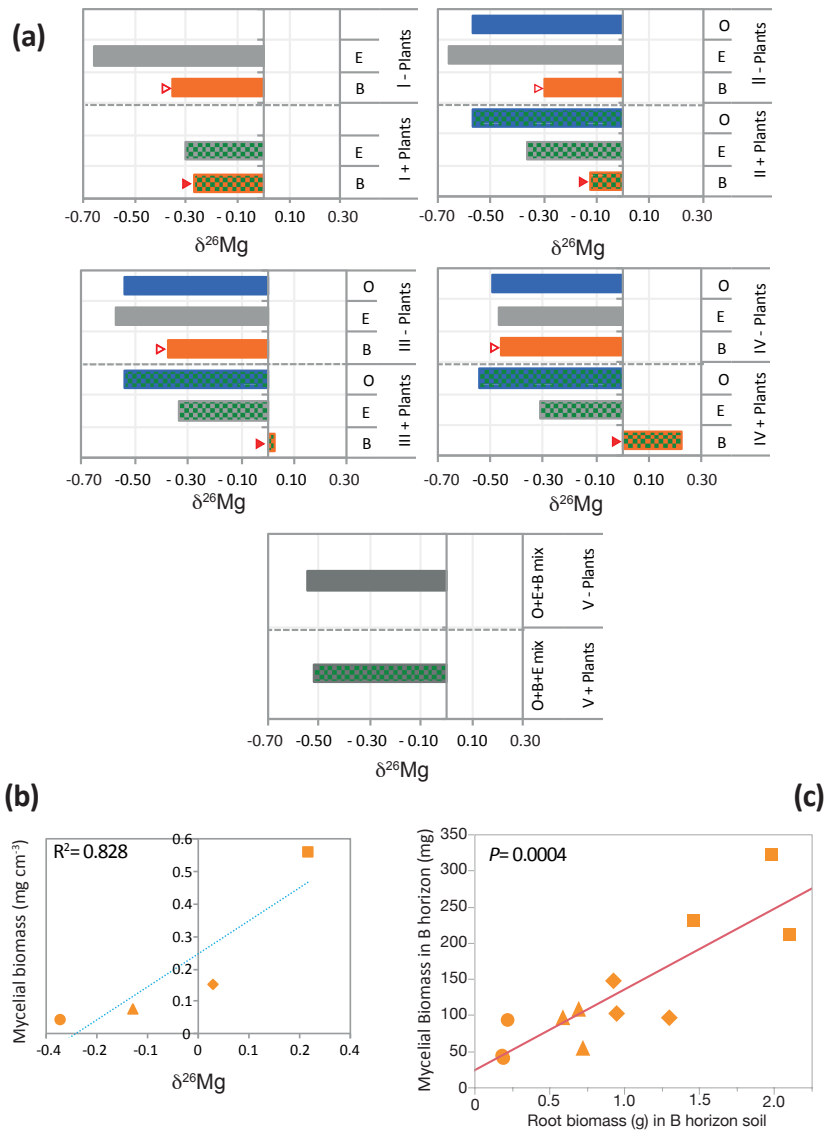


Figure 14. (a) δ^{26}Mg isotopic ratio of soil solutions collected from organic (O), eluvial (E), illuvial (B) and mixed soil horizons in microcosms containing podzol soils with different relative amounts of organic soil. **I** - no organic horizon, **II** - reduced (½) organic horizon, **III** - normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the soils were completely mixed. Six *Pinus sylvestris* seedlings were grown in each microcosm. Soil solution was collected by centrifugation from four replicates that were pooled for δ^{26}Mg isotopic measurements. **(b)** & **(c)** show regressions between mycelial biomass and δ^{26}Mg and between mycelial biomass and root biomass. Circles, triangles, diamonds and squares represent treatments I-IV, respectively.

