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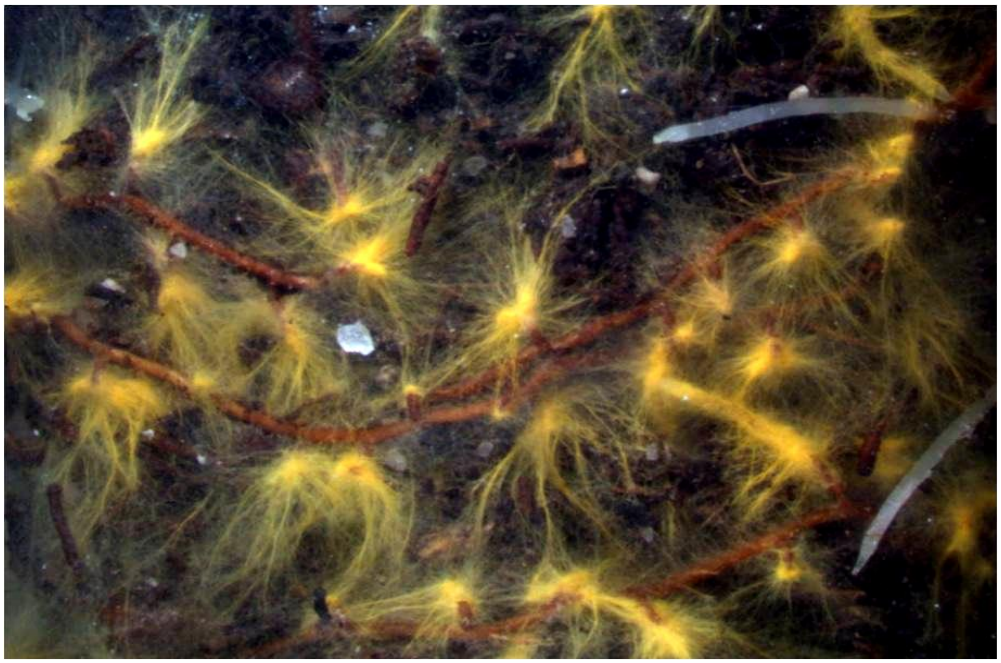

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Microbial communities in a boreal forest podzol profile and responses to the presence of *Pinus sylvestris* seedling roots

Johanna Jernberg

Supervisor:	Shahid Mahmood, Swedish University of Agricultural Sciences, Forest Mycology and Plant Pathology
Assistant Supervisor:	Roger Finlay, Swedish University of Agricultural Sciences, Forest Mycology and Plant Pathology
Examiner:	Björn Lindahl, Swedish University of Agricultural Sciences, Forest Mycology and Plant Pathology

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Abstract

The boreal biome consists of coniferous forests that cover much of the northern hemisphere, and constitute an important natural resource for the forest industry, but has also gained attention in the light of global warming in acting as potential sinks of atmospheric carbon dioxide. Both forest production and carbon sequestration are closely connected to the activities of soil microorganisms, which play major roles in nutrient cycling and decomposition of plant biomass. Boreal forest soils are commonly classified as podzols, which are characterized by their clear horizon development with depth. Ecological surveys of soil biota commonly focus on the topsoil. The aim of this experiment was to examine if the organic, eluvial and illuvial horizons of a Swedish boreal forest podzol harboured distinct communities of fungi, bacteria and archaea, and further if roots of Scots pine seedlings, through input of rhizodeposition, would induce shifts in microbial communities in the different soil categories. The study was conducted as a microcosm experiment in combination with denaturing gradient gel electrophoresis (DGGE) fingerprinting of phylogenetic marker sequences, ITS rDNA for fungi and 16S rDNA for archaea and bacteria. Soil sampled at the time of experimental set-up served as control for community shifts caused by incubation time alone.

All soil horizons harboured distinct communities of fungi, bacteria and archaea. Fungi and bacteria displayed complex community profiles for all soil samples, but richness for both groups tended to be highest in the organic soil. The archaeal community profiles were relatively simple for all soil samples, though their richness appeared to be highest in the eluvial soil. Fungi was the microbial group that displayed the strongest response to the presence of Scots pine seedling roots, and ectomycorrhizas were observed in all microcosms with seedling, although the organic, eluvial and illuvial horizons harboured distinct morphotypes. In contrast, neither bacteria nor archaea responded much to the presence of Scots pine seedlings, but the bacterial communities underwent a clear shift in composition during the incubation period, especially in the organic soil. These results highlight the importance of including all layers in a soil profile to obtain a holistic view on the ecology of soil microorganisms and their roles in ecosystem functioning.

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1. Introduction

Humans have long tried to understand the nature of their surroundings, and in doing so have laid the foundation for the discipline of ecology. The goal of ecology is to seek patterns and trends that can explain and predict how living entities are distributed and how they interact with the biotic and abiotic environment in which they dwell (Begon *et al*. 2006). The subject of microbial ecology is no exception in this endeavour (Prosser *et al.* 2007), though the methods of ecological studies of microorganisms in some aspects differ from those of macroorganisms. Microbiologists have traditionally relied on laboratory cultivation and microscopy to elucidate the everyday life of microbes (Madigan *et al*. 2012). However, phylogenetic classification is difficult to achieve by observing cell morphologies alone (Woese, 1987), and commonly only a small fraction of the microbial taxa present in natural samples are readily cultivated on standard laboratory media (Staley & Konopka, 1985). Instead, a vast array of cultivation-independent molecular methods has been developed and are commonly applied in microbial ecology surveys to resolve the phylogenetic composition of microbial communities and to examine the activities of community members (van Elsas & Boersma, 2011). These methods are often based on comparison of the primary structure of nucleic acids or polypeptides (Zuckerkandl & Pauling, 1965), and these informational molecules, especially ribosomal DNA sequences, act as taxonomic identities for any cellular system (Woese, 1987). Application of cultivation-independent methods has allowed us to recognize that Earth harbours an immense microbial diversity (Hawksworth, 2001; Torsvik *et al*. 2002), and a microbial biomass that might hold as much carbon as is stored in plant biomass globally (Whitman *et al*. 1998).

Forest ecosystems form conspicuous features in terrestrial landscapes and collectively cover about one third of Earth´s terrestrial surface (FAO, 2010). These plant communities are in many areas important resources for food, fuel and forestry products (FAO, 2010), but are also of interest in the light of global warming in acting as potential sinks of atmospheric carbon dioxide (Pan *et al*. 2011). There is a close connection of both forest production and carbon sequestration to the activities of soil microorganisms, especially in boreal forest ecosystems where over 60 percent of the carbon stock is stored in the soil (Kasischke, 2000; Pan *et al*. 2011), and substantial amounts of nutrients are locked up in soil organic matter (see Read & Perez-Moreno, 2003). In the soil, microorganisms partake in an array of important processes such as decomposition of organic matter (Read & Perez-Moreno, 2003), element transformations (Gadd, 2010), and development of soil structure (Rillig & Mummey, 2006). Microbial ecology studies of forest soils commonly focus on the topsoil where the highest density of biomass is usually encountered (Fritze *et al*. 2000; Hartmann *et al*. 2012). However, it has been estimated that in Swedish podzols the bulk of soil organic carbon occurs in the mineral horizons (Olsson *et al*. 2009), thus constituting a potential resource for microbial metabolism (Karhu *et al*. 2010). Even though the knowledge of

microorganisms inhabiting boreal forest soils is steadily increasing, their ecological roles in many cases remain an enigma.

2. Background

2.1 Boreal forests

The boreal forest biome, also called Taiga, forms a circumpolar belt mainly located between latitudes 50°N and 60°N (Taggart & Cross, 2009). In these northern regions the climate is characterized by low annual mean temperatures (Whittaker, 1975), and high seasonal variation with cold and dry winters, and moist and mild summers (see Taggart & Cross, 2009). Boreal plant communities commonly display low species diversity (Whittaker, 1975) and a uniform physical structure throughout Northern America and Eurasia (Larsen, 1980), with both regions having many tree genera and field layer species in common (Larsen, 1980). Coniferous trees of genera such as pine (*Pinus*), spruce (*Picea*), fir (*Abies*) and larch (*Larix*) usually dominate older forest stands, while deciduous broad leaf tree genera, such as birch (*Betula*), aspen (*Populus*) and alder (*Alnus*) are more frequent in earlier successional stages (Taggart & Cross, 2009). In Fennoscandian boreal forests, Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) are the most common coniferous tree species, where Scots pine tend to predominate in drier areas while Norway spruce tend to grow at mesic to moist sites (Esseen *et al*. 1997). Many coniferous trees share a similar growth form, with an evergreen triangularly shaped canopy that allows for effective capture of solar rays coming in at low angles (Taggart & Cross, 2009). The light that reaches the forest floor supports a field layer dominated by ericaceous dwarf shrubs belonging to the genera *Calluna*, *Vaccinium* and *Empetrum* (Esseen *et al*. 1997), which are underlain by a bottom layer consisting of mosses of genera such as *Hylocomium*, *Pleurozium* and *Dicranum*, and lichens mainly belonging to the genera *Cladina* and *Cladonia* (Nilsson *et al*. 1999; Nilsson & Wardle, 2005). The composition of the forest floor flora is largely influenced by canopy closure, and hence the intensity of light reaching the forest floor (Persson, 1980). Mosses are typically more abundant in moist and shady areas while lichens tend to dominate in drier areas with a higher influx of sunlight (Bonan & Shugart, 1989). Boreal forests generally lack a well developed shrub layer, and the scattered large shrubs tend to consist of suppressed trees (Persson, 1980).

2.2 Podzols – a description

Large areas of Earth´s terrestrial surface are covered with soil, which can be defined as the layer of unconsolidated rock fragments and organic matter that can support plant growth (Hartel, 2005). The identity of any soil profile is the result of pedogenesis - the transformation of parent material by climate and biota through time. Boreal forest soils are commonly classified as podzols, which are characterized by their typical vertical horizon formation with depth (Figure 1) (Sauer *et al*. 2007). Podzols develop in humid and cold

climates, usually on coarse-grained parent material of felsic composition (Lundström *et al*. 2000). The prevailing low annual mean temperatures and production of decomposition resistant plant litter in boreal forests allow for the development of a superficial horizon of organic matter overlying the mineral parent material (DeLuca & Boisvenue, 2012). This organic (O) horizon can be subdivided into a litter (L), a fermentation (F), and a humus (H) layer, which differ in the degree of litter decomposition (Lindahl *et al*. 2007; Hilli, 2011).

Throughout the organic horizon various organic acids, including fulvic acids, humic acids and low molecular weight organic acids, are released into the soil solution by soil biota and decaying plant litter (Lundström *et al*. 2000). These acids are leached down into the underlying mineral soil where they assist in weathering of primary minerals by ion-exchange, dissolution and formation of metal-organic complexes (Eriksson *et al*. 2005). When protons replace cations in the crystal lattice of primary minerals, the surface of the mineral grains turns white in colour (Eriksson *et al*. 2005). This process is responsible for the characteristic pale ash colour of the eluvial (E) horizon which gives this group of soils their name, derived from the Russian words “pod” and “zola” which means “under ash” (see Sauer *et al*. 2007). Weathering products are transported down the soil profile with the percolating soil solution, leaving the eluvial horizon poor in nutrients (Eriksson *et al*. 2005). Despite the proximity to the organic horizon, levels of dissolved organic matter are usually low in the eluvial soil (van Hees *et al*. 2000), which might indicate high decomposition rates of this fraction in the eluvial horizon (Buurman & Jongmans, 2000). As water percolates further down the soil profile, aluminum, iron and organic substances are precipitated and accumulate in a zone called the illuvial (B) horizon (see Lundström *et al*. 2000). Buurman and Jongmans (2000) outline three processes that contribute to the precipitation and accumulation of illuvial material; (1) weathering of silicate minerals allows for the formation and precipitation of allophanic material, (2) charge saturation of metal-organic complexes eventually hamper their migration further down the soil profile, and (3) decomposition of the organic component of metal-organic complexes releases iron and aluminum ions that subsequently precipitate as sesquioxides. In well-drained boreal podzols, sesquioxides are responsible for the red to brown colouration of the illuvial horizon (Eriksson *et al*. 2005). Compared with the eluvial horizon, the illuvial zone contains higher levels of both nutrients and organic matter, the latter can occur as coatings over mineral grains or as up to sand-sized pellets whose composition indicate substances of plant root origin (see Buurman & Jongmans, 2000). Podzol soils are typically acidic throughout the profile with the lowest pH values in the organic horizon, and an increasing pH with depth (Fritze *et al*. 2000; Eriksson *et al*. 2005).

Figure 1. A podzol profile of a coniferous boreal forest soil at Jädraås, Sweden. An organic layer has accumulated on top of the glaciofluvial parent material. Weathering and leaching in the upper mineral soil have resulted in the development of the pale ash coloured eluvial horizon, while precipitation of organic matter, aluminum and iron gives the underlying illuvial horizon its rust coloured appearance. (*Photo Johanna Jernberg*).

2.3 Podzols as microbial habitats

Soil is a dynamic medium consisting of solid, liquid and gaseous phases that collectively define the physicochemical environment that soil organisms encounter. All living entities each have a unique set of inherent traits enabling optimal growth at a particular set of environmental conditions (Madigan *et al*. 2012). Factors regarded as important determinants for growth and distribution of soil microorganisms include resource availability, matrix, soil chemistry, dispersal and biotic interactions such as competition, symbiosis and predation (Tiedje *et al*. 1999). Microorganisms experience the soil matrix as a very heterogeneous environment that provides a vast array of so called microniches, and microbial communities commonly display a much higher diversity in soil than in many other natural environments such as water columns and aquatic sediments (Torsvik *et al*. 2002).

Terrestrial ecosystems are ultimately driven by electromagnetic radiation emitted by the sun. Plants harness this energy and transform it into chemical forms via the process of photosynthesis, where organic compounds are produced by reduction of carbon dioxide. A substantial amount of produced photosynthate is allocated belowground, either as shed litter or as rhizodeposition by plant roots, and thus forms a resource base for any soil food

web (Cheng & Gershenson, 2007). Plant litter differs widely in nutrient content and resistance to degradation (Aerts, 1995). Most members of boreal plant communities are evergreen (i.e. conifers and ericaceous dwarf shrubs), and invest in production of long lasting tissues that are rich in structural polymers and low in nutrient content (Aerts, 1995), which leads to low decomposition rates of boreal plant litter (Hilli, 2011). In addition, boreal plants contain high levels of secondary metabolites, such as terpenes and phenolic compounds, that further might retard decomposition rates and nutrient extraction from organic matter (Smolander *et al*. 2012). However, labile organic compounds in fresh litter are degraded quickly (Karhu *et al*. 2010; Hilli, 2011), leading to a gradual accumulation of decomposition resistant compounds (Karhu *et al*. 2010; Hilli, 2011) with lower carbon to nutrient ratio with age, and hence depth (Lindahl *et al*. 2007). This vertical fractionation of resources is especially pronounced in boreal forest podzols due to low levels of biogenic mixing of litter within the organic horizon and with underlying mineral soil (Eriksson *et al*. 2005). Carbon distribution in soil is also influenced by rhizodeposition, which encompass sloughed root material as well as root exudates that are rich in easily utilised compounds (Cheng & Gershenson, 2007). This root-influenced zone, called the rhizosphere, is densely populated with soil biota and is characterized by intense biological interactions (Walker *et al*. 2003) and high spatial and temporal environmental variability (Hawkes *et al*. 2003). A study of the root distribution in boreal podzols has revealed a vertical separation of roots from different members of the plant community, where the highest fine root density of ericaceous dwarf shrubs could be found in the fermentation and humus layers, while the fine root density of Scots pine was highest in the upper mineral soil (Persson, 1983). In another survey, Rosling and colleagues (2003) found the highest density of coniferous tree fine roots in the organic horizon of a boreal forest podzol, and a lower density but a higher total number of fine roots in the mineral soil.

