Effects of organic matter depletion on fungal communities in a reconstructed boreal forest podzol system

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Abstract

The boreal forest biome serves as a valuable resource, both as a source of wood, pulp and biofuel, and as a sink of atmospheric carbon (C). A large proportion of C is sequestered to the soil of the boreal biome, however belowground processes are still poorly understood. Biogeochemical processes occurring in the soil are influenced by fungal communities and activity. Different processes and microbial communities exist throughout the soil profile due to differences in physico-chemical and biological properties. Boreal forest soils are commonly podzolized, with stratified layers of organic and mineral soil referred to as horizons. With intensifying forest harvesting practices, it is important to build our knowledge of fungal community function in each horizon and establish predictions of the effects intensified forest harvesting may have on these communities. The main objectives of this microcosm-based study were to characterise the fungal communities present in each horizon and investigate how these are affected by different degrees of organic matter depletion, simulating a gradient of intensity in forest harvesting.

We used a microcosm experiment with *Pinus sylvestris* seedlings and reconstructed soil profiles containing decreasing amounts of organic material to simulate increasing intensification of forestry. Fungal communities were characterised by high throughput DNA sequencing and statistical analysis. The 20 most abundant fungi accounted for over 80% of the DNA sequences, and there were statistically distinct communities in the O, E and B horizons. *Piloderma sphaerosporum* was the most abundant species in the O horizon and *Suillus bovinus* the most abundant in both the mineral horizons. Fungal species richness was significantly higher in the E horizon layers with an overlying O horizon compared to systems where the O horizon was removed completely (simulating extreme loss of organic matter due to intensive biomass harvesting). Fungal species richness was not otherwise affected by the treatments during the 14 month duration of the experiment, but they caused significant changes in community structure in the E and B horizons.

Parallel studies of the same experimental system showed that plant growth was proportional to the amount of organic material and it is likely the reduced growth in systems with reduced amounts of organic material was due to reduced supply of organic N mobilised and transported to plants by mycorrhizal fungi, and lower C allocation by these small plants to their mycorrhizal symbionts. Published studies suggest that mycorrhizal fungi can access Mg from mineral weathering in the B horizon and the present study shows that the relative abundance of ectomycorrhizal fungi is high in the B horizon. Further studies of the distribution and activity of soil microorganisms in all soil horizons are therefore necessary to improve understanding of their contributions to sustainable forest growth and management.

Keywords: ectomycorrhizal fungi, fungal community structure, fungal diversity, high throughput sequencing, podzol, soil horizons

Popular science summary

Boreal (Northern) forests serve as a valuable resource, both as a source of wood, pulp and biofuel, and as a "sink" (a reservoir which takes up a compound and removes it from the natural cycle) for atmospheric carbon (C). Much of this C is stored in the soil, however belowground processes are still poorly understood. Boreal forest soils are acidic, making them inhospitable to earthworms that normally cause mixing. The soil therefore forms distinct layers, with a surface organic (O) horizon overlying a pale, leached (Eluvial) (E) mineral horizon, and beneath this a darker "Illuvial" mineral horizon (B) where substances leached from the overlying layers are deposited.

Different microorganisms form diverse communities and play important roles in biogeochemical processes in these soils, but their patterns of distribution and activity are still poorly understood. The O horizon, composed of decomposing plant and fungal debris, is a key store of organic C, and source of plant nutrients such as nitrogen (N), phosphorus (P) and base cations including calcium (Ca); potassium (K); and magnesium (Mg). Fungi play important roles in making these nutrients available to plants, both through decomposition of organic material, and as symbiotic "mycorrhizal" partners of plant roots.

In forestry removal of tree stems for timber has long been practiced and is well studied. However, in recent years the harvesting of whole trees, removing branches, foliage and stumps to be used as biofuels, has become more common. This practice reduces the amount of debris in forests and over time will reduce the volume of the organic horizon. This reduces the availability of nutrients and can affect fungal community composition and activity, and plant growth. The roles of microorganisms in other horizons may then become more important and new knowledge about the effects of intensified forest harvesting on fungal community structure and function in each horizon is needed to improve the sustainable management of forests.

We used a microcosm experiment with Scots pine seedlings and reconstructed soil profiles containing decreasing amounts of organic material to simulate increasing intensification of forestry. Fungal communities were characterised by DNA sequencing and statistical analysis. The 20 most abundant fungi accounted for over 80% of the DNA sequences, and there were statistically distinct communities in the O, E and B horizons. *Piloderma sphaerosporum* was the most abundant species in the O horizon and *Suillus bovinus* the most abundant in both the mineral horizons. Fungal diversity was significantly higher in the E horizon layers with an overlying O horizon compared to systems where the O horizon was removed completely (simulating extreme loss of organic matter due to intensive biomass harvesting). Fungal diversity was not otherwise affected by the treatments during the 14 month duration of the experiment, but they caused significant changes in community structure in the E and B horizons.

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Abbreviations

ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
B	Illuvial
CH	Conventional Harvesting
CWDE	Cell Wall Degrading Enzymes
E	Eluvial
EB	Elution Buffer
EPS	Extracellular Polymeric Substances
HMW	High Molecular Weight
ITS	Internal Transcribed Spacer
LMW	Low Molecular Weight
MAMPs	Microbe Associated Molecular Patterns
NMDS	Dimensional Scaling
O	Organic
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis of Variance
PRR	Pattern Recognition Receptor
QIIME	Quantitative Insights Into Microbial Ecology
ROS	Reactive Oxygen Species
SIP	Stable Isotope Probing
WTH	Whole Tree Harvesting
WTHSR	Whole Tree Harvesting and Stump Removal

1. Background

Boreal forest soils are typically stratified into organic (O) horizons overlying distinct mineral eluvial (E) and illuvial (B) horizons. The trees in boreal forests rely on symbiotic ectomycorrhizal fungi to mobilise N and P from organic substrates. New insights into this process have been gained during recent years from studies using genomics, transcriptomics and spectroscopy (Lindahl and Tunlid, 2015) but interactions of ectomycorrhizal fungi with mineral substrates are less well studied. There is increasing evidence from evolutionary biology, paleontology, geochemistry, and earth system sciences that the coevolution of plants and mycorrhizal fungi has led to progressive intensification of pedogenesis and biogeochemical cycles and that interactions with minerals can affect ocean chemistry, atmospheric composition, and global climate (Leake and Read, 2017; Finlay *et al.*, 2020). However, many community studies of ectomycorrhizal fungi ignore the mineral horizons. There is mounting evidence that mineral weathering of silicates may promote C sequestration through driving export of calcium to ocean carbonates, driving the reduction of global atmospheric CO_2 (Quirk *et al.*, 2012; Quirk *et al.*, 2014; Schmalenberger *et al.*, 2015). The mobilisation of minerals through weathering is also important because it replaces nutrient elements lost through leaching and biomass harvesting. This is important because increased interest in biofuel harvesting from forests has led to a transition from conventional clear felling (CH) where only the trunk is harvested, to whole tree harvesting (WTH) and stump removal (SR). WTH and SR involve the removal of branches, foliage, stumps and roots, and reduce the amount of organic material, and thus base cations, returning to the soil (Sverdrup and Rosen, 1998; Brandtberg and Olsson, 2012). There is concern that current intensification of forestry may not be sustainable with respect to continued supply of base cations (Axelsson *et al.,* 2019). A microcosm experiment was therefore conducted to simulate different degrees of intensification of forestry by removing different amounts of organic material from a reconstructed boreal forest podzol. Patterns of distribution of stable isotopes of Mg have already been published (Finlay *et al*., 2020) but no information is yet available on the effects of these treatments on the fungal microbiome colonising different horizons. This was the primary aim of the work described in this thesis.

Originally, we intended to analyse the allocation of C by the plants to fungal symbionts and by fungal symbionts to associated bacteria using $^{13}CO_2$ pulse labelling and stable isotope probing (SIP) of fungal DNA and RNA. DNA/RNA extraction and density

gradient centrifugation and fractionation was started, however due to the onset of the covid-19 pandemic this experimental work had to be halted and the project was adapted to involve bioinformatic analysis of samples that had already been sequenced at SciLIfeLab, Uppsala. In the present study bioinformatic and statistical analyses were performed to characterise the fungal communities occupying different soil horizons and analyse their response to differences in the availability of organic material. Patterns of alpha and beta diversity were also determined. The information gathered is an important complement to existing analyses of patterns of distribution of stable Mg isotopes in the same experimental system (Finlay *et al.*, 2020), and unpublished data on spatial patterns of carbon allocation following ^{13}C-labelling as well as the SIP analyses, when they are completed.

2. Introduction

2.1. Boreal forests

2.1.1. General characteristics, climate, dominant tree species

Geography, size, and location

Boreal forests are the largest of the forest biomes, accounting for one third of the world's forest area (15.09×10^8 ha) (Taggart and Cross, 2009). They are situated in the Northern hemisphere from 50 °N to 60 °N, covering much of North America and Eurasia, stretching around the globe in a circumpolar fashion (Bonan and Shugart, 1989).

Climate

The boreal climate is relatively cold, with a mean annual temperature of -5 to +5 °C with annual precipitation ranging between 20 and 200 mm. High rainfall, low rates of evaporation and the presence of bogs make boreal forests a valuable water resource (Bonan and Shugart, 1989). Winters are cold, dry and long with short days whilst summers are mild, wet and short with long days. The growing season is therefore short – typically only 3 months or less (Taggart and Cross, 2009). This combined with low temperatures and poor nutrients results in slow plant growth (Bonan and Shugart, 1989).

Origin and age

Fossil records of extant boreal forest species indicate that boreal forests originated between the late Cretaceous and early Tertiary periods. Global "icehouse" conditions after the Eocene epoch are thought to have driven the southward migration of montane conifer forest to lowland areas, giving rise to the boreal forests of today (Taggart and Cross, 2009). Though the youngest of the forest biomes, they are by far the most economically important as a key source of timber and biofuel (Egnell and Valinger, 2003; Egnell, 2011; Lundmark *et al.*, 2013).

Flora – Canopy, Understory and Floor

The flora of boreal forests is typically less diverse than that of other forests, with conifers of the family Pinaceae making up the majority of the canopy in climax communities (Lindahl *et al.*, 2007). Broad-leaved trees are also abundant, particularly in successional zones where climax communities have not yet been reached (Taggart and Cross, 2009). The dominant tree genera in boreal forests are; *Pinus* (pine), *Picea* (spruce), *Abies* (fir), *Larix* (larch), *Betula* (birch), *Alnus* (alder) and *Populus* (aspen) (Egnell and Valinger, 2003; Taggart and Cross, 2009). *Pinus*, *Picea* and *Abies* are evergreen – a life strategy that does not require the regeneration of foliage – a very energy demanding process. All three are present in climax communities (Axelsson and Bråkenhielm, 1980). *Larix* – the only non-evergreen conifer – *Betula*, *Alnus* and *Populus* are all deciduous, with larch present in climax communities, birch present in semi-climax and successional communities and alder and aspen present in successional communities (Taggart and Cross, 2009).

The understory consists of small, supressed trees and saplings, and ericaceous shrubs, resembling a post-fire successional community (Esseen *et al.*, 1997; Nilsson and Wardle, 2005). Forest floors are commonly dominated by bryophytes and lichens, with bryophytes preferring wetter habitats and lichens preferring drier habitats. Bryophytes play an essential role in suppressing growth by other plants, preventing seeds from reaching the soil and germinating, and shading seedlings that do succeed in germinating (Esseen *et al.*, 1997).

Fennoscandian boreal forests

Boreal coniferous forests are the dominant biome across Fennoscandia, situated between 56 °N and 69 °N (Esseen *et al.*, 1997). The dominant tree species in Fennoscandia are *Pinus sylvestris* (Scots pine) and *Picea abies* (Norway spruce) (Esseen *et al.*, 1997). Both species are capable of establishing populations in both dry and wet environments, with typical habitats including rocky outcrops, alluvial heaths, forested wetlands and mires (Esseen *et al.*, 1997). There is a small distinction in habitat preference between *Pinus sylvestris* and *Picea abies*, namely that the former is dominant on drier soils and the latter is slightly -more dominant on wetter soils (Esseen *et al.*, 1997).

The understory of Fennoscandian boreal forests typically contains small, suppressed trees and saplings; *Salix starkeana*, *Salix xerophila* and *Salix caprea* (all willows); *Juniperus communisis* (common juniper); and *Sorbus aucuparia* (rowan) – and dwarf shrubs; *Calluna vulgaris* (common heather), *Empetrum hermaphroditum* (crowberry), *Vaccinium vitis-idaea* (lingonberry) and *Vaccinium myrtillus* (European blueberry) (Esseen *et al.*, 1997; Nilsson and Wardle, 2005). On the forest floor *Hylocomium splenden* and *Pleurozium schreberi* (both feather mosses) form carpets in wetter areas,

with *Cladonia* and *Stereocaulon* (white-grey powder lichen) colonising drier areas (Ahti, 1977; Oksanen and Ahti, 1982; Ahti and Oksanen, 1990).

2.1.2. Soils- physical, chemical and biological features

Boreal forest soils commonly form podzols – stratified layers (referred to as horizons) which are physically and chemically distinct from each other. Horizons form due to the acidic nature of the soil (Rayner and Boddy, 1988), resulting in leaching and creating a hostile environment for earthworms – which are responsible for mixing in other soils. Podzols are composed of an organic horizon (O), overlying the mineral soil, that consists of an eluvial (E) horizon and an illuvial (B) horizon. Beneath the B horizon lies the parent material, or C horizon (Figure 1).

The O horizon overlies the E and B horizons and is formed from the organic litter shed by plants. With depth, the O horizon gradually transitions from distinguishable litter to humus becoming dark brown or black when fully decomposed. The organic substrates influence water retention, nutrient cycling and soil acidity, in turn influencing the productivity of a forest and the microbial communities present. The recalcitrant nature of leaf litter and plant debris in boreal forests provides a multitude of complex components to be utilised as nutrient sources, promoting microbial diversity (Bonan and Shugart, 1989). Decomposers such as saprotrophic fungi reside in the upper O horizon, where there is a plethora of fresh organic matter for them to degrade and utilise. Further down in the O horizon matter is depleted of carbon (C) through the action of saprotrophs. Here, mycorrhiza – fungal-root symbionts able to tap into an independent C source – dominate, outcompeting saprotrophs as they mine the substrate for nutrients. (Bonan and Shugart, 1989; Lundström *et al.*, 1999; Lundström *et al.*, 2000; Rosling *et al.*, 2003; Lindahl *et al.*, 2007; Baldrian *et al.*, 2012; Clemmensen *et al.*, 2013; McGuire *et al.*, 2013; Finlay and Clemmensen, 2017).

The E horizon is composed of recalcitrant minerals with a slow weathering rate. It lies beneath the O horizon and has very low levels of organic matter. Microbial degradation of minerals and high acidity results in the leaching of aluminium (Al), iron (Fe) and magnesium (Mg) from the E horizon, depleting it of heavy metals and leaving it ash-grey in colour. The B horizon is also composed of recalcitrant minerals. It too has low levels of organic material and lies beneath the E horizon. Al, Fe and Mg leached from the E horizon precipitate in the B horizon. The high levels of heavy metals give it a rich orange-brown colour. In the mineral horizons' ectomycorrhizal fungi – with an independent C source, and mineral-weathering bacteria and fungi – capable of mobilising base cations and phosphorus from minerals, dominate. (Bonan and Shugart, 1989; Lundström *et al.*, 1999; Lundström *et al.*, 2000; Rosling *et al.*, 2003; Lindahl *et al.*, 2007; Baldrian *et al.*, 2012; Clemmensen *et al.*, 2013; McGuire *et al.*, 2013; Finlay and Clemmensen, 2017).

The aforementioned microbial degradation of minerals and low acidity contribute to the process of podzolisation. Podzolisation is the process of stratification of soils into podzols. It is governed by a number of processes involving the formation and degradation of complexes through the actions of biological, mechanical and chemical weathering. There are two main theories of podzolisation; adsorption and precipitation theory; and biodegradation theory (see Figure 1). These two processes work in unison to influence podzolisation.

In the theory of adsorption and precipitation, high molecular weight (HMW) fulvic organic acids present in the O horizon leach downwards to the E horizon. In the theory of biodegradation, low molecular weight (LMW) organic acids exuded by plant roots and mycorrhizal hyphae in the E horizon are degraded by soil microbes, releasing Al and Fe ions into the substrate. Al and Fe ions form either AlSiOH and FeOH or they form complexes with the HMW fluvic organic acids. Al and Fe ions continue to be added to complexes as they continue to leach downwards until they reach a certain C/metal ratio where they precipitate in the B horizon. AlSiOH, MgOH and FeOH also precipitate in the B horizon (McKeague *et al.*, 1971; Petersen, 1976; Aristovskaya and Zykina, 1977; Lundström, 1993; Lundström *et al.*, 1995; Lundström *et al.*, 1999).

These processes contribute to the loss of metals such as Fe and Al in the E horizon and their build-up in the B horizon. The above processes, and therefore podzol formation, are influenced by climate, topography, parent material, soil age and the overlying flora (Jenny, 1941; Jauhianen, 1973; Lundström *et al.*, 2000).