5.5 Study V

The mean mycelial standing biomass in mesh bags that were buried in organic and mineral horizon soils was 3, 2 and 7 g m^{-2}. These estimates were based on 6, 4 and 20 cm depths for O, E and B horizons respectively. The mean mycelial biomass was statistically higher in B than in E (P=0.0239) but did not differ from O horizon soil. Mycelial biomass of E and O horizon soils did not differ significantly. Mycelial biomass per 1 cm^3 substrate (glass beads) of this experiment did not differ statistically between the three horizons (50 µg for O and E, 30 µg for B horizon soils), but was far less than mycelial biomass we found in our laboratory experiment (Paper IV- treatment III) that was supposed to reflect Jädraås forest in terms of proportions of O, E and B soils. Mycelial biomass cm^{-3} in paper IV was 160, 220 and 150 µg for O, E and B horizon soils respectively. Different environmental factors might affect mycelial biomass production such as seasonality. In controlled settings such as the phytotron, the temperature and humidity were fixed along 13 months incubation, while in field settings during winter, a low temperature and light shortage should affect photosynthesis rate, consequently allocation of less C belowground for both mycelial growth and activity. In the forest, rain events are frequent and that might play an important role in hydrology and pH of the soil, or nutrient availability for fungi to grow.

Mean mycelial elemental content for B horizon soil was generally higher than for O or E horizon soil. Mg, Na, K, Al and Fe of B horizon mycelia were statistically higher than in O and E horizon soil (Mg P= 0.0024, Na P= 0.0012, K P= 0.0007, Al P<0.0001, Fe P= 0.0083). Mn content in mycelia growing in B horizon soil differed statistically than in E but not from O horizon soil (P= 0.0214). Mycelial Ca content did not differ statistically between the three soil horizons.

Söderström (1979) in a field study estimated total fungal biomass using agar film technique and active fungal biomass using fluorescein diacetate staining, and compared fungal biomass grown in O, E and 10 cm depth of B horizon soils in a Scots pine stand in Jädraås forest during 27 months, and found that fungal biomass was the highest during autumn and early spring. The results showed that active fungi per square meter were equally distributed between organic and mineral soils and varied between 0.5 to 2.4 g m^{-2}. Total fungal biomass was estimated 42, 8.6 and 33 g m^{-2} for O, E and B horizon soils. This big difference between Söderström (1979) study and our study is might be because we buried the mesh bags in field soils for 12 months only, while the other study the incubation time was 27 months.

Our approach was to clean the mycelia thoroughly because our estimation is based on biomass measurements, while the author used mycelial length which

was converted to biomass with the assumption that fungal water content is 15%. We believe that we lost at least about 10-20% mycelium during washings and cleaning under dissecting microscope to remove any secondary minerals or surface contaminants, since these mycelia were to be used for elemental composition and ^{26}Mg isotopic analysis.

This study showed that despite the three-fold greater amount of fungal biomass in B than in O and E horizon soils, elemental content is also of a magnitude that we should consider when we study C cost or estimate mineral weathering rates.

6 Conclusions and future perspectives

The main conclusions of the studies described in this thesis are as follows:

1. The microcosm study on decomposition of ^{13}C-labelled ectomycorrhizal mycelium revealed rapid evolution of $^{13}CO_2$ during the first week suggesting rapid decomposition. The decomposition rate was similar in soil from O, E and B horizons and did not differ significantly between systems with and without plants.

2. Incorporation of C derived from the decomposing mycelium was followed using ^{13}C-RNA stable isotope probing. In systems containing O horizon soil *Betaproteobacteria* were active in assimilating ^{13}C from the dead mycelium but they were not active in the absence of added mycelium irrespective of the presence or absence of plants. This effect was also apparent for *Gammaproteobacteria* but only occurred in the presence of plants. In systems containing E horizon soil *Betaproteobacteria* were also highly active in assimilating ^{13}C from the dead mycelium. The same effect was observed for *Sphingobacteria* although this group was less active. In the presence of plants, *Gammaproteobacteria* also demonstrated some capacity to assimilate ^{13}C from the dead mycelium.