In essence, boreal forest podzols display a clear vertical division of resources and living environments for soil organisms to inhabit, where plant roots locally intercept and create living conditions that differ from those in the surrounding bulk soil – the soil volume presumed to be unaffected by the presence of plant roots. The often thick moss and lichen carpet that cover the forest floor acts as an insulating layer over the organic horizon, and dampens temperature and moisture oscillations and hence relieves soil biota from some environmental stress (Bonan & Shugart, 1989). Also, slow decomposition of plant litter allows for the organic layer to supply soil organisms with a continuous stock of resources throughout the year (Lindahl *et al*. 2010). Hence, boreal forest soils provide a rather stable environment with distinct vertical differences in character, a feat that is reflected in previous comparisons of the vertical distribution of soil microbial communities (Fritze *et al*. 2000; Heinonsalo *et al*. 2001; Rosling *et al*. 2003, Lindahl *et al*. 2007, Hartmann *et al*. 2009; Baldrian *et al*. 2012).

2.4 Soil fungi

Fungi form a separate kingdom within the domain Eukarya, with 98 000 described species (Moore *et al*. 2011) and an estimated total of 1.5 million species worldwide (Hawksworth, 2001). All fungi are heterotrophs that acquire metabolic resources by external enzymatic breakdown of substrates, followed by absorption of the enzymatic products (Moore *et al*. 2011). Their lifestyles vary and are commonly assigned to symbiotic, saprotrophic or pathogenic trophic strategies (see Finlay, 2007). Fungi can exist as unicellular forms such as yeast- or chytrid cells, or as multicellular mycelia (Morton, 2005). Even though a fungal mycelium can occupy a large volume of substrate, fungi are regarded as microorganisms due to the microscopic diameter of individual hyphae (Lindahl *et al*. 2010). Mycelial growth is an effective life strategy in a heterogeneous environment such as soil where resources are patchily distributed. Growth patterns of mycelia are dynamic and respond to resource availability. Foraging hyphae in nutrient poor substrates exhibit extension growth, while hyphae that encounter a resource switch to a growth pattern with an increased branching frequency to increase utilisation efficiency (Moore *et al*. 2011). Within a single mycelium, resources can be reallocated to meet local needs in order to sustain an effective overall mycelial growth (Boberg, 2009). This internal transport of resources is enhanced by the production of hyphal cords or rhizomorphs which function as transport pipes and exploratory organs (Moore *et al*. 2011).

Boreal forest soils are generally regarded as poor in easily available (i.e. inorganic) nutrients, as the bulk of nutrients occur in organic forms (Read & Perez-Moreno, 2003). Microorganisms gain access to these nutrients by breaking down organic polymers into entities that cells manage to absorb (Lindahl *et al*. 2002 and references herein). In boreal forest soils, decomposition of organic matter can largely be assigned to fungi, as they are efficient in degrading structural polymers by using hyphae to penetrate cell wall material, and they have an arsenal of enzymes adapted to perform optimally in low pH environments (De Boer *et al*. 2005 and references herein). In addition, some basidiomycetes have a near monopoly on lignin degradation (De Boer *et al*. 2005 and references herein). Saprotrophic fungi obtain carbon, energy and nutrients by direct digestion of organic matter, and this lifestyle can be found in many fungal groups such as basidiomycetes, ascomycetes and members grouped within the zygomycetes (Moore *et al*. 2011), but saprotrophs in boreal forests mainly belong to the ascomycetes (Lindahl *et al*. 2010). Mycorrhizal fungi require a constant supply of energy-rich carbon compounds via symbiotic root connections with a plant partner, an association occurring in about 80 percent of all terrestrial plants (Smith & Read, 2008). Mycorrhizas are traditionally grouped into seven categories (Smith & Read, 2008), two of which are of major importance in boreal ecosystems (Read & Perez-Moreno, 2003). The first category is called ectomycorrhiza (ECM), and commonly involves a symbiotic relationship between forest trees and mycorrhiza forming basidiomycetes, although some ECM fungi belong to the ascomycetes. The second type is referred to as ericoid mycorrhiza (ERM) and forms between ericaceous shrubs and some ascomycetes. Both ECM and ERM

fungi possess degradative capabilities that enable them to extract nutrients for themselves and their plant associates (Read & Perez-Moreno, 2003; Bödeker, 2012).

Several studies of coniferous forest soils point to a vertical stratification of fungal taxa (Rosling *et al*. 2003; Lindahl *et al*. 2007; Hartmann *et al*. 2009; Baldrian *et al*. 2012; Bödeker, 2012; Hartmann *et al*. 2012), correlating well with differences in life strategies (Rosling *et al*. 2003; Lindahl *et al*. 2007; Bödeker, 2012). In the upper litter, endophytic fungi and opportunistic saprotrophs thrive (Lindahl *et al*. 2007; Bödeker, 2012), as do lichen forming fungi (Baldrian *et al*. 2012). Further down in the soil profile, plant roots provide mycorrhizal fungi with energy-rich carbon compounds to explore aging organic matter for nutrient mining (Read & Perez-Moreno, 2003). Evidence for this activity is supported by an observed increase in C:N ratio of organic matter explored by mycorrhizal fungi (Lindahl *et al*. 2007), and by detection of a high activity of humus oxidizing enzymes produced by mycorrhizal taxa under nitrogen limited conditions (Bödeker, 2012). The majority of mycorrhizal taxa in boreal podzols display restricted vertical distribution throughout the profile (Heinonsalo *et al*. 2001, Rosling *et al*. 2003; Hartmann *et al.* 2009). The commonly observed dominance of ectomycorrhizal fungi in the lower organic horizon and mineral soil may reflect a competitive advantage over saprotrophic fungi. In a microcosm study it was shown that extramatrical mycelia of an ectomycorrhizal fungus inhibited advancement of mycelia of a wood-decomposing fungus, where the competitive outcome depended on the amount of substrate available for the latter group (see Lindahl *et al*. 2002). In addition, disconnection of the carbon flow between ectomycorrhizal fungi and their plant partners has been shown to result in a decreased abundance of ectomycorrhizal fungi and an increased abundance and activity of saprotrophic and possibly ericoid mycorrhizal fungi (Yarwood *et al*. 2009; Lindahl *et al*. 2010). A decomposition experiment conducted by Bödeker and colleagues (see Bödeker, 2012) showed that the vertical location of buried substrate bags was a stronger determinant of fungal communities colonizing the substrates than was substrate quality. Their results also imply that saprotrophic fungi are more effective decomposers of fresh litter than ectomycorrhizal fungi, but not in decomposition of more recalcitrant substrates such as humus. Taken together, these observations might indicate that the availability of easily utilised substrates in 'young' litter give saprotrophic fungi a competitive advantage in the litter layer, while ectomycorrhizal fungi fare better than most saprotrophic taxa, due to the supplement of energy-rich carbon compounds from plant associates, in environments otherwise depleted in high quality substrates.

Thus, forest soil dwelling fungi provide many services essential to boreal ecosystem functioning. With their efficient decomposition of plant residues, fungi release resources for other soil organisms to utilise, and help to overcome resource isolation in the soil matrix by mycelial translocation (see Simard *et al*. 2012). Their ability to utilise organic forms of nutrients short-circuit and make nutrient cycling in boreal forest ecosystems more effective by inhibiting the mineralization step and hence potential leaching. Also, fungi are believed

to be involved in biogenic weathering of minerals by release of low molecular weight organic acids and other chelating agents to gain access to base cations and phosphorus (Jongmans *et al*. 1997). Like the rhizosphere, the mycorrhizosphere creates zones of microniches for other microorganisms to occupy, and there are indications that mycorrhizal associations also may involve interactions with bacteria (Frey-Klett *et al.* 2007) and archaea (Bomberg & Timonen, 2007; Bomberg *et al.* 2010).

2.5 Two domains of prokaryotes – Archaea and Bacteria

Commonly, all cells of prokaryotic character are referred to as bacteria. Prokaryotic cells differ from eukaryotic cells in many aspects both structurally and physiologically (Madigan *et al*. 2012). The term prokaryote ("pro"-before, "karyon"-nucleus) itself highlights one major difference between these two cell types. In all eukaryotic cells, the genome is contained in a membrane bound structure called the nucleus, which is absent in prokaryotic cells where the genome is freely suspended in the cytoplasm. Also, eukaryotic cells harbour membrane-bound organelles such as mitochondria and chloroplasts, which are both of bacterial origin (Sagan, 1967). Although eukaryotic cells can be regarded as more complex in structure, it is claimed that prokaryotic cells have been around on earth and experienced evolution for at least 2 billion years longer than eukaryotic cells, giving rise to a high phylogenetic and functional diversity of prokaryotes on Earth (Torsvik *et al*. 2002; Falkowski *et al*. 2008).

Since prokaryotic cells exhibit asexual reproduction, the classical biological species concept does not apply (Achtman & Wagner, 2008). Instead a polyphasic approach to define a prokaryotic species is used and has resulted in the description of over 7000 species (Achtman & Wagner, 2008). Traditionally, prokaryotic classification has been based on observable characteristics, a procedure called phenotypic classification (Woese, 1987). Today it is common practice in microbial ecology to employ the phylogenetic concept, which is based on the notion that the degree of similarity in primary structure of informational biomolecules, such as nucleic acids and polypeptides, mirrors the degree of evolutionary ancestry (Zuckerkandl & Pauling, 1965). In 1977, Woese and Fox reported that based on studies of 16S rRNA genes, methanogenic bacteria seemed to deviate from other bacteria and they were proposed to be assigned a separate "urkingdom" then referred to as archaebacteria. This eventually led to the development of the three domain classification system used to group organisms today, where the domain **Eukarya** encompass all eukaryotic organisms while the two other domains, **Bacteria** and **Archaea**, both encompass prokaryotic cells (Woese, *et al*. 1990). Even though the two prokaryotic cell types resemble each other morphologically they are at the structural and molecular level no more related to each other than they are to eukaryotic cells (Woese *et al*. 1990). In fact, evidence points to a shared ancestry between the archaea and eukarya, the nature of which is still debated (Gribaldo *et al*. 2010).

Prokaryotic cells display a wide range of metabolic strategies with regard to sources of energy (phototroph or chemotroph), electrons (organotroph or lithotroph) and carbon for anabolism (heterotroph or autotroph) (Madigan *et al*. 2012). In addition, prokaryotic cells can use a wide variety of both external (respiration) and internal (fermentation) electron acceptors (Madigan *et al*. 2012). To bring order into the functional diversity, microorganisms have been assigned to functional groups according to growth strategies or metabolic capabilities (van Elsas *et al*. 2007; Fierer *et al*. 2007; Prosser *et al*. 2007). For example, microbes that grow slowly and display stable population sizes are referred to as autochthonous. These cells can often be described as oligotrophic which means that they are well adapted to growing under nutrient poor conditions. At the other end of the spectrum, zymogenous microorganisms have fluctuating populations, with high abundances under nutrient rich conditions that decline rapidly when nutrients become limiting. Zymogenous cells commonly depend on easily utilised (labile) substrates for growth, a strategy referred to as copiotrophic. In addition, many microorganisms possess some degree of phenotypic plasticity, being able to switch between trophic strategies or even enter dormancy to cope with changes in environmental conditions (see Prosser *et al*. 2007).

2.6 Bacteria in coniferous forest soils

The domain *Bacteria* encompasses at least 80 phyla (Madigan *et al*. 2012), and displays a high genotypic diversity in soil environments (Torsvik *et al*. 2002; Aller & Kemp, 2008). In coniferous forest soil, the presence of over 20 bacterial phyla has been recorded (Baldrian *et al*. 2012), where the most abundant phyla include *Actinobacteria*, *Proteobacteria* and *Acidobacteria* (Hartmann *et al*. 2009, Yarwood *et al*. 2009; Lindahl *et al*. 2010, Eilers *et al*. 2012; Baldrian *et al*. 2012, Hartmann *et al*. 2012) which together can constitute up to 80% of bacterial 16S rDNA sequences detected in a soil sample (Baldrian *et al*. 2012). However, the majority of bacterial taxa occur in rather low abundances (Hartmann, *et al*. 2009; Baldrian *et al*. 2012), and other bacterial phyla commonly encountered in coniferous forest soils include *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia* and *Planctomycetes* (Hartmann *et al*. 2009, Yarwood *et al*. 2009; Lindahl *et al*. 2010, Baldrian *et al*. 2012, Hartmann *et al*. 2012). Both field- and microcosm studies have previously shown that the bacterial biomass in coniferous forest soil is higher in the organic horizon than in underlying mineral soil (Heinonsalo *et al*. 2001; Hartmann *et al*. 2012), and that bacterial communities tend to shift in composition and decrease in complexity with depth (Hartmann *et al*. 2009; Eilers *et al*. 2012).

It is claimed that the majority of soil bacteria maintain themselves through heterotrophic metabolism (Alexander, 2005). And indeed, many soil bacteria have responded to addition of organic substrates in manipulation experiments, indicating that different carbon compounds either promote or deter growth of certain groups of soil bacteria (Fierer *et al*. 2007; Goldfarb *et al*. 2011; VanInsberghe *et al*. 2013). Actinobacteria and proteobacteria commonly give a positive growth response to the addition of labile carbon compounds,

while growth of acidobacteria is negatively affected (Fierer *et al*. 2007; Goldfarb *et al*. 2011). In an attempt to isolate bacteria from forest soil, VanInsberghe and colleagues (2013) noted that bacteria readily grown on different standard media, such as proteobacteria and actinobacteria, were present in low abundances in corresponding soil samples, as investigated by deep sequencing of 16S rDNA sequences. In contrast, bacteria with a high and uniform abundance of 16S rDNA sequences, such as acidobacteria, could only be grown on nutrient poor cultivation media (VanInsberghe *et al*. 2013). These studies clearly suggest that substrate quality and quantity affect the composition of bacterial communities in soil, with more pronounced changes induced by changes in easily utilised carbon compounds (Karhu *et al*. 2010; VanInsberghe *et al*. 2013). However, Fierer and Jackson (2006) also found a strong positive correlation between soil pH and the overall diversity of soil bacterial communities. Many gram-negative bacteria thrive in topsoils and in the rhizosphere, which both are environments enriched in easily degraded substrates (Fierer *et al*. 2007; Hartmann *et al*. 2009; Yarwood *et al*. 2009; Karhu *et al*. 2010), while bacteria that manage to degrade more complex molecules, such as actinobacteria, are abundant in the humus and mineral layers in podzol profiles (Fritze *et al*. 2000; Hartmann *et al*. 2009; Baldrian *et al*. 2011).