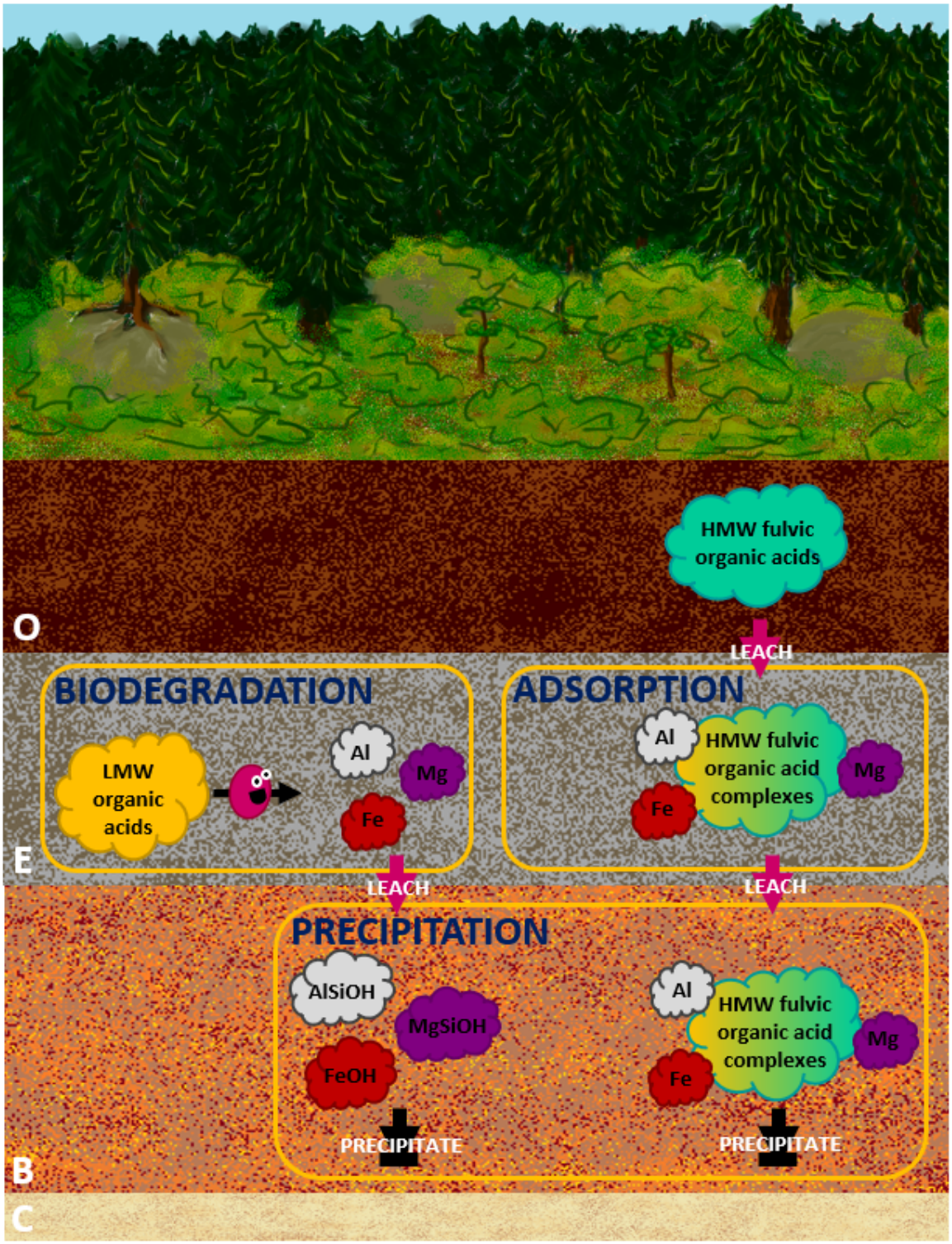

Figure 1 – Schematic diagram of the two contributing processes of podzolisation. Fungi and bacteria degrade low molecular weight (LMW) organic acids, releasing Fe, Mg and Al ions. In the adsorption and precipitation process ions released by biodegradation adhere to high molecular weight (HMW) fulvic organic acids (which leach down from the O horizon) to form HMW fulvic organic acid complexes in the E horizon. Ions also form oxides in the E horizon. Complexes and oxides leach down from the E horizon and precipitate in the B horizon. Below is the C horizon.

2.2. Biological components

A plethora of organisms exist in boreal forest soils including, archaea, bacteria, fungi and soil animals. Through their interactions with their biotic and abiotic environment they can directly and indirectly influence each other and the overall health of the forest. In boreal forests these interactions come into play with different organisms displaying beneficial, mutualistic, parasitic or pathogenic behaviour – or a combination of these. Behaviour is partially dependent on environmental conditions and can change with varying availability of nutrients, water and the condition of surrounding organisms and those they are associated with.

2.2.1. Archaea

Though poorly studied, archaea account for a significant proportion of microorganisms in boreal soils (Kemnitz *et al.*, 2007), with estimates of 10^5 archaeal cells g^{-1} in mycorrhizal soils and 10^2 archaeal cells g^{-1} in non-mycorrhizal O horizon soil (Bomberg, 2016). Like prokaryotes and eukaryotes, archaea are difficult to identify in the environment as very few are culturable.

Of the detectable archaea, the most common in boreal soils are members of the phylum Thaumarchaeota. Thaumarchaeota, previously included in the phylum Crenarchaeota, are nonthermophilic – unable to survive at high temperatures, or mesophilic – preferring temperatures between 20 and 45 °C (Kemnitz, *et al.*, 2007; Bomberg and Timonen, 2009; Offre *et al.*, 2013; Bomberg, 2016).

Group I.1c Thaumarchaeota are the most common archaea in acidic boreal soils. Groups I.1a and I.1b are also present, though to a lesser degree. Groups I.1a and I.1b have been reported to form non-specific associations with numerous plants, whilst group I.1c forms specific associations with boreal plants. Groups I.1a and I.1b have ammonia oxidation properties, but these properties have not been found in group I.1c. I.1c is however, prevalent in decaying wood in boreal forests, indicating a preference for organic nitrogen. (Bomberg *et al.*, 2011; Rinta-Kanto, *et al.*, 2016; Bomberg, 2016).

Archaea are more prevalent on plant roots colonised by ectomycorrhizal fungi compared to non-colonised roots. Furthermore, community composition between colonised and non-colonised roots, and between different ectomycorrhizal species differs. This indicates that relationships between ectomycorrhizal fungi and archaea may be specific which may impact community composition and plant and forest health (Bomberg and Timonen, 2008; Bomberg, 2016).

2.2.2. Bacteria

Boreal forest soils are relatively "unworked" compared to agricultural soils – their nutrient poor and acidic nature makes them unattractive for cultivation purposes, resulting in less human disturbance (Uroz *et al.*, 2011; Uroz *et al.*, 2016). This has enabled the establishment of diverse stable habitats, in which an abundance of bacteria reside. These habitats include decaying necromass, plant roots, mycelium and mineral surfaces.

Easily accessible nutrients are sparse, with most essential nutrients locked in recalcitrant matter (Uroz *et al.*, 2009). Bacteria in boreal forest soils display a diverse range of enzymatic capabilities beneficial to their colonisation and survival. Many of these enzymes aid in the degradation of recalcitrant substrates like plant cell walls, woody debris and minerals, releasing nutrients for utilisation by bacteria.

Actinobacteria, Acidobacteria, Proteobacteria dominate in boreal forest soils. Other common phyla include, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes and Verrucomicrobia. (Uroz *et al.*, 2013; Llado *et al.*, 2017).

Members of the Actinobacteria, Acidobacteria, Proteobacteria and Bacteroidetes have been shown to produce and exude carbohydrate-active enzymes, including glycosidic hydrolases and auxiliary activity enzymes (Žifčáková *et al.*, 2017). Such enzymes facilitate the degradation of C-rich polymers such as cellulose, chitin and plant and fungal exudates such as oxalates. These phyla therefore have high abundance in the O horizon.

Actinobacteria, Proteobacteria and Bacteriodetes are copiotrophic, with some species being oxalotrophic – better adapted to utilising C-rich organic substrates (Stursova *et al.*, 2012; Llado *et al.*, 2016; Llado *et al.*, 2017), and better adapted to surviving on oxalate (Sun *et al.*, 2019). Actinobacteria, Proteobacteria and Firmicutes possess ligninolytic capabilities, degrading woody substrates (Llado *et al.*, 2017). Acidobacteria grow better in acidic soils and are highly versatile, capable of surviving on C from a range of both easily degradable and recalcitrant matter (Llado *et al.*, 2017). Planctomycetes and Verrucomicrobia have been shown to utilise the exopolysaccharides of other bacteria, making them reliant on other bacterial communities for survival (Wang *et al.*, 2015; Llado *et al.*, 2017).

Though present in all soil horizons, Chloroflexi and Firmicutes are dominant within the mineral horizons as they are better adapted to mobilise nutrients from inorganic sources (Uroz *et al.*, 2016). Bacteria residing in the mineral horizons are often capable of mineral weathering. Biological mineral weathering is a key process in boreal forest soils, through which base cations and P are released from recalcitrant materials and made available to plants and microbes. Bacteria involved in mineral weathering produce organic acids and other chelating molecules, which can degrade minerals into smaller

usable components. This process involves oxidoreduction reactions, acidolysis and chelation. Genera known to possess mineral weathering capabilities include; *Agrobacterium*, *Arthrobacter*, *Streptomyces*, *Rhodococcus*, (Actinobacteria), *Burkholderia*, *Pseudomonas*, *Rhizobium* (Proteobacteria), *Pedobacter*, *Sphingobacteriaceae* (Bacteroidetes), *Bacillus*, *Lysinibacillus*, *Paenibacillus*, and *Viridibacillus* (Firmicutes) (Uroz *et al.*, 2009; Lepleux *et al.*, 2012).

Another key process is nitrogen (N) fixation. Whilst N is plentiful in the environment it is most commonly in the form of N_2, NO_2^- and NH_3 – unusable by plants and microbes (Blasko *et al.*, 2015). N fixation is the process by which unusable forms of N are converted into usable forms such as NO_3^- and NH_4^+ (Blasko *et al.*, 2015). Genera of bacteria capable of mitigating this process are; *Azorhizobium*, *Azospirillium*, *Azotobacter*, *Beijerincka*, *Bradyrhizobium*, *Burkholderia*, *Desulfobacter*, *Derxia*, *Methylocapsa*, *Methylocella* and *Methylococcus* – which are all Proteobacteria (Izumi *et al.*, 2006). In boreal forests cyanobacteria present in feather mosses are a main source of biological N fixation (Ackermann *et al.*, 2012).

2.2.3. Fungi

Appearance, strategies, biomass and numbers

Fungi are predominantly filamentous multicellular eukaryotes, growing by hyphal extension by anastomosis (Finlay and Thorn, 2019). There are 75,000 described species of an estimated 1.5 million (Finlay and Thorn, 2019). In individual boreal forest habitats as many as1000 taxa are typically found (Kyaschenko *et al.*, 2017) amounting to 1600-2500 kg/ha estimated biomass (Ekblad *et al.*, 1998; Yuan *et al.*, 2008; Wallander *et al.,* 2010; Clemmensen *et al.*, 2013; Sterkenburg *et al.,* 2015) and contributing at least 50% of total soil respiration (Högberg *et al.,* 2001). Fungi cannot photosynthesize and must therefore attain their C heterotrophically – primarily from plants. Three life strategies can be employed to achieve this; consume dead plant matter, i.e. decomposers; consume live plant matter through an exchange of resources, i.e. as mycorrhizal symbionts; or consume live plant matter by weakening or killing the host, i.e. pathogens.

Saprotrophs

The dominant decomposers in boreal forest soils are saprotrophic fungi. Saprotrophs reside primarily in the O horizon where they use plant litter as a C source. They can be categorised as fungi with primary resource capture strategies and fungi with secondary resource capture strategies – those which colonise a substrate in the absence of competitors and those which compete and succeed fungi already present, respectively (Woodward and Boddy, 2012). During the decomposition of litter, substrates are successively degraded from most easily degraded to most recalcitrant. Differences in

substrate quantity and quality, microbial community and abiotic factors affect both primary colonisation and succession (Rayner and Boddy, 1988). During competition, fungi release toxins, antifungal compounds and enzymes, and can interfere and parasitise at the hyphal level (Woodward and Boddy, 2012). Polymer-degrading saprotrophs include *Fusarium* and *Trichoderma* species and the soft-root fungi *Chaetomium* spp. and *Ceratosystis* spp. (Rayner and Boddy, 1988).

Brown-rot fungi degrade recalcitrant matter chemically by Fenton chemistry. Hydrogen peroxide, produced by brown-rot fungi, oxidise Fe^{2+}, releasing reactive oxygen species which degrade cellulose, hemicellulose and pectin. Prominent brown-rot fungi are *Serpula lacrymans* and *Schizophyllum commune* (Finlay and Thorn, 2019).

White-rot fungi also degrade recalcitrant matter by both enzymatic and chemical means. Cellulases, laccases and peroxidases degrade cellulose and lignin respectively (Baldrian, 2004; Finlay and Thorn, 2019). Manganese peroxidases oxidise manganese to Mn^{3+} which chelate with white-rot produced organic acids and form complexes. These complexes interact with phenolics and other compounds in the soil and disrupt the lignin structure, aiding degradation.

Mycorrhizal fungi

Mycorrhizal fungi are important symbionts in boreal forests. They are allocated C in the form of photoassimilates by their plant hosts. In exchange, mycorrhizal fungi acquire and transport nutrients and water to plant hosts. In all mycorrhizal associations, hyphae of the fungal symbiont form close connections with plant roots, maintaining an interface through which communication and nutrient exchange can occur. These hyphae extend into the soil, providing the plant host with access to nutrients it could not acquire by itself. Soil animals, pollen and saprotrophic mycelium have all been reported as sources of phosphorus (P) or nitrogen (N) utilised by mycorrhizal fungi (Lindahl *et al.*, 1999; Perez-Moreno and Read, 2001a, b; Klironomos and Hart, 2001; Finlay 2008). Frequently, exudates such as organic acids are released into the soil to aid degradation of substrates and make nutrients more accessible. In addition to gathering nutrients, mycorrhizal fungi are capable of priming the soil, by releasing pulses or a continuous stream of organic substances rich in C. This creates a nutrient-rich niche, attracting microbes. Many of these microbes possess beneficial qualities to the mycorrhizal fungi and the plant (Kuzyakov, 2010). As such it is important to study the interface between plant roots, mycorrhizal fungi and other microbial partners holistically as a ‘mycorrhizosphere’ (Finlay and Thorn, 2019).

Within the functional guild of mycorrhizal fungi there are different types including arbuscular mycorrhizal, orchid-, ectomycorrhizal and ericoid mycorrhizal fungi – each forming symbiotic relationships with different groups of plants. Arbuscular mycorrhizal

fungi form symbiotic relationships primarily with angiosperms and are the most widespread, and ancient, of the mycorrhizal groups, forming symbiotic relationships with 80 % of plants (Karandashov *et al.*, 2004; Smith and Read, 2008; Zeilinger *et al.*, 2015). Hyphae grow inter- and intracellularly establishing arbuscules – dichotomously branched hyphae which maximise surface area for transfer of nutrients. Orchid mycorrhizal fungi only form symbiotic relationships with orchids, forming a mantle around orchid seeds and providing nutrients during the early stages of development. In boreal forests ecto- and ericoid mycorrhizal fungi dominate.

In boreal forests, ectomycorrhizal fungi form symbioses mainly with members of the Pinaceae and Betulaceae families. Fungal hyphae colonise roots in an ectotrophic manner, forming dense sheaths around fine roots. Thick sheaths act both as storage organs for nutrients and physical and chemical barriers against non-beneficial microbes, such as pathogens and root grazers. Hartig nets develop between epidermal and cortical root cells acting as the interface between the plant host and fungus, providing a large surface area through which communication and transfer of nutrients can occur. Extraradical mycelia extend extensively into the soil, colonising and mining pockets of nutrients. The lower O horizon and E and B horizons are densely colonised by ectomycorrhizal fungi, able to mine nutrients in C poor conditions whilst supplied C by their plant host. The fungi can attain a mass of 160-500 kg/ha in boreal forest soils (Ekblad *et al.*, 1998; Yuan *et al.*, 2008; Wallander *et al.,* 2010; Clemmensen *et al.*, 2013; Sterkenburg *et al.,* 2015).

Ericoid mycorrhizal fungi form symbioses with Ericaceous shrubs, penetrating the root hair cells of their host plant and developing coil-like structures within. From here, ericoid mycorrhiza modify incoming nutrients into usable forms before transferring them to their plant host. Extraradical mycelia do not grow extensively into the soil, leading to the assumption that their role is directed towards converting nutrients into usable forms rather than maximising acquisition of nutrients from a large soil volume (Read and Perez-Moreno, 2000).

Pathogens

Pathogens of plants and fungi in boreal forest soils exploit their hosts, parasitizing them and in some cases killing them. Pathogens can be categorised into three groups: biotrophs, hemibiotrophs and necrotrophs. Necrotrophs use an aggressive chemical arsenal to bombard their target, overwhelming it. Cell wall degrading enzymes (CWDEs), reactive oxygen species (ROS) and toxins are produced, resulting in damage to the host organism's cells (Wang *et al.,* 2014). Biotrophs supress the host immune system by secreting effectors – this often being a very specific molecular interaction between pathogen and host (Perfect and Green, 2001; Yi and Valet, 2013). Hemibiotrophs initially behave as biotrophs, but later switch to a necrotrophic lifestyle (Struck, 2006; Gardiner, Kazak and Manners, 2013).

Biotrophic pathogens that are problematic in boreal forests include: *Erysiphe alphitoides*; *Phaeocryptopus gaeumannii*; and rust fungi of the genus *Melampsora*. Necrotrophic pathogens in boreal forests include: *Heterobasidion annosum*; *Armillaria* sp.; *Cryphonectria parasitica*; and *Cytospora chrysosperma* (Daniel *et al.*, 1998; Oliva *et al.*, 2014).

2.2.4. Soil animals

Soil animals in boreal forest soils exist mainly in the O horizon where there is a rich source of organic C, in the form of detritus, plants, fungi and other animals. Microfauna in boreal forest soils include; the Rotifera; the Nematoda; the Tardigrada; the Collembola; the Acari; and the Enchytracidae (Huhta *et al.*, 1998).

Rotifera are worm-like cyst-forming vortex feeders, numbering up to 10^5 m^{-2} in organic soils. Nematoda are also worm-like in their appearance and are amongst the most numerous of the microfauna (4.1×10^6 m^{-2}) (see Coyne, 2001). Nematodes (members of Nematoda) can be herbivores, fungivores, bacterial predators, animal predators and omnivores. Herbivores and fungivores have been shown to possess cell wall degrading enzymes and to reside mainly in the rhizosphere. Tardigrada are "water-bears", hardy organisms which can survive in extreme conditions. These reside in the upper 10 cm of the soil and number on average 200 m^{-2}. Collembola are springtails, numbering at more than 10^5 m^{-2} and feeding on fungi. Mites (phylum Acari) and have been measured to number 500/100 g soil. Acari may be herbivores, fungivores, detrivores, parasites surviving on other animal or predators. Enchytracidae are small unpigmented worms capable of surviving on both organic and mineral soil. Enchytracidae have been estimated to number between 100 and 140,000 m^{-2}.