3. In systems containing O horizon soil, the active fungi decomposing the dead mycelium were mainly non-ectomycorrhizal saprotrophic fungi, even in the presence of plants. The activity of *Umbelopsis* sp.*, Umbelopsis angularis, Mortierella humilius,* and *Penicillium spinulosum, Sugiyamael paludigena, Trichoderma viride* and *Mucor silvaticus* increased greatly following the addition of dead mycelium. Ectomycorrhizal fungi including *Cenococcum geophilum, Piloderma olivaceum,* in the O horizon and *Piloderma sphaerosporum*, in the E horizon, were active in systems containing plants, but their activity was greatly reduced in systems to which dead mycelium was added.

Umbelopsis dimorpha, Mortierella sp. and an unidentified fungal species (SH234809), in the E horizon, and *Sugiyamaella paludigena, Trichoderma viride,* and *Mucor silvaticus*, in the O horizon, were highly active in assimilating ^{13}C from the dead mycelium. Mycelial biomass is a potential nutrient source that is decomposed rapidly, mainly by fungal saprotrophs and bacteria that that are not dependent on plant-derived C in organic and mineral soils. Decomposition of fungal necromass by some microbial taxa appears to be stimulated by the presence of plants. The data provide detailed, functionally based evidence that supports the suggestion that the metabolic C demand of ectomycorrhizal fungi is not met by organic matter decomposition but supplied by the host plant in exchange for N.

4. *In vitro* experiments designed to study the capacity of different fungi to fractionate and assimilate stable isotopes of Mg revealed that the mycorrhizal fungi were significantly depleted in heavy isotopes of Mg with the lowest $\Delta^{26}Mg$ values compared with nonmycorrhizal fungi, when grown on mineral substrates containing granite particles.

5. The ectomycorrhizal fungi accumulated significantly higher concentrations of Mg, K and P than the nonmycorrhizal fungi and there was a highly significant statistical relationship between $\delta^{26}Mg$ tissue signature and mycelial concentration of Mg, with a clear separation between most ectomycorrhizal fungi and the nonmycorrhizal fungi.

6. These results are consistent with the idea that ectomycorrhizal fungi have evolved efficient mechanisms to mobilise, transport and store Mg within their mycelia but further studies, including a wider range of fungi with different trophic status need to be performed to test this idea.

7. A third study was designed to examine patterns of C allocation to, and base cation mobilisation from, organic and inorganic substrates via ectomycorrhizal mycelium growing from *P. sylvestris* seedlings in compartmentalised microcosms. Shoot biomass was significantly higher where fresh organic soil was added to the systems than other systems. Root biomass was significantly higher in granite and organic containing systems than other systems. But fungal nutrient mobilisation was reflected in roots elemental contents of Ca, K, Mg, and P of plants in granite treatment that were significantly higher than in control roots suggesting that the mycorrhizal mycelium was effectively able to support growth of their host.

8. Concentrations of Ca in soil solution were significantly higher in the granite compartment than the other treatments. Concentrations of Mg, K and P were higher in the organic soil compartment than in the other treatments.

9. K and Ca contents in mycelium were much higher in granite containing systems than in other substrate treatments.

10. Carbon allocation to the soil solution was highest in systems that contained organic soil, and there was a statistically significant (P<0.001) relationship that showed more ^{13}C was exuded into soil solution per unit base cations assimilated by plants in systems containing O soil than in systems containing granite or E/B interface substrate.

11. The results obtained so far suggest that elemental concentrations of soil solution alone are a poor predictor of plant nutrient status since elements may be mobilised in remote locations and translocated to the plant through ectomycorrhizal mycelium. When seedlings are grown in 'semi-depleted' organic soils, mobilisation and translocation of nutrients from (relatively fresh) organic matter probably requires more C investment than uptake of nutrients from a mineral source, presumably because organic matter is a source of, not only P and base cations, but also N.

