FUNGAL AND BACTERIAL

CONTRIBUTIONS TO HYPHOSPHERE

ENZYME ACTIVITY

by

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Abstract

In temperate forests, trees form symbiotic associations with fungi on their roots; the majority being an ectomycorrhizal alliance. Ectomycorrhizal fungi (EMF) secrete phosphatase enzymes which mobilize phosphorus from soil organic matter to their host. Soil bacteria also contribute to phosphorus mobilization through phosphatase release. I characterized bacterial and EMF contributions to phosphatase activity in forest soils regenerating after stand-replacing wildfire or from clearcut logging followed by broadcast burning. Fire can cause ecosystem phosphorus loss and can change microbial community structure, but it is unclear what effects these changes have on phosphorus availability.

To link EMF hyphae with phosphorus mobilization in forest soil, I developed a novel method for visualizing fine-scale soil enzyme activity *in-situ*. Visualization of phosphatase activity across a chronosequence forest stands demonstrated a change in the pattern of *in-situ* soil phosphatase activity; areas of phosphatase activity were smaller in stands less than 61 years-old (stand initiation to canopy closure) and became larger in stands 61 to 103 years-old (stem exclusion to post stem exclusion). To link EMF with areas of high and low phosphatase activity I also developed a new soil sampling method where enzyme activity was first visualized and then used to guide small, targeted, soil samples from the soil profile for molecular (T-RFLP) analysis. The number of EMF molecular signatures was not different between the high and low-phosphatase soil microsites in stands less than 61 years-old, but in older stands, there were typically more EMF signatures in areas of low phosphatase activity.

Bacteria also contribute to soil phosphatase activity; therefore, I investigated the effect of EMF hyphae on the enzyme activities of nearby soil bacteria by trapping bacteria and EMF hyphae *in-situ* using sand-filled mesh bags. Bacteria from the bags with hyphal ingrowth had lower phosphatase activities than bacteria from bags without hyphae. Given the higher number of EMF signatures present in low compared to high phosphatase microsites and the lower phosphatase activities of bacteria near hyphae, it is possible that EMF species may be excluded from organic phosphorus or may compete for phosphorus by excluding other EMF and selecting for less competitive soil bacteria.

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Dedication

To my children

Co-authorship statement

Chapter 2: A method for linking in situ activities of hydrolytic enzymes to associated organisms in forest soils

D. Brooks was responsible for all work related to the chronosequence of forest stands. D. Brooks produced the enzyme imprints, wrote the program that generated the data for the analysis of phosphatase activity, analyzed all data related to the chronosequence, wrote all material related to the analysis of enzyme imprints from the three studies presented, and formatted and submitted the manuscript for publication.

Chapter 3: Mapping ectomycorrhizal fungi to fine-scale soil phosphatase activity in regenerating forests

D. Brooks developed the research question; designed the microsampling technique used to localize microbial communities to *in situ* phosphatase activity, performed the soil microsampling and sample processing, analyzed all data presented, and wrote, formatted, and submitted the manuscript for publication.

Chapter 4: Ectomycorrhizal hyphae interact with soil bacteria to reduce organic phosphorus mobilization

D. Brooks developed the research question, designed the study, made and collected the sand bags, developed the enzyme assays, analyzed all data presented, and wrote, formatted, and submitted the manuscript for publication.

Appendix A Imprinting soil enzyme activity

D. Brooks formatted, organized and edited the content of the sections included in Appendix A; wrote sections A-2.2, A-8.3, and A-9. D; and developed the methods detailed in A-2.2, A-8.3 and A-9.

1   Introduction

Our ability to predict impacts of climate change is hindered by methodologies that treat soil as a homogeneous medium because measurements made on bulk soil samples do not accurately represent whole-soil processes (Schimel & Bennett, 2004; Litton & Giardina, 2008). Soil represents the largest pool of terrestrial carbon in the biosphere, more than plants and atmosphere combined (Schlesinger, 1997). In forests, below-ground carbon can account for three times the carbon of above-ground biomass (Mackey *et al*., 2008), yet below-ground carbon dynamics remain the least well understood (Litton & Giardina, 2008). The predictions of climate change models are affected by incorporation of both above-ground and belowground feedback. For example, by including the effects of vegetative feedback in climate change models, the predicted levels of atmospheric CO_2 concentrations by 2100 increased by 34% over models that did not include feedback (Cox *et al.*, 2000). In addition, climate change models that do not take into account the belowground feedback involved in nutrient acquisition from soil by plants overestimate net primary productivity and ecosystem carbon storage (Kirschbaum *et al*., 2003; Roxburgh *et al*., 2004). Given that soil microbes are responsible for 80 to 90% of the reactions that drive soil processes (Nannipieri *et al.*, 2003), are tightly coupled with plant physiology and productivity (Ekblad & Högberg, 2001; Giardina *et al.*, 2004; Högberg *et al.*, 2008,2009), and are sensitive and not highly resilient to disturbance (Allison & Martiny, 2008), a better understanding of the distribution and function of microbial populations in soil is needed to incorporate soil microbial feedback into models of climate change (Wardle *et al.*, 2004; van der Heijden *et al.*, 2008).

One goal of ecological forest management is to mimic natural processes in order to maintain spatial and genetic diversity in managed ecosystems. Understanding the effects of natural forest processes, such as fire, on the potential nutrient mobilizing abilities of soil microbes and the resultant carbon storage from growth of above-ground and belowground biomass is essential for sustainable forest management. Wildfire varies in its impact across a landscape creating a patchwork of effects (van Wagner, 1983), and the effects of fire on soil and soil microorganisms vary depending on fire severity and the type of forest burned

(reviewed by Certini, 2005). Changes in soil properties and loss of organic matter affects soil organisms at different depths in the soil profile (Prieto-Fernández *et al.*, 1998; Neary *et al.*, 1999), and changes in soil communities can subsequently change soil properties affecting above-ground forest regeneration. This feedback between belowground and above-ground ecosystems can have profound effects on forest regeneration, changing both the composition and development of forest ecosystems (Dahlberg *et al.*, 2001; Tuininga & Dighton, 2004).

Fire has a profound effect on nutrient cycling. Fire causes ecosystem nutrient loss through volatilization, the mineralization of organic nutrients, and by lowering the production of nutrient mobilizing enzymes. Fire induced loss of phosphorus is especially important because unlike carbon and nitrogen which can be fixed from the atmosphere, phosphorus must be either mobilized from the soil mineral matrix or enzymatically recycled from soil organic matter (Burns, 1978; McGill & Cole, 1981; Paul & Clark, 1989; Attiwill & Adams, 1993). Soil microbes, including ectomycorrhizal fungi (EMF), tightly control phosphorus availability through immobilization of phosphorus into living biomass. Fire can cause changes in both bacterial and EMF community structures, and it is unknown what effects these changes may have on both the long term and short term availability of phosphorus, and by extension, to forest productivity. Therefore, understanding the effects of natural forest processes, such as fire, on the potential nutrient mobilizing abilities of bacteria and EMF is essential for sustainable forest management.