Macrofauna include; the Isopoda – woodlice, surviving mainly on detritus; the Diplopoda – millipedes living as saprophages; the Chilipoda – centipedes, living in a predaceous manner; the Scorpionida – scorpions living as predaceous carnivores; the Araneae – spiders, also living as predaceous carnivores; the Opiliones – harvestmen, residing and predating in the litter; the Coleoptera – beetles, surviving as herbivores, fungivores detrivores and carnivores; the Hymenoptera – ants, also surviving as herbivores, fungivores detrivores and carnivores; and the Diptera – flies, feeding on plants, fungi and detritus. (Coleman *et al.*, 2018).

2.3. Biological interactions

Biological interactions are traditionally viewed as a two-way "conversation" between two organisms, but realistically interactions occur over a complex network with diverse

inputs from multiple sources contributing to a single organism's actions (Deveau *et al.*, 2018).

For simplicity, selected components of the vast network of interactions are discussed whilst bearing in mind that they are only a part of a wider interaction.

2.3.1. Plant-fungal interactions

Plants and fungi have a multitude of interactions, influencing each other and their environment. These interactions can be mutualistic –both parties benefiting equally, beneficial – one or more parties benefiting, parasitic – one part benefitting at the expense of the other, or pathogenic – one party exploiting the other until it dies. Depending on environmental conditions, developmental stages and health of participating parties and the physical and chemical dialogue between them, the nature of these interactions can change (Grigoriev, 2013; Deveau *et al.*, 2018). Plants interact with mycorrhizal fungi, saprotrophs and pathogens – all members of diverse fungal communities residing in boreal forest soils.

Plants and fungi begin their interactions in the soil. From their roots, plants exude LMW molecules; ions, free oxygen, amino acids, organic acids, sugars and phenolics, and HMW molecules; mucilage and proteins (Buscot *et al.*, 2000; deBoer *et al.*, 2005; Bais *et al.*, 2006; Zeilinger *et al.*, 2015). The abundance of these exudates makes the rhizosphere a rich source of nutrients, attracting many microbes and potential fungal partners (Philippot *et al.*, 2013). Some volatile exudates exhibit antimicrobial and antiherbivore qualities toward unfavourable microbes (Baetz and Martinoia, 2014; Zeilinger *et al.*, 2015).

Fungi in the vicinity of the rhizosphere grow chemotropically towards it, attracted by plant exudates. Plants identify the array of molecules exuded by fungi through cell surface localised pattern recognition receptor (PRR) proteins. Fungi are identified as non-threatening or threatening based on the composition of the molecular array they exude, with non-threatening fungi by displaying microbial-associated molecular patterns (MAMPs) and pathogenic fungi displaying pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Depending on the fungi perceived, plants will either allow colonisation or increase their defences, producing more phenolics and reactive oxygen species (ROS) and increasing the expression of defence-related genes (Zeilinger *et al.*, 2015). Mycorrhizal fungi and some pathogens are capable of masking or supressing defence responses of their target host plant to aid their colonisation (Zamioudis and Pieterse, 2012; Zeilinger *et al.*, 2015). In some cases, enzymes are secreted to degrade physical barriers and detoxify toxic components. Chemical messengers capable of interfering with plant signally pathways may be produced, helping to obscure fungal identity (Zeilinger *et al.*, 2015).

If a non-beneficial fungus gets past initial defences, plants have an arsenal of strategies to protect themselves. Fungi that fail to supply nutrients to their host plant can be "sanctioned" through regulation of hexose transporters, leading to redirection of sugars to the host rather than the colonising fungus (Nehls, 2008). Detected pathogenic fungi are exposed to ROS, phenolics and terpenes. Host plants increase the expression of defence-related genes and may enter hypersensitive response (HR) inducing the death of cells surrounding the infection site. An increased incidence of cross-linking cell wall proteins may also occur, strengthening the cells physical defences against pathogens.

Mycorrhizal fungi support their host plants, supplying them with water and nutrients in exchange for photoassimilates in the form of sugars (Brownlee *et al.*, 1983; deBoer *et al.*, 2005; Finlay, 2005; Finlay, 2008; Zeilinger *et al.*, 2015; Finlay and Clemmensen, 2017; Deveau *et al.*, 2018). Mycorrhizal fungi can also support plant defences, physically and chemically (Zeilinger *et al.*, 2015). In the case of ectomycorrhiza, a thick fungal sheath forms, physically shielding the plant root from pathogens and grazers (Smith and Read, 2008). Mycorrhizal fungi also provide chemical protection, detoxifying harmful compounds, removing antibiotics and in some cases altering gene expression of assailants (Duffy *et al.*, 2003; deBoer *et al.*, 2005). Endophytes, (fungal endosymbionts), also form symbiotic relationships with plants. Mycorrhizal fungi and endophytes provide additional resistance to stress by influencing the gene expression of defence or stress-tolerance genes (Strobel and Daisy, 2003; Brundett, 2004; Zeilinger *et al.*, 2015).

Fungal partners of plants also release exudates into the soil. Enzymes, ROS and peroxidases degrade substrates, releasing key nutrients like N, phosphorus (P) and potassium (K) from the soil. Sugars and other energy rich molecules are also released into the soil, attracting microbes that aid in substrate degradation, nutrient mobilisation and defence (Deveau *et al.*, 2018). In this way plants and their fungal partners can cultivate their surrounding communities in the "mycorrhizosphere".

Pathogens can also evade plant defences, either colonising or directly degrading their hosts. Necrotrophs use cell-wall degrading enzymes (CWDEs), ROS and toxins to chemically and enzymatically degrade the cell walls of their target. This causes the cell contents to erupt which the necrotroph then utilises as nutrients. (Wang *et al.*, 2014). Biotrophs secrete effectors which interact with the plant to suppress its immunity, enabling the biotroph to colonise its target host and exploit it (Perfect and Green, 2001). These interactions are usually very specific, between two single species, as a high level of specificity is required to influence the hosts immune system (Yi and Valet, 2013). Hemibiotrophs begin interactions as biotrophs and switch to a necrotrophic lifestyle at a later stage (Struck, 2006; Gardiner *et al.*, 2013). It is unclear whether this transition is due to the failure of the biotrophic strategy and the successive transition to necrotrophy, or a successful biotrophic infection followed by necrotrophy once the plant is weakened or when the fungus in question intends to propagate (Zeilinger *et al.*, 2015). Mycorrhizal

fungi and pathogens use appressoria to physically adhere to and penetrate the host cell wall. This is common in arbuscular- and orchid mycorrhizal fungi, as well as pathogens (Emmet and Parbery, 1975; Zeilinger *et al.*, 2015).

Symbiotic, decomposer and pathogenic fungi compete for space, resources and beneficial partners, leading to complex interactions within these communities which can be beneficial, mutualistic or antagonistic (Finlay, 2008). Gadgil and Gadgil (1971, 1978) showed that ectomycorrhizal fungi supress decomposition by saprotrophs in forest soils. Saprotrophs decompose organic matter to gain C and mycorrhizal fungi gain C from their plant hosts, but both groups of fungi compete for N (Lindahl and Tunlid, 2014). In the presence of ectomycorrhizal fungi, saprotrophs gain a competitor for substrate – with ectomycorrhizal fungi decomposing organic matter to attain N and P and saprotrophs decomposing to attain C (Lindahl and Tunlid, 2014).

2.3.2. Fungal-bacterial interactions

Fungi dominate terrestrial habitats, being better adapted to dry conditions. In soils, air between substrate particles prevent single-celled organisms from moving freely. Fungi are well adapted to this, with hyphae able to cross these gaps (de Boer *et al.*, 2005). Fungi therefore have an advantage over bacteria.

Bacteria compete by way of antifungal strategies. Bacteria weaken fungi by producing fungal inhibitory factors, lytic enzymes and volatiles, and strengthen their ability to gather valuable substrate by producing iron-chelating siderophores (Handelsman and Stabb, 1996; Whipps, 2001; Weller *et al.*, 2002; Wheatley, 2002; de Boer *et al.*, 2005). Fungi counteract by influencing bacterial gene expression, detoxifying bacterial volatiles and washing away antibiotics by efflux (Duffy *et al.*, 2003; de Boer *et al.*, 2005).

Whilst fungal exudates can play a defensive role, they can also attract bacteria. So-called “helper” bacteria have been reported to assist mycorrhizal colonisation of their plant host (Labbe *et al.*, 2014 Deveau and Labbe, 2016). Mineral weathering and decomposer fungi often form associations with mycorrhizal fungi, degrading substrates in return for energy-rich fungal exudates (Koele *et al.*, 2009). Endosymbionts also assist fungi, influencing gene expression to better withstand stresses (Strobel and Daisy, 2003; Brundett, 2004; de Boer *et al.*, 2005; Zeilinger *et al.*, 2015).

Fungi also assist bacteria, providing “mycelial highways” which bacteria can use to move more easily through the soil (Warmink *et al.*, 2011). Fungi also provide new niches – mycelia, fruiting bodies, spores and mycorrhizal fungi can all be colonised by bacteria, with different species of fungi offering different niches (de Boer *et al.*, 2005). Each niche harbours distinct microbial communities which likely contributes to the biology of the organism colonised (Zagriadskaia *et al.*, 2013; Deveau *et al.*, 2016; El-Jurdi and Ghannoum, 2017; Deveau *et al.*, 2018). These niches are moist – providing a water film

rich in exudates which allows bacteria to move around easily (Neal *et al.*, 1964; Oswald and Ferchau, 1968; Timonen *et al.*, 1998; deBoer *et al.*, 2005). In addition to exudates, bacteria can utilise fungal necromass as an energy source, particularly in the E and B horizon where there is less organic material (de Boer *et al.*, 2005).

Bacteria do not only aid, but also exploit fungi. Bacteria are can degrading simple polymers (e.g. chitin or cellulose), however fungi are better at degrading more complex substrates (e.g. cell walls). Mobile hyphae of saprotrophs physically penetrate woody substrates, breaking lignin and cellulose apart, making it available for bacteria to utilise. ROS, peroxides and lytic enzymes of fungi degrade substrates releasing cell contents and breaking polymers into sugars. Bacteria can then access these nutrients and compete for fungal-produced sugars (de Boer *et al.*, 2005).

Bacteria also directly use fungi as an energy and nutrient source through macrophagy. Filamentous bacteria, e.g. *Streptomyces* sp., produce CWDEs which results in the penetration of fungal cells. Non-filamentous bacteria, e.g. *Myxobacteria* sp. and *Paenibacilli* sp., produce lytic enzymes and spread in a biofilm on the fungus surface. Both of these strategies can result in partial damage or death of the target fungus (Deveau *et al.*, 2018).

2.4. Biogeochemical processes

2.4.1. Carbon flow

Plants are autotrophs, capable of using solar energy and atmospheric CO_2 to make C-rich photoassimilates that are used for growth, resulting in structural and non-structural organic substrates of differing recalcitrance that can be used by other groups of organisms (Finlay and Clemmensen, 2017). Boreal forests are an important sink for atmospheric CO_2, harbouring 625 Pg (petagrams) of soil C with just 78 Pg shared between other forests globally (Kasischke, 2000; Taggart and Cross, 2009).

Non-structural forms of C can enter the soil through plant and fungal exudates, and physical disturbance leading to damage of roots and mycelium, and extraction of plant-derived C by opportunists (Lindahl *et al.*, 2010). Plants secrete exudates into the soil from their root tips, functioning both as a form of defence and as priming agents that stimulate rhizosphere colonisation by beneficial microbes. Sugars, organic acids and other non-structural C rich molecules are utilised by these microbes for energy (Buscot *et al.*, 2000; deBoer *et al.*, 2005; Bais *et al.*, 2006; Zeilinger *et al.*, 2015). Plants supply mycorrhizal fungi with photoassimilates in the form of sugars in exchange for water and nutrients which the fungal symbionts can then use to build structural C, respire, produce chemicals

for their own use and as exudates, and pass on to their associated microbes in exchange for services (deBoer *et al.*, 2005; Finlay, 2005; Finlay, 2008; Zeilinger *et al.*, 2015; Finlay and Clemmensen, 2017; Deveau *et al.*, 2018). Pathogens effectively "steal" photoassimilates from plants by either disguising themselves as "friendly" microbes or destroying cell walls and feeding on the cell contents (Perfect and Green, 2001; de Boer *et al.* 2005; Wang *et al.*, 2014; Zeilinger *et al.*, 2015). In each of these scenarios, the receiver of photoassimilates may use them to make other metabolic compounds or build structural C.

Structural C used by plants, fungi and bacteria can enter the soil in the form of necromass. There, it is slowly degraded by bacteria and saprotrophs – the components used for energy and to build structural polymers like chitin and glucans (Lindahl, Taylor and Finlay, 2002). Non-structural C can also enter the soil in the form of enzymes produced by plants – to defend, and fungi and bacteria– to defend and degrade substrates (de Boer *et al.*, 2005; Zeilinger *et al.*, 2015).

Fungal mycelium is an important C sink with an estimated biomass of 400-900 kg/ha with 125-200 kg/ha being produced annually (Wallander *et al.*, 2001; Wallander *et al.*, 2004; Ekbland *et al.*, 2013). Complete turnover is estimated to occur once every 6-24 months (Brunner *et al.*, 2012; Finlay and Clemmensen, 2017) and mycelium has been reported to contribute 40-50 % of total C stocks in soils by Högberg and Högberg (2002).

Some bacteria and fungi are capable of building and degrading minerals. Minerals are a store of nutrients which fungi and bacteria utilise in place of organic substrates. In mineral soils, where organic matter content is low, minerals can act as nutrient reserves (Koele *et al.*, 2009; Uroz *et al.*, 2009; Lepleux *et al.*, 2012).

Whilst plants fungi and bacteria are key sinks of C, they also release C in the form of CO_2 during respiration (Lindahl *et al.*, 2002). Fungi are reported to account for 50 % of total soil respiration by Högberg *et al.* (2001) with plants and bacteria making up the remaining half. Figures on soils animals are unclear due to studies focusing on only a few species at a time, however they do appear to have a stimulating effect on the respiration levels of other organisms (Huhta *et al*., 1998). Levels of respiration compared to C sequestration are dependent on the species involved and their relationships with one another. An increase in nutrient acquisition by mycorrhizal fungi may increase plant biomass in the form of structural polymers but respiration by mycorrhizal fungi may also increase. Saprotrophic fungi may release more or less C depending on the substrate they are degrading. Additionally, respiration levels will fluctuate with growth rate and exudate production. As such, C flow in forest ecosystems is a complex, ever-fluctuating process (Lindahl *et al.*, 2002).

2.4.2. Weathering and pedogenesis

All nutrients required by plants, apart from C and N, originate from minerals, making weathering and pedogenesis key processes in boreal forest soils (Smits and Wallander, 2017). Weathering and pedogenesis are biogeochemical processes which occur simultaneously. Weathering involves the disintegration of rock into its smaller parts, the dissolution of minerals into solutions and the precipitation of mineral compounds forming secondary minerals – all of which contribute to the creation of soil: pedogenesis. Both processes are influenced by rainwater, temperature, oxidative conditions and microbial community activity and composition (Uroz *et al.*, 2009; Leake and Read, 2017; Smits and Wallander, 2017).

Physical agents of mineral weathering are thermal stresses and mechanical forces (Smits and Wallander, 2017). Mechanical forces include: the freezing and thawing of water in cracks; growth of and penetration by plant roots; and the growth and expansion of hyphae through osmotic pressure of up to 20 μN/μm (Howard *et al.*, 1991; Bonneville *et al*., 2016). The physical fragmentation of rock increases the surface area of minerals aiding and accelerating chemical and biological weathering.

Chemical weathering disrupts the ionic bonds between elements, leading to the dissolution of minerals. Ionic bond disruption can be mediated by cation-driven and anion-driven reactions. The soil solution provides a liquid environment which facilitates chemical reactions such as hydrolysis, a proton-driven reaction involving the attack of ionic bonds by protons and hydroxides (Smits and Wallander, 2017). An important limiter of chemical weathering is the formation of complexes on mineral surfaces (Furrer and Stumm, 1986; Wieland *et al.*, 1988; Smits and Wallander, 2017). This can be mitigated by the inclusion of microorganisms.

Physical and chemical weathering is slow, but biological factors accelerate it (Smits and Wallander, 2017). Biological weathering is similar to physical and chemical weathering, but it is instigated by the activities of plants, fungi, bacteria and other organisms (Smits and Wallander, 2017). Plant necromass and debris contribute to the acidic nature of the soil aiding weathering (Rayner and Boddy, 1988; Leake and Read, 2017). The selective uptake of water and elements by plants both alters the chemical composition of the soil and provides a sink, influencing weathering and preventing the build-up of mineral compounds (van Schöll *et al.*, 2006a; 2006b).

Both plants and fungi produce exudates used for communication, degradation of substrates and priming the soil for associative organisms (Uroz *et al.*, 2009; Leake and Read, 2017). These exudates alter the pH of the surrounding soil, influencing mineral weathering – though it is unclear whether this is a primary function of the exudates, or a side-effect (Hoffland *et al.*, 2004; Rosling, 2009; Schmalenberger *et al.*, 2015; Smits and

Wallander, 2017; Finlay *et al.*, 2020). Organic acids such as oxalic and citric acid, and protons are strong weathering agents, capable of interfering with the ionic bonds between elements in minerals. Oxalic and citric acids can be deprotonated to oxalates and citrates respectively, which also interfere with ionic bonds (Smits and Wallander, 2017).

Along with organic acids and protons, fungi (and bacteria) exude siderophores – compounds capable of forming complexes with metal ions – an important component of many minerals (Handelsman and Stabb, 1996; Whipps, 2001; Weller *et al.*, 2002; Wheatley, 2002; de Boer *et al.*, 2005; Ahmed and Holmström, 2014; Kraemer *et al.*, 2014; Smits and Wallander, 2017; Finlay *et al.*, 2020). Fungi also produce extracellular polymeric substances (EPS) which form structural mats on mineral and substrate surfaces. These structures provide a fluid construct for fungi and bacteria to adhere to, leading to the formation of biofilms. These biofilms enable close proximity to weatherable mineral surfaces and a high concentration of weathering agents and signalling molecules, facilitating communication and quorum sensing (Balogh-Brunstad *et al.*, 2008; Saccone *et al.*, 2012; Smits and Wallander, 2017; Finlay *et al.*, 2020).