12. Microcosms were designed to study the relationship between organic matter decomposition and mineral weathering in relation to sustainable supply of base cations under contrasting forest management regimes (partial or whole tree harvesting). A gradient of organic matter depletion was simulated by altering the proportions of organic and mineral soil. Total plant and fungal biomass was positively related to the amount of organic soil in this experiment.

13. In the presence of plants and associated ectomycorrhizal mycelium the $\delta^{26}Mg$ signatures of soil solution samples in B horizon soil increased successively with increasing plant and fungal biomass. This suggests that increased Mg uptake from the B horizon, associated with increased plant and fungal biomass and discrimination against the heavier isotope (see paper II) resulted in higher enrichment of ^{26}Mg in the soil solution.

14. Organic matter availability enhanced plant growth, generating a sink for nutrients that were supplied from both organic and mineral substrates through roots and associated ectomycorrhizal mycelia. Plants allocated significantly more C to roots that were growing in

mineral soils than in organic soil, and this also resulted in higly significant enrichment of ^{13}C in mineral soil than in organic layer, suggesting that weathering of minerals probably costs more recently fixed C than recycling of nutrients through decomposition of organic matter.

15. The removal/depletion of organic matter in boreal forests might be compensated for temporarily through intensified weathering but since this process is ultimately driven by the input of plant-derived C, N limitation will eventually restrict biological weathering. Ectomycorrhizal fungi are efficient at mobilising N and P from recalcitrant organic residues in nutrient poor soils and the C they receive from their host trees "primes" both this N extraction and the complementary mobilisation of base cations and P from a range of mineral sources. These processes are "sustainable" for the trees and fungi.

15. A field experiment revealed that mycelial standing biomass was significantly higher in B than in E but did not differ from O horizon soil. Elemental composition of mycelium collected from in organic and mineral soils did not vary significantly except that mycelia from B horizon soil had much higher contents of Ca, Mg, K, Al and Mn in other soils. The results imply that mycelial biomass is an important pool of nutrients in both organic and mineral horizons.

Our studies show the importance of ectomycorrhizal roots and mycelia in regulating nutrient mobilisation from different horizon soils, while doing this, part of the photoassimilates will be translocated to the soil and might fuel further biogeochemical processes. One of the challenges to quantify the role of ectomycorrhizal fungi in natural forest soil is the difficulty in estimating fungal standing mass and nutrient uptake in a very clean condition. We were able to estimate and show that ectomycorrhizal mycelial standing biomass in laboratory and field settings, was significantly high in mineral soils where root growth is limited by nutrient availability, this mass function was to assist plant growth by compensating for nutrients through weathering. The result we found where the substrate was inaccessible to roots, is not new finding, but we were able to measure the chemical composition of the fungal mycelium by using isolated buffer zone compartment or double compartment mesh bags to collect clean mycelium that hold nutrients scavenged directly from soil solution or mined from the soil substrate.

Elemental concentration in mycelium generally did not differ between the horizons, which indicates that fungal nutrient uptake from organic and mineral substrates and play an important role in nutrient cycling in forest ecosystems. This finding reinforces the need to consider two things, a) account for C allocation in deeper soil horizons and b) quantify the nutritional value of ectomycorrhizal necromass in boreal forest. Decomposition of ectomycorrhizal necromass appears to be a fast process, carried out by decomposer fungi and bacteria that require C and other nutrients for their growth and metabolism. Our study also shows that soil type and plant presence have an effect on microbial activity. RNA-SIP study enabled us to show that ectomycorrhizal fungi when they are in decent supply of plant C, or even when they are disconnected from the plant, they do not take up C from fungal necromass. We have found that ectomycorrhizal fungi weather granite and discriminate against the heavy Mg isotope and preferentially take up the lighter isotope. The finding has ecological application in studying different biogeochemical cycles in forest ecosystems, not only because of their ability to fractionate Mg but also due to their ability to assimilate base cations and P in much higher amount than free-living fungi.