1.1   Fire and microbes

Fire changes soil factors that affect soil microorganisms and can have a profound effect on soil nutrient pools. Fire can vaporize soil organic matter, resulting in the loss of phosphorus and biomass from the ecosystem, or soil organic matter can be mineralized, causing a temporary phosphorus fertilization effect. Losses of phosphorus caused by fire can be significant. More than 50% of the total phosphorus contained in combusted fuel can be lost to the atmosphere during fire (Raison *et al.*, 1985). After fire, more phosphorus can be lost when ash is eroded by wind and rain. Fire intensity also changes the distribution of phosphorus in the soil profile; influencing the amount of phosphorus occluded through sorption to soil minerals or

through formation of insoluble salts (Kwari & Batey, 1991; Polglase *et al.*, 1992; Romanyà *et al.*, 1994; Saa *et al.*, 1993; Cade-Menun, 1995; Ström *et al.*, 2005). In contrast, the effect of clearcutting on phosphorus pools and cycling appear to be variable and site specific (Kreutweiser *et al.*, 2008). In a recent review of logging impacts on soil biogeochemistry, Kreutweiser *et al*. (2008) reported that most studies have shown that logging will have relatively small effects on soil nutrient pools and cycling in comparison to other disturbances, such as fire.

The intensity of the fire will determine the depth to which soil microbes are killed and the extent to which the biological sink for phosphorus mineralized by fire will be reduced (Pietikainen & Fritze, 1992; Fritze *et al.*, 1998; Tuininga & Dighton, 2004). However, even light burning can create toxic compounds that cause significant shifts in the microbial community (Fritze *et al.*, 1998). Other fire-induced changes in soil factors that affect soil microorganisms include temporary increases in soil pH (Ulery *et al.*, 1993), increased soil moisture and temperature fluctuations (Bissett & Parkinson, 1980; D'Ascoli *et al.*, 2005) or decreased soil moisture due to fire induced hydrophobicity (DeBano, 2000; Robichaud, 2000; Ice *et al.*, 2004). Despite variations in the effects of fire on soil factors, microbial biomass is typically reduced immediately after fire (Pietikäinen & Fritze, 1995; Vázquez *et al.*, 1993; De Marco *et al.*, 2005; Hart *et al.*, 2005; Smith *et al.*, 2008). After this initial reduction, microbial biomass may increase rapidly to several times higher than unburnt forest soil, followed by biomass decreases to below unburnt levels, until an eventual return to original levels (Ahlgren & Ahlgren, 1965; Prieto-Fernández *et al.*, 1998). Equally, biomass may recover slowly without a short-term spike in growth (Wright & Bollen, 1961). One year after fire, microbial community structure can still differ significantly from communities in unburnt forest soil (Bissett & Parkinson, 1980; Acea & Carballas, 1996; Staddon *et al.*, 1998; Yeager *et al.*, 2005; Hamman *et al.*, 2007; Smith *et al.*, 2008), and it may take up to 12 years for microbial biomass to recover to pre-fire levels (Dumontet *et al.*, 1996; Fritze *et al.*, 1993). However, it is unclear how the initial changes in microbial community structure, or the slow recovery of microbial biomass, may influence forest regeneration (Neary *et al.*, 1999).

The transformation of organic to inorganic phosphorus by fire also disrupts the illuviation of organic phosphorus normally seen in podzolic soils, which, in turn, reduces the amount of organic phosphorus that can then be biologically mineralized and subsequently used by soil microbes and plants. Biological processes have long been recognized as essential in determining the unique illuviation patterns seen in podzolic forest soils (Crawford, 1963; Berthelin & Dommerques, 1976; Boudot *et al.*, 1989). Soil sterilization can cause changes in these patterns that are similar to those seen after fire, and typical patterns can be restored through inoculation of soil with fungi and bacteria (Aristovaskaya & Zykina, 1977). Therefore, it is likely that the effect of fire on the illuviation patterns of organic phosphorus and on the long term availability of phosphorus for use by plants and microbes is related to the death of active soil microbes. Furthermore, it has been hypothesized by Cade-Menum *et al.* (1995) that destruction of soil organic layers by both clearcutting and burning may be responsible for long-term changes in illuviation patterns that reduce organic phosphorus in podzolic soils, resulting in growth checks during later forest regeneration.

1.2   Biological control of phosphorus

Unlike carbon, nitrogen, and sulfur, which can be added to ecosystems either through biological fixation or rain deposition, phosphorus must either be mobilized from the soil mineral matrix or enzymatically recycled from soil organic matter (Burns, 1978; McGill & Cole, 1981; Paul & Clark, 1989; Attiwill & Adams, 1993). Even though a small amount of phosphorus can be deposited from the atmosphere as ash (Olander & Vitousek, 2004), the phosphorus cycle is basically a closed system. Plants and microbes liberate phosphorus from soil minerals through the production of organic acids, which destabilize the mineral matrix to release bound phosphorus, or through the production of phosphatase enzymes, which cleave phosphorus from organic molecules. Because phosphorus is necessary for the formation of ATP used for the fixation of carbon or nitrogen, the mobilization of phosphorus represents a critical ecosystem process in which the carbon and nitrogen cycles converge. Thus, the activities of plants and soil microbes that replenish phosphorus lost from the system through leaching or volatilization by fire are critical to ecosystem function and sustainability.

Phosphatase enzymes encompass a broad group of enzymes that are active in soil. These enzymes release pyrophosphate by catalyzing the hydrolysis of ester bound phosphorus and anhydrides of phosphate (Schmidt & Laskowski, 1961). These enzymes include phosphoric monoester hydrolases (phosphomonoesterases) that cleave ester-bound P (O-P) from organic compounds releasing orthophosphate. The phosphoric diester hydrolases (phosphodiesterases) cleave one of the ester bonds of diester-bound P (O-P-O). Diester bound P links nucleotides in nucleic acids, and the action of phosphodisterase produces smaller nucleotide units or mononucleotides, but does not release inorganic phosphate. Triphosphoric monoester hydrolases cleave ester-bound triphosphate groups (O-P-P-P) from organic compounds. Additional groups include enzymes that act on phosphoryl-containing anhydrides (e.g. ATP phosphohydrolase), and enzymes acting on P-N bonds, such as the phosphoamidase (Tabatabai, 1994). Phosphatases are classified acid or alkaline depending on the pH where they show optimum activity, and acid phosphatases predominate in acid soils (Eivazi & Tabatabai, 1977; Juma &Tabatabai, 1977, 1978). Acid phosphatases are secreted by plants, fungi, bacteria and microfauna, but while alkaline phosphatases can be secreted by bacteria and fungi, they are not secreted by plants (Dick et al., 1983; Juma & Tabatabai, 1988a,b,c). While many phosphatase enzymes play a role in phosphorus nutrition, the phosphomonoesterases (acid phosphatase and alkaline phosphatase) have been studied extensively because these enzymes contribute directly to phosphorus nutrition by mineralizing phosphorus as orthophosphate, and assays based on artificial substrates, such α-naphthyl phosphate (Dinkelaker & Marschner, 1992), facilitate the characteriziation of these enzymes in a complex soil environment (Cosgrove, 1967; Ramirez-Martinez, 1968; Kiss et al., 1975; Speir & Ross, 1978).