Mycorrhizal and bacterial activities to increase mineral weathering increase the nutrients available to plants, which in turn increases plant biomass. Bigger and healthier plants allocate more photoassimilates to their partners, supporting a more extensive mycelial and bacterial network capable of a greater production of weathering agents. As weathering products build up, they either inhibit the production of weathering agents or are removed by mycorrhizal mycelium (Leake and Read, 2017). This balance highlights the importance of biological components, mineral weathering and forest health to each other.

2.4.3. Organic matter decomposition

A combination of chemical reactions and enzymatic activity is utilised in decomposition. Decomposition is a vital process to both C and nutrient circulation (Lindahl *et al*, 2007; Gadd, 2017; Op De Beeck *et al.*, 2020) and primarily takes place in the O horizon. It is largely driven by saprotrophic fungi (see section 2.2.3) though bacteria and mycorrhizal fungi also play a role (de Boer *et al.*, 2005; Rineau *et al.*, 2012). Substrates are decomposed from easiest to degrade to most difficult to degrade. A succession of saprotrophic fungi converges with the order of substrates decomposed beginning with pioneer saprotrophs and followed by soft-rot fungi, brown-rot fungi and finally white-rot fungi (de Boer *et al.*, 2005). Two important chemical reactions that occur during decomposition by fungi are the Fenton reaction, used by brown-rot fungi, and peroxidase reactions, used by white-rot fungi (de Boer *et al.*, 2005). The Fenton reaction (see below), requiring Fe ions (Op De Beeck *et al.*, 2020), produces free radicals – unstable atoms with an incorrect number of electrons. These free radicals then interact

with surrounding material and loan electrons from bonds in cellulose, hemicellulose and lignin polymers, breaking the bonds and degrading the polymers (de Boer *et al.*, 2005).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$$

Cellulose and hemicellulose are depolymerised by free radicals, cleaving them randomly along the chain (de Boer *et al.*, 2005). This allows greater accessibility to enzymes produced by brown-rot fungi, and other microbes. Free radicals do not directly degrade lignin, but its side-chains. Fenton chemistry is commonly used by brown-rot fungi but has also been found in ectomycorrhizal fungi, thought to facilitate access to N present in organic matter. This further accelerates decomposition, breaking up large polymers and making them more accessible to other microbes. (Rineau *et al.*, 2012; Op De Beeck *et al.*, 2018; Op De Beeck *et al.*, 2020)

Peroxidase reactions are used primarily by white-rot fungi during the degradation of wood material and take place in aerobic conditions (Kirk and Farrel, 1986; de Boer *et al.*, 2005; Op De Beeck *et al.*, 2020). White-rot fungi produce enzymes with high oxidation potential (Op De Beeck *et al.*, 2020). These include lignin peroxidases, manganese peroxidases, phenol peroxidases and H_2O_2 peroxidases which work in concert to degrade woody substrates (Kirk and Farrel, 1986). Some ectomycorrhizal fungi, descended from fungi with saprotrophic abilities, have retained genes for the production of manganese peroxidases (Bödecker *et al.*, 2014; 2016). Peroxidases induce the addition of O_2 to C-centered radicals, produced during the cleaving of polymers, and one-electron oxidation and reduction. In the absence of O_2 radicals they interact with each other forming complexes, thus preventing them from interacting with substrates and slowing decomposition. These reactions are ruled by the kinetics of the environment and their occurrence is therefore somewhat random. The diversity of reactions which take place explains the diversity of decomposition products found. (Kirk and Farrel, 1986).

2.5. Forest management

2.5.1. Weathering & base cation supply

Base cations are key nutrients present in soil. They have roles in many processes and are taken up by plants and fungi. Weathering breaks down minerals, releasing base cations into the soil ready for uptake. Weathering alone, however, is not able to replenish base cation stocks at the rate they are depleted (Brandtberg and Olsson 2012). Decomposition ensures the return of base cations removed by plants and fungi, thus

replenishing the soil stock. It is therefore important that dead plants and fungi remain at forest sites.

Today, most forests are heavily managed, with perimeters and sites of succession carefully managed, floods mitigated, fires quelled and trees harvested by the forestry industry (Carlson *et al.*, 2015; Kellomaki, 2017). These factors, combined with climate change, place strain on forest ecosystems (Sverdrup and Rosen, 1998). The forestry industry operates by conventional stem-only harvesting (CH), whole tree harvesting (WTH) or whole tree harvesting and stump removal (WTHSR), all of which disturb the circulation of base cations in forest ecosystems (Lundmark *et al.*, 2013). Forestry is strictly regulated in accordance with various Forest Acts in different countries.

Currently WTH and WTHSR are gaining popularity as opportunities to create sustainable biofuels (Egnell and Valinger, 2003). WTH is different from CH in that branches and foliage are harvested in addition to the trunk. WTHSR takes this one step further and removes the stumps as well (Akselsson *et al.*, 2007; Merilä *et al.*, 2014). Foliage, branches and roots are rich in base cations and the complete extraction of trees removes 2-4 times more base cations than CH (Joki-Heiskala *et al.*, 2003; Brandtberg and Olsson, 2012).

Presumably as a result of this, conifer growth rates in WTH and WTHSR stands have been observed to be reduced when compared to CH stands (Proe *et al.*, 1996; Egnell and Leijon, 1999; Egnell, 2011). WTH and WTHSR have also been reported to increase soil acidification, decrease soil nutrients, and mineralisation of C and N (Nykvist, 1985; Staaf and Olsson, 1991; Olsson *et al.*, 1996; Johnson and Todd, 1998; Belleau *et al.*, 2006; Thiffault *et al.*, 2006, 2011; Smolander *et al.*, 2008; Wall, 2008; Walmsley *et al.*, 2009; Saarsalmi *et al.*, 2010; Vanguelova *et al.*, 2010; Brandtberg and Olsson, 2012).

Processes such as mineral weathering, N fixation and deposition release base cations and other nutrients, however the rate of uptake by plants and fungi far exceeds the rate of base cation release. This can lead to long-term exhaustion of resources in forest soils negatively impacting future growth rates and forest health (Svedrup and Rosén, 1998; Akselsson *et al.*, 2007). Additionally, an absence of base cations can lead to soil acidification (Akselsson *et al.*, 2016). However, forest ecosystems can rectify imbalances induced by CH, WTH and WTHSR through nutrient cycling processes if given sufficient recovery time (Brandtberg and Olsson, 2012). Mineral weathering rates are capable of accelerating, replenishing the deficit in base cations and other nutrients following CH, WTH and WTHSR (Högberg *et al.*, 2006; Brandtberg and Olsson, 2012). Often however, natural processes alone are not enough, and artificial measures are taken to increase base cations and nutrients to healthy levels. In Sweden for example, the Swedish Forest

Agency recommends the deposition of wood-ash used in biofuels and artificial N fertilisation (Anonymous, 2008).

2.5.2. N deposition

Nitrogen is the major growth limiting factor in plants and fungi (Merilä *et al.*, 2014; Sponseller *et al.*, 2016). Growing boreal forests can take up 10-50 kg N ha^{-1} $year^{-1}$ (Sponseller *et al.*, 2016) acquiring accessible N from soil. N sources come from atmospheric deposition, bacterial N fixation and organic matter decomposition (Merliä *et al.*, 2014; Sponseller *et al.*, 2016). Soil stores of N have been reported to range between 1000 and 8000 kg N ha^{-1} (Egnell *et al*, 2015a,b; Sponseller *et al.*, 2016), however, most of this is bound in complex recalcitrant matter and therefore inaccessible. To release usable forms of N, recalcitrant matter must first be degraded, making this process a growth rate limiting factor (Schimel and Bennet, 2004).

Human activity heavily influences the global N cycle – particularly through the intensive use of fertilisers and large N emissions (Galloway *et al.*, 2008; Sponseller *et al.*, 2016). The effect of these activities has resulted in a large loss of biodiversity (Bobbink *et al.*, 2010) and soil acidification (Moldan and Wright, 2011). Large additions of N can have a large impact on microbial communities, greatly reducing their diversity, particularly in boreal forests (Marroufi *et al.*, 2015). Activities such as forestry remove large quantities of organic matter from their natural environment, disturbing the normal cycling of N and reducing the amount of N returning to the soil (Merlilä *et al.*, 2014; Sponseller *et al.*, 2016). This has long term effects on tree growth in areas of repeated tree removal, potentially slowing the growth rates of stands in the future. It has been reported that stands subjected to CH are capable of recovering normal N levels with time, however, WTH and WTHSR are not (Akselsson *et al.*, 2007; Merilä *et al.*, 2014). This is supported by numerous studies using ecosystem models which predict a similar outcome (Bengtsson and Wikström, 1993; Rolff and Ågren, 1999; Brandtberg and Olsson, 2012).

Large stocks of N exist in understory vegetation and humus and, given sufficient time, natural processes – bacterial N fixation, mineral weathering, decomposition of organic matter and atmospheric deposition – can return accessible N stocks to normal levels (Gundale *et al.*, 2013; Merilä *et al.*, 2014; Maaroufi *et al.*, 2015). This, however, may not be possible in stands subjected to WTH and WTHSR (Akselsson *et al.*, 2007; Merilä *et al.*, 2014). The stumps, branches, roots and foliage which remain in CH have been reported to account for 50-70% of whole tree N stock by Merilä *et al.* (2014), significantly decreasing the recovery time of these stands.

In some cases, particularly those of WTH and WTHSR, artificial measures are taken to aid N stock recovery. As stated above, wood-ash deposition and N fertilisation are recommended and commonplace (Anonymous, 2008; Brandtberg and Olsson, 2012),

however, N fertilisation, whilst in many cases beneficial, can have repercussions. A beneficial outcome of N fertilisation is that an increase in N leads to an increase in C stocks in trees and soils with reports of 26 kg C kg^{-1} N (Gundale *et al.*, 2014; Merilä *et al.*, 2014; Marroufi *et al.*, 2015; Sponseller *et al.*, 2016). Elevated N has also been reported to raise soil respiration and C turnover (Hasselquist *et al.*, 2012; Sponseller *et al.*, 2016). If too much N is deposited it can lead to a decrease in biomass and respiration rates in microbial and fungal communities (Janssens *et al.*, 2010; Merilä *et al.*, 2014; Marroufi *et al.*, 2015). The use of fertilisers must therefore be in line with policies guided by research to prevent unnecessary damage to forest ecosystems (Sponseller *et al.*, 2016).

2.5.3. Climate change

Boreal forests play a critical role in the regulation of carbon and nitrogen cycles, and global temperatures (Bhatti *et al.,* 2003), however, they are also very sensitive to climactic change (Kuusela 1990; Ogden and Innes, 2007). As forests adapt or become damaged in the face of climate change, their ability to function in these roles may become impaired.

Global temperatures are rising at a rapidly quickening pace. Mean temperatures have increased by 1°C since 1900 (Gauthier, *et al.*, 2014), and are predicted to increase by a further 1.4 – 5.8 °C by 2100 (IPCC 2001, 2007a; Ogden and Innes, 2007; Gauthier, *et al.*, 2014). These predicted temperature increases, combined with other consequences of climate change, will occur at a faster rate and by larger magnitudes than forests have faced before (IPCC, 2001, 2007a,b,c; Gauthier, *et al.*, 2014). Northern latitudes are expected to suffer the biggest increase in temperature, predicted to be 40 % more than the global mean temperature increase (IPCC 2001, 2007a; Ogden and Innes, 2007; Macias-Fauria and Johnson, 2008; Gauthier, *et al.*, 2014).

Distribution of organisms is predicted to be affected by changes in local environmental conditions, induced by climate change (Nitschke and Innes, 2006; Ogden and Innes, 2007; Nitschke and Innes, 2008). Organisms will move to remain in preferential conditions, resulting in tree shifts (Ogden and Innes, 2007) that will alter natural insect dynamics (Volney and Fleming, 2000; Volney and Hirsch, 2005; Ogden and Innes, 2007). Non-native invasive species may find changes in local environmental conditions favourable and outcompete native species or disrupt ecosystem balance (Iverson and Prasad, 2001; ACIA, 2004; Ogden and Innes, 2007).

Boreal forests are expected to migrate northwards and to higher latitudes to remain in cooler climates (Ogden and Innes, 2007; Nitschke and Innes, 2008). Climate change may result in quicker shifts than species dispersal rates can keep up with (Malcolm and Markman, 2000; Malcolm *et al.*, 2002), meaning some species may be lost with the rapidly changing climate. Additionally, changes in climate may disturb the timing of

flowering, seed production and dispersal and insect life cycles – disrupting pollinator activity and further impeding species dispersal abilities (Ogden and Innes, 2007).

Climate change may increase the frequency and intensity of disturbances occurring in forests (ACIA, 2004; Ogden and Innes, 2007). There may be an increase in both fires and the duration of the fire season (Macias-Fauria and Johnson, 2008; Gauthier, *et al.*, 2014); an increase in the incidence of disease and attack by pest insects (ACIA, 2004; Ogden and Innes, 2007; Gauthier, *et al.*, 2014); competition for resources and habitat by invasive species (Iverson and Prasad, 2001; ACIA, 2004; Ogden and Innes, 2007); and extreme weather events such as flooding, drought and storms (Gauthier, *et al.*, 2014). Additionally, poor soil conditions like excessive dryness or wetness caused by fires and extreme weather events will impede reestablishment of species (Hogg and Wein, 2005).

The effects of climate change on forest health are already apparent and are heavily linked to rising temperatures. Current strategies employed involve the improvement of forest management and continuously adapting to the current situation (Ogden and Innes, 2007). Better knowledge on the effect of climate change on all forest components is key to success in both strategies.

2.6. Aims and Hypotheses of this Study

The aim of the present study was to examine whether there are differences between fungal communities colonising distinct soil horizons of boreal forest podzol from Jädraås, Sweden and to investigate the effect of successive depletion of organic horizon material (intended to simulate intensified forest harvesting) on these communities. We used reconstructed podzol profiles in laboratory microcosms, varying the amount of organic horizon material that was present, as well as including another treatment in which the soils horizons were mixed before planting of *Pinus sylvestris* seedlings.

The soil horizons of stratified boreal forest soils differ in physico-chemical composition. We therefore expected that fungal communities of each horizon would be distinct from each other.

A transition to WTH and WTHSR is taking place, however research into the impact of these practices on fungal communities is limited. WTH and WTHSR involve the removal of branches and foliage form the environment, reducing the amount of organic matter contributing to the O horizon. Over time it is expected that this will result in a thinner O horizon and a reduction of available N and base cations. This is expected to

have a negative impact on plant growth. WTHSR also induces localised soil mixing, the implications of which on fungal community are unknown.

The role of fungal communities in the health and function of the forest is often underestimated, however research indicative of their importance is mounting (Lindahl *et al.*, 1999; Perez-Moreno and Read, 2001a, b; Klironomos and Hart, 2001; Finlay 2008). Soil fungi, and ectomycorrhizal fungi in particular, are capable of mobilising base cations and other nutrients, making them available to plants. It is important to discern whether soil fungi are able to maintain this function when harvesting practices are intensified

To explore the effects of intensified harvesting practices on soil fungal communities, microcosms subjected to five treatments – each simulating different soil conditions – were constructed. Treatment I was devoid of any organic horizon material, simulating extreme soil conditions that could theoretically be brought about by long-term branch and foliage removal. Treatment II retained 50% of the organic horizon, simulating short-term branch and foliage removal. Treatment III simulated a natural podzol with material from the O and E horizons in their natural proportions. Treatment IV contained an O horizon that was increased in thickness by 50%. Treatment V simulated the disruption following stump removal resulting in mixing and homogenisation of the natural podzol horizons. DNA was extracted from harvested soil and following high throughput sequencing fungal communities in each soil type and treatment were analysed bioinformatically and statistically. Microcosm soil was also used to compare fungal communities in O, E and B soil horizons within each treatment.

We hypothesized that:

1. Fungal communities from the O, E and B horizons would be significantly different from each other.
2. Fungal communities of different treatments would differ significantly from each other, (possibly reflecting differences in C and N supply).
3. Some fungi might be sensitive to physical, chemical or biological disturbance induced by mixing, and that the overall fungal diversity might be lower in the mixed treatment.

3. Materials and Methods

3.1. Material

Soil samples from a previously conducted microcosm experiment (described by Fahad, 2017) were used for high throughput analysis of fungal communities. The original experimental design and the molecular and bioinformatic analyses that I performed are described below.

O, E, B and E/B interface soil was collected from three separate 20 x 20 m plots in Jädraås, Sweden (60°49'N, 16°30'E). – a nutrient poor, dry-pine – dwarf-shrub – lichen heathland forest, which is representative of boreal forests common in Scandinavia. Jädraås stands on Sveco-Fenno-Karelian bedrock, specifically composed of leptite, gneiss-granite and gneiss sediments, and is overlain by sandy till and boulders. Soils are podzolised, are uniformly porous, and water primarily moves vertically with no influence from groundwater. It is situated at the border between the boreal and boreo-nemoral zones and contains both Northern and Southern flora and fauna. Jädraås has hilly terrain with elevation variations of 100 m. Mean annual air temperature is 3.8 °C and annual precipitation is 600 mm. It has an abundance of lichen and dwarf shrubs mainly composed of *Vaccinium myrtillus*, low herbs, grasses and mosses. Tree stands vary in age from clear-felled areas to 160 year old stands with variable tree densities. (Axelsson and Bråkenhielm, 1980)

Soils from each plot were collected from each soil horizon and within each horizon the samples were pooled and then homogenised using 5 mm and 3 mm mesh sieves, for organic and mineral horizon soils respectively. Water content and water holding capacity of different soils were also estimated. There are much higher quantities of C and N present in the O horizon due to the deposition of organic material. Mg, Fe and Al are highest in the B horizon, where they precipitate (Table 1).