Plant communities can affect soil microorganisms by providing litter and rhizodeposits of varying character, but the selective pressure by plant roots can be difficult to discern due to the close association with mycobionts in mycorrhizas (Prescott & Grayston, 2013). However, different forest types (Hackl *et al*. 2004) and plant species (Priha *et al*. 2001) have been shown to invoke distinct soil bacterial communities, with the largest differences often observed when comparing soils under broadleaf and coniferous trees (reviewed by Prescott & Grayston, 2013). In a microcosm study, Priha and colleagues (1999) found plant roots of different tree species to stimulate microbial activity in both the organic and mineral soils collected from a podzol, but a selective effect by tree species on bacteria could only be noted in the organic soil. The mycorrhizosphere provides many niches for bacteria to occupy, and a higher density of bacteria has been observed in the mycorrhizosphere than in corresponding bulk soils (Heinonsalo *et al*. 2001). Fungi can promote bacterial growth by enhancing decomposition of organic matter, and by providing substrates as necromass or exudates, but also reduce bacterial growth by competition for substrates or production of bactericides (see De Boer *et al*. 2005). Commonly, the mycosphere is populated with a distinct (Warmink *et al*. 2009; Kluber *et al*. 2011) and sometimes less diverse community of bacteria (van Elsas *et al*. 2011 and references herein) that demonstrate separate substrate utilisation patterns than bacteria from rhizosphere and bulk soil (Heinonsalo *et al*. 2001; Timonen & Hurek, 2006; Warmink *et al*. 2009). Bacteria often encountered in the mycosphere include genera such as *Pseudomonas*, *Burkholderia* and *Bacillus* (De Boer *et al*. 2005), many of which have been reported to facilitate mycorrhiza establishment (reviewed by Frey-Klett *et al*. 2007). Thus, the spatial distribution of bacterial taxa in a boreal soil profile is probably dictated by a combination of prevailing edaphic conditions and the multitude of biological interactions that occur between soil biota.

2.7 Archaea in moderate soil environments

Archaeal diversity has been found to be much lower than bacterial diversity in a wide range of habitats (Aller & Kemp 2008; Auguet *et al*. 2010), and their abundance usually constitutes a few percent of the total prokaryotic abundance in soils (Ochsenreiter *et al*. 2003; Bates *et al*. 2011). Members of the domain *Archaea* have traditionally been divided into two major phyla – *Crenarchaea* and *Euryarchaea* (Woese *et al*. 1990). Recently however, mesophilic crenarchaeota group 1 have been assigned a phylum of their own, the *Thaumarchaea* (Brochier-Armanet *et al*. 2008). Former reports on crenarchaeota will therefore in this report be referred to as thaumarchaeota. Initially archaea were believed to be restricted to extreme environmental conditions such as high or low pH, high salinity, anoxia and extreme temperatures (Woese *et al*. 1978). Prevalence of moderate archaea was first discovered in marine environments (DeLong, 1992), but was soon thereafter detected in an agricultural soil (Bintrim *et al*. 1997) and in the organic horizon of a boreal forest soil (Jurgens *et al*. 1997). Since then, the presence of archaea in a range of non-extreme terrestrial environments has been confirmed (Ochsenreiter *et al*. 2003; Bates *et al*. 2011). The majority of soil archaea affiliate with the thaumarchaeota, with a dominance of thaumarchaeota group 1.1b in most soil environments (Ochsenreiter *et al*. 2003; Auguet *et al*. 2010, Bates *et al*. 2011). However, forest soils and other acidic soils seem to harbour a high abundance of thaumarchaeota group 1.1c and affiliated phylotypes (Jurgens *et al*. 1997; Bomberg & Timonen, 2007; Kemnitz *et al*. 2007; Nicol *et al*. 2007; Bomberg *et al*. 2010, Bates *et al*. 2011). The contribution of euryarchaeota to total soil archaeal communities is usually very low (Pesario & Widmer, 2002; Ochsenreiter *et al*. 2003; Bates *et al*. 2011), but 16S rDNA sequences affiliated with the euryarchaeal order *Thermoplasmales* have previously been reported from the organic horizon in forest soils (Pesario & Widmer 2002; Kemnitz *et al*. 2007). Bomberg and colleagues (2007; 2010) investigated archaeal functions in boreal forest humus soil and detected both thaumarchaeota and euryarchaeota in the mycorrhizosphere of Scots pine seedlings. The highest levels of archaea were detected on ectomycorrhizal root tips while non-mycorrhizal root tips and bulk humus appeared to display a lower archaeal abundance (Bomberg & Timonen, 2007). When comparing selective pressures of several tree species, Bomberg *et al*. (2009; 2011) also found that different tree species selected for distinct archaeal communities on non-mycorrhizal fine roots, although the selective pressure from fungi appeared stronger on mycorrhizal fine roots. Thaumarchaeota group 1.1c was the most abundant archaeal group in both bulk humus and all mycorrhizosphere compartments (Bomberg & Timonen, 2007; 2009; Bomberg *et al*. 2011), and sequences belonging to euryarchaeota were mainly confined to the mycorrhizosphere and were affiliated with the order *Halobacteriales* and with the methanogenic order *Methanosarcinales* (Bomberg *et al*. 2011). Different field studies investigating the distribution of soil archaea in relation to plant communities and the rhizosphere report contradictory results. Sliwinski and Goodman (2004) compared the rhizosphere and bulk soil of 76 plant species and found a tendency to higher archaeal richness in rhizosphere soil. When examining a glacier foreland, Nicol and colleagues (2005) did not find any major

difference in archaeal community DGGE profiles between rhizosphere soil from several plant species and corresponding bulk soils, but they noted a major increase in thaumarchaeal diversity with soil age and development of plant cover. In another study, it was shown that two species of mangrove trees growing in the same area harboured distinct rhizosphere archaeal consortia that also differed from the bulk sediments, where one tree species had lower and the other higher richness than the bulk sediments (Pires *et al*. 2012). While thaumarchaeota group 1.1b has been found to thrive on tomato plant roots (Simon *et al*. 2000), another study suggested that archaea respond negatively to both root and fungal exudates (Karlsson *et al*. 2012). Archaeal soil communities, as identified by 16S rRNA gene sequences, are commonly reported to be simple (Pesaro & Widmer, 2002; Hartmann *et al*. 2009), and to shift in composition (Pesaro & Widmer; Eilers *et al*. 2012) and increase in richness and abundance with depth (Hartmann *et al*. 2009; Eilers *et al*. 2012).

The ecology and functional roles of soil archaea are not well established. The first archaeal group to be studied was the euryarchaeal methanogens (Woese *et al*. 1978), which produce methane as a metabolic by-product in anoxic environments (Juottonen *et al*. 2008). An insight into the metabolic traits of thaumarchaea was revealed from metagenomic studies of sea water (Venter *et al*. 2004) and soil (Treusch *et al*. 2005), where archaeal DNA sequences affiliated with thaumarchaeota were found to harbour putative ammonia monooxygenase genes. The role of archaea in ammonia oxidation was first confirmed when Könneke and colleagues (2005) from a marine aquarium isolated a thaumarchaeon capable of autotrophic growth with ammonia as sole energy source, indicating a chemolithoautotrophic lifestyle. This finding has been followed by several molecular studies indicating that mesophilic thaumarchaea capable of ammonia oxidation are prevalent in a wide range of soil environments, and generally outnumber their bacterial counterparts (Leininger *et al*. 2006), especially in acidic soils (Kemnitz *et al*. 2007, Nicol *et al*. 2008). However, this metabolism was not established in moderate soil archaea until two thaumarchaeal ammonia oxidizers were cultivated from soil (Lehtovirta-Morley *et al*. 2011; Tourna *et al*. 2011). The thaumarchaeon *Nitrososphaera viennensis* was isolated from a garden soil and affiliated with thaumarchaea group 1.1a, and showed an optimal growth at pH 7.5 and tolerated high ammonium concentrations (Tourna *et al*. 2011). The thaumarchaeon *Nitrosotalea devanaterra* was enriched from an acidic agricultural soil and was affiliated with thaumarchaea group 1.1b, and displayed optimal growth at pH between 4 and 5 under oligotrophic conditions (Lehtovirta-Morley *et al*. 2011). The presence of co-enriched bacteria, most of which have the potential to fix dinitrogen, was necessary to sustain growth of *Nitrosotalea devanaterra* (Lehtovirta-Morley *et al*. 2011), and growth rates of *Nitrososphaera viennensis* was greatly improved either by co-culture with bacteria or addition of pyruvate (Tourna *et al*. 2011). These findings suggest that soil thaumarchaea might be mixotrophic and that they possibly require symbiotic partners for growth. Bomberg and colleagues (2010) enriched archaea from humus and Scots pine ectomycorrhizas collected in a boreal forest in Finland, and were able to enrich

thaumarchaea both under aerobic and anaerobic incubation conditions, and euryarchaeota under anaerobic conditions. However, they did not manage to amplify any archaeal *amoA* genes, which code for the active site of ammonia monooxygenase (see Rotthauwe *et al*. 1997), in this study (Bomberg *et al*. 2010). In contrast, Long *et al*. (2012) detected *amoA* genes of both bacteria and archaea in topsoil from a Swedish boreal forest, and found both bacterial 16S rRNA and *amoA* genes in higher abundances than their archaeal counterparts. Hence, a picture emerges of soil archaea to be metabolically diverse and that they possibly need syntrophic partners for growth, but more studies are warranted to draw further conclusion about their ecological roles.

2.8 Denaturing gradient gel electrophoresis (DGGE) – an introduction

One major challenge in microbial ecology is to make interpretations about microbial communities from environmental samples. For this purpose, various fingerprinting techniques can be used to elucidate trends in community composition of phylogenetic or functional target groups. Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting technique that has been extensively used in microbial ecology surveys (van Elsas *et al*. 2011), and was in this context first described by Muyzer *et al*. (1993). In DGGE, PCR amplified DNA sequences of phylogenetic markers or functional genes are separated in a gel containing a linear denaturing gradient. Separation of amplicons occurs due to differences in guanine – cytosine (GC) content and the relative position of these base pairs along the DNA sequences. In DNA, the nucleotides thymine (T) and adenine (A) are held together by two hydrogen bonds while guanine (G) and cytosine (C) are held together by three hydrogen bonds, and GC-rich regions are therefore more strongly bound than AT-rich regions. In an electrophoresis gel containing a denaturing gradient, the double stranded DNA fragments will reach a point where the denaturing agent is strong enough for melting to occur. When amplicons become partly single stranded, migration stops and similar fragments assemble at a particular location in the gel that after staining becomes visible as a band. In the case of phylogenetic marker sequences, it is generally assumed that each band represents a unique taxonomic identity that often is referred to as an operational taxonomic unit (OTU) or phylotype. The pattern of bands obtained from each PCR reaction represents a fingerprint of the genotypic variety of the amplified target sequence in that sample.

2.9 Aims of the study

The goal of this study was to investigate if the organic, eluvial and illuvial horizons of a Swedish boreal forest podzol soil harboured different communities of fungi, bacteria and archaea. It was further tested how the presence of Scots pine seedling roots, through input of rhizodeposition, would affect the microbial communities in the different soil categories. This was tested in a microcosm study combined with molecular analysis of soil samples after 10 weeks of incubation. Microbial community composition was investigated by denaturing gradient gel electrophoresis (DGGE) fingerprinting of PCR amplified phylogenetic marker sequences, ITS rDNA for fungi and 16S rDNA for archaea and bacteria. Soil samples from

experimental set-up served as control for community shifts caused by incubation time alone.

3. Materials and methods

3.1 Site description and soil collection

The soils were collected from Jädraås, which is a research site in Central Sweden (60°49´N, 16°30'E) within the Swedish Coniferous Forest Project (SWECON), and is described by Axelsson & Bråkenhielm (1980). In brief, Scots pine (*Pinus sylvestris*) dominates in drier areas while moister sections contain mixed stands of Norway spruce (*Picea abies*) and Scots pine (Figure 2a). The field layer consists of ericaceous dwarf shrubs such as *Empetrum* spp., *Calluna vulgaris*, *Vaccinium myrtillus* and *Vaccinium vitis-idaea*. The forest floor is covered with a mixed carpet of lichens, mainly *Cladina* spp. and occasional *Cladonia* spp., and mosses such as *Pleurozium schreberi* and *Dicranum* spp. (Figure 2b).

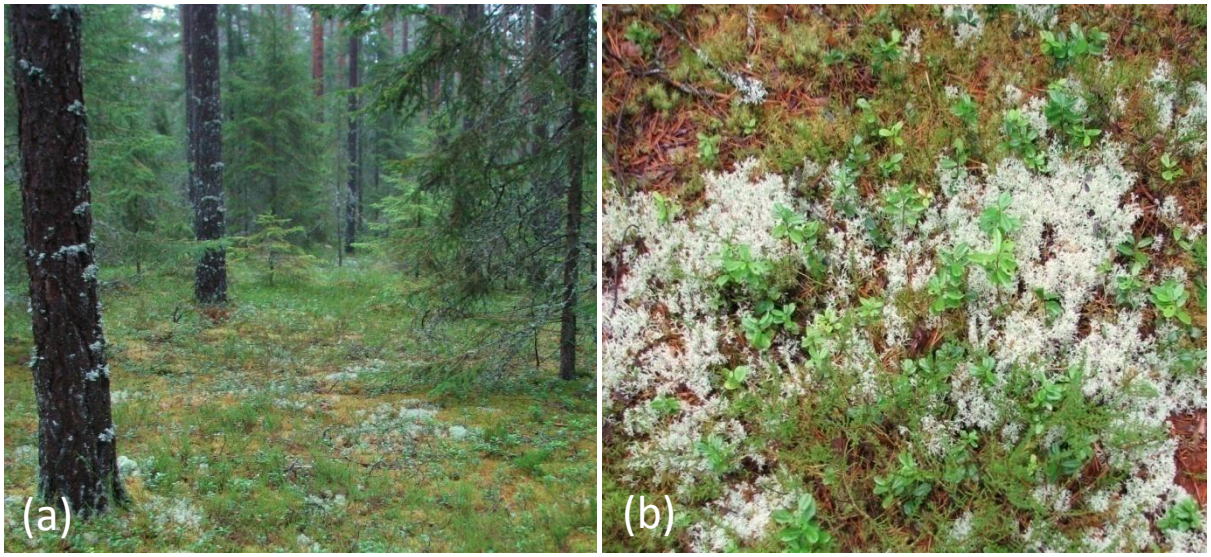

Figure 2. a) A mixed boreal forest stand of Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) at Jädraås, Sweden. b) The field- and bottom layer consisted of ericaceous dwarf shrubs, mosses and lichens. (*Photo Johanna Jernberg*)

The boreal forest soil examined in this study is classified as an iron-podzol developed on glaciofluvial sediments of a sorted sandy texture and a felsic composition (Axelsson and Bråkenhielm, 1980). Soil chemical characteristics were analyzed by Agrilab AB (Uppsala, Sweden) and are summarised in Table 1. Soils for this experiment were collected in October 2011, from three separate locations within a 20 x 20 m area in a mixed forest stand of Norway spruce and Scots pine by digging with shovel. Each soil column comprised an iron-podzol profile of about 25 cm depth, encompassing the organic (O), eluvial (E) and illuvial (B) horizons (Figure 1). The three soil horizons were separated and any transition zone removed

so that each soil category exhibited the typical identity of each horizon to be studied. Horizon thickness in the profiles varied between sampling locations, but was approximately 4 to 5 cm for the organic horizon when the superficial litter was removed, 6 to 10 cm for the eluvial horizon, and from here the illuvial horizon stretched downwards to a depth of 25 cm, not reaching the unchanged parent material (i.e. C horizon). Triplicate samples of each soil horizon were pooled into one batch, and stored at 4°C in plastic boxes with ventilation holes until further processing.