Extrapolating results from lab scale to field condition should be done with caution, though we used small seedlings planted in natural soils with natural microbial inoculum for not further than two years, and at fixed growth conditions of temperature and humidity, our systems evince the potential dual role of ectomycorrhizal fungi in nutrient uptake and C allocation in boreal forests. We supported our laboratory results with a field study to conclude that mycelial standing biomass in mineral soil horizons is not less important than the upper organic layer, and by taking into account the total mycelial biomass in each layer, we realise that fungal mycelium in forest soil profile could be a considerably big pool of organic nutrients (base cations, P and N) and therefore should be taken into consideration while estimating the elements that were mobilised either through biogeochemical weathering of minerals or recycled through decomposition of organic matter. Mycelial elemental concentrations exceeded the concentration of the same elements in the root growing in the same horizon, particularly in B soil in our laboratory paper IV. Organic N supply (through decomposition) appears to be regulating the weathering process in paper IV. Future studies, should therefore aim at studying the interactions between weathering and decomposition processes in forest ecosystem. Such studies should be conducted using stable isotopes of C, N and Mg to track the links between different biogeochemical processes.

Microbial community analysis (papers III-V) are in progress, and will reveal functional role of different bacterial and fungal communities.

The data on chemical composition of soil solution, plant, fungal mycelium and soil mineralogy are currently being analysed for estimation of weathering rates in our simulated podzol systems using a combination of modelling and mass balance approaches.

List of illustrations

was added to the buffer zone/mycelial compartment, and the outer 'nutrient' compartment contained either granite particles (1), fresh organic horizon soil (2), soil from the E/B interface layer (3) or glass beads as control (4). Fungal mycelia were able to colonise the 'nutrient' substrates in the outermost compartments by crossing the glass beads' compartment. Clean mycelium was harvested from the glass beads for elemental and δ^{26}Mg isotopic analyses. The microcosms were wrapped with thick black plastic sheet and incubated in a phytotron for 11 months. **(b)** aerial view of the actual microcosms.

Figure 6a. Microcosm system consisting of five treatments representing a stratified podzol soil with different degrees of organic matter depletion: I - no organic horizon, II - reduced (1⁄2) organic horizon, III -normal organic horizon, IV - increased (x1.5) organic horizon. In treatment V the O, E and B soil horizons were completely mixed. The O, E and B layers were separated by 2 mm nylon mesh. Lysimeters were inserted in each horizon for soil solution sampling and dual compartment mesh bags were placed in each horizon. Six *Pinus sylvestris* seedlings were grown in each microcosm. The photo was taken after 14 months of growth. (Photo: Shahid Mahmood)

Figure 6b. Side view of microcosm showing O, E & B soil layers and mycorrhizal colonisation of roots in treatment III. (Photo: Shahid Mahmood)

Figure 7. $^{13}CO_2$ evolution rate (^{13}C excess nmol min^{-1}) over 28 days as a proxy for decomposition of ^{13}C-labelled *Piloderma fallax* mycelium added to O, E and B horizon soils collected from a boreal forest at Jädraås, Sweden. The soil microcosms were either without plants **(a)** or planted with *Picea abies* seedlings **(b)**. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled mycelium was added one month prior to harvesting. Data points represent the mean ± SE (n = 3). Error bars are not shown when smaller than the symbols.

Figure 8. Abundance of the 20 most active bacterial classes including the ones that were able to incorporate ^{13}C in their RNA following addition of ^{13}C-labelled *Piloderma fallax* mycelial necromass into O horizon **(a)** and E horizon **(b)** soil microcosms that were either without plants or planted with *Picea abies* seedlings. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled mycelium was added one month prior to harvesting. RNA was extracted from three biological replicates and pooled for ^{13}C-RNA stable isotope probing and 454 pyrosequencing analyses.