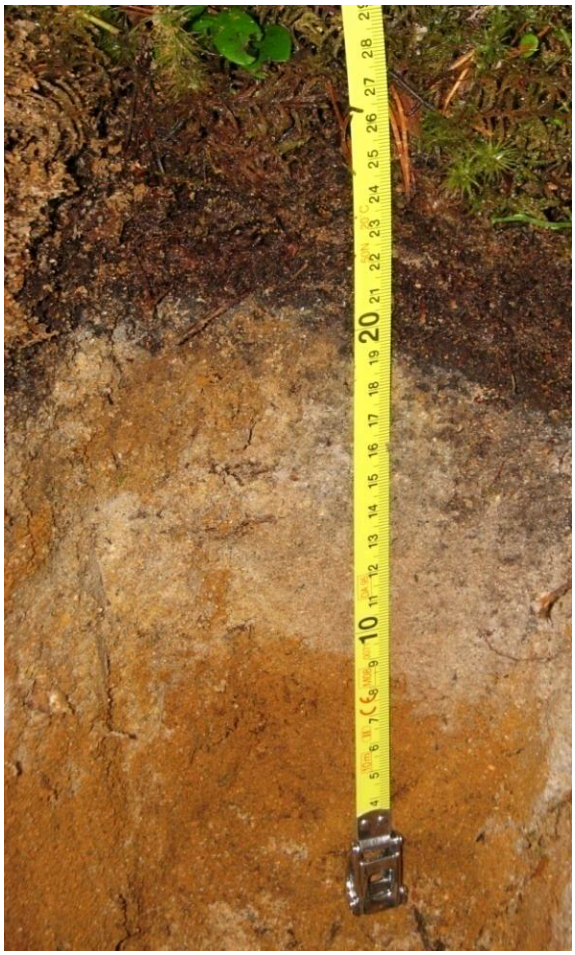

Figure 2. Podzol profile located in Jädraås forest. A thin layer of organic material is deposited on the podzol surface, forming the organic (O) horizon. Below this is the eluvial (E) horizon, subjected to leaching, removing mobile elements and nutrients. Beneath the E horizon is the illuvial (B) horizon. Elements including Fe and nutrients leached from the E horizon precipitate here, giving the B horizon its rich orange-brown colour. (Photograph taken by Johanna Jernberg)

Element	O horizon	E horizon	B horizon
C	451600	13900	16100
N	11200	700	700
Si	81000	345000	35600
K	609	149	334
Ca	1474	446	601
Mg	381	243	1517
P	494	53	507
S	839	38	176
Na	133	92	59
Mn	119	8	108
Fe	1401	1856	11524
Al	1207	1851	13395

Reproduced from Marupakula et al. 2017.

Table 1. Elemental composition of ten mixed soil samples from each of the organic (O), eluvial (E), and illuvial (B) horizons in a boreal forest podzol profile from Jädraås, Sweden. All values are given as mg per kg dry weight of soil.

3.2. Plant preparation

Pinus sylvestris seeds were surface sterilised with 33 % hydrogen peroxide for 30 minutes at room temperature. They were then rinsed with milliQ water and allowed to air dry. Prepared seeds were scattered over a 0.3-0.4 cm thick layer of non-sterile vermiculite, in a 20 x 30 cm enclosed propagator with a clear ventilated lid. Three propagators were prepared and incubated in a phytotron set to a 250 μmol m^{-2} s^{-1} PAR 18h/6h light/dark cycle with a "day" temperature of 18 °C and a "night" temperature of 16 °C. The germinating seedlings were incubated under these parameters for 4 months.

3.3. Establishment of microcosms

Soil from the different horizons was loaded into 40 2.1 L (200 x 100 x 142 mm (L x W x H)) transparent acrylic containers (see Figure 3). One to three 5 mm holes were drilled into one side of each container to allow for soil solution sampling – the number and orientation depending on treatment. Additional holes were drilled into the base to prevent waterlogging. Prior to the addition of soil, two nylon mesh sheets (0.5 mm thick, 0.6 mm pore size) were placed at the base of each microcosm to prevent soil loss through drainage holes and to aid harvesting. Different horizons were separated by a nylon mesh sheet (1.0 mm thick, 2 mm pore size), maintaining stratification and aiding harvesting. Two mesh bags containing corresponding soil were embedded into each soil type, assisting the extraction of root-free mycelium and allowing the sampling of pure mycelium communities. A Rhizon® pore water sampler (Rhizon Research Products, The Netherlands) was also embedded in each soil type, allowing the collection of soil solution via the lateral holes in the microcosm. (See Fahad 2017 for a description of mesh bag and Rhizon® water pore sampler design and set up). Five treatments (**I**, **II**, **III**, **IV** and **V**) were prepared, each replicating real-life soil conditions in managed forests, with treatments **I-IV** modelling podzolized soils and treatment **V** modelling a mixed soil. A total of eight microcosms were constructed per treatment – four with six *Pinus sylvestris* seedlings, and four without (as controls).

In the construction of microcosms containing stratified podzol profiles, two 0.5 mm thick/0.6 mm pore size nylon mesh sheets were first placed at the base followed by the loading and compaction of B horizon soil in variable quantities depending on the treatment. Upon this a 1.0 mm thick/2.0 mm pore size nylon mesh sheet was placed, followed by 800 g E horizon soil, which remained constant regardless of treatment. Again, a 1.0 mm thick/2.0 mm pore size nylon mesh was placed upon the soil and O horizon soil, again in variable quantities depending on the treatment, was deposited. The combined total volume of O and B horizon soils is the same in every treatment. Within each layer two mesh bags and a Rhizon pore water sampler were placed, facilitating extraction of root-free mycelium and soil solution respectively. Each microcosm was

wrapped in aluminium foil and the soil surface was covered in a thick black plastic sheet to prevent exposure of developing mycelia to light and to prevent growth of algae and mosses.

Microcosms containing mixed soil required only the two base 0.5 mm thick/0.6 mm pore size nylon mesh sheets, upon which mixed O/E/B soil was deposited. Within the O/E/B soil, two mesh bags and a Rhizon™ pore water sampler were placed. As with the podzolized microcosms, the soil surface was covered with a thick black plastic sheet and the sides were wrapped in aluminium foil.

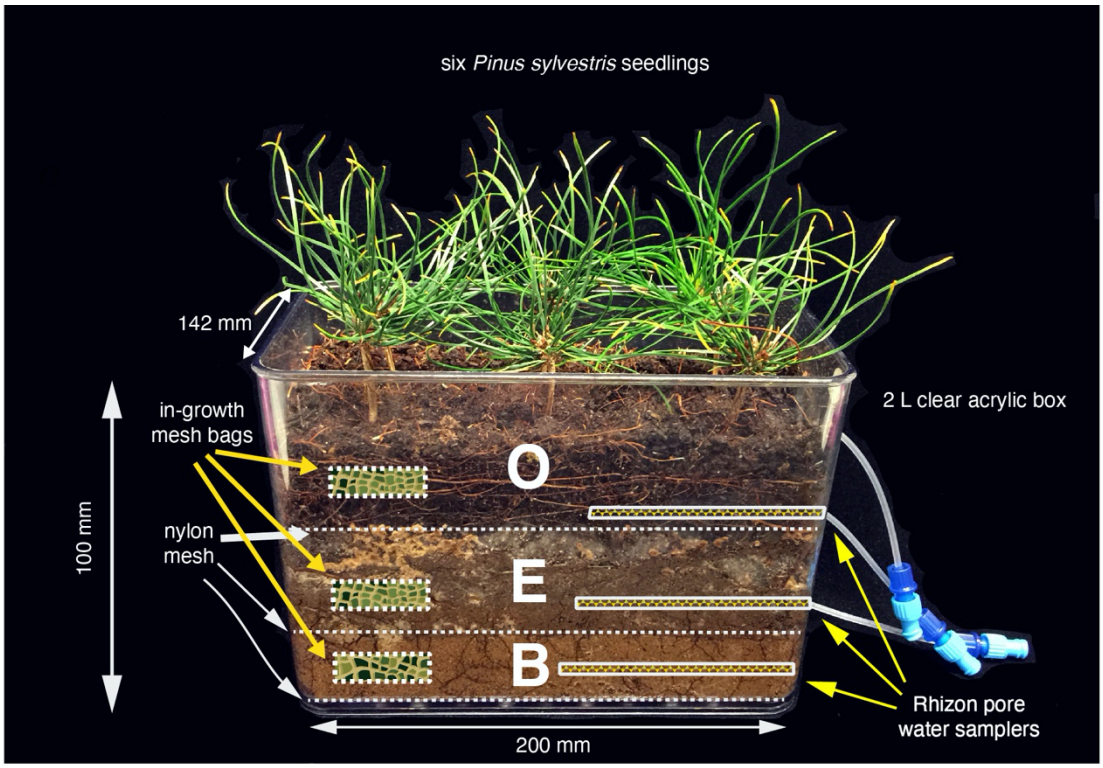

Figure 3. Microcosm design. Annotated photo of a microcosm containing a reconstructed podzol soil using soil from a boreal forest at Jädraås, Sweden. Two 0.5 mm thick nylon mesh sheets with 0.6 mm pores are at the base of a 2.1 L 200 x 100 x 142 mm clear acrylic box. B horizon soil rests on top of this mesh, followed by E and then O horizon soil. Between each horizon is a 1 mm thick nylon mesh sheet with 2 mm pores. Planted into the O horizon are six *Pinus sylvestris* seedlings. Embedded in each horizon is a mesh bag containing soil of that horizon and a porous Rhizon pore water sampler. Water samplers are affixed to silicon tubes which are threaded through a rubber plug fitted into 50 mm holes drilled into the side of the microcosm. Nozzles enabling soil water extraction are attached to the end of the silicon tube. (Photograph by Shahid Mahmood)

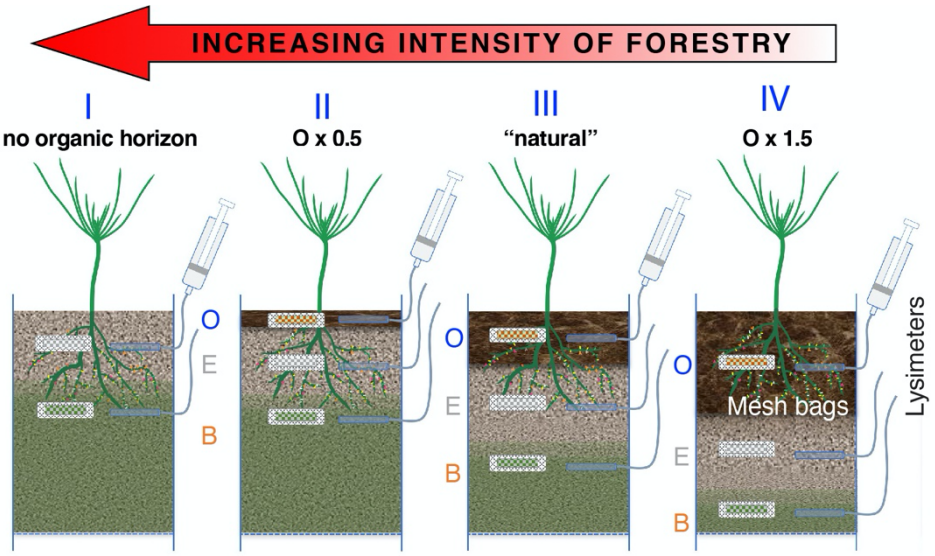

Figure 4. Schematic diagram showing the experimental design of a microcosm experiment designed to investigate the effects of differing degrees of intensity of forestry simulated by removal of increasing amounts of organic matter. The amount of eluvial material (E) was kept constant but the ratio of organic material (O) to illuvial material (B) was varied to simulate different degrees of organic matter depletion. Treatment **III** normal thickness of O horizon, Treatment **II** 50% reduction in thickness of O horizon, Treatment **I** no O horizon, Treatment **IV** O horizon thickness increased by 50%. Soil solution was sampled using lysimiters buried in each horizon and fungal mycelial growth was sampled using in-growth mesh bags buried in each horizon. These excluded plant roots but allowed penetration by fungal hyphae.

Treatments were designed to simulate soil conditions resulting from WTH (treatments **I** and **II**) and WTHSR (treatment **V**) practices, as well as soil conditions of a natural forest (treatment **III**) and an old growth unmanaged forest (treatment **IV**) (see Figure 4). In treatment **I** 800 g of E horizon soil overlay 1450 g B horizon soil. The O horizon was absent, replicating soil conditions resulting from long-term WTH practices. In treatment **II** 1050 g of B horizon soil underlay 800 g of E horizon soil. 200 g of O horizon soil composed the thin topsoil, imitating forest soils undergoing partial WTH. In treatment **III** 650 g of B horizon soil underlay 800 g of E horizon soil. 400 g of O horizon soil completed the podzol, depicting normal soil conditions of an unmanaged forest. Treatment **IV** represents a podzol found in an old growth unmanaged forest, with a thicker O horizon of 600 g and a thinner B horizon of 250 g. The E horizon remained constant at 800 g. Treatment **V** represented soil conditions local to sites of stump removal, with mixed soil of the same composition as in treatment **III**. Water-retention by each soil treatment was estimated by the construction of four plant-free microcosms prior to the experiment. These microcosms were also used to optimise the systems.

Microcosms were incubated in a phytotron for 14 months with the same set conditions under which the *Pinus sylvestris* seedlings developed. Soil moisture was kept constant through the use of a gravimetric watering regime using deionised water. Air moisture was maintained through natural evaporation from randomly placed plastic cups filled with deionised water. The positions of the microcosms were shuffled randomly on a weekly basis to reduce the impact of environmental differences within the phytotron.

3.4. $^{13}CO_2$ pulse labelling

In the final month of incubation, one replicate of each treatment was moved to a separate phytotron to await $\delta^{13}C$ natural abundance measurements. The remaining microcosms were placed into a transparent airtight acrylic chamber and subjected to $^{13}CO_2$ pulse labelling. The chamber was equipped with fans to promote air circulation and additional lights to support seedling growth. Continuous $^{13}CO_2$ exposure occurred in 8-hour episodes over the course of 3 days, coinciding with the peak photoperiod. During these 8-hour periods total CO_2 concentration was maintained at an average 480 ppm – measured by an infra-red gas analyser (IRGA – EMG-4 Environmental Gas Monitor for CO_2 by PP Systems, United Kingdom). Upon depletion to 300 ppm, additional $^{13}CO_2$ gas was added by manual injection via an airtight syringe attached to a canister of 99 % $^{13}CO_2$ gas. Prior to the initial pulse of $^{13}CO_2$, seedlings were allowed to deplete $^{12}CO_2$ to 200 ppm to aid a higher concentration of $^{13}CO_2$ within the chamber for the duration of the $^{13}CO_2$ exposure episode. The average rate of $^{13}CO_2$ assimilation by seedlings was 124 ml h^{-1}, determined by IRGA. Following the 3-day course of $^{13}CO_2$ pulse exposure microcosms were incubated for a further week – a chase period – allowing assimilated ^{13}C to migrate through the plant to microbial associates in the soil in the form of sugars. After this chase period microcosms were harvested.

3.5. Harvesting of microcosms

Microcosms were harvested destructively. During dismantling microcosms were placed on ice to limit microbial respiration and potential losses of ^{13}C. Microcosms reserved for natural $\delta^{13}C$ abundance measurements were dismantled first and the remaining microcosms were dismantled in a random order.

Disassembly began with cutting the seedlings at the base of their stem. Cut seedlings were washed in deionised water, dried at 80 °C for 48 hours and then homogenised using a ball mill (Retsch MM2, Germany). Milled seedlings were then stored at room temperature in glass vials to await further analysis.

Soil was harvested layer by layer, beginning with the uppermost horizon and ending with the B horizon. Upon excavation Rhizon pore water samplers were removed and mesh bags were retrieved. Mesh bags from each soil type and microcosm were stored separately in sealed plastic bags at -20 °C. Soil and roots of each layer were removed together, with roots being cut at nylon mesh at the base of each layer. Soil was collected on a plastic sheet, over which excavated roots were shaken to remove firmly adhering soil.

Retrieved soil was sieved – O horizon soil through a 5 mm mesh, E and B horizon and O/E/B homogenised soils through a 3 mm mesh – to remove any remaining roots. Soil

subsamples for water content and soil solution extractions were taken and stored at -20 °C. Soil subsamples for $\delta^{13}C$-natural abundance/enrichment analyses were freeze dried at -90 °C for 120 hours and milled using a ball mill (Retsch MM2, Germany). Homogenised soil was stored at -20 °C in airtight falcon tubes. Roots collected during excavation were examined and washed thoroughly as described in Fahad (2017), freeze dried at -90 °C for three days and, after milling, stored in glass vials at -20 °C.

3.6. DNA and RNA Extraction and Purification

DNA and RNA from soil samples were co-extracted using RNA PowerSoil Total RNA Isolation Kit and RNA PowerSoil DNA Elution Accessary Kit (MoBio Laboratories, CA, USA), following the manufacturer protocol. DNA and RNA concentration were measured by nanodrop and stored in PCR grade H_2O at -80 °C until further processing.

3.7. Polymerase Chain Reaction

The fungal ITS region was PCR amplified using primers fITS9 (5'-GAACGCAGCRAAIIGYGA-3') (Ihrmark *et al.*, 2012) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The ITS4 primer contained an 8-base barcode sequence unique for each sample. Each 25 µl reaction volume contained: 2 µl template DNA, 2.5 µl each of fITS9 (10 µM) and ITS4 (10 µM) primers, 12.5 µl MyTaq Red Mix, 2x (Bioline, UK) and 5.5 µl H_2O.

Three technical replicates were run for each sample in a thermal cycler using the following thermocycling programme: DNA was first denatured at 95 °C for 5 minutes; Following this, primers were annealed and strands were extended by heating to 94 °C for 30 seconds, 55 °C for 45 seconds and the 72 °C for 45 seconds. This was repeated for 35 cycles. Reactions then remained at 72 °C for 10 minutes before being cooled to 4 °C. Following PCR amplifications, PCR products were analysed by gel electrophoresis on 1 % wt/vol agarose gels pre-stained with Nancy-520 DNA gel stain (sigma-Aldrich, USA).

The triplicate PCR products from each sample were then pooled together, and cleaned with Agencourt AMPure kit (Beckman Couter, USA) according to manufacturer's recommendations. Using a Qubit Fluorometer (Invitrogen, USA) DNA concentration was determined in each sample by Qubit dsDNA HS Assay. All the PCR products from differently barcoded samples were pooled together in equal concentrations and purified using the EZNA Cycle Pure Kit (Omega Bio-Tek, USA) and then eluted twice with 50 µl EB. The quality of resulting pool and fragment size distribution were analysed using

Agilent DNA 7500 Kit with the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA).