Table 1. Elemental composition of soil samples collected from the organic, eluvial, and illuvial horizons in a boreal forest podzol profile at Jädraås, Sweden. All values are given as mg element per kg dry weight of soil.

Element	Organic	Eluvial	Illuvial
C	451600	13900	16100
N	11200	700	700
P	494	53	507
S	839	38	176
Si	81000	354000	365000
Na	133	92	59
K	609	149	334
Ca	1474	446	601
Mg	381	243	1517
Mn	119	8	108
Fe	1401	1856	11524
Al	1207	1851	13395

3.2 Preparation and set-up of microcosms

In January 2012, the soils were processed for the experiment. The mineral soils were sieved through a 3 mm mesh sieve to remove larger mineral and litter particles. The organic soil was prepared by removal of roots by hand and thereafter sieved through a 5 mm mesh sieve to homogenize the substrate. The soil batches were moved from 4°C storage to a growth chamber with a temperature set to 16°C, to activate soil microorganisms. During a storage period of four weeks at 16°C, soils were aerated twice a week by mixing to avoid extensive development of anaerobic conditions within the soil batches.

Seeds of Scots pine were rendered free from potential pathogens by immersion in hydrogen peroxide (30%) for 30 minutes, and then rinsed several times with sterile double distilled water (ddH_2O). Seeds were sprinkled on plastic trays filled with non-sterile vermiculate and covered with transparent plastic lids with adjustable air slits. The plant growth propagators were incubated in a phytotron with a photoperiod of 18 hours light (250 µmol m^{-2} s^{-1} PAR) at 18°C, and 6 hours darkness at 16°C. Watering was performed by spraying with ddH_2O. When seedlings had grown for 14 days they were ready to be transplanted to microcosms.

Triplicates of each treatment were prepared in microcosms consisting of 50 ml Falcon tubes (Figure 3). Treatments were as follows; organic soil with (OP) and without (ON) Scots pine seedlings, eluvial soil with (EP) and without (EN) Scots pine seedlings, and illuvial soil with (BP) and without (BN) Scots pine seedlings, a total of 18 microcosms. At the time of microcosm establishment, triplicate soil samples were taken from each soil batch and frozen at -20°C to serve as zero-time samples for examination of community changes caused by incubation time alone. Zero-time samples were designated OZ for the organic, EZ for the eluvial and BZ for the illuvial soil samples. Soil pH was measured with a pH meter in soil suspensions with ddH_2O (1:5 v/v) which were homogenized by vortexing and left to settle for 4 h, and mixed again shortly before measurement. The measured pH values were 4.0, 4.4 and 4.8 for the organic, eluvial, and illuvial soils respectively. Microcosms containing soil from the same horizon received the same fresh weight of soil, 13.5 g for the organic soil and 26.0 g for both mineral soils, which were compressed lightly to a soil volume of 25 ml. Scots pine seedlings had at the time of transplantation developed a primary root, a few centimeters in length, without lateral roots. Treatments with plant roots received one Scots pine seedling per microcosm. Seedlings were inserted by sliding the root through a hole drilled in the side of the Falcon tubes, leaving the photosynthesizing part of the seedling on the outside of the microcosms. Microcosms without seedlings also had a hole drilled in the side to ensure equal gas exchange abilities in all treatments. All microcosms had caps with tree ventilation holes and were wrapped in aluminum foil to exclude any light during the incubation.

Figure 3. Microcosms consisting of 50 ml Falcon tubes, where each microcosm had a hole drilled half way up on one side of the tube to fit the seedling stem and also three ventilation holes in the cap to allow gas exchange with the surrounding air. (*Photo Johanna Jernberg*)

Microcosms were placed vertically standing in racks in plastic plant growth propagators with transparent lids with adjustable ventilation slits. To keep the environment moist inside the propagators, cups of ddH_2O were placed between the racks. All microcosms were incubated in a phytotron with a photoperiod of 18 hours light (250 µmol m^{-2} s^{-1} PAR) at 18°C, and 6 hours darkness at 16°C. Microcosms were watered gravimetrically twice a week by adding ddH_2O with a syringe to maintain a soil moisture content corresponding to 65% of water holding capacity for each soil category. Water holding capacity and moisture content at experimental set-up were determined gravimetrically by drying at 105°C overnight (see Hartel, 2005).

3.3 Sampling and extraction of nucleic acids

Microcosms were harvested destructively after 10 weeks of incubation, a time point at which all microcosms with Scots pine seedling harbored ectomycorrhizal root tips. The uppermost few mm of all soil cores were removed to exclude growth of algae and bryophytes. For microcosms without seedling, the remainder of the soil cores, here referred to as bulk soil samples, were immediately stored at -20°C. For microcosms with seedling, soil that easily separated from the root system was assigned as bulk soil samples. Soil particles held by the roots and mycorrhizal hyphae were gently shaken off the root system in separate containers and assigned as rhizosphere soil. All soil samples, as well as whole seedlings, were immediately stored at -20°C after sampling.

Nucleic acids were extracted from 2.0 g fresh weight of bulk soil from each microcosm core and each zero-time sample with a kit based method, RNA PowerSoil® Total RNA Isolation Kit (MoBio Laboratories) and RNA PowerSoil®DNA Elution Accessory Kit (MoBio Laboratories), according to manufacturer´s instructions. DNA extracts were further used for analysis of microbial communities in the soil samples. DNA concentrations in all extracts (Figure 9d) were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

3.4 Polymerase chain reaction (PCR)

To evaluate if differences in microbial community composition existed between treatments, DNA sequences of phylogenetic marker regions were amplified by nested polymerase chain reaction (PCR) with primers targeting specific taxonomical groups. Primers used in this study are summerised in Table 2. Bacterial 16S rDNA was amplified by a nested PCR approach using primer pair 27F and 1492R in the first run with thermo cycling conditions: 95°C, 5 min; 10 x (94°C, 30 s; 55°C, 30 s; 72°C, 90 s); 25 x (92°C, 30 s; 55°C, 30 s; 72°C, 90 s); 72°C, 10 min. Secondary PCR for bacterial 16S rDNA was performed with primers 357F-GC and 518R with thermo cycling conditions: 95°C, 5 min; 10 x (94°C, 30 s; 55°C, 30 s; 72°C, 30 s); 25 x (92°C, 30 s; 55°C, 30 s; 72°C, 45 s); 72°C 10 min. Archaeal 16S rDNA was amplified with two different nested approaches, both targeting the total archaeal community. In the first nested approach primary PCR was performed with primers Arch21F and Arch958R with thermo cycling conditions 95°C, 5 min: 35 x (94°C, 45 s; 56°C, 45 s; 72°C, 60 s); 72°C 10 min,

and secondary PCR with primer pair ARC344F-GC and Arch806R under cycling conditions: 95°C, 5 min; 30 x (94°C, 45 s; 50°C, 45 s; 72°C, 60 s); 72°C 10 min. The second nested PCR approach targeting archaeal 16S rDNA was run with primer pair A109F and Ar9R in the first round under cycling conditions: 95°C, 5 min; 5 x (94°C, 30 s; 55°C, 30 s; 72°C, 60 s); 30 x (92°C, 30 s; 55°C, 30 s; 72°C, 60s); 72°C, 10 min, and secondary PCR with primer pair rSAF-GC and PARCH519R with cycling conditions: 95°C, 5 min; 5 x (94°C, 30 s; 63°C, 30 s; 72°C, 60 s); 30 x (92°C, 30 s; 63°C, 30 s; 72°C, 60 s); 72°C, 10 min. The internally transcribed spacer region (ITS) was targeted as a phylogenetic marker sequence for comparison of fungal communities. For fungal ITS amplification, a nested PCR was performed with primer pair ITS1F and ITS4 in the primary run and primer pair ITS1F-GC and ITS2 in the secondary run, both carried out with the thermo cycling program 95°C, 5 min; 35 x (94°C, 30 s; 55°C, 30 s; 72°C, 30s); 72°C, 10 min.

Table 2. Primers used in this study to amplify ribosomal DNA sequences by PCR.

Primer	Target	Sequence 5´- 3´	Reference
A109f	Archaeal 16S rDNA	ACKGCTCAGTAACACGT	Großkopf *et al*. 1998
Ar9R	Archaeal 16S rDNA	CCCGCCAATTCCTTTAAGTTTC	Jurgens *et a*l. 1997
rSAF(i)[a]	Archaeal 16S rDNA	[GC][b]CCTAYGGGGCGCAGCAG	Nicol *et al*. 2005
rSAF(ii)[a]	Archaeal 16S rDNA	[GC][b]CCTACGGGGCGCAGAGG	Nicol *et al*. 2005
PARCH519R	Archaeal 16S rDNA	TTACCGCGGCKGCTG	Øvreås *et al*. 1997
Arch21F	Archaeal 16S rDNA	TTCCGGTTGATCCYGCCGGA	DeLong 1992
Arch958R	Archaeal 16S rDNA	YCCGGCGTTGAMTCCAATT	DeLong 1992
ARC344F[GC]	Archaeal 16S rDNA	[GC][b]ACGGGGYGCAGCAGGCGCGA	Casamayor *et al*. 2000; Raskin *et al*. 1994
Arch806R	Archaeal 16S rDNA	GGACTACVSGGGTATCTAAT	Takai and Horikoshi 2000
27F	Bacterial 16S rDNA	AGAGTTTGATCMTGGCTCAG	Lane 1991
1492R	Bacterial 16S rDNA	ACCTTGTTACGACTT	Lane 1991
357F[GC]	Bacterial 16S rDNA	[GC][b]CCTACGGGAGGCAGCAG	Muyzer *et al*. 1993
518R	Bacterial 16S rDNA	ATTACCGCGGCTGCTGG	Muyzer *et al*. 1993
ITS1F/ITS1F[GC]	Fungal ITS	[GC][b]TCCGTAGGTGAACCTGCGG	White 1990
ITS2	Fungal ITS	GCTGCGTTCTTCATCGATGC	White 1990
ITS4	Fungal ITS	TCCTCCGCTTATTGATATGC	White 1990

a. Primer rSAF is a composite 2:1 ration mix of primers rSAF(i) and rSAF(ii), according to Nicol *et al*.(2005)
b. GC-clamp [CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG] (Muyzer *et al*. 1993), not part of primer target DNA sequence

All PCR reactions were performed with a Veriti 96 well thermocycler (Applied Biosystems) as 50 µl reactions in Milli-Q water, each containing 1 U BIOTAQ^{TM} DNA Polymerase (Bioline), 5 µl 10 x NH_4 reaction buffer, 1,5 mM $MgCl_2$, 250 µM of dNTPs, 200 nM of each primer and 20 µg bovine serum albumin (only for primary PCR runs). In primary runs for bacteria and both nested approaches for archaea, each reaction received 2 µl of 50-fold dilutions of all DNA extracts as template. Secondary PCR for archaea with primers rSAF-GC and PARCH519R received 1 µl of undiluted primary PCR product as template. Secondary runs for archaea with primers ARC344F-GC and Arch806R and bacteria each received 2 µl of undiluted primary PCR product per reaction. For fungal primary PCR, each reaction received 2 µl of 100-fold dilutions of all DNA extracts as template, and the secondary run 1 µl of 10-fold dilution of primary products as template. All PCR runs had a negative control (sterile ddH_2O) to ensure uncontaminated PCR reactions, and a positive control consisting of DNA extracted

from an agricultural soil known to contain all target groups. Success of PCR amplifications were checked by running electrophoresis on agarose (1%) gels, and amplicon sizes were estimated by comparison with the GeneRuler^{TM} DNA Ladder Mix (Fermentas Life Sciences).

3.5 DGGE procedure

Preparation and processing of DGGE gels were performed according to the methods described by Mahmood *et al*. (2006). In brief, 15 µl secondary PCR products and 4 µl of loading dye was loaded per well on 8% (v/v) acrylamide gels, with a 20-50 % denaturing gradient for fungi, 35-65% for bacteria, 45-75% for archaea (primers rSAF-GC and PARCH519R), where 100% denaturing strength correspond to 40% (v/v) formamide and 7 M urea. Gels were cast by using a gradient mixer connected to a peristaltic pump. All gels were run for 16 h at 75 V using a DCode Universal Mutation Detections system (Bio-RAD), where gels were submerged in 1XTAE buffer with a set temperature of 60°C.

After electrophoresis, DGGE gels were briefly washed with ddH_2O prior to silver staining. Gels were submerged in a fixing solution (10% v/v ethanol, 0.5% v/v acetic acid and ddH_2O) for 15 minutes and thereafter in a silver staining solution ($AgNO_{3(s)}$ 0.2% w/v and fixing solution) for 15 minutes. Then gels were rinsed three times with ddH_2O before being submerged in developing solution ($NaOH_{(s)}$ 3% w/v, formaldehyde 0.5% v/v, and ddH_2O) until clear banding patterns emerged. Finally gels were washed with ddH_2O and covered with fixing solution for another 10 minutes. Processed gels were scanned with an Epson Perfection V700 PHOTO scanner (Epson America) to obtain digital DGGE gel images.

3.6 Numerical extraction and statistical analysis

DGGE gel images were analyzed with the computer software Phoretix 1D (TotalLab), and DGGE banding patterns were transformed to binary matrices, representing presence/absence of bands in each PCR reaction. Statistical differences in DNA yields and OTU richness between treatments were tested by One-way analysis of variance (ANOVA) with a Tukey test, preceded by a Bartlett´s and a Levene´s test of equal variances ($p > 0.05$ indicate equal variances), using Minitab 16 statistical software (Minitab). Differences in community composition between treatments were explored by non-metric multidimensional scaling of binary matrices using the statistical software PC-ORD version 5.33d (MjM Software Design). Ordinations were run in the autopilot “slow and thorough” mode using Sørensen distance measure, random starting coordinations, a maximum number of 500 iterations and an instability criterion set to 0.00001. Significance of clustering was evaluated by a Monte Carlo test with 250 runs of randomized and real data respectively.

4. Results

4.1 Microscopic observations of ectomycorrhizal development

During the ten weeks of incubation, microcosms with Scots pine seedling were examined weekly under dissecting microscope to observe the development of ectomycorrhizas. The first ectomycorrhizal fine roots appeared in the eluvial soil after one week of incubation. After two weeks, both the organic and eluvial soils harboured ectomycorrhizal fine roots, though of different morphotypes. Seedlings growing in the organic soil had ectomycorrhizas with a commonly non-bifurcated fine root structure covered by a mantle of yellow hyphae and an extramatrical mycelium characterized by straight, dry looking and radially growing hyphae (Figure 4a). Mycorrhizal formation occurred much later in the illuvial soil, where ectomycorrhizal fine roots first appeared after three weeks of incubation. Scots pine seedlings growing in both mineral soils had the same apparent morphotype which was characterized by both non-bifurcated and bifurcated fine roots and a dense mantle of white, sticky hyphae that turned dark brown in colour with age (Figure 4c). In addition, these ectomycorrhizal fungi displayed extensive extramatrical growth consisting of cotton-looking, entangled hyphae close to infected fine roots, and thick hyphal cords running through the soil matrix and commonly along the roots axis between mycorrhizal fine roots (Figure 4d). These fungi produced exudates that upon drying resulted in deposition of sticky crystals on the inside surface of the Falcon tubes. After 6 weeks of incubation, seedlings growing in the organic soil also hosted ectomycorrhizal fungi forming a morphotype with a black mantle covering bifurcated fine roots and an extramatrical mycelium exhibiting thin, radially growing hyphae (Figure 4b). At the time of harvest, which was performed after 10 weeks of incubation, seedlings growing in the organic soil harboured two distinct ectomycorrhizal morphotypes, the dominating yellow morphotype and the less abundant black morphotype. Seedlings growing in the eluvial and illuvial soils hosted ectomycorrhizas dominated by the white morphotype, but the eluvial soil also harboured occasional fine roots with the yellow morphotype.