Figure 9. Abundance of the 20 most active fungal taxa including the ones that were able to incorporate ^{13}C in their RNA following addition of ^{13}C-labelled *Piloderma fallax* mycelial necromass into O horizon **(a)** and E horizon **(b)** soil microcosms that were either without plants or planted with *Picea abies* seedlings. The microcosms were incubated in a phytotron for eight months and ^{13}C-labelled mycelium was added one month prior to harvesting. RNA was extracted from three biological replicates and pooled for ^{13}C-RNA stable isotope probing and 454 pyrosequencing analyses.

Figure 10a. Mean values of Δ26Mg (‰) for four ectomycorrhizal and four nonmycorrhizal fungi grown either on a mineral free Modified Melin-Norkrans (MMN) medium amended with granite particles, mineral free MMN medium amended with solubilised organic matter from the organic layer of a boreal podzol or MMN medium. Vertical bars represent ± SE (n = 4). The fungi were grown on cellophane membranes covering the growth substrates in Petri dish microcosms.

Figure 10b. Mean values of Mg, K, P, Ca, Fe, S concentration (mg g^{-1}) in ectomycorrhizal and nonmycorrhizal fungal mycelia grown on mineral free Modified Melin-Norkrans (MMN) medium amended with granite particles or MMN medium. Vertical bars represent ± SE (n = 4). The fungi were grown on cellophane membranes covering the growth substrates in Petri dish microcosms.

Figure 11. (a) Mean shoot and root biomass (g) of *Pinus sylvestris* seedlings grown in 'semi-depleted' organic soil in the centre of partitioned microcosms containing organic soil, E/B interface soil or granite in outer compartments accessible only to fungal hyphae. The soil was collected from a boreal forest at Jädraås, Sweden. Each bar represents the mean of four replicates ± SE. Different letters indicate statistically significant differences between treatments (P<0.05). **(b)** Mean contents of Ca, K, Mg and P in shoots and roots of seedlings and concentrations of the same elements soil solution of the outer compartments in the experiment described above. **(c)** Bivariate plot of ^{3}C concentration (μg ml^{-1} day^{-1}) allocated to soil solution of outer substrate versus mean total base cation and P (Ca+Mg+K and P) content (mg) of *P. sylvestris* seedlings. Organic soil (blue), E/B interface soil (brown) or granite (grey), (n=3).

Figure 12. Mean shoot, root and ectomycorrhizal fungal biomass in microcosms with a gradient of organic matter depletion. **I** - no organic horizon,

II - reduced (1⁄2) organic horizon, **III** - normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the O, E and B soil horizons were completely mixed. Vertical bars = ± 1 SE (n = 4). Six *Pinus sylvestris* seedlings were grown in each microcosm. O, E and B horizon soils were from a boreal forest podzol at Jadraås, Sweden.

Figure 13. Canonical variate analysis of root concentrations of Ca, Mg, K, Fe, Mn, P, S and N in *Pinus sylvestris* seedlings growing in different podzol horizon soils in microcosms with gradient of organic matter depletion. **I** - no organic horizon, **II** - reduced (1⁄2) organic horizon, **III** -normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the O, E and B soil horizons were completely mixed.

Figure 14. (a) δ^{26}Mg isotopic ratio of soil solutions collected from organic (O), eluvial (E), illuvial (B) and mixed soil horizons in microcosms containing podzol soils with different relative amounts of organic soil. **I** - no organic horizon, **II** - reduced (1⁄2) organic horizon, **III** - normal organic horizon, **IV** - increased (x1.5) organic horizon. In treatment **V** the soils were completely mixed. Six *Pinus sylvestris* seedlings were grown in each microcosm. Soil solution was collected by centrifugation from four replicates that were pooled for δ^{26}Mg isotopic measurements. **(b)** & **(c)** show regressions between mycelial biomass and δ^{26}Mg and between mycelial biomass and root biomass. Circles, triangles, diamonds and squares represent treatments I-IV, respectively.

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