3.8. High throughput DNA Sequencing

The pooled sample was sequenced at SciLifeLab, NGI-Uppsala, Sweden, and underwent high throughput sequencing, using 3 SMRT cells of PacBio Sequel System (Pacific Biosciences, USA) according to manufacturer's recommendations. The library preparation and adaptors ligation were also carried out at SciLifeLab, NGI-Uppsala, Sweden.

3.9. Bioinformatic Analyses

Using the command 'fastq.info' in MOTHUR (Schloss *et al*., 2009) the sequencing data file (fastq) was converted to a fasta and quality files that were used in QIIME pipeline (Caporaso *et al*., 2010). In brief, command 'demultiplex_fasta.py' was used to demultiplex sequences based on barcodes and 'adjust_seq_orientation.py' to reverse complement sequences that remained unassigned. After combining both forward and reverse complemented reads in one file, the command 'split_libraries.py' was used to split libraries according to sample barcodes as listed in the mapping file. Chimeric sequences were identified using VSEARCH (Rognes *et al*., 2016) in MOTHUR and a UCHIME reference dataset (version 7.2; 2017-06-28) (Nilsson *et al*., 2015). The sequences were clustered into Operational Taxonomic Units (OTUs) by UCLUST (Edgar, 2010) using the command 'pick_otus.py' with the *denovo* option, as implemented in QIIME, using a 97 % sequence similarity criterium. The representative sequences of all OTUs were chosen with command 'pick_rep_sets.py' and an OTU table was constructed with command 'make_otu_table.py'. Individual sequences were classified taxonomically using the "classify.seqs" command in MOTHUR (Schloss *et al*. 2009) (confidence threshold 80) using the UNITE fungal ITS reference database (release ver. 7.2; UNITE_public_mothur_full_10.10.2017) (Kõljalg *et al*. 2013). Non-fungal OTUs were removed manually from the OTU table and the singletons were removed in QIIME using command 'filter_otus_from_otu_table.py'. The filtered OTU table was rarefied using command 'single_rarefaction.py' and from this rarefaction curves were generated using command 'multiple_rarefactions.py'. Taxa summary tables for downstream analyses of sequences were generated using 'summarize_taxa.py' and taxa plots were generated using 'summarize_taxa_through_plots.py'.

3.10. Statistical Analysis

Alpha diversity indices were calculated using 9999 runs of bootstrapping (PAST 4.03 statistical package) and the differences due to treatments were evaluated by pair-wise t-tests using JMP Pro 15 software. Beta diversity patterns were analysed by nonmetric multidimensional scaling (NMDS) ordinations with Bray-Curtis dissimilarity measure. The differences in fungal community composition in O, E and B horizon soils within a reconstructed podzol microcosm or in a particular horizon soil across the treatments were estimated using nonparametric analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations. The fungal taxa that differed significantly ($P < 0.05$) in abundance across the treatments, were identified using the command 'group_significance.py' with ANOVA implementation in QIIME. For the Venn charts a fungal OTU was considered to be 'present' only when present in all the three replicates of a particular sample, otherwise considered 'absent'. Venn plots were generated using the VENNY 2.1 tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

4. Results

In total 1,010,556 reads were obtained from sequencing of DNA from podzol horizon soils and mycelial mesh bags collected from this experiment. After quality filtering and removal of chimera 516,998 reads remained. Since the analysis fungal communities of the O, E and B horizon soils was the main aim of my thesis project, sequencing data from the mycelial mesh bags was not analysed further. After removal of non-fungal sequences and singletons from the soil sequencing data, 263,435 sequences were obtained for alpha and beta diversity analyses of fungal communities. Upon analysis, rarefaction curves of fungal taxa versus number of sequences, NMDS graphs and stack graphs visually displaying the 20 most abundant fungal taxa were constructed (see below).

4.1. Plant growth

At the time of harvesting *Pinus sylvestris* seedlings showed a statistically significant increase in shoot and root biomass from treatment **I** to **IV** (Figure 5a,b). Seedlings in treatment **V** had significantly higher biomass than treatment **III** but lower than seedlings growing in treatment **IV** microcosms.

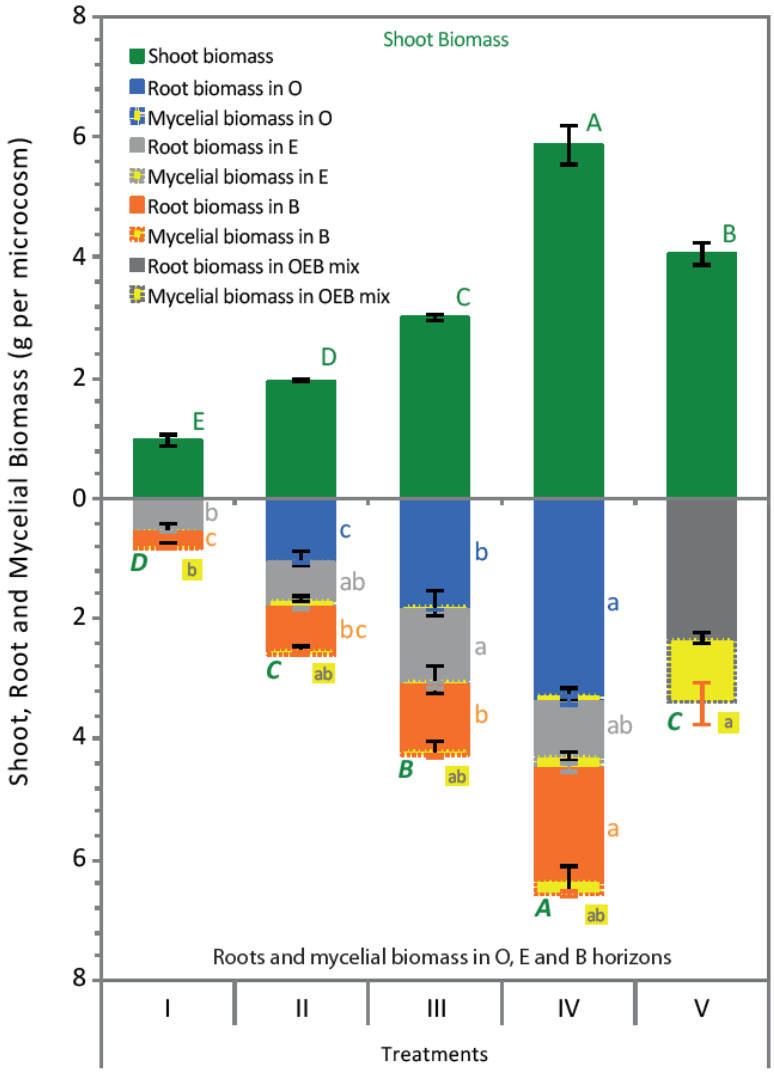

Figure 5a. Mean shoot, root and ectomycorrhizal mycelial biomass in microcosms with a gradient of organic matter depletion. **I** - no organic horizon, **II** - reduced (x 0.5) organic horizon, **III** - normal organic horizon, **IV** - increased (x 1.5) organic horizon. In treatment **V** the O, E and B soil horizons were completely mixed. Vertical bars represent ± standard error, (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 taken from Fahad, 2017)

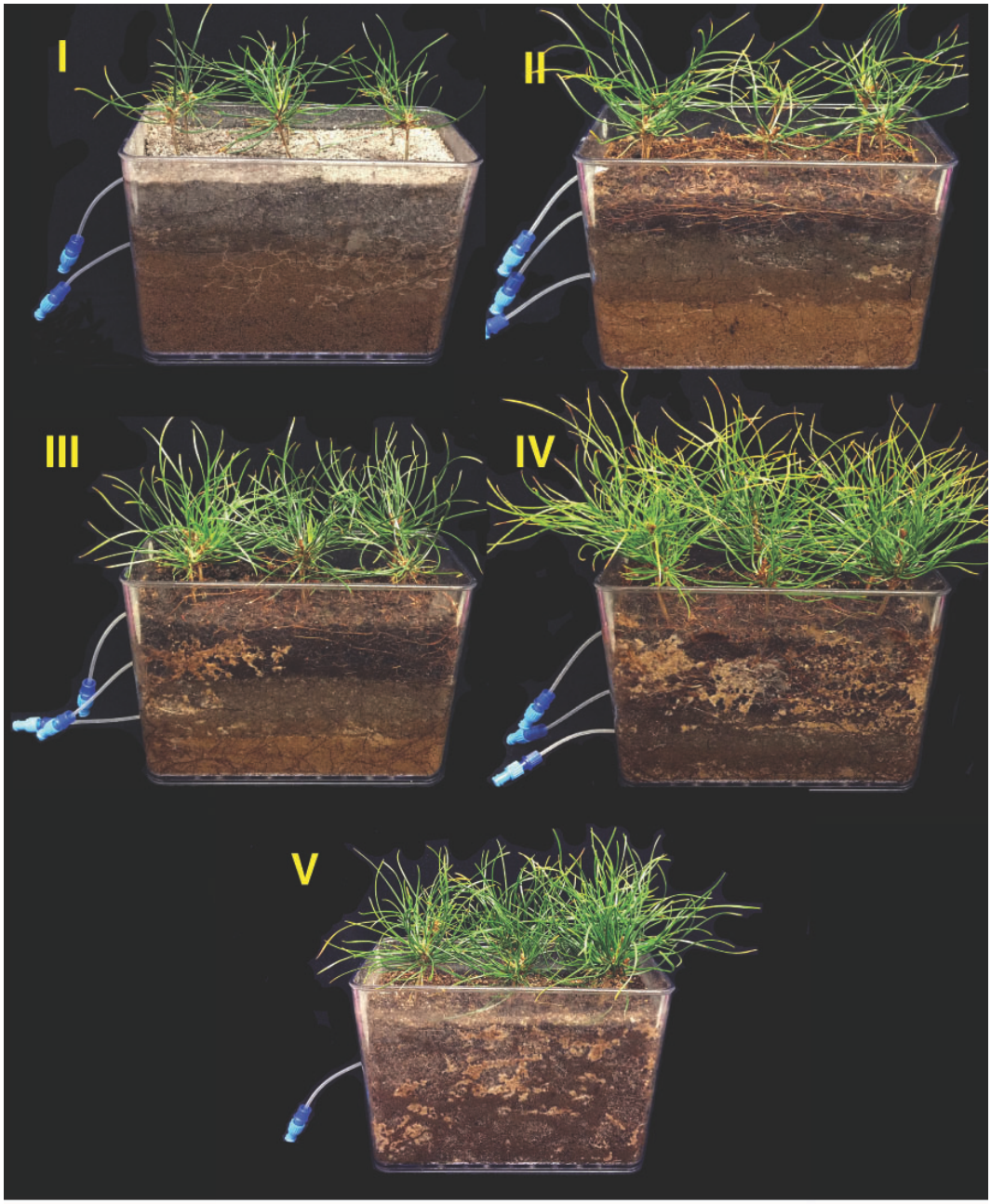

Figure 5b. Representative microcosms from different treatments one week before harvesting - showing differences in plant growth. Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: Treatment **I**, no organic horizon; Treatment **II**, organic horizon reduced by 50%; Treatment **III**, natural soil profile; Treatment **IV**, organic horizon increased by 50%; Treatment **V**, natural soil horizons (Treatment **III**) fully mixed.

4.2. Patterns of fungal diversity

Samples were rarefied to an equal number of sequences (5303), before further analysis. All rarefaction curves indicate that a large proportion, but not all taxa, were detected within the 5303 sequences.

Rarefaction curves comparing species richness between different soil horizons within each treatment (Figure 6) show no significant difference in number of OTUs in treatments **I** and **IV**. In treatment **II**, O, E and B horizons have significantly different numbers of OTUs. In treatment **III**, E horizon material had significantly more OTUs than O and B horizons. In all treatments the E horizon had the highest number of OTUs, followed by O horizon, then the B horizon.

OTUs found in each treatment were compared using Venn diagrams (Figure 6) and some general trends that could be seen clearly were: (a) the relative proportion of OTUs that were specific/unique to a particular horizon soil was much greater than the ones that were shared, (b) there was relatively greater overlap of (shared) OTUs between E and B horizons than between O and E or B horizon.

Rarefaction curves comparing species richness between treatments within each horizon (Figure 7) show no significant (ANOVA) difference in number of OTUs in O horizons and B horizons. In E horizons treatment **I** has significantly fewer OTUs than in other treatments.

OTUs found in each soil horizon were compared across the treatments using Venn diagrams (Figure 7). More OTUs were shared in each horizon soil when compared across the treatments, e.g., in O horizon soils 28% OTUs were shared between the treatments and the proportion of OTUs that were unique to a particular treatment, did not vary markedly (15-17%). OTUs in E and B horizons followed the same general pattern of abundance of shared OTUs but the OTUs that were specific to different treatments exhibited some variation, particularly treatment II had greater numbers of OTUs in E and B horizon soils than other treatments.

A comparison of fungal species richness between each horizon of treatment **III** and mixed OEB horizon soil of treatment **V** (Figure 7) showed significantly more OTUs in the E horizon than in O and B horizons. OEB mixed horizon soil had no significant difference in number of OTUs when compared to the E horizon or O and B horizons of treatment **III**.

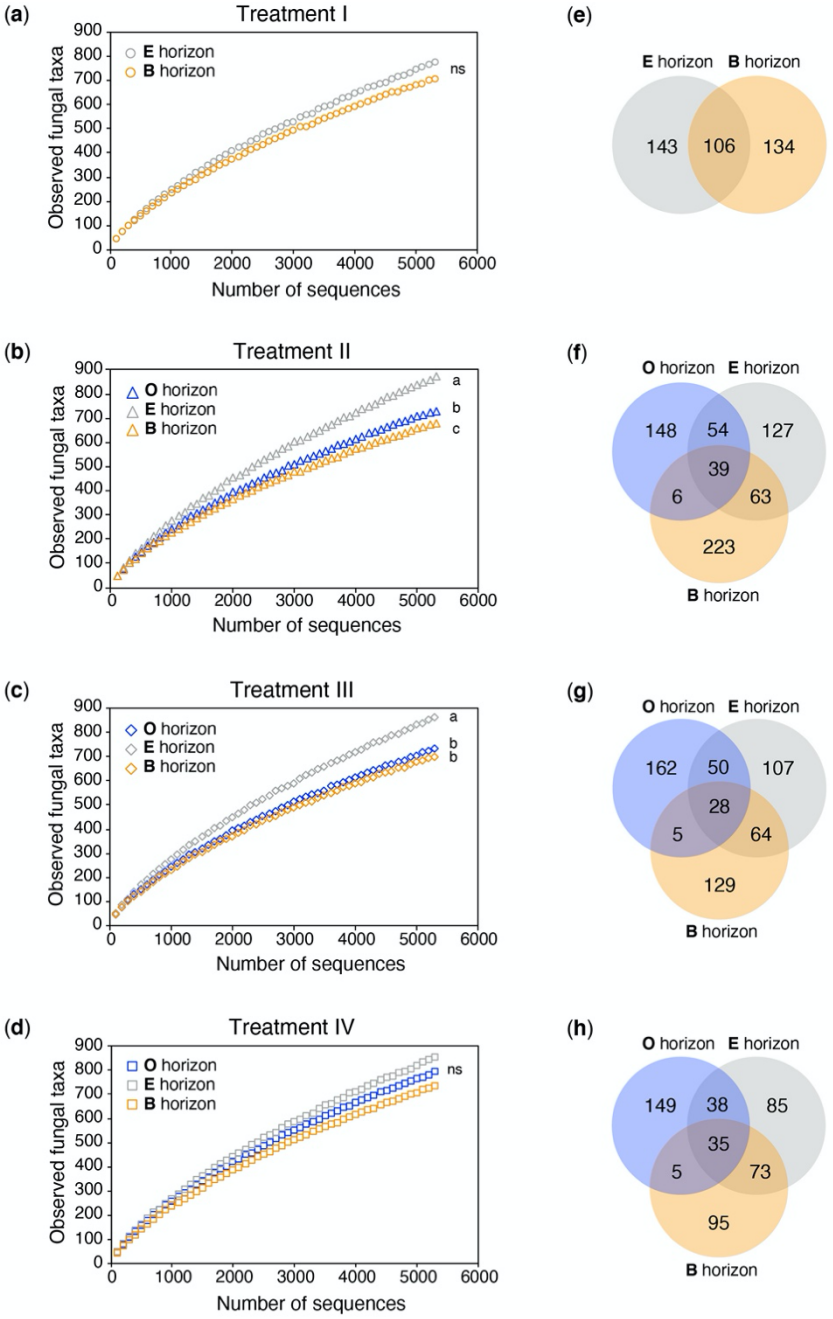

Figure 6. Rarefaction curves and Venn diagrams showing numbers of fungal taxa in O, E and B horizons from microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: (**a**, **e**) Treatment I, no organic horizon; (**b**, **f**) Treatment II, organic horizon reduced by 50%; (**c**, **g**) Treatment III, natural soil profile; (**d**, **h**) Treatment IV, organic horizon increased by 50%. Different letters beside the rarefaction curves indicate statistically significant (P<0.05) differences between the numbers of taxa, or no significant difference (ns) (n = 3).