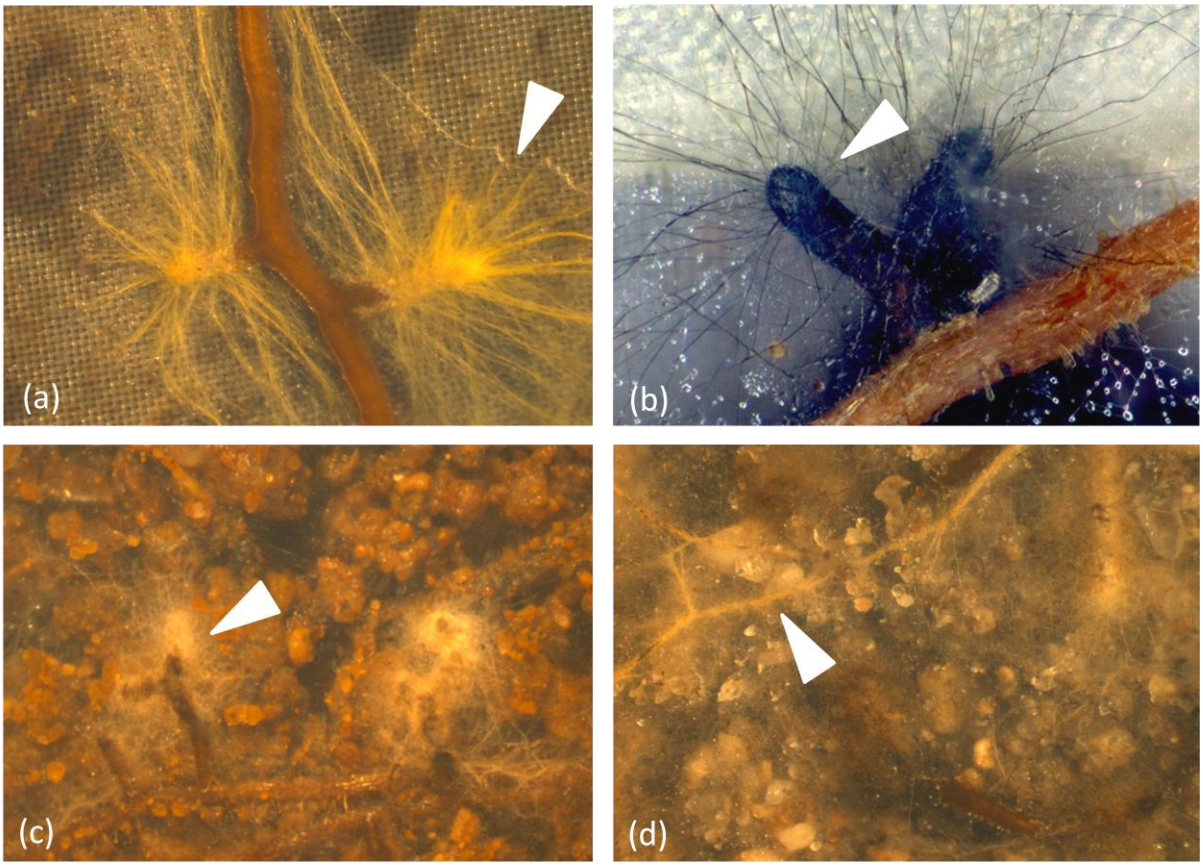

Figure 4. Ectomycorrhizas on Scots pine seedling roots observed in this study. Objects of interest are indicated by white arrowheads. Seedlings growing in the organic soil were associated with ectomycorrhizal fungi forming yellow (a) and black (b) morphotypes. The ectomycorrhizal fungi forming the dominant white morphotype (c) on seedling roots growing in the eluvial and illuvial soils produced extensive extramatrical mycelia partly consisting of hyphal cords (d). (*Photo Shahid Mahmood (a, c, & d) Johanna Jernberg (b)*)

4.2 Analysis of DGGE gels

The community composition of fungi, bacteria and archaea residing in the organic, eluvial and illuvial horizons of a boreal forest podzol were examined by DGGE fingerprinting. DGGE gels were analyzed both visually and with the gel analysis computer software Phoretix 1D (TotalLab Ltd.) to extract numerical data. Since neither phylogenetic identity nor taxonomic rank were assigned to the bands in the DGGE profiles, they are here referred to as operational taxonomic units (OTUs). The DGGE profiles for fungi and bacteria were complex, and therefore non-metric multidimensional scaling ordinations were performed to assess differences in community composition between treatments. The DGGE profiles for archaea were simple enough to extract information without further ordination analysis.

4.2 Fungal DGGE profiles

DGGE profiles of fungal ITS amplicons (Figure 5) revealed distinct and reproducible banding patterns for the organic, eluvial and illuvial soils collected from a boreal forest podzol. Non-metric multidimensional scaling, based on presence/absence of bands in the DGGE profiles, further supported clustering of fungal communities according to soil horizon (p=0.004). In

the ordination plot (Figure 7b), data points representing the organic soil showed a stronger aggregation and a larger separation from data points representing the two mineral soils, indicating a larger distinction in fungal community composition between the organic and the mineral soils. Clustering of data points representing the eluvial and illuvial soils was weaker than for the organic soil, suggesting a tendency to larger variation in fungal community composition in the mineral soils than in the organic soil.

Banding patterns in the fungal DGGE gel also revealed that fungal communities residing in the different soil horizons responded differently to incubation time and the presence of Scots pine seedling roots. In the organic soil, two bands were present in zero-time samples only (a), and decreased to below detection level on the DGGE gel after 10 weeks of incubation. At the same time two other bands (c) increased in intensity with incubation time, irrespective of the presence of Scots pine seedlings. The two bands assigned (d) had the same intensity for zero-time samples and microcosms with seedling, but a reduced intensity in soil samples from microcosms without seedling. These were the only fungal taxa that responded to the presence of Scots pine seedlings in the organic soil. For the eluvial soil, community shifts due to treatments were difficult to discern because of the lower reproducibility of the triplicate treatment samples, a trend that also could be seen in the ordination plot as lower clustering for eluvial data points according to treatment (Figure 7b). The only community shifts that could be discerned between treatments in the eluvial soil were the four bands (b) that gave a positive response to the presence of Scots pine seedlings. All illuvial soil samples produced highly similar DGGE profiles with no differences in band intensities except for one band (a) that reduced in intensity with incubation time. However, in the ordination plot (Figure 7b) data points representing the organic and illuvial soil samples tended to cluster according to treatments.

The fungal OTU richness (Figure 9a), as measured by the total number of bands detected per soil sample in the DGGE profiles, seemed to be highest in the organic horizon and lower in both mineral horizons, though this difference was not statistically significant for any of the treatments. The average number of OTUs was lower in the eluvial soil than in the organic and illuvial soils, but the large standard deviation reflects the lower reproducibility of banding patterns in the eluvial soil. Thus, the organic horizon appeared to harbour a diverse community of fungi that responded to both incubation time and presence of Scots pine seedling roots. In the eluvial soil detectable shifts in fungal communities occurred exclusively as a response to the presence of seedling roots. The illuvial fungal community did not respond much to either incubation time or presence of Scots pine seedlings, indicating a stable fungal community that might be relatively insensitive to environmental disturbances.

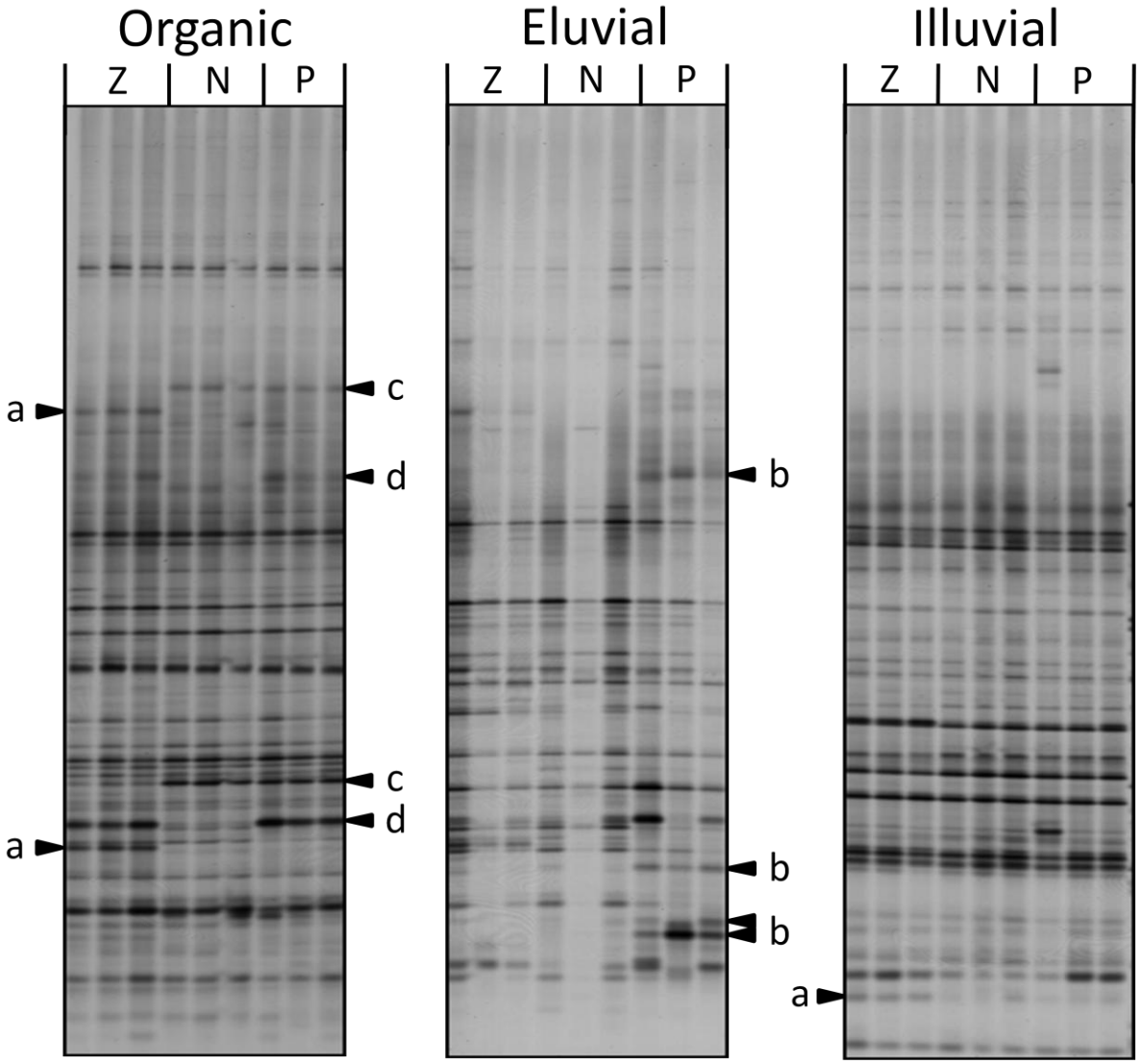

Figure 5. Denaturing gradient gel electrophoresis profiles of fungal ITS rDNA sequences amplified from the organic, eluvial and illuvial soils collected from a boreal forest podzol. For each soil horizon, triplicate treatment samples are assigned (Z) for zero-time soil samples, (N) for microcosms without Scots pine seedling, and (P) for microcosms with Scots pine seedling. Arrows show treatment induced differences in community composition within each soil. Letter (a) indicates the presence of a band in zero-time samples but an absence/reduction of the same band in both microcosm treatments. A positive response to the presence of Scots pine seedling roots is designated by letter (b), while bands that increase in intensity during incubation are assigned letter (c). Letter (d) indicates bands present in zero-time soil samples and in microcosms with Scots pine seedling.

4.3 Bacterial DGGE profiles

The bacterial DGGE profiles (Figure 6) generated from 16S rDNA sequences revealed complex and reproducible banding patterns throughout all soil horizons and across treatments. Bacterial richness, as measured by the number of detectable bands (OTUs), appeared to be highest in the organic soil and lower in both mineral soils (Figure 9b). Though this difference was only significant for the organic soil samples that had been

incubated for 10 weeks and the illuvial soil samples. Visual inspection of the bacterial DGGE profiles showed that the organic, eluvial and illuvial soils harboured distinct bacterial communities that responded strongly to incubation time. In the organic soil many bands either declined (a) or increased (c) in intensity during the 10 week incubation period, irrespective of presence of Scots pine seedlings. Only one reproducible band (b) increased in intensity in the presence of Scots pine seedlings, and three bands (d) were only detected in soil samples from microcosms without Scots pine seedling. A region with lower reproducibility (v) could be discerned in the DGGE profiles of the eluvial soil samples, making shifts in the bacterial community composition difficult to estimate. However, reproducibility of treatment triplicates for the eluvial soil was high in the lower region of the gel. One band in the eluvial and several bands in the illuvial profiles increased in intensity during incubation (c), regardless of presence or absence of seedlings. These were the only bacterial community shifts that could be detected by DGGE fingerprinting in the mineral soil samples. The observed difference in bacterial community composition between soil horizons and treatments was confirmed by non-metric multidimensional scaling ordination of presence/absence banding patterns in the bacterial DGGE profiles ($p=0.004$). In the ordination plot (Figure 7a) all data points showed a strong clustering according to soil horizon. Further, clustering could be observed in consonance with treatment for the organic and illuvial soil samples, but not for the eluvial soil samples. For the organic soil, a large divergence in distance could be seen between zero-time samples and all samples incubated in microcosms for 10 weeks. Data points defining the illuvial soil samples also displayed treatment induced clustering, with the largest distance between zero-time samples and soil incubated for 10 week in microcosms with Scots pine seedling. These results indicate that the organic horizon in a boreal forest podzol supports a rich flora of bacteria whose populations may respond strongly to environmental changes. The mineral soils on the other hand seem to contain less diverse communities of bacteria with relatively stable populations that display rather weak responses to disturbances.