Due to the closed nature of the phosphorus cycle and the relative immobility of easily absorbable phosphorus in mineral soil (Bhat & Nye, 1966; Lewis & Quirk, 1967; Farr & Vaidyanathan, 1972), phosphorus availability in natural soils is low (McGill & Cole, 1981). Phosphate ($H_2PO_4^-$) is the predominant form of available phosphorus in acidic soils, and it is the interaction between the biological immobilization of mobilized $H_2PO_4^-$ and the immobilization of both $H_2PO_4^-$ and organic phosphorus through sorption to clay minerals and binding to aluminum and iron oxides that determines the total available phosphorus for biological uptake.

Short-term biological demand for phosphorus is met primarily through biological and biochemical mineralization of organic phosphorus in the organic soil horizons, while long term phosphorus retention and cycling is controlled in the mineral soil horizons through adsorption of both inorganic and organic phosphorus to clay minerals (Wood *et al.*, 1984).

Plants and microbes tightly control phosphorus in undisturbed forests; losses of inorganic phosphorus from undisturbed stands can be less than 1 μg liter^{-1} year^{-1} (Wood *et al.*, 1984). Because the production of enzymes requires both metabolic energy and the commitment of scarce nitrogen resources, plants and microbes strictly regulate phosphatase production Plants and microbes preferentially absorb available phosphorus from the soil solution before engaging in phosphatase production, maintaining low levels of available phosphorus levels in forest soil (Speir & McGill, 1979; McGill & Cole, 1981; Olander & Vitousek, 2000). Measurements of available phosphorus in soil may reflect neither the extent nor the rate of actual phosphorus cycling in an ecosystem because the level of available, inorganic phosphorus levels do not indicate the extent or rate of the biological activity that drives phosphorus mobilization and uptake. Because phosphatase activity results from biological activity in soil, measurement of phosphatase activity may provide a better indication of phosphorus cycling in the forest ecosystem.

When phosphorus is lost from a system, it must be replaced through mobilization of inorganic phosphorus from the mineral substrate or through mineralization of organic phosphorus from soil organic matter. Plants and microbes can mobilize phosphorus from organic compounds through the secretion of phosphatase, and it can be mineralized by saprotrophic microbes during the oxidation of carbon compounds for energy. In addition to biological mineralization of organic matter, plant roots, fungi, and bacteria can release phosphorus from soil minerals through biochemical solubilization. In this process, low molecular weight organic acids are secreted, which chelate metal cations and destabilize soil minerals causing phosphorus to be released (Whitelaw, 2000; Dakora & Phillips, 2002; Ström *et al.*, 2005). These acids can also act to help keep liberated phosphorus in circulation by binding to phosphorus-sorbing sites, preventing sorption onto secondary minerals (Parfitt, 1978), or reacting with free calcium, iron, and aluminum ions, to inhibit the formation of insoluble

precipitates (Stevenson, 1967). While biological mineralization of organic phosphorus may result as a byproduct of the search for energy by saprotrophs, both biochemical solubilization of phosphorus through the production of organic acids and the production of extra-cellular phosphatase enzymes are strongly controlled by the supply of, and need for, phosphorus (McGill & Cole, 1981).

1.3   Ectomycorrhizal fungi

While many macro and micro-organisms contribute to the production of phosphatase enzymes in soil, in forests with acidic, podzolic soils, fungi dominate the microbial community, and these fungi are largely ectomycorrhizal fungi (EMF) (Finlay & Söderström, 1989; Hackl et al., 2005; Hendricks et al., 2006). Most conifer tree species form symbiotic associations with these fungi on their roots, relying on them to access soil moisture and nutrients. Ecomycorrhizal fungi create a direct connection between soil microbial processes and forest productivity because they share the nutrients they mobilize with their hosts in exchange for recently fixed carbon. It has been estimated that they supply approximately 15% of the phosphorus absorbed by host trees (reviewed by Read & Perez-Moreno 2003). The activities of ectomycorrhizal mycelia directly influence forest productivity and plant ecology. Mycorrhizal hyphae not only absorb phosphorus from the soil solution, but extract inorganic phosphorus by actively weathering soil minerals (Cumming & Weinstein, 1990; van Breemen *et al.*, 2000; Nilsson & Wallander, 2003; Wallander *et al*., 2005) and to mobilize organic phosphorus from organic matter through the production of phosphatases (Leake & Read, 1997; Smith & Read, 1997; Perez-Moreno & Read, 2001; Koide & Wu, 2003; Read & Perez-Moreno, 2003). Additonally, inorganic phosphorus-solubilizing bacteria have been found to be selectively associated with mycorrhizal hyphae (Toro *et al.*, 1997; Kim *et al.*, 1998; Muthukumar *et al.*, 2001; Frey-Klett *et al.*, 2005; Calvaruso *et al*., 2007; Uroz *et al*., 2007). The potentially synergistic interaction between inorganic phosphorus-solubilizing bacteria and ectomycorrhizal hyphae would further support a sustainable inorganic phosphorus supply to ectomycorrhizal hosts. By producing extra-cellular phosphatase enzymes that extract phosphorus from organic matter, solubilizing and absorbing inorganic phosphorus, and interacting with nearby soil bacteria for enhanced inorganic

phosphorus mobilization, ectomycorrhizal hyphae participate in both the biochemical and geochemical aspects of phosphorus mobilization and transfer much of that phosphorus to their hosts; creating a direct connection between soil microbial processes and forest productivity.

1.4   Ectomycorrhizal mycelia and hyphae

The mycorrhizal structures EMF form with the root tips of trees (ectomycorrhizae) produce large quantities of extra-matrical mycelia and hyphae that interact with soil microbes, organic matter, and minerals. The hyphae are microscopic, intermingled with closely related saprotrophic fungi (Hibbett et al., 2000), and share functions with other saprobes (Leake et al., 2002). Despite acting as the main nutrient-absorbing interface between mycorrhizae and the soil (Smith & Read, 1997), the extra-matrical mycelia and hyphae are the most poorly understood and difficult-to-study component of the mycorrhizal symbiosis (Staddon et al., 2003; reviewed by Anderson & Cairney, 2007).