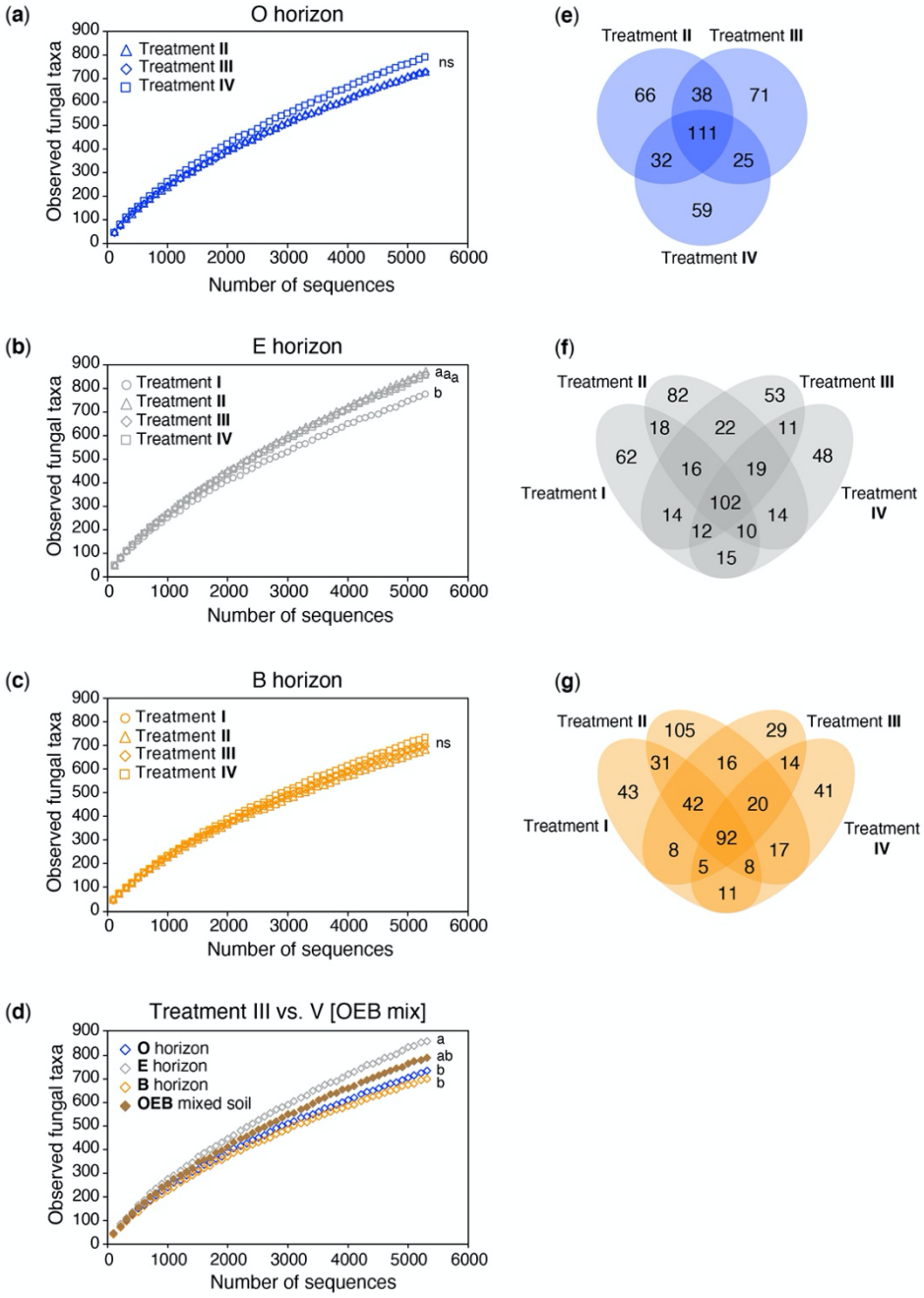

Figure 7. Rarefaction curves and Venn diagrams showing numbers of fungal taxa in different treatments in microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: Treatment I, no organic horizon; Treatment II, organic horizon reduced by 50%; Treatment III, natural soil profile; Treatment IV, organic horizon increased by 50%; Treatment V, Organic (O), Eluvial (E) & Illuvial (B) horizons (corresponding to Treatment III) fully mixed. (**a, e**) O horizon; (**b, f**) E horizon; (**c, g**) B horizon; (**d**) Comparison of O, E & B horizons (Treatment III) with mixed soil (V). Different letters beside the rarefaction curves indicate statistically significant (P<0.05) differences between the numbers of taxa, or no significant difference (ns) (n = 3).

Alpha diversity patterns of fungal taxa were compared between treatments within each horizon (Table 2). No differences in fungal species richness in O and B soils were found (Table 2a,c). Moreover, different diversity indices and measures of dominance or evenness of fungal taxa in O and B horizons did not show any significant effect of the treatments. In E horizons, treatment **I** had significantly ($p < 0.05$) reduced fungal species richness (based on Taxa S, Menhinick, Margalef estimates) than in other treatments (Table 2b). Measures of species evenness (Evenness $(e^H)/S$, Simpson 1-D) and diversity indices (Shannon H, Fisher's alpha) showed significantly reduced values in treatment **I**, whereas species dominance (Dominance D, Berger-Parker) index values were significantly higher in this treatment (Table 2b).

Table 2. Alpha diversity indices of fungal communities in (**a**) Organic (O), (**b**) Eluvial (E) and (**c**) Illuvial (B) horizons from microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Diversity estimates are based on sequence abundance data that have been rarefied to 5303 sequences per sample. Singletons were removed prior to analysis. The values are means of three replicates and ± represent standard error (SE). Different letters denote significant differences among means ($p < 0.05$; ANOVA, pair-wise comparisons using Student's t).

(**a**)

O horizon soils	Treatment II	Treatment III	Treatment IV
Species richness			
Taxa S	736.33 ± 21.11 a	734.33 ± 16.74 a	789.33 ± 31.80 a
Menhinick	10.11 ± 0.29 a	10.08 ± 0.23 a	10.84 ± 0.44 a
Margalef	85.74 ± 2.46 a	85.51 ± 1.95 a	91.92 ± 3.71 a
Dominance			
Dominance D	0.07 ± 0.01 a	0.07 ± 0.00 a	0.07 ± 0.02 a
Berger-Parker	0.21 ± 0.03 a	0.17 ± 0.01 a	0.18 ± 0.05 a
Evenness			
Evenness (e^H)/S	0.09 ± 0.01 a	0.09 ± 0.00 a	0.09 ± 0.01 a
Equitability J	0.63 ± 0.01 a	0.63 ± 0.01 a	0.63 ± 0.03 a
Simpson 1-D	0.93 ± 0.01 a	0.93 ± 0.00 a	0.93 ± 0.02 a
Diversity indices			
Shannon H	4.19 ± 0.09 a	4.15 ± 0.07 a	4.23 ± 0.21 a
Fisher's alpha	232.37 ± 9.57 a	231.40 ± 7.52 a	257.00 ± 14.82 a

(**b**)

E horizon soils	Treatment I	Treatment II	Treatment III	Treatment IV
Species richness				
Taxa S	777.67 ± 26.74 b	876.67 ± 11.86 a	868.67 ± 18.70 a	851.67 ± 9.94 a
Menhinick	10.68 ± 0.37 b	12.04 ± 0.16 a	11.93 ± 0.26 a	11.70 ± 0.13 a
Margalef	90.56 ± 3.12 b	102.10 ± 1.39 a	101.16 ± 2.17 a	99.17 ± 1.15 a
Dominance				
Dominance D	0.14 ± 0.03 a	0.06 ± 0.01 b	0.06 ± 0.02 b	0.06 ± 0.01 b
Berger-Parker	0.35 ± 0.05 a	0.16 ± 0.01 b	0.18 ± 0.05 b	0.21 ± 0.03 b
Evenness				
Evenness (e^H)/S	0.06 ± 0.01 b	0.09 ± 0.01 a	0.10 ± 0.01 a	0.09 ± 0.01 ab
Equitability J	0.58 ± 0.02 b	0.65 ± 0.01 ab	0.66 ± 0.02 a	0.64 ± 0.02 ab
Simpson 1-D	0.86 ± 0.03 b	0.94 ± 0.01 a	0.94 ± 0.02 a	0.94 ± 0.01 a
Diversity indices				
Shannon H	3.85 ± 0.16 b	4.40 ± 0.07 a	4.49 ± 0.13 a	4.34 ± 0.13 a
Fisher's alpha	251.40 ± 12.60 b	299.30 ± 5.99 a	295.33 ± 9.45 a	286.80 ± 4.90 a

(**c**)

B horizon soils	Treatment I	Treatment II	Treatment III	Treatment IV
Species richness				
Taxa S	717.67 ± 1.76 a	681.00 ± 12.58 a	696.00 ± 13.61 a	732.67 ± 51.34 a
Menhinick	9.86 ± 0.02 a	9.35 ± 0.17 a	9.56 ± 0.19 a	10.06 ± 0.71 a
Margalef	83.57 ± 0.20 a	79.29 ± 1.47 a	81.04 ± 1.59 a	85.31 ± 5.99 a
Dominance				
Dominance D	0.06 ± 0.00 a	0.06 ± 0.01 a	0.10 ± 0.01 a	0.14 ± 0.06 a
Berger-Parker	0.21 ± 0.01 a	0.20 ± 0.03 a	0.29 ± 0.03 a	0.32 ± 0.10 a
Evenness				
Evenness (e^H)/S	0.09 ± 0.00 a	0.10 ± 0.01 a	0.08 ± 0.01 a	0.07 ± 0.02 a
Equitability J	0.64 ± 0.00 a	0.64 ± 0.01 a	0.61 ± 0.01 a	0.58 ± 0.05 a
Simpson 1-D	0.94 ± 0.00 a	0.94 ± 0.01 a	0.90 ± 0.01 a	0.86 ± 0.06 a
Diversity indices				
Shannon H	4.19 ± 0.02 a	4.20 ± 0.06 a	3.97 ± 0.09 a	3.84 ± 0.35 a
Fisher's alpha	223.80 ± 0.79 a	207.77 ± 5.45 a	214.30 ± 5.96 a	231.47 ± 22.87 a

4.3. Fungal community structure

Non-metric multidimensional scaling (NMDS) was used to visualise fungal community structure in each treatment and in each horizon across treatments. All NMDS graphs show biologically distinct communities between treatments and soil types (Figures 8, 9 and 10).

There was no statistically significant difference between E and B horizon fungal communities in treatment I (Figure 8a). In treatments II (Figure 8b), III (Figure 8c) and IV (Figure 8d) fungal community composition of O, E and B horizon soils within each treatment showed statistically significant differences (ANOSIM $P \leq 0.003$; PERMANOVA $P \leq 0.004$). Moreover, a comparison of the mixed OEB soil (treatment V) with O, E and B horizons of treatment III (Figure 8e) also revealed that statistically distinct fungal communities colonise these soils (ANOSIM $P = 0.0001$; PERMANOVA $P = 0.0001$). Bonferroni-corrected pairwise comparisons based on ANOSIM and PERMANOVA however, did not suggest that fungal community composition was statistically different within the treatments.

NMDS analysis of the O horizon soils collected from treatments II, III and IV indicated that fungal communities were marginally significantly different based on PERMANOVA ($p = 0.0345$) whereas ANOSIM suggested no significant differences ($p = 0.1687$) (Figure 9a). NMDS analysis of the effects of treatments I-IV on fungal community structure in E (Figure 9b) and B (Figure 9c) horizon soils depicted statistically significant differences within each horizon due to the treatments (ANOSIM $p \leq 0.005$; PERMANOVA $p \leq 0.003$).

To get an overview of the soil horizon effect on the fungal community structure, O, E and B horizon samples from different treatments were pooled within each soil horizon and analysed using NMDS ordination (Figure 10) which showed a highly significant effect of the horizons on fungal community structure (ANOSIM $p = 0.0001$; PERMANOVA $p = 0.0001$).

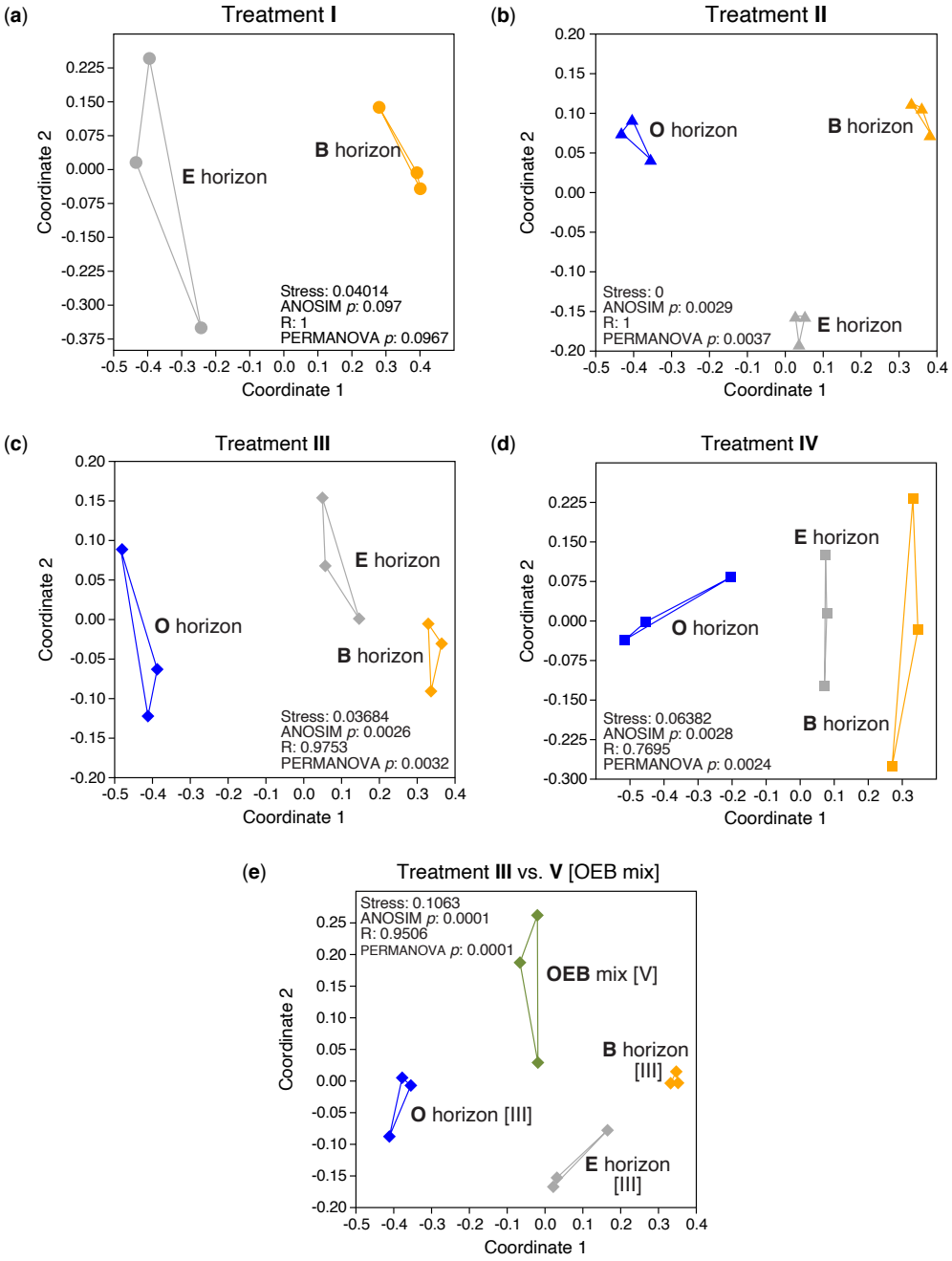

Figure 8. Nonmetric multidimensional scaling (NMDS) ordinations of fungal community structure in Organic (O), Eluvial (E) and Illuvial (B) horizons from microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Significance levels of analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) are also shown (n = 3). Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: (**a**) Treatment I, no organic horizon; (**b**) Treatment II, organic horizon reduced by 50%; (**c**) Treatment III, natural soil profile; (**d**) Treatment IV, organic horizon increased by 50%; (**e**) Treatment V, natural soil horizons fully mixed. (**b**), (**c**) and (**d**) show statistically significant differences in community structure between different soil horizons within each treatment, and (**e**) shows statistically significant differences in fungal community structure between the different horizons in Treatment III and the Mixed soil in Treatment V.

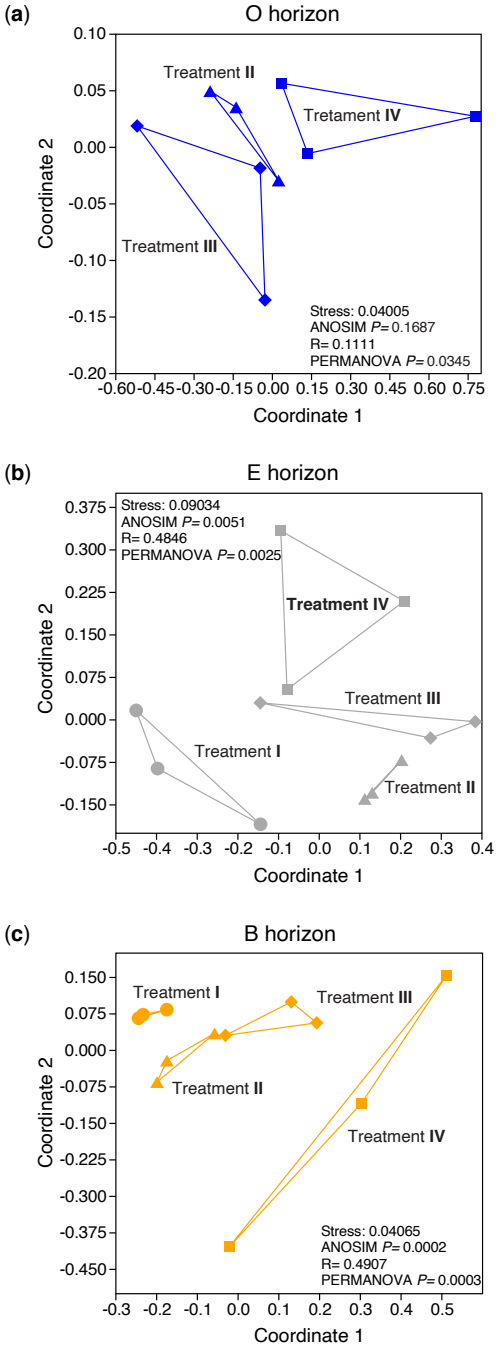

Figure 9. Nonmetric multidimensional scaling (NMDS) ordinations of fungal community structure in (**a**) Organic (O), (**b**) Eluvial (E) and (**c**) Illuvial (B) horizons from microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Significance levels of analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) are also shown (n = 3). Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: Treatment I, no organic horizon; Treatment II, organic horizon reduced by 50%; Treatment III, natural soil profile; Treatment IV, organic horizon increased by 50%; Treatment V, natural soil horizons fully mixed.