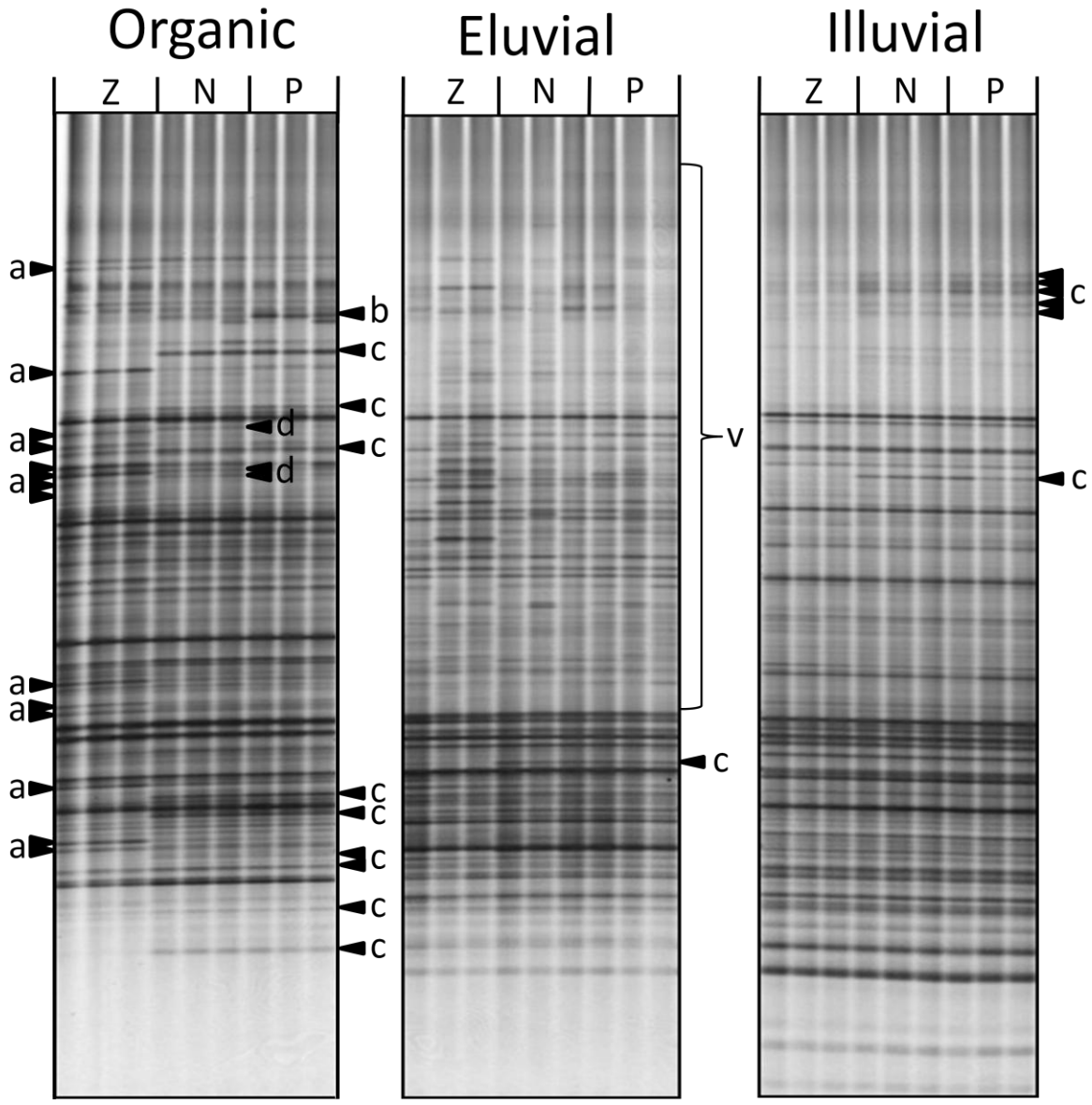

Figure 6. Denaturing gradient gel electrophoresis profiles of bacterial 16S rDNA sequences amplified from the organic, eluvial and illuvial soils collected from a boreal forest podzol. For each soil horizon, triplicate treatment samples are assigned (Z) for zero-time soil samples, (N) for microcosms without Scots pine seedling, and (P) for microcosms with Scots pine seedling. Arrows show treatment-induced differences in community composition within each soil. Letter (a) indicates the presence of a band in zero-time samples but an absence/reduction of the same band in both microcosm treatments. A positive response to the presence of Scots pine seedling roots is designated by letter (b), while bands unique to microcosms without seedling are assigned (d). Bands that increase in intensity during incubation are assigned letter (c). The region designated (v) represents variable banding patterns among triplicate treatment samples.

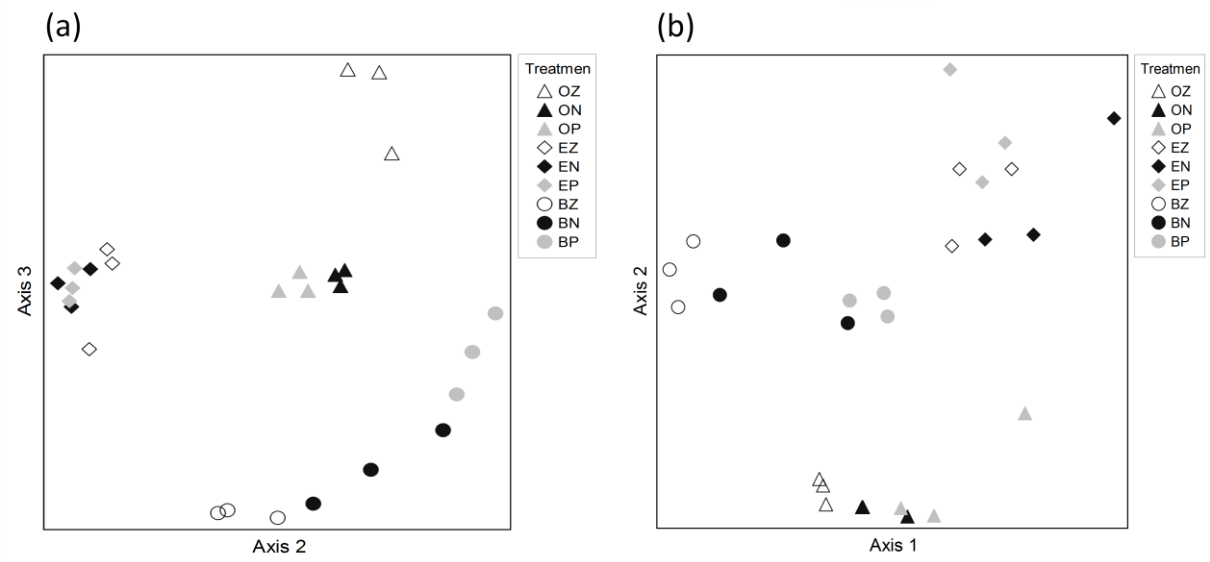

Figure 7. Non-metric multidimensional scaling ordination plots displaying treatment-induced community clustering of bacterial 16S rDNA sequences (a) and fungal ITS rDNA sequences (b), as defined by presence/absence banding patterns of DGGE profiles. Ordinations for both bacteria and fungi were executed with Sørensen distance measure, and had the lowest final stress, 10.2 for bacteria and 8.9 for fungi, at 3D solutions for both ordinations. The two axes that best depict the distance relationship between data points in 3D space were chosen for construction of 2D plots. A Monte Carlo test with 250 runs with real and randomized data respectively confirmed statistical significance of clustering patterns for both bacteria and fungi (p=0.004). The organic (O), eluvial (E) and illuvial (B) soils are represented by triangles, diamonds and circles respectively. Open symbols represent zero-time samples (Z), while soil samples from microcosms with (P) and without (N) Scots pine seedling are shown as gray and black symbols respectively.

4.5 Archaeal DGGE profiles

Two nested PCR approaches, both targeting archaeal 16S rDNA sequences, were performed to examine archaeal communities residing in the organic, eluvial and illuvial soils collected from a boreal forest podzol. PCR products from both nested strategies formed clearly visible bands of expected sizes when checked on agarose (1%) electrophoresis gels, and were further used for DGGE analysis. However, DGGE runs with amplicons generated from secondary PCR with primers ARC344F-GC and Arch806R were unsuccessful as black smears appeared in lanes after silver staining, which obscured possible banding patterns. On the other hand, products obtained from secondary PCR with primers rSAF-GC and PARCH519R generated reproducible DGGE profiles for all soil samples (Figure 8). Each soil horizon appeared to harbour a distinct archaeal community, where no bands were found to respond to either incubation time or presence of Scots pine seedling roots. Both rSAF-GC and PARCH519R contained degeneracies that altered the GC-content of amplicons, resulting in replicate bands possibly representing a single genotype. This posed a problem when estimating archaeal richness (Figure 9c), but each single band was assigned as an OTU due to inconsistency in the number of replicate bands that clustered in the DGGE profiles. The lowest richness of archaea was found in the organic horizon, where two band triplets assigned (oe) were present in all organic soil samples. In addition, a few faint bands

occurred in some of the organic soil samples. The eluvial horizon displayed the highest richness of archaea, and had three unique band clusters (e), two band clusters (oe) shared with the organic horizon, and two band clusters (eb) that were common with the illuvial horizon. The illuvial soil samples also had two unique band clusters (b). Together these data point to an increased diversity of archaea with depth in a boreal forest podzol profile, where some archaea were unique to either the eluvial or illuvial horizon while other archaea seemed to display a wider vertical distribution.

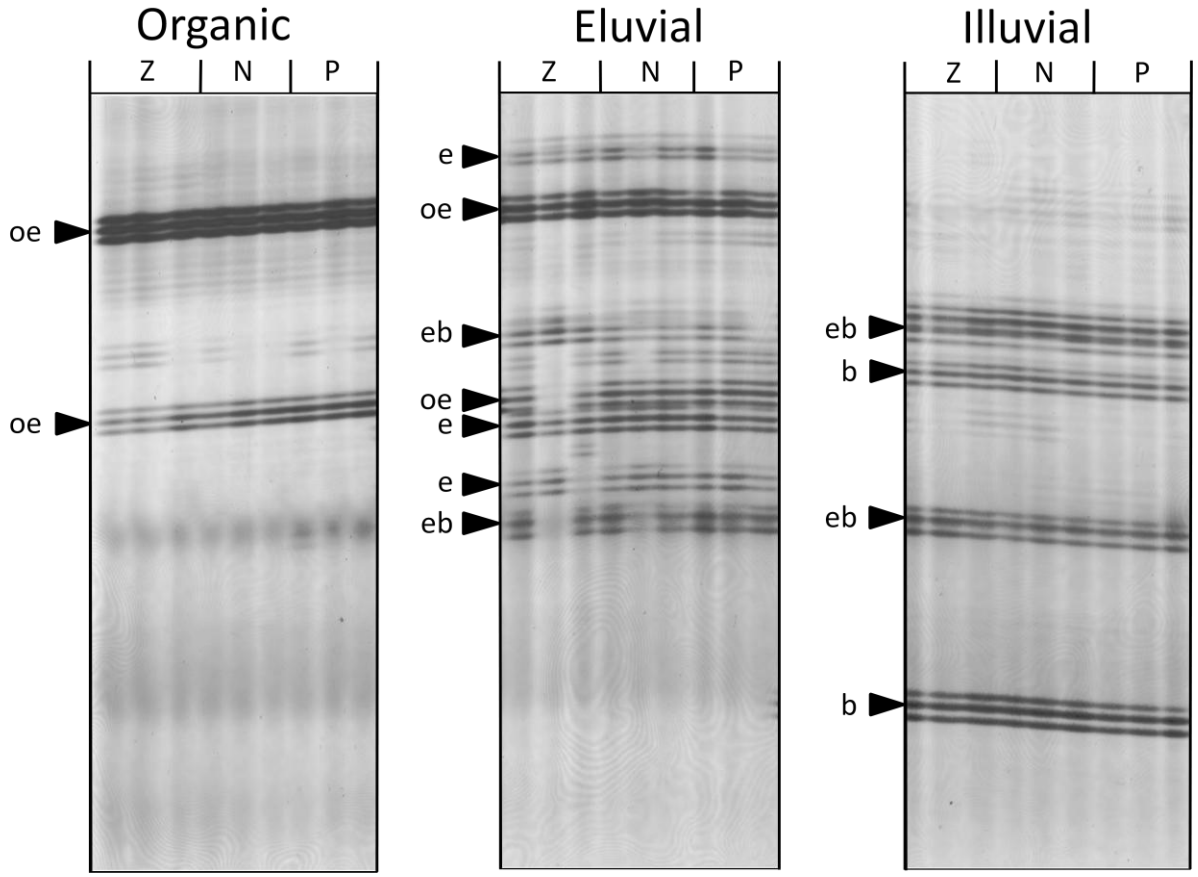

Figure 8. Denaturing gradient gel electrophoresis profiles of archaeal 16S rDNA sequences amplified from the organic, eluvial and illuvial soils collected from a boreal forest podzol. For each soil horizon, triplicate treatment samples are assigned (Z) for zero-time soil samples, (N) for microcosms without Scots pine seedling and (P) for microcosms with Scots pine seedling. Both the forward (rSAf) and reverse (PARCH519r) primers contained degeneracies that changed the GC-content of amplicons. Each sequence type is therefore represented by replicate band in the archaeal DGGE profiles. Arrows indicate band clusters which are assigned according to affiliation with the organic (o), eluvial (e) and illuvial (b) soils, or a combination when a cluster occurred in more than one soil horizon.

4.6 DNA concentrations

DNA extracts from the organic soil had significantly higher DNA concentrations than extracts from both mineral soils (Figure 9d). Within the organic soil treatments, zero-time samples and soil from microcosms with Scots pine seedling yielded significantly higher DNA

concentrations than soil from microcosms without seedling. For the eluvial soil, DNA concentrations were slightly higher in extracts obtained from microcosms with Scots pine seedling than in extracts from zero-time samples and microcosms without seedling. Illuvial soil sampled from both microcosm treatments yielded extracts with similar and slightly higher DNA concentrations than extracts from illuvial zero-time samples.

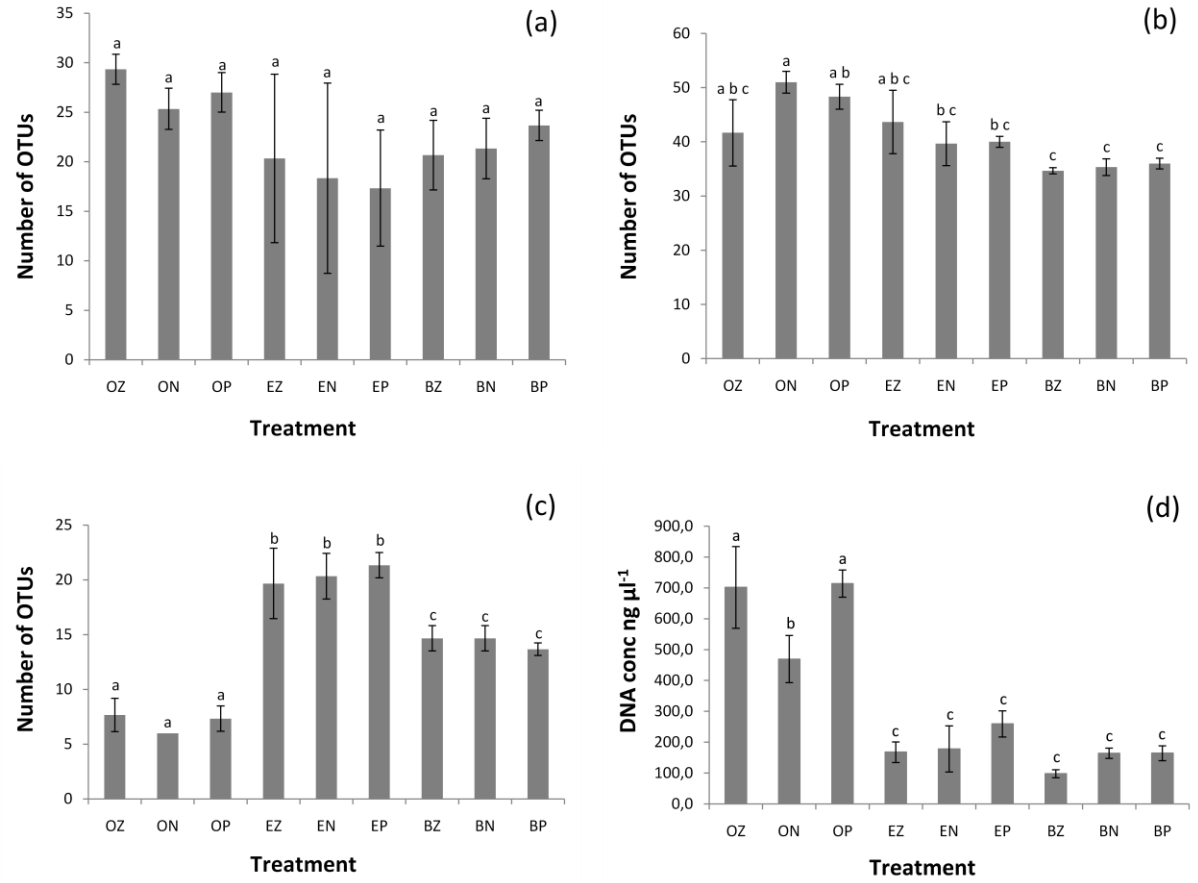

Figure 9. OTU richness for fungi (a), bacteria (b) and archaea (c) in the different soil horizons and treatments, obtained from the number of detectable bands per soil sample in the DGGE profiles. Figure (d) shows DNA concentrations in extracts (100 µl) obtained from 2 g fresh weight of soil per sample. Error bars represent standard deviation of mean values of triplicate samples. In each figure, treatments that share the same letter above error bars are not significantly different from each other ($p < 0.05$) as tested by one-way ANOVA.