Ectomycorrhizal mycelial growth patterns vary, but the ecological functions associated with mycelial structures are unclear. Some species produce hyphal mats; others produce coarser hyphae, and broad range of species produce specialized rhizomorphs or hyphal strands (Read, 1991; Agerer 2001, 2006). In addition, ectomycorrhizal mycelia form structurally complex nets of interconnected hyphae, which are capable of simultaneous bidirectional transport of materials within individual hyphae (Ashford & Allaway, 2002). The cost of maintaining this large mycelial biomass has been found to be offset by increased leaf area (Read & Perez-Moreno, 2003), increased photosynthesis (Wright *et al.*, 1998), protection from root pathogens (Rasanayagam & Jeffries, 1992), carbon transfers through ectomycorrhizal mycelial connections between plant roots (reviewed by Selosse *et al.*, 2006), and increased uptake of nitrogen and phosphorus (reviewed by Read & Perez-Moreno, 2003). While the genome of the host tree may have some effect on the production of complex fungal structures such as rhizomorphs (Rosado *et al.*, 1994), there is still little known about factors that affect mycelial growth patterns in the field (Cairney, 1999; Baier *et al.*, 2006).

While it is accepted that EMF largely benefit their host plants, there have been mixed reports regarding which aspects of the symbiosis result in these benefits. It does not appear

that the percent EMF colonization is strongly correlated with host response (Karst *et al.*, 2008), and while Lamhamedi *et al.*, (1992) found that host benefit corresponded with the extent of mycelial exploration; Ek (1997) found no relationship between mycelial growth and either nitrogen transfer to host or soil respiration. Furthermore Lamhamedi *et al.* (1992) found that while a greater length of mycelia correlated with better protection of EMF hosts from water stress, this protection was not correlated with rhizomorph production. This finding underscores the need for more studies of ectomycorrhizal mycelia and hyphae in the field because the transfer of water to the EMF host has long been considered one of the primary functions of rhizomorph formation.

1.5 Relationship between ectomycorrhizal community structure and ecosystem functions

Ectomycorrhizal fungi are extremely diverse, especially when compared to their host community (Visser, 1995; Dahlberg et al., 1997; Durall et al., 1999), with 100-2000 EMF species found in Douglas-fir forests (Zak, 1973; Trappe, 1977). Diversity of EMF species is influenced by soil conditions (Marx, 1981; Bougher & Malajczuk, 1990; Bougher et al., 1990; Thomson et al., 1994b), host specificity (Marx, 1981; Bougher et al., 1990; Jacobson & Miller, 1992; Molina et al., 1992; Bonfante et al., 1998), tree age (Mason et al., 1982; Deacon et al., 1983; Last et al., 1983), distance of roots from stem (Ford et al., 1980; Mason et al.,1982; Last et al., 1983), and disturbances such as fire or clearcut logging (Gibson & Deacon, 1988; Taylor & Bruns, 1999). Variations in fire intensity and frequency influence the composition and development of EMF communities (Dahlberg *et al.*, 2001; Tuininga & Dighton, 2004). Certain EMF have been found to be more prevalent after fire, while other species are noticeably reduced (Visser, 1995; Grogan *et al.*, 2000). Even though the species that dominate the EMF community after fire or disturbance associate largely with young trees, the majority of these fungal species continue to be present in later stages of stand regeneration (Visser, 1995; Bradbury *et al.*, 1998; Jonsson *et al.*, 1999). While there are usually fewer EMF species present after fire or disturbance, Tuininga & Dighton (2004) found no noticeable decrease in percent root tip colonization by EMF in mature pine-oak forests subjected to prescribed burn. While the diversity of EMF species can

be positively correlated with such functions as total phosphorus uptake (Baxter & Dighton, 2001), the factors that control fungal diversity are poorly understood (Bruns, 1995).

Because EMF differ in their abilities to explore, access, and control soil resources, it is possible that EMF taxonomic diversity is related to EMF functional diversity; hence, efforts have been made to classify fungi according to their perceived ecological roles. The observation that certain species of fungi predominately colonized seedlings, while other species associated with mature trees, led to the classification of these fungi as either "early" or "late" stage. Fungal species that were found to be present throughout the tree lifecycle were termed "multi-stage" (Mason et al., 1982; Danielson, 1984). Later classifications such as protein, non-protein, and intermediate (Abuzinudah & Read, 1986) or 'r', 'K' selected (Smith & Read, 1997) attempted to link metabolic functions or reproductive strategies of EMF to their ecological role. These classification systems were based largely on observations of fungal fruiting bodies in the field (Mason et al. 1982, Danielson 1984), ectomycorrhizal root tips from soil cores (Visser, 1995), or the in vitro substrate preferences of a few EMF isolates (Abuzinudah & Read, 1986; Hutchison, 1990a,b). Because it is EMF mycelia and hyphae that explore, encounter, and access soil resources, to effectively link EMF taxonomic diversity with these ecological functions, further studies are needed to assess the activities of naturally occurring EMF mycelia and hyphae *in situ*.

In situ studies have shown that EMF species cluster in specific habitats (Dickie *et al.*, 2002; Koide & Wu, 2003; Rosling *et al.*, 2003; Tedersoo *et al.*, 2003). This clustering indicates that EMF diversity may be driven by niche differentiation (Bruns, 1995). The high diversity of EMF species seen in even-aged stands indicates that EMF may be characterized by a wide range of eco-physiologies. For example, the proteolytic abilities EMF species, as well as their production of other nutrient-mobilizing enzymes, such as phosphatases, are greatest in northern forests and decrease along a gradient moving south (Tibbett *et al.*, 1998a,b; Taylor *et al.*, 2000). Phosphatase production varies even within the same EMF species in a forest stand (Buée *et al.*, 2007); however, variation of production is still greater between forests than within species (Zhu *et al.*, 1988; Sen, 1990; El Karkouri *et al.*, 1996; Courty *et al.*, 2005).

Ectomycorrhizal fungal isolates from deciduous hosts also produce more phosphatase activity than isolates from coniferous hosts (Meysselle *et al.*, 1991).

Because soil chemical and mineralogical properties change with depth in the soil profile, soil pedogenesis offers a number of specialized niches for EMF species, especially in unmixed podzols (Lundstrom *et al.*, 2000; van Breemen *et al.*, 2000). Root tips are colonized by EMF at all depths in the soil profile (Malajczuk & Hingston, 1981; Rosling *et al.*, 2003) with significant differences in species composition between organic and mineral layers (Danielson & Visser, 1989; Goodman & Trofymow, 1998; Fransson *et al.*, 2000; Heinonsalo *et al.*, 2001; Rosling *et al.*, 2003; Gebhardt *et al.*, 2009). Although the density of ectomycorrhizal root tips is highest in organic horizons; up to two thirds of the total ectomycorrhizal roots can be located in mineral horizons (Rosling *et al.*, 2003). EMF species on root tips in soil mineral horizons can account for half of the total EMF taxa in a forest stand (Rosling *et al.*, 2003).

Despite evidence for niche differentiation of EMF, the ecological role of niche differentiation in forest ecosystems remains unclear. There is evidence that the soil horizon in which the mycorrhizae of a particular EMF species is located corresponds to some extent with the soil horizon in which its mycelia and hyphae actively forage (Landeweert *et al.*, 2003; Baier *et al.*, 2006; Genney *et al.*, 2006), but there is also evidence that the mycelia and hyphae forage widely. In microcosm studies, the location of mycelial activity was not correlated with location of ectomycorrhizal root tips; mycelia and hyphae foraged widely and proliferated in nutrient rich patches independent of root tip location (Bending & Read, 1996). Ectomycorrhizal mycelia have been observed to extend several meters from roots with 1 meter of root supporting 300 to 800 meters of mycelia (Jones *et al.*, 1990; Smith & Read, 1997). In order to evaluate the activity of EMF and the potential for, and role of, EMF niche differentiation in forest ecology, the identity, location, and activity of mycelia and hyphae must be determined.

1.6 Ectomycorrhizal fungi and carbon cycling

Ectomycorrhizal fungi are important to carbon cycling. Extra-matrical mycelia are long-lived in soil, with some individual mycelia conservatively estimated to live at least 300 years (Dahlberg *et al.*, 1997). The absorptive area and biomass of mycelia and hyphae is very large,

ranging from 3–600 m g soil^{-1} (reviewed by Leake *et al.*, 2004). One genet can spread vegetatively from root to root over an area as large as 50 m^2, with genet size varying widely by species, forest age, and time since disturbance (Guidot *et al.*, 2002). Ectomycorrhizal fungi account for at least 32% of microbial biomass in forest soils (Högberg & Högberg, 2002), and EMF mycelia and hyphae make up at least 80% of EMF biomass (Wallander *et al.*, 2001). In lab studies, up to 30% of net carbon fixation by ectomycorrhizal seedlings went to ectomycorrhizal mycelia (Finlay & Söderström, 1992; Durall *et al.*, 1994; Bidartondo *et al.*, 2001). Extra-matrical mycelia of different species grow at different rates in axenic culture (Cline *et al.*, 1987), and similar differences in growth rate may occur in the field (Colpaert *et al.*, 1992; Lamhamedi *et al.*, 1992; Thomson *et al.*, 1994a). Differences in growth rate may influence host carbon allocation to fungi and fungal CO$_2$ production (Ek, 1997). Roots, mycorrhizal fungi and other root-associated microbes that depend on recent photosynthate can contribute up to 53% to total soil respiration in boreal forests and up to 44% in temperate forests (Högberg *et al.*, 2009), and mycorrhizal root tips are the strongest sinks for photosynthate in forests (Högberg *et al.*, 2008). In addition, these mycelia are sensitive to disturbance and responses to changes in soil properties are seen in mycelia before the EMF community as a whole (Wallenda & Kottke, 1998; Brunner, 2001; Erland & Taylor, 2002; Kabir & Koide, 2002). Techniques have been developed to measure mycelial density *in situ* (Söderström, 1979; Finlay and Söderström, 1989; Jones *et al.*, 1990; Rousseau *et al.*, 1994), but to determine the functions of mycelia in soil, we must determine the species identities, distribution, and soil processes associated with EMF mycelia *in situ* as well (Horton & Bruns, 2001).

1.7   Interactions between ectomycorrhizal fungi and bacteria

There is an area of enhanced microbial activity in the soil around mycorrhizae analogous to the area of enhanced activity in rhizosphere soil around non-mycorrhizal roots (Linderman, 1988). This zone of enhanced microbial activity has been described by Linderman (1988) as the mycorrhizosphere. The ectomycorrhizosphere is further separated into the area influenced by ectomycorrhizal root tips, the area of hyphal extension by means of specialized bundles of hyphae (rhizomorphs) and other mature mycelial strands, and the area of active

exploration by the hyphal fan (Finlay and Read, 1986; Bending & Read, 1995). These three areas of the ectomycorrhizosphere are associated with distinct functions such as nutrient transport through rhizomorphs, and nutrient mobilization by hyphal fans (Landeweert *et al.*, 2001). Timonen *et al.* (1998) demonstrated a distinct profile of enzyme production in each of these areas of ectomycorrhizospheres when developed in non-sterile soil microcosms. Interestingly, these enzyme activities were either absent, or much reduced, in axenically grown fungi of the same species.

The "mycorrhizosphere effect" has been used to explain some of the effects of mycorrhization on plants, soil quality, and the soil microbial community (Linderman, 1988). Mycorrhizal fungi affect both plant and soil factors that, in turn, affect soil microbial communities. Mycorrhizal fungi increase root branching (reviewed by Linderman, 1988), affect root exudation and plant metabolic functions (Rambelli, 1973; Norton *et al.*, 1990; Berthelin, 1993; Rygiewicz & Anderson, 1994; Grayston & Campbell, 1996; Leyval & Marschner *et al.*, 1997; Wright *et al.*, 2000; Leake *et al.*, 2001), increase root branching (reviewed by Linderman, 1988), and improve soil aggregation (reviewed by Schreiner and Bethlenfalvay, 1995). Mycorrhizal fungi affect the structure of many soil communities including protozoa (Jentschke *et al.* 1995), microarthropods (Cromack *et al.*, 1988), microfungi (Neal *et al.*, 1964), and bacteria (Ames *et al.*, 1984; Christensen & Jakobsen, 1993; Grayston & Campbell, 1996; Olsson *et al.*, 1996; Olsson & Wallander, 1998; Timonen *et al.*, 1998; Heinonsalo *et al.*, 2000, 2001; Wamberg *et al.*, 2003). Because the interrelationship between mycorrhizal fungi and a diverse community of soil microbes is important to the plant-fungus symbiosis at physical, metabolic, and functional levels, the ectomycorrhizal symbiosis has been described as a multitrophic ectomycorrhizal complex (Frey-Klett & Garbaye, 2005; Frey-Klett *et al.*, 2007); emphasizing the importance of the interaction of the EMF with the soil community.

The three areas of the ectomycorrhizosphere support unique microbial communities with distinct functional profiles (Bomberg *et al.*, 2003; Frey-Klett *et al.*, 2005). Bacteria colonize all areas of the ectomycorrhizosphere as well as EMF fruiting bodies (Nurmiaho-Lassila *et al.*, 1997; Mogge *et al.*, 2000), and the structures of the associated bacterial communities vary by EMF species and with soil conditions (Timonen *et al.*, 1998; Mogge *et al.*, 2000; Poole *et al.*,

2001). Ectomycorrhizal fungi can either reduce (Olsson *et al.*, 1996; Olsson & Wallander, 1998) or increase (Olsson & Wallander, 1998) bacterial metabolic activity, and quantitatively alter the composition of the soil bacterial community (Olsson & Wallander, 1998; Probanza *et al.*, 2001). The genetic and functional profiles of the pseudomonad community associated with each area of the ectomycorrhizosphere can be "positively structured" to support increased host plant health and nutrition (Frey-Klett *et al.*, 2005). Pseudomonads isolated from the ectomycorrhizosphere of Douglas-fir seedlings ectomycorrhizal with *Laccaria bicolor* S238N, more efficiently solubilized inorganic phosphorus, chelated free iron, and suppressed growth of pathogenic fungi, than pseudomonads isolated from bulk soil. The possibility of positive feedback between mycorrhizal fungi and soil microbial communities highlights the importance of evaluating EMF function together with the associated soil microbial community and not in isolation.

Positive feed-back between EMF and saprophytic bacteria and fungi can positively affect host plant nutrition, growth, and health through a number of mechanisms. A wide variety of bacteria can promote mycorrhization, with potential benefits to host plant nutrient acquisition, especially acquisition of phosphorus (Frey-Klett *et al.*, 2007). Five mechanisms have been proposed for the promotion of mycorrhization by these bacteria (termed mycorrhizal helper bacteria, or MHB, by Garbaye, 1994): (1) effect on root receptivity to mycorrhizal fungi; (2) effect on root–fungus recognition and attachment; (3) effect on fungus survival and growth; (4) effect on physico-chemical properties of the soil; and (5) effect on germination of fungal propagules. Of these five mechanisms, it has been mechanism 3, the support and promotion vegetative growth of EMF in soil that has received the most attention. Schrey *et al.* (2005) have demonstrated the strain-specific stimulation of *Amanita muscaria* genes related to growth and metabolism by a strain of *Streptomyces*. Another way that bacterial and fungal soil saprotrophs can promote host plant nutrition is through enhanced nutrient mobilization to mycorrhizal fungi through their metabolic processes and extracellular enzyme production (Colpaert & van Laere, 1996; Colpaert & vanTichelen, 1996). This physiological complementarity allows mycorrhizal mycelia growing in close proximity to soil saprotrophs to absorb nutrients

mobilized by saprotrophic activity, those which would otherwise not be available to the mycorrhizal fungi (Colpaert & van Laere, 1996).

Interaction of soil saprotrophic microbes with EMF can have an additive or synergistic effect on the nutrition and growth of ectomycorrhizal host plants (Koide & Kabir, 2001), yet the interactions between EMF and soil saprotrophs are complex and variable. Although Garbaye (1994) has reviewed the documented positive effects of mycorrhizal helper bacteria in the ectomycorrhizosphere, negative effects have also been reported (Bowen & Theodorou, 1973; Bowen & Theodorou, 1979; Shaw *et al.*, 1995). The increased plant growth associated with certain bacterial strains can also occur regardless of the degree of host mycorrhization (Shishido *et al.*, 1996; Chanway, 1997; Probanza *et al.*, 2001). Negative interactions include competitive effects. Koide *et al.* (2001) demonstrated that forest-floor microbes could reduce the nutrients available to *Pinus resinosa* seedlings mycorrhizal with *P. tinctorius* when nutrients were limiting, indicating that *P. tinctorius* was unable to effectively compete against saprotrophic soil microbes for soil nutrients. Direct antagonistic interactions between EMF and saprotrophic fungi have also been documented (Shaw *et al.*, 1995); however, the antagonistic interaction can turn in favor of either the EMF or the saprotroph depending on the available nutrient resources (Lindahl *et al.*, 1999).

1.8   Thesis objectives

The studies presented in this thesis address the following overarching question: Do ectomycorrhizal fungi influence access by plants and bacteria to organic phosphorus as forests age, and thereby have the potential to alter forest productivity? These studies focus on the following three objectives:

i.   To visualize and characterize patterns of soil phosphatase activity at a fine scale *in situ* and determine how these patterns change as forests age following regeneration after fire.

ii.   To associate ectomycorrhizal fungi with these patterns of organic phosphorus mobilization (soil phosphatase activity) and determine whether this relationship changes as forests age.

iii.   To characterize the effect of ectomycorrhizal hyphae on the community structure and enzymatic activities of nearby soil bacteria.

1.9   Thesis overview

In Chapter 2, to address objective (i), I developed an enzyme imprinting technique that visualizes areas of phosphatase activity on soil profiles. I applied this method, together with computer image processing, to characterize the soil phosphatase of a chronosequence of forests regenerating from stand replacing wild-fire or clearcut logging. This chronosequence of forest stands was located in the Interior Cedar-Hemlock biogeoclimatic zone in B.C. and was dominated by Douglas-fir and paper birch trees. The chronosequence consisted of four stand age classes representing important stages of stand development. The stands were generated by stand-replacing fires or by clear-cut logging followed by broadcast burning (two of the youngest stands). The stages of stand development represented in the study were: stand initiation (5-6 years-old), canopy closure (24-30 years-old), stem exclusion (61-71 years-old), and post-stem exclusion (90-103 years-old).

In Chapter 3, to address objective (ii), I developed a soil micro-sampling method that utilized the enzyme imprinting method developed in Chapter 2. I tested the hypothesis that ectomycorrhizal fungi are associated with the mobilization of organic phosphorus (phosphatase activity) in forest soil by identifying molecular signatures of terminal restriction fragment length polymorphisms of EMF in small soil samples (microsamples) taken from areas of high and low phosphatase activity present on the soil profiles of the chronosequence stands. Because replicate forest stands of each age class were located long distances from each other and were largely interspersed across with landscape, I evaluated the efficacy of this microsampling method for detecting the landscape-scale effect of stand age on the general fungal as well as the EMF community present in the microsamples. I also evaluated community differences between the organic and mineral layers. I identified specific EMF species that were indicators of high or low phosphatase microsites and determined the frequency of ectomycorrhizal molecular signatures detected at these microsites.

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In Chapter 4, to address objective (iii), I trapped ectomycorrhizal hyphae on site using mesh sand bags and then isolated bacteria from bags containing hyphae and bags that excluded hyphae. Bacteria were isolated from replicate bags placed at one stand in the stem-exclusion stage of stand development where my phosphatase imprinting had indicated the highest levels of soil phosphatase activity. The isolation methods were selected for orders of bacteria thought to compete with EMF hyphae in soil. Isolated bacteria were identified to genus for characterization of differences in bacterial community structure between bag types. A clone library was developed using DNA from all sand bags to determine the prevalence of the isolated bacterial genera in the general bacterial community present in the bags. I also tested the effectiveness of this hyphal trapping method by incubating hyphae-ingrowth and hyphae-exclusion sandbags at six stands in the forest chronosequence.

1.10   Bibliography

Abuzinadah RA, Read DJ. 1986. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. III: Protein utilization by Betula, Picea and Pinus in mycorrhizal association with *Hebeloma crustuliniforme. New Phytologist* **103:** 507-514.

Acea MJ, Carballas T. 1996. Changes in physiological groups of microorganisms in soil following wildfire. *FEMS Microbiology Ecology* **20:** 33-39.

Agerer R. 2001. Exploration types of ectomycorrhizae. *Mycorrhiza* **11:** 107-114.

Agerer R. 2006. Fungal relationships and structural identity of their ectomycorrhizae. *Mycological Progress* **5:** 67-107.

Ahlgren IF, Ahlgren CE. 1965. Effects of prescribed burning on soil microorganisms in a Minnesota Jack pine forest. *Ecology* **46(3):** 302-310.

Allison SD, Martiny JBH. 2008. Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 11512-11519.

Ames RN, Reid CPP, Ingham ER. 1984. Rhizosphere bacterial population responses to root colonization by a vesicular arbuscular mycorrhizal fungus. *New Phytologist* **96:** 555-563.

Anderson IC, Cairney JWG. 2007. Ectomycorrhizal fungi: exploring the mycelial frontier. *FEMS Microbiology Reviews* **31:** 388-406.

Aristovaskaya TV, Zykina LV. 1977. Biological factors of aluminium migration and accumulation in soils and weathering crusts. In: *Problems in Soil Science.* Nauka, Moscow: .

Ashford AE, Allaway WG. 2002. The role of the motile tubular vacuole system in mycorrhizal fungi. *Plant and Soil* **244:** 177-187.

Attiwill PM, Adams MA. 1993. Tansley Review No. 50. Nutrient cycling in forests. *New Phytologist* **124:** 561-582.

Baier R, Ingenhaag J, Blaschke H, Göttlein A, Agerer R. 2006. Vertical distribution of an ectomycorrhizal community in upper soil horizons of a young Norway spruce (Picea abies [L.] Karst.) stand of the Bavarian Limestone Alps. *Mycorrhiza* **16:** 197-206.

Baxter JW, Dighton J. 2001. Ectomycorrhizal diversity alters growth and nutrient acquisition of grey birch (*Betula populifolia*) seedlings in host-symbiont culture conditions. *New Phytologist* **152:** 139-149.

Bending GD, Read DJ. 1995. The structure and function of the vegetative mycelium of ectomycorrhizal plants.V. Foraging behaviour and translocation of nutrients from exploited litter. *New Phytologist* **130:** 401-409.

Bending GD, Read DJ. 1996. Nitrogen mobilization from protein-polyphenol complex by ericoid and ectomycorrhizal fungi. *Soil Biology and Biochemistry* **28:** 1603-1612.

Berthelin J, Dommerques Y, Nriagu JO. 1976. The role of heterotrophic microorganisms in the deposition of iron and carbon in soil profiles. In: *Environmental Biochemistry.* Ann Arbor, MI: Ann Arbor Science Publishing, 609-631.

Bhat KKS, Nye PH. 1973. Diffusion of phosphate to plant roots in soil I. Quantitative autoradiography of the depletion zone. *Plant and Soil* **38:** 161-175.

Bidartondo MI, Ek H, Wallander H, Söderström B. 2001. Do nutrient additions alter carbon sink strength of ectomycorrhizal fungi? *New Phytologist* **151:** 543-550.

Bissett J, Parkinson D. 1980. Long-term effects of fire on the composition and activity of the soil microflora of a subalpine, coniferous forest. *Canadian Journal of Botany/Revue Canadienne de Botanique* **58:** 1704-1721.

Bomberg M, Jurgens G, Saano A, Sen R, Timonen S. 2003. Nested PCR detection of Archaea in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. *FEMS Microbiology Ecology* **43:** 163-171.

Bonfante P, Balestrini R, Martino E, Perotto S, Plassard C, Mousain D. 1998. Morphological analysis of early contacts between pine roots and two ectomycorrhizal *Suillus* strains. *Mycorrhiza* **8:** 1-10.

Boudot J-, Bel Hadj Brahim A, Steiman R, Seigle-Murandi F. 1989. Biodegradation of synthetic organo-metallic complexes of iron and aluminium with selected metal to carbon ratios. *Soil Biology & Biochemistry* **21:** 961-966.

Bougher NL, Malajczuk N. 1990. Effects of high soil moisture on formation of ectomycorrhizas and growth of karri (*Eucalyptus diversicolor*) seedlings inoculated with *Descolea maculata*, *Pisolithus tinctorius* and *Laccaria laccata. New Phytologist* **114:** 87-91.

Bowen GD, Theodorou C. 1979. Interactions between bacteria and ectomycorrhizal fungi. *Soil Biology and Biochemistry* **11:** 119-126.

Bowen GD, Theodorou C, Marks GC, Kozlowski TT. 1973. Growth of ectomycorrhizal fungi around seeds and roots. In: *Ectomycorrhizae, their ecology and physiology.* New York, USA: Academic Press, 107-150.

Bradbury SM, Danielson RM, Visser S. 1998. Ectomycorrhizas of regenerating stands of lodgepole pine (*Pinus contorta*). *Canadian Journal of Botany/Revue Canadienne de Botanique* **76:** 218-227.

Brunner I. 2001. Ectomycorrhizas: their role in forest ecosystems under the impact of acidifying pollutants. *Perspectives in Plant Ecology, Evolution and Systematics* **4:** 13-27.

Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil* **170:** 63-73.

Buée M, Courty PE, Mignot D, Garbaye J. 2007. Soil niche effect on species diversity and catabolic activities in an ectomycorrhizal fungal community. *Soil Biology and Biochemistry* **39:** 1947-1955.

Burns RG. 1978. *Soil Enzymes.* Great Britian: Academic Press.

Cairney JWG. 1999. Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. *Mycorrhiza* **9:** 125-135.

Calvaruso C, Turpault MP, Leclerc E, Frey-Klett P. 2007. Impact of ectomycorrhizosphere on the functional diversity of soil bacterial and fungal communities from a forest stand in relation to nutrient mobilization processes. *Microbial Ecology* **54:** 567-577.

Certini G. 2005. Effects of fire on properties of forest soils: a review. *Oecologia* **143:** 1-10.

Chanway CP. 1997. Inoculation of tree roots with plant growth promoting soil bacteria: An emerging technology for reforestation. *Forest Science* **43:** 99-112.

Christensen H, Jakobsen I. 1993. Reduction of bacterial-growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis-Sativus* L). *Biology and Fertility of Soils* **15:** 253-258.

Cline ML, France RC, Reid CPP. 1987. Intraspecific and interspecific growth variation of ectomycorrhizal fungi at different temperatures. *Canadian Journal of Botany/Revue Canadienne de Botanique* **65:** 869-875.

Colpaert JV, Van Assche JA, Luijtens K. 1992. The growth of the extramatrical mycelium of ectomycorrhizal fungi and the growth response of *Pinus sylvestris* L. *New Phyologist* **120:** 127-135.

Colpaert JV, vanTichelen KK. 1996. Decomposition, nitrogen and phosphorus mineralization from beech leaf litter colonized by ectomycorrhizal or litter-decomposing basidiomycetes. *New Phytologist* **134:** 123-132.

Cosgrove DJ. 1967. Metabolism of organic phosphate in soil. In: McLaren AD, Peterson GH, eds. *Soil biochemistry, Vol 1.* New York, USA: Marcel Dekker, 216-228.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167:** 309-319.

Cox PM, Betts RA, Jones CD, Spall SA, Totterdell IJ. 2000. Acceleration of global warming due to carbon-cycle feedbacks in a coupled climate model. *Nature* **408:** 184-187.

Crawford DV. 1963. Some processes of podzolization. In: *Proceeding of the Easter School in Agricultural Science.* London: 267-281.

Cromack K, Fichter BL, Moldenke AM, Entry JA, Ingham ER. 1988. Interactions between soil animals and ectomycorrhizal fungal mats. *Agriculture Ecosystems & Environment* **24:** 161-168.

Cumming JR, Weinstein LH. 1990. Utilization of $AlPO_4$ as a phosphorus source by ectomycorrhizal *Pinus-rigida* Mill. seedlings. *New Phytologist* **116:** 99-106.

D'Ascoli R, Rutigliano FA, De Pascale RA, Gentile A, De Santo AV. 2005. Functional diversity of the microbial community in Mediterranean maquis soils as affected by fires. *International Journal of Wildland Fire* **14:** 355-363.

Dahlberg A, Jonsson L, Nylund JE. 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. *Canadian Journal of Botany-Revue Canadienne De Botanique* **75:** 1323-1335.

Dahlberg A, Schimmel J, Taylor AFS, Johannesson H. 2001. Post-fire legacy of ectomycorrhizal fungal communities in the Swedish boreal forest in relation to fire severity and logging intensity. *Biology Conservation* **100:** 151-161.

Dakora FD, Phillips DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient enviroments. *Plant and Soil* **245:** 35-47.

Danielson RM. 1984. Ectomycorrhizal associations in jack pine stands in north-eastern Alberta. *Canadian Journal of Botany/Revue Canadienne de Botanique* **62:** 932-939.

Danielson RM, Visser S. 1989. Effects of forest soil acidification on ectomycorrhizal and vesicular-arbuscular mycorrhizal development. *New Phytologist* **112:** 41-47.

Deacon JW, Donaldson SJ, Last FT. 1983. Sequences and interactions of mycorrhizal fungi on birch. *Plant and Soil* **71:** 257-262.

DeBano LF. 2000. The role of fire and soil heating on water repellency in wildland environments: a review. *Journal of Hydrology* **231-232:** 195-206.

Dick WA, Tabatabai MA. 1983. Activation of soil pyrophosphate by metal ions. *Soil Biology and Biochemistry* **15:** 359-363.

Dickie IA, Xu B, Koide RT. 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist* **156:** 527-535.

Dinkelaker B, Marschner H. 1992. *In vivo* demonstration of acid phosphatase activity in the rhizosphere of soil-grown plants. *Plant and Soil* **144:** 199-205.

Dumontet S, Dinel H, Scopa A, Mazzatura A, Saracino A. 1996. Post-fire soil microbial biomass and nutrient content of a pine forest soil from a dunal mediterranean environment. *Soil Biology and Biochemistry* **28(10/11):** 1467-1475.

Durall DM, Jones MD, Tinker PB. 1994. ^{14}C allocation in ectomycorrhizal willow. *New Phytologist* **128:** 109-114.

Durall DM, Jones MD, Wright EF, Kroeger P, Coates KD. 1999. Species richness of ectomycorrhizal fungi in cutblocks of different sizes in the Interior Cedar-Hemlock forests of northwestern British Columbia: sporocarps and ectomycorrhizae. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **29:** 1322-1332.

Ek H. 1997. The influence of nitrogen fertilization on the carbon economy of *Paxillus involutus* in ectomycorrhizal association with *Betula pendula*. *New Phytologist* **135:** 133-142.

Ekblad A, Högberg P. 2001. Natural abundance of ^{13}C in CO_2 respired from forest soils reveals speed of link between tree photosynthesis and root respiration. *Oecologia* **127:** 305-308.

El Karkouri K, Cleyet Marel JC, Mousain D. 1996. Isozyme variation and somatic incompatibility in populations of the ectomycorrhizal fungus *Suillus collinitus. New Phytologist* **134:** 143-153.

Erland S, Taylor AFS, van der Heijden MGA, Sanders IR. 2002. Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. In: *Mycorrhizal ecology.* Berlin.: Springer-Verlag, 163-200.

Eivazi F, Tabatabai MA. 1977. Phosphatases in soils. *Soil Biology and Biochemistry* **9:**167-172.

Farr E, Vaidyanathan LV. 1972. The supply of nutrient ions by diffusion to plant roots in soil IV. Direct measurement of changes in labile phosphate content in soil near absorbing roots. *Plant and Soil* **37:** 609-616.

Finlay R, Söderström B. 1989. Mycorrhizal mycelia and their role in soil and plant communities. In: *Ecology of arable pand: Perspectives and challenges.* Dordrecht, Netherlands: Kluwer, 139-148.

Finlay RD, Read DJ. 1986. The structure and function of the vegetative mycelium of ectomycorrhizal plants. 2. The uptake and distribution of phosphorus by mycelial strands interconnecting host plants. *New Phytologist* **103:** 157-165.

Finlay RD, Söderström B, Allen MF. 1992. Mycorrhiza and carbon flow to the soil. In: *Mycorrhizal functioning.* London: Chapman and Hall, 134-160.

Ford ED, Mason PA, Pelham J. 1980. Spatial patterns of sporophore distribution around a young birch tree in three successive years. *Transactions of the British Mycological Society* **75:** 287-296.

Fransson PMA, Taylor AFS, Finlay RD. 2000. Effects of continuous optimal fertilisation upon a Norway spruce ectomycorrhizal community. *Tree Physiology* **20:** 599-606.

Frey P, Frey-Klett P, Garbaye J, Berge O, Heulin T. 1997. Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with the Douglas fir *Laccaria bicolo*r mycorrhizosphere. *Applied and Environmental Microbiology* **63:** 1852-1860.

Frey-Klett P, Garbaye J. 2005. Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal-bacterial interactions. *New Phytologist* **168:** 4-8.

Frey-Klett P, Garbaye J, Tarkka M. 2007. The mycorrhiza helper bacteria revisited. *New Phytologist* **176:** 22-36.

Fritze H, Pennanen T, Kitunen V. 1998. Characterization of dissolved organic carbon from burned humus and its effects on microbial activity and community structure. *Soil Biology and Biochemistry* **30:** 687-693.

Fritze H, Pennanen T, Pietikäinen J. 1993. Recovery of soil microbial biomass and activity from prescribed burning. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **23:** 1286-1290.

Garbaye J. 1994. Helper bacteria - a new dimension to the mycorrhizal symbiosis. *New Phytologist* **128:** 197-210.

Gebhardt S, Wöllecke J, Münzenberger B, Hüttl RF. 2009. Microscale spatial distribution patterns of red oak (*Quercus rubra* L