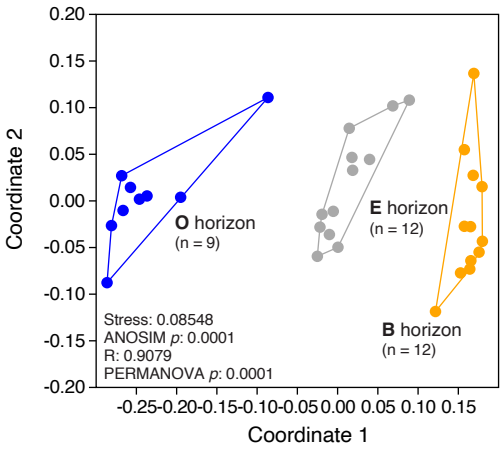

Figure 10. Nonmetric multidimensional scaling (NMDS) ordination of fungal community structure in Organic (O), Eluvial (E) and Illuvial (B) horizons from microcosms containing reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Significance levels of analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) are also shown. Treatments simulating different degrees of intensification of forestry are pooled within each soil horizon. The figure demonstrates statistically significant differences in fungal community structure between horizons.

4.4. Fungal taxa

The 20 most abundant taxa, accounted for above 85 % of all detected taxa, when the effects of treatments were assessed on O, E or B horizon soils separately (Figure 11).

The fungal taxa in O horizon soils appeared to be the least affected by the treatments. Fifteen taxa were significantly ($p < 0.05$) affected by the treatments and only 2 of them (*Hypocreales* and an unclassified fungus) were among the top 20 most abundant taxa in O horizon soil (Figure 11a). *Hypocreales* had highest relative abundance in treatment II but its abundance declined significantly in treatments III and IV. In contrast to this, an unclassified fungus that had highest abundance in treatment IV but declined significantly in treatments with relatively thinner O horizon (i.e. II and III). *Piloderma sphaerosporum* was the most abundant species, showing a trend of decreasing abundance from treatment II to IV. Conversely, relative abundance of *Suillus bovinus, Suillus* sp. and *Mortierella pulchella* declined markedly in treatments II and III.

Eighteen taxa were significantly ($p < 0.05$) affected by the treatments and 10 of them were among the top 20 most abundant taxa in E horizon soil (Figure 11b). *S. bovinus* was the most abundant taxon in E soils, its relative abundance was significantly higher in treatment I compared to the other treatments. It is followed by *P. sphaerosporum* which had greater in treatment II than the other treatments but the differences were not

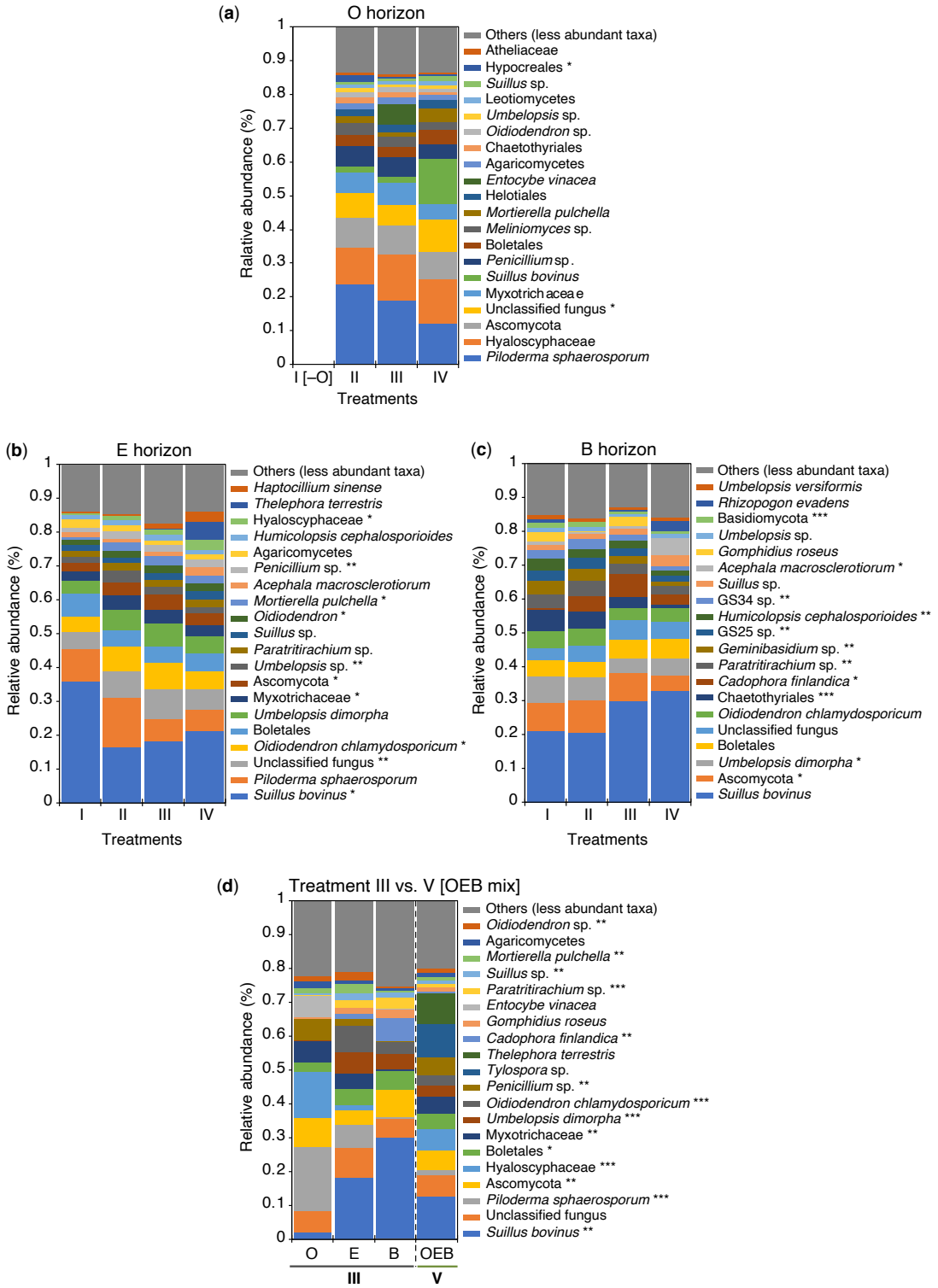

Figure 11. Histograms showing the relative abundance of ITS sequences of the 20 most abundant fungal taxa colonising different soil horizons in reconstructed podzol profiles using soil from a boreal forest at Jädraås, Sweden. Different degrees of intensification of forestry are simulated by removal of different amounts of organic material: Treatment I, no organic horizon; Treatment II, organic horizon reduced by 50%; Treatment III, natural soil profile; Treatment IV, organic horizon increased by 50%; Treatment V, natural soil horizons (Treatment III) fully mixed. (**a**) Organic (O) horizon, (**b**) Eluvial (E) horizon, (**c**) Illuvial (B) horizon, (**d**) comparison of O, E & B horizons of Treatment III with mixed soil (Treatment V). Asterisks indicate significant variation in abundance of individual taxa, based on ANOVA * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$; the values are means of three replicates.

statistically supported. An unclassified fungus, *Myxotrichaceae,* Ascomycota taxon, *Oidiodendron, Haptocillum sinense* and *Thelephora terrestris* displayed a trend of highest abundance in treatment IV but declined markedly in other treatments.

The fungal taxa in B horizon soils appeared to be the most affected by the treatments. Twenty-eight taxa were significantly ($p < 0.05$) affected by the treatments and 11 of them were among the top 20 taxa in B horizon soil (Figure 11c). *S. bovinus* was also the most abundant taxon in B horizon soils collected from different treatments and displayed greater relative abundance in treatments III and IV than in I and II. *Cadophora finlandica* and *Acephala macrosclerotiorum* had significantly lower abundance in treatment I than in the other treatments whereas an unclassified Ascomycota taxon, *Umbelopsis dimorhpa, Chaetothyriales, Paratritirachium* sp. *Geminibasidium* sp. *GS25* sp. *Humicolopsis cephalosporioides, GS34* sp. and an unclassified Basidiomycota taxon, all had significantly lower abundance in treatment IV compared to the other treatments.

Almost all the dominant taxa that we found in the O, E and B horizons of treatment III were represented in the OEB mixed soil of treatment V, but with varying proportions. Apparently there was no evidence to suggest that any taxa were lost due to disturbance caused by mixing of podzol horizons. Since the relative abundances of different taxa have changed in the mixed soil, it suggests that most of these taxa were actively growing and have responded to the soil disturbance event. Rarefaction curves (Figure 7d) also suggest that there was no loss of species diversity during the course of this short-term (14 months) experiment.

5. Discussion & Conclusions

Our aim was to understand whether fungal communities in different horizons are significantly different from each other and investigate the effects of simulated intensified forest harvesting on these fungal communities. We expected to find distinct fungal communities in O, E and B soils and in O, E and B soils of different treatments. We also expected that fungal diversity might be reduced in treatment V since some ectomycorrhiza fungi are sensitive to physical disturbance.

Horizons in boreal forest podzols are physico-chemically distinct from one another, providing diverse and distinct niches. Microorganisms adapt to these different niches, leading to different communities in each horizon. Species richness and community structure in O, E and B horizon soils are significantly different regardless of treatment, with the exception of treatment **I**. This is in keeping with findings in previous studies (López-Mondéjar *et al.*, 2015; Lladó *et al.*, 2016; Marupakula *et al.*, 2017) where microbial communities in each horizon are significantly different but with many overlapping taxa. Other than in treatment **I**, E horizons possess higher species richness, probably due to their direct contact with both O and B horizons, allowing colonization from both above and below.

The ectomycorrhizal fungi *Piloderma sphaerosporum* and *Suillus bovinus* were dominant in the organic and mineral horizons respectively, indicating these species are better adapted to these environments. A previous study conducted by Fahad *et al.* (2016) showed high levels of uptake of base cations by *Suillus variegatus* and *Piloderma fallax*. *Cadophora finlandica*, a root associated endosymbiont, and *Oidiodendron chlamydosporicum* – an ericoid mycorrhizal fungus, also appear to have a higher affinity for mineral horizons. *Hyaloscyphaceae* – a saprotrophic family growing on dead wood and decaying plant matter, are more abundant in the organic horizon.

Excluding treatment **I**, E and B horizons of different treatments have significantly different community structure. Alterations in the uppermost O horizon are likely to affect the soil chemistry of underlying horizons. This may explain the distinction between treatments in E and B horizon soils and the lack of distinction between E and B soils horizons of treatment **I**. The much lower species richness of the E horizon of treatment I may also be explained by the absence of the overlying O horizon, both altering the soil

chemistry of the E horizon and removing any taxa that would typically live on the O-E border. Intensive harvesting of organic residues may reduce the diversity of fungal communities colonizing mineral horizons lower in the soil profile. Additionally, increased competition between taxa due to the reducing volume of the B horizon from treatments **I**-**IV** may result in alterations in community structure.

There is an increase in abundance and colonisation by *S. bovinus* in the B horizon from treatments **I**-**IV**. This intensified colonisation is likely explained by the increased volume of the O horizon and therefore an increased supply of N. As the seedlings grow larger, a larger sink is created for nutrients, and more carbon can be allocated to their associated fungi, resulting in the denser colonisation of soils by mycelium and an increased rate of nutrient harvesting. The decrease in the saprotroph *Myxotrichaceae* in E horizon treatment **I** is likely due to the lack of O horizon and an absence of organic matter. The abundance of *Myxotrichaceae*, *Oidiodenron spp.*, *Mortierella pulchella*, *Penicillium sp.*, *Acephala chlamydosporicum* and *Hyaloscyphaceae* appears to be positively linked to the volume of O horizon, most likely due to their saprotrophic lifestyle.

The measured species richness of OEB mixed horizons in treatment **V** was not significantly reduced when compared to O, E and B horizons of treatment **III** although the community structure was changed (Figure 8e). This could be due to differences in soil chemistry. Seedlings in treatment **V** were larger than those of treatment **III** which may be due to the more even distribution of different nutrients resulting from mixing, providing better access to nutrients stimulating growth.

If ectomycorrhizal fungi had compensated for the lack of organic matter by increasing colonisation and mineral weathering we would have expected *Pinus sylvestris* seedlings to be of a similar size regardless of treatment, however this did not happen, within the time scale of this experiment. Evidently there was a confounding effect on the availability of N of reducing the amount of organic material. N and C cycles are closely linked, and it has been shown on numerous occasions, as summarised by Sponseller *et al.* (2016) that an increase in N results in an increase in C sequestration in soils as plants allocate more photoassimilates to their microbial partners. A shortage of N from the O horizon will limit plant growth and thus the amount of C that plants can allocate belowground to roots and mycorrhizal fungi. This will limit the ability of ectomycorrhizal to allocate C to mineral substrates and thus limit their capacity to mobilise base cations and P through weathering.

Fahad *et al.* (2016) studied mobilisation of P and base cations from granite particles *in vitro* and found evidence that ectomycorrhizal fungi were more efficient at mobilising nutrients, accumulating significantly higher concentrations of Mg, K and P than non-mycorrhizal fungi. The ectomycorrhizal fungi fractionated stable Mg isotopes and were significantly depleted in heavy ^{26}Mg, displaying a significant inverse relationship

between the δ^{26}Mg signature in mycelial tissue and the concentration of total Mg accumulated (Fahad *et al.* 2016). Thus, the greater the uptake of total Mg, the more negative the δ^{26}Mg signature became. This property was used by Finlay *et al.* (2020) to analyse Mg uptake in the same experimental system investigated in this thesis. These authors found that the signature of δ^{26}Mg in soil solution became successively enriched as growth and total Mg uptake increased due to increasing amounts of O horizon material. Discrimination against the heavy isotope of Mg led to accumulation of ^{26}Mg in solution but this only occurred in systems containing plants and only in the B horizon suggesting that this was the principal site of Mg mobilisation. Table 1 indicates that there are high concentrations of Mg in the B horizon soil at Jädraås and, together with the high biomass allocation to roots and ectomycorrhizal mycelium (Figure 5a) suggest that carbon allocation to ectomycorrhizal mycelium may have been important in mobilisation of Mg. No information on composition of fungi colonising the different horizons was given by Finlay *et al.* (2020) but the data in the present study indicate that *Suillus bovinus, Rhizopogon evadens, Acephala macrosclerotiorum,* another unidentified *Suillus* species and other unidentified species belonging to the Ascomycota, Boletales and Basidiomycota, were common in the B horizon soil, accounting together for more than 50% of the ITS sequences. A previous study (Marupakula *et al.,* 2017), using the soil from the same forest, but based on fungal colonisation or root tips, found that *S. bovinus* accounted for almost 25% of the sequences in the E horizon but that the B horizon roots were dominated (85% relative abundance) by *Suillus variegatus* which was replaced by *Rhizopogon bacillisporus* (64%) when the soil was fertilised with N in the form of urea. This study was conducted over a shorter six-month period and in much smaller microcosms than the present study, possibly influencing the composition of the fungal communities at the point of harvesting. Many recent field studies have not sampled the B horizon but an early study by Lindahl *et al.* (2007) in the same forest found that *Piloderma reticulatum* had a relative abundance of 42% in the B horizon soil. A recent field study in a similar, nearby forest at Lamborn, Sweden (Marupakula *et al.,* 2020) found that *Piloderma lanatum*, *Meliniomyces bicolor* and *Tricholoma portentosum* were common in B horizon soil and that *P. lanatum*, *M. bicolor*, *T. portentosum* and *Rhizopogon evadens* were also dominant ectomycorrhizal colonisers of roots in the B horizon.

The dominance of these taxa suggests that they are actively receiving plant assimilates and could be important in contributing to the mobilisation of base cations, including Mg, that has been demonstrated in the B horizon, but further experiments are required to provide more direct evidence of patterns of C allocation. The ^{13}C-SIP analyses we were originally planning are still in progress and should provide some relevant evidence. Unpublished data from our laboratory (Mahmood *et al.* personal communication) using ^{13}C labelling suggests that ^{13}C enrichment in the soil of the B horizon is much higher than that in the O horizon soils and that it is highest in treatment IV. Interestingly the residual amounts of ^{13}C label in the plants in this treatment were lowest, suggesting that a higher

proportion of the assimilated ^{13}C had been allocated belowground to the mycelium and subsequently the soil and soil solution. Additional unpublished results that are still in preparation support this idea and the idea that the carbon allocation is related to mobilisation of Mg in the B horizon since the enrichment of ^{13}C in both the soil and the soil solution is significantly related to the mycelial biomass in the B horizon and both of these parameters increase from treatment I to IV. The $\delta^{26}Mg$ signature in soil solution is also positively correlated with plant Mg content, plant biomass and ^{13}C enrichment in the soil solution. All these results are consistent with the idea that the fungi we have identified in the present study are playing an important role in mobilisation of Mg from the B horizon soil. The question of how the C is allocated and in what form it is ultimately sequestered will require additional experiments.

Further information is required about the capacity of mycorrhizal fungi to weather mineral nutrients and how this activity affects long term carbon sequestration. "Enhanced weathering" has been suggested as a strategy to mitigate climate change by reducing the amount of CO_2 entering the atmosphere (Beerling *et al.*, 2018). Although much progress has been made in understanding the important roles ectomycorrhizal fungi play in mobilising organic N, increasing numbers of studies suggest that ectomycorrhizal symbioses may play important roles in pedogenesis and carbon storage that involve interactions in the mineral soil (Leake and Read, 2017). In the present study the relative abundance of ectomycorrhizal fungi was highest in the B horizon soil, however it is important to consider the limitations of this experiment. As shown by the rarefaction curves, sampling was incomplete and likely omitted many rarer species. Microcosms were only incubated for 14 months, meaning that it is unlikely that fungal communities reached their climax. Furthermore, the *Pinus sylvestris* seedlings are very young. Trees at different developmental stages harbour different communities. In this instance fungal communities investigated would only apply to other seedlings. Also, this study was conducted in small microcosms with no understory vegetation, and whilst it provides important insights into processes occurring following depletion of organic substrates, it does not fully reflect the complexity present in forest soils.

Future studies of management strategies involving N fertilization (as well as inadvertent, anthropogenic N deposition) and removal of organic material during biofuel harvesting should include the bacteria and fungi in this horizon. Better knowledge of the identity, distribution and functional characteristics of these many different microbial taxa, as well as the chemical forms in which C is sequestered, are a pre-requisite for development of sustainable biomass harvesting, coupled with new carbon dioxide technologies.

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