5. Discussion

5.1 Soil characteristics

Boreal forest podzols can be regarded as rather stable soil environments in which a superficial carpet of mosses, lichens and slowly degrading plant litter moderates oscillations in moisture and temperature (DeLuca & Boisvenue, 2012) and provides a constant reservoir of metabolic resources for soil organisms (Lindahl *et al*. 2010). The commonly observed low abundance of larger soil mixing animals, such as earthworms, in podzols allows for

development of soil horizons with strongly shifting physicochemical characters (Eriksson *et al*. 2005), which could dictate growth patterns and hence distribution of different soil microorganism. Elemental analysis of the organic, eluvial and illuvial soils in this experiment (Table 1) revealed compositional differences that agree with previous descriptions of podzol profiles (Lundström *et al*. 2000; Eriksson *et al*. 2005). Not surprisingly, carbon was the most abundant element in the organic horizon while silica dominated in the eluvial and illuvial horizons. When comparing the eluvial and illuvial soils, slightly higher levels of both silica and carbon could be found in the illuvial soil, indicating deposition of these elements following weathering and leaching of material from overlying soil layers (Eriksson *et al*. 2005). On a dry weight basis, the organic horizon contained the highest levels of N, S, Na, K and Ca. The amounts of Mg, Fe and Al in the organic and eluvial horizons were much lower than in the illuvial horizon, reflecting precipitation of these elements with depth (Buurman and Jongmans, 2005). The nitrogen level in the organic soil was about 16 times higher than in both mineral soils, which signifies the organic horizon as a major nitrogen stock in boreal forest soils (Read & Perez-Moreno, 2003). The eluvial horizon is usually described as nutrient poor (Lundström *et al*. 2000; Eriksson *et al*. 2005), and had indeed the lowest abundance of most elements including P, S, Na, K, Ca, Mg and Mn. Interestingly P was equally abundant in the organic and illuvial horizons and had about 10 times lower abundance in the eluvial horizon, reflecting the ability of organic matter, Fe and Al to adsorb and hold phosphorus in soil (Eriksson *et al*. 2005). The measured pH values for the organic, eluvial and illuvial soils were 4.0, 4.4 and 4.8 respectively, and a pH increase with depth is a commonly mentioned characteristic of podzol profiles (Fritze *et al*. 2000; Eriksson *et al*. 2005).

The main goal of this study was to investigate if the organic, eluvial and illuvial horizons of a boreal forest iron-podzol harboured distinct communities of fungi, bacteria and archaea. It was further examined if Scots pine seedlings, as suppliers of rhizodeposition, would induce shifts in community composition of the different target groups. The experiment was conducted as a microcosm study in which microcosms, with and without Scots pine seedling, were incubated for 10 weeks. In addition, triplicate samples from the organic, eluvial and illuvial soils were frozen at the time of experimental set-up to serve as control for community shifts caused by incubation time alone. Differences in microbial community composition were assessed by denaturing gradient gel electrophoresis (DGGE) profiling of phylogenetic marker sequences, ITS rDNA for fungi and 16S rDNA for archaea and bacteria.

5.2 Fungal communities

The composition of fungal communities residing in the organic, eluvial and illuvial horizons of a boreal forest podzol profile was in this study investigated by comparing denaturing gradient gel electrophoresis (DGGE) profiles of fungal ITS sequences and by running non-metric multidimensional scaling (NMS) ordinations of presence/absence banding patterns. Visual analysis of DGGE profiles (Figure 5) and the NMS ordination plot (Figure 7b) showed

the soil horizons to harbour distinct fungal communities. This agrees well with previous studies reporting on limited vertical distributions of many fungal taxa in coniferous forest soils (Rosling *et al*. 2003; Lindahl *et al*. 2007; Hartmann. *et al*. 2009, Hartmann *et al*. 2012), a finding that often coincides with different carbon acquisition strategies (Lindahl *et al*. 2007). Fresh litter contains labile compounds and tends to be colonized by endophytes and opportunistic saprotrophs, while later successional saprotrophs and mycorrhizal taxa are able to metabolize more recalcitrant substrates located further down in podzol profiles (Lindahl *et al*. 2007). Mycorrhizal fungi obtain energy-rich carbon compounds from plant associates (Smith & Read, 2008), and are believed to explore soil resources primarily for nutrient mining (Read & Perez-Moreno, 2003; Bödeker, 2012). Mycorrhiza forming fungi have been found at all depths in coniferous forest soils (Rosling *et al*. 2003; Lindahl *et al*. 2007, Baldrian *et al*. 2012), although many mycorrhizal taxa seem to be confined to particular soil layers (Heinonsalo *et al*. 2001; Rosling *et al*. 2003, Lindahl *et al*. 2007, Hartmann *et al*. 2009). In this study the fungal community residing in the organic soil seemed to display a larger divergence in composition in comparison with the fungal communities inhabiting the two mineral soils, possibly reflecting a higher degree of similarity in physicochemical characteristics of the eluvial and illuvial soils.

All Scots pine seedlings had at the time of transplantation to microcosms equally developed root systems consisting of a few cm long primary root without lateral roots, but mycorrhizal development proceeded differently in the organic, eluvial and illuvial horizons. Ectomycorrhizas first formed in the eluvial soil by fungi producing a white morphotype that could belong the genus *Suillus* (Figure 4c) (Agerer, 1987-2002), which commonly dominates mycorrhizal root tips of Scots pine growing in podzol mineral soils (Heinonsalo *et al*. 2001; Rosling *et al*. 2003). The same apparent morphotype dominated all mycorrhizal root tips in the illuvial soil, but here mycorrhizal root tips could be observed first after three weeks of incubation. The quick mycorrhiza formation in the eluvial soil could be a response to the low nutrient status of this horizon (see Table 1). The ectomycorrhizal fungi that developed in both mineral soils exhibited extensive growth of extramatrical mycelia, partly consisting of hyphal cords spanning through the soil matrix (Figure 4d). A similar observation was also made by Heinonsalo and colleagues (2001) who identified the fungus as belonging to the genus *Suillus*. Extensive growth of extramatrical mycelia could be a foraging behaviour in ectomycorrhizal fungi induced under low nutrient conditions, as observed by Wallander & Nylund (1992). Scots pine seedlings growing in the organic soil became mycorrhizal after two weeks of incubation by fungi forming a yellow morphotype that could belong to the genus *Piloderma* (Figure 4a) (Agerer, 1987-2002), and this morphotype was also found on a few root tips in the eluvial soil. A black ectomycorrhizal morphotype, possibly belonging to the genus *Cenococcum* (Figure 4b) (Agerer, 1987-2002), was observed on some fine roots in the organic soil after six weeks of incubation. Both *Piloderma* spp. and *Cenococcum geophilum* are ectomycorrhizal fungi frequently detected in the organic horizon of

coniferous forest soils (Heinonsalo *et al*. 2001; Rosling *et al*. 2003), and have been shown to co-exist in mycelial mats formed by *Piloderma* spp. (Kluber *et al*. 2011).

In microcosms containing the organic soil, both incubation time and presence of Scots pine seedling roots caused slight shifts in DGGE banding patterns. Bands that were present in zero-time samples but decreased in intensity after 10 weeks of incubation (a) could represent early successional saprotrophs whose populations declined along with diminished levels of easily utilised substrates. At the same time, bands that increased in intensity with incubation time (c) may reflect an increased abundance of later successional saprotrophs better able to utilise more recalcitrant substrates, or fungi whose growth previously was repressed by competition with early successional fungi. In a microcosm study, Karhu and colleagues (2010) measured respiration rates and soil organic matter quality in soils sampled from different depths of two boreal forest soil profiles. They found that respiration rates in the organic soils declined rapidly during the first 100 days of incubation, and explained this observation by a loss of labile carbon compounds. In contrast, respiration rates in corresponding mineral soils did not change much with incubation time, which indicated a low soil organic matter quality already from the beginning of the incubation (Karhu *et al*. 2010). Competition suppression by dominant functional groups and changes in substrate availability are two factors that could cause shifts in fungal community composition in boreal forest soils. For example, Lindahl *et al*. (2010) observed an increased abundance of saprotrophic fungi after severing the root connection for ectomycorrhizal fungi which then declined in biomass. The observed increase in saprotrophic activity was partly explained by a reduced competition from ectomycorrhizal fungi and partly by the newly available resource in the increased necromass of ectomycorrhizal fungi (Lindahl *et al*. 2010). In the DGGE profiles of the organic soil, two bands (d) displayed the same intensity in zero-time samples and in presence of Scots pine seedlings, but a decreased intensity in soil samples from microcosms without seedling. These fungi might depend on labile carbon compounds for proliferation, which was depleted during the 10 weeks of incubation in microcosms without seedling, but supplied anew into the soil by Scots pine seedling roots. It is tempting to suggest that these bands represent the two ectomycorrhizal fungi observed in the organic soil, which might have been able to survive as free living mycelia in zero time samples.

In the eluvial soil, all reproducible shifts observed in the fungal DGGE profiles were represented by four bands (d) that increased in intensity in the presence of Scots pine seedlings. This suggests that rhizodeposition could be an important resource and thus a determinant of fungal community composition in this soil horizon. These bands probably represent ectomycorrhizal fungi or opportunistic saprotrophs whose growth was stimulated by input of labile carbon compounds by seedling roots. However, fungal DGGE banding patterns for the eluvial horizon were difficult to interpret due to a high variability between samples. This phenomenon has been reported before and was explained as low abundances

of some taxa in DNA extractions, leading to stochastic PCR amplification and possibly reduced reproducibility of replicate samples in DGGE profiles (see Lehtovirta *et al*. 2009). Hence, microcosms with eluvial soil might have contained natural inocula with slightly different community compositions, where some taxa occurred at very low abundances to begin with. Fungi are often reported to exhibit patchy distributions in soils (Rosling *et al*. 2003; Baldrian *et al*. 2011; Hartmann *et al*. 2012), a pattern explained by the large size of some mycelia and by the movement of individuals through the soil volume (Baldrian *et al*. 2011), which could contribute to reduced reproducibility of DGGE banding patterns. However, frequent mixing of soils prior to set-up of this experiment ought to have levelled out an uneven distribution of fungal taxa within the soil batches. The fungal community profiles for the illuvial horizon were nearly identical across treatments, except for one band (a) that decreased in intensity during incubation. This is a surprising result since all Scots pine seedlings harboured ectomycorrhizal fine roots, and detection of bands responding to the presence of seedling roots could be expected. However, only bulk soil samples were analyzed in this experiment, and differences in fungal species composition in DNA extracts from mycorrhizal fine roots and corresponding soil samples have been observed before (see Rosling *et al*. 2003). Most bands in the fungal DGGE profiles appeared with the same intensity in all samples of the same soil horizon, indicating stable populations of possibly slow growing or dormant fungi. Fungal richness was in this study assessed by the number of bands detected in the DGGE profiles (Figure 9a), and tended to be higher in the organic soil than in both mineral soils. This is in accordance with the observation by Heinonsalo and colleagues (2001) who found the organic horizon of a boreal forest podzol to contain a higher number of ECM morphotypes and RFLP-taxa than did mineral soil from the same soil profile.

5.3 Bacterial communities

The bacterial DGGE profiles (Figure 6) of 16S rDNA sequences revealed complex banding patterns for all soil samples, indicating a high phylogenetic diversity of bacteria throughout the examined boreal forest podzol profile. Previous studies have found coniferous forest soils to harbour a high bacterial diversity (Baldrian *et al*. 2012; VanInsberghe *et al*. 2013) that tend to decrease with soil depth (Hartmann *et al*. 2009; Eilers *et al*. 2012). The same trend was observed for the three soil horizons in this study, with the highest richness detected in the organic soil and the lowest richness in the illuvial soil (Figure 9b). Visual analysis of the bacterial DGGE profiles suggests that the organic, eluvial and illuvial soils house distinct communities of bacteria. This observation was confirmed by non-metric multidimensional scaling analysis of presence/absence DGGE banding patterns, which showed a strong clustering according to soil horizon (Figure 7a). Vertical differences in bacterial community composition have also been reported from other coniferous forest soil profiles (Hartmann *et al*. 2009; Eilers *et al*. 2012), where some bacterial taxa tended to either increase or decrease, while others attained a more uniform abundance with soil depth (Eilers *et al*. 2012).

Data points in the NMS ordination plot (Figure 7a) clustered according to treatment in the organic and illuvial soil, but less so in the eluvial soil due to the lower reproducibility of banding patterns for these soil samples. In the organic soil many bacterial taxa responded to incubation time by either decreasing (a) or increasing (c) in abundance (Figure 6). Although the metabolic diversity within the domain *Bacteria* is immense (Madigan *et al*. 2012), most soil bacteria rely on organic compounds for both energy and carbon (Alexander, 2005), and often display different resource utilisation patterns (Goldfarb *et al*. 2011; VanInsberghe *et al*. 2013). Without a constant input of new organic matter, the contents of labile substrates and nutrients in the organic soil decline during the incubation of microcosms (Karhu *et al*. 2010), which potentially lead to successional shifts in bacterial community composition. Karhu and colleagues (2010) found clear shifts in microbial community composition in organic soils collected from two boreal forest soil profiles, where gram-negative bacteria in particular declined in abundance with incubation time. When levels of labile substrates and nutrients are high, copiotrophic bacteria such as *Proteobacteria* and *Bacteroidetes* thrive (Fierer *et al*. 2007; VanInsberghe *et al*. 2013; Goldfarb *et al*. 2013), and are often abundant in organic topsoils (Eilers *et al*. 2012). As the soil substrate becomes depleted of nutrients and more resistant to degradation, copiotrophic bacteria may decline in abundance giving way to oligotrophic bacteria such as *Acidobacteria* that are able to cope under more nutrient poor conditions (Fierer *et al*. 2007; VanInsberghe *et al*. 2013), or bacteria with high metabolic plasticity such as *Actinobacteria* (VanInsberghe *et al*. 2013). Only one bacterial taxon (b) increased in abundance in the presence of Scots pine seedlings growing in the organic soil, which was rather unexpected considering the large shifts in community composition due to incubation time. There may be several explanations for the low response to the presence of plant roots. Firstly, Scot pine seedlings might not have exuded large amounts of photosynthate at this stage, or mycorrhizal fungi could to a large degree have monopolized the resources that were provided by the seedling roots. Secondly, rhizosphere soil was not analyzed in this study, and this is the soil compartment expected to display the clearest response to the rhizosphere effect. Thirdly, rhizosphere bacteria might have been reduced in the soil inocula as root material was removed prior to set-up of the experiment, which possibly reduced detection of these bacteria (see Hartmann *et al*. 2009). The organic soil in microcosms without seedling had three bands (d) that only were detected in this treatment. These bands could represent oligotrophic bacteria whose populations could increase to detection levels only when alleviated from competition from more copiotrophic bacteria.

Bacterial community shifts in the mineral soils were less pronounced than in the organic soil, and occurred only as increased abundances of taxa with incubation time irrespective of the presence of Scots pine seedlings. Bacterial taxa residing in the mineral horizons are probably well adapted to metabolize recalcitrant substrates and to live under nutrient poor conditions. Possibly the mixing of soils prior to experimental set-up hampered growth of

these bacteria by disruption of biofilms, filaments or syntrophic associations needed for efficient growth. Actinomycetes commonly increase in abundance with soil depth (Hartmann *et al*. 2009; Eilers *et al*. 2012), and some of these bacteria exhibit filamentous growth (Alexander, 2005) and could hence pose as potential candidates for taxa that increased in abundance during incubation of microcosms. In the DGGE profiles of the eluvial soil a region with lower reproducibility (v) could be discerned, possibly caused by stochastic PCR amplification due to low abundances of some bacterial taxa (see Lehtovirta *et al*. 2009). Many bands in the DGGE profiles appeared with the same intensity in all samples of each soil horizon, especially in the mineral soils, implying stable populations of these bacteria. This observation suggests that many soil bacteria may be autochthonous, being well adapted to grow under the oligotrophic conditions that the mineral soils in general, and the eluvial horizon in particular provide. Alternatively, these bands could represent bacterial cells in resting stages, as the majority of cells in soil environments have been estimated to be dormant at any one time (Lennon & Jones, 2011). The pronounced community shifts in the organic soil might point to a bacterial community more sensitive to disturbances compared with bacterial communities residing in the mineral soils, a trend that has been proposed for coniferous forest soil bacteria before (Karhu *et al*. 2010; Hartmann *et al*. 2012). In this study the organic, eluvial and illuvial horizons were shown to harbour distinct bacterial communities, probably reflecting differences in living environments. Substrate quality and quantity are just two of many edaphic factors, including nutrient status, local redox and water potential, pH and biotic interactions (see Tiedje *et al*. 1999), that could affect the composition of bacterial communities in the different soil layers.

5.4 Archaeal communities

In this experiment two nested PCR approaches were employed to amplify archaeal 16S rDNA sequences, and both strategies yielded amplicons of expected sizes when controlled on agarose (1%) electrophoresis gels. PCR products from secondary amplification with primers ARC344F-GC and Arch806R yielded unsatisfactory DGGE profiles due to formation of dark smears in all lanes after silver staining, and neither re-amplification nor template dilution improved the results. It may be that this primer combination displayed low amplification efficiency, leading to loading of unamplified DNA that possibly formed smears during migration in the DGGE gel. However, the nested PCR approach using primers rSAF-GC and PARCH519R in the secondary run resulted in reproducible banding patterns, revealing distinct archaeal communities in the organic, eluvial and illuvial horizons of a boreal forest podzol profile (Figure 8). The primers rSAF-GC and PARCH519R both contained degeneracies that influenced the GC-content of amplicons, so that one sequence type was displayed as band replicates (see Nicol *et al*. 2005). This posed a problem when estimating archaeal richness, but each single band was counted as an OTU due to variation in the number of replicate bands that clustered in the DGGE profiles. Archaeal richness was relatively low in all soil samples (Figure 9c), which is accordance with earlier reports on low diversity of archaeal 16S rRNA genes in soil environments (Aller & Kemp, 2008; Auguet *et al*. 2010). The

organic horizon had lower OTU richness than both mineral soils, suggesting an increased diversity of archaea with soil depth. A similar observation was made by Hartmann and colleagues (2009) who found archaeal richness to increase with depth in a Lodgepole pine forest soil. The archaeal DGGE profiles further revealed that some archaea were present in two contiguous horizons, while other archaea were unique to either the eluvial or illuvial soil.

There were no detectable changes in band intensities induced by incubation time or presence of Scots pine seedling roots, indicating stable archaeal populations of possibly slow growing or dormant cells. Previous studies report contradictory results on archaeal responses to plant roots. A high abundance of archaea has been found on tomato plant roots (Simon *et al*. 2000), and mycorrhizal root tips of Scots pine (Bomberg & Timonen, 2007), suggesting that some archaea thrive in mycorrhizosphere environments, while Karlsson and colleagues (2012) found a tendency of archaea to decrease in abundance when exposed to root and fungal exudates. Plant roots have previously been shown to house archaeal consortia distinct from corresponding bulk soil or sediments (Bomberg and Timonen, 2007; Pires *et al*. 2012), while another study found no major difference in archaeal community composition when comparing rhizosphere and bulk soils from several plant species (Nicol *et al*. 2005). Interestingly, Nicol and colleagues (2005) found thaumarchaea to increase in diversity and shift in community composition with successional stage of plant cover and soil age, with increasing abundances of thaumarchaea group 1.1c and 1.3. These two thaumarchaeal groups were also observed in boreal oligotrophic peat wetlands (Juottonen *et al*. 2008), and group 1.1c is commonly found in forest soils and other acidic soils (Jurgens *et al*. 1997; Nicol *et al*. 2007). Archaea have been detected in the organic layer of boreal forest podzols before (Jurgens *et al*. 1997, Bomberg *et al*. 2007), where the community mainly consisted of thaumarchaeota group 1.1c and associated sequences and to a lesser extent euryarchaeota affiliated with the orders *Halobacteriales* and *Methanosarcinales* (Bomberg *et al*. 2011). However, little is known about the functional roles of archaea inhabiting boreal forest podzols, and it is tempting to speculate what edaphic factors that could select for archaeal communities in the different soil horizons. Two recent cultivations of thaumarchaeota from moderate soil environments confirmed the ability to oxidize ammonia (Lehtovirta-Morley *et al*. 2011; Tourna *et al*. 2011). However, co-culture with possibly syntrophic bacteria (Lehtovirta-Morley *et al*. 2011; Tourna *et al*. 2011) or addition of pyruvate (Tourna *et al*. 2011), seemed to be necessary to sustain efficient growth, suggesting a mixotrophic, as opposed to purely lithoautotrophic, life strategy of these archaea. In this study the functional gene *amoA*, coding for the active site of the enzyme ammonia monooxygenase involved in oxygenic ammonia oxidation (see Rotthauwe *et al*. 1997), could not be amplified for either archaea or bacteria from any of the soil samples, except for the positive control which yielded products in all PCR runs. This finding agrees with Bomberg *et al*. (2010) who also failed to detect archaeal *amoA* genes in the humus layer of a boreal forest podzol, but stands in contrast to the finding by Long and

colleagues (2012) who found *amoA* genes from both archaea and bacteria in boreal forest topsoil. The functional roles of boreal soil archaea and what factors that determine their consortia in different soil layers remains yet to be explored.

5.5 Summarising discussion and future prospects

Soils are complex environments that pose a challenge to study, and disturbance of the soil system is inevitable whenever sampling is performed. Commonly, the aim of soil microbial surveys is to examine the ecology of particular target groups, often by manipulation experiments where treatments are compared with a reference control. Field studies undoubtedly present the most natural approach but also introduce slight variations in local conditions between experimental plots, thus introducing an element of uncertainty in result analysis. On the other hand, laboratory cultivation is performed under highly controlled conditions and is often required to confirm functional roles of microorganisms (Tourna *et al*. 2011), but cultivation of soil microorganisms is usually difficult to achieve with standard laboratory media (Staley & Konopka, 1985). Instead microcosm experiments can provide an intermediate method where soil substrates can serve as highly complex and undefined cultivation media (see Read & Perez-Moreno, 2003), that still allows for manipulation under controlled conditions. However, microcosm studies suffer from the same drawbacks as laboratory cultivations in depletion of substrates and nutrients, and accumulation of metabolic by-products that eventually alter the substrate chemistry (Madigan *et al*. 2012).

Both fungi and bacteria in the organic soil reacted strongly to incubation time, and this finding probably reflects a successional shift of functional groups with different abilities to cope with changes in substrate quality and quantity. The organic horizon in a boreal forest podzol is composed of distinct soil layers - the litter, fermentation and humus layers – each characterized by the decompositional state of the organic matter (Lindahl *et al*. 2007; Hilli, 2011). There are also indications that fungi and bacteria display vertical separation in community composition and activities within the organic horizon (Lindahl *et al*. 2007; Baldrian *et al*. 2012). In this study, soil preparation encompassed removal of roots, sieving and mixing, which could have led to an increased availability of labile compounds. This disturbance was probably more pronounce in the organic soil than in the mineral soils, and may have led to an increased abundance of zymogenous taxa, which decreased in abundance once most of the labile substrates and nutrients were metabolized. Hence, microbial communities in the organic horizon might be more adapted to fluctuating conditions than taxa residing in the mineral horizons. This observation is in accordance with results from a microcosm study of boreal forest soils where shifts in microbial community composition and loss of labile substrates were more pronounced in the organic soil than in the mineral soil during the first 100 days of incubation (Karhu *et al*. 2010). It would therefore be advisable in this kind of experiments to sample in a time series to elucidate more subtle temporal changes in community composition of target organisms. The high richness of bacteria and fungi that was observed in the organic soil may thus be an artifact

induced by soil preparation, and that in field represent taxa from the different layers within the organic horizon. Archaeal richness was in general low, but highest richness was observed in the eluvial soil where most bands also were present either in the organic or the illuvial soil. Such transient shifts in community compositions with depth have also been found for ectomycorrhizal fungi in a boreal forest podzol profile (Rosling *et al*. 2003).

It has been proposed that DNA yields from soil extractions can serve as a rough estimate of the amount of biomass present in soil samples (Hartmann *et al*. 2012). In this study, the average DNA concentration in extracts suggests that the organic horizon holds a much higher biomass than both minerals horizons (Figure 9d), which is in agreement with previous observations of decreased biomass with depth in coniferous forest soils (Eilers *et al.* 2012; Hartmann *et al*. 2012). The biomass seemed to decline in the organic soil during the 10 week incubation period, while Scots pine seedlings managed to maintain a higher biomass of soil biota, possibly through input of rhizodeposition. For the mineral soils, DNA yields were not significantly different between the eluvial and illuvial horizons or treatments. However, Scots pine seedlings seemed to stimulate biomass production in the eluvial soil while incubation time alone caused slight increases in biomass in the illuvial soil. Thus, rhizodeposition might be able to raise the local carrying capacity in the organic and eluvial horizons.

The main objective of this experiment was to examine if the organic, eluvial and illuvial horizons supported distinct communities of fungi, bacteria and archaea, and further if Scots pine seedlings would induce community shifts in the different soils. All three groups of microorganisms displayed distinct community compositions in the different soil horizons, overshadowing responses to incubation time and the presence of Scots pine seedling roots. The fungal response to the presence of Scots pine seedlings was expected considering the observed development of ectomycorrhizal associations. However, the presence of seedling roots did not lead to any discernible changes in either bacterial or archaeal communities, except for one bacterial band in the organic soil that increased in intensity. In this study bulk soil samples were analyzed and it may be that more pronounced changes in prokaryotic community composition would have been detected if the rhizoplane had been examined. Indeed, previous studies indicate that Scots pine fine roots harbour communities of fungi (Rosling *et al*. 2003), bacteria (Timonen & Hurek, 2006) and archaea (Bomberg and Timonen, 2007) that differ in composition compared with communities in the surrounding soil. The archaeal communities did not respond to either incubation time or presence of Scots pine seedlings in any of the soil horizons, and the same observation was made for many bands in the fungal and bacterial profiles. These taxa may be dormant or display a lifestyle that do not directly depend on the availability of labile substrates, such as slow growing saprotrophs that degrade recalcitrant compounds or cells that have a lithoautotrophic life strategy.

Detection of microorganisms by PCR amplification of phylogenetic marker sequences indicates the presence of taxa in natural samples, but tells little of their metabolic status. Starvation is the prevailing condition that soil microorganisms experience and most of them exist as dormant stages (Lennon & Jones, 2011). A complementary method that can be used to target the active community is to amplify corresponding RNA molecules by reverse transcript PCR. Studies that include both DNA and RNA in the analysis have shown some abundant taxa to be inactive, while less abundant taxa might be highly active (Baldrian *et al*. 2012). RNA based surveys can thus highlight more subtle community shifts than DNA based profiling (Juottonen *et al*. 2008). Denaturing gradient gel electrophoresis (DGGE) of PCR amplicons is a fingerprinting technique widely employed in microbial ecology (van Elsas *et al*. 2011). However, DGGE profiling provides depiction of the most abundant members of the examined target group. If 'universal' primers are used to amplify bacteria and fungi, rare taxa may be lost in the analysis, and deep sequencing has shown that most soil microorganisms occur in low abundances (Hartmann *et al*. 2012). Another drawback when using 'universal' primers is that DGGE profiles may turn out too complex to discern individual bands. When examining natural samples expected to harbour complex communities, such as soil, it would be advisable to use primers targeting subgroups to simplify profile analysis. The archaeal community was relatively simple, but here degeneracies in both the forward and reverse primers generated replicate bands that compromised resolution of individual phylotypes. DGGE and other fingerprinting techniques provide rough indications of the genotypic diversity of the amplified target sequence, but do not provide phylogenetic information unless combined with sequencing. Sequencing ensures that fingerprinting profiles consist of sequences of targeted members only, which is a concern as PCR primers do sometimes amplify non-target sequences as well (Mahmood *et al*. 2006; Bomberg *et al*. 2007). In this survey sequencing was not performed, but DGGE profiling alone did answer the main question of interest.

This study clearly indicated a vertical segregation of microbial communities in a boreal forest podzol profile. It can only be speculated what edaphic factors that contribute to shape this zonation of the microflora, since each soil differed in texture, chemistry and pH, but probably in organic matter quality and quantity as well. Further, Scots pine seedling roots seemed to have a stronger stimulating effect on fungi than on prokaryotic groups, a surprising finding that might need further investigation to understand the rhizosphere biology in the different soil horizons. These results provide an exciting starting point for further investigation of microorganisms in boreal podzols, and strongly suggest that the mineral soil should be included in such surveys. Deep sequencing is becoming increasingly popular, but may not necessarily provide more information than fingerprinting techniques about the functional roles of individual taxa (Prosser, 2012). It would therefore be exciting if experiments aimed at unveiling the functional diversity of soil microbiota, and to examine the connection between phenotypic and phylogenetic diversity in the different podzol horizons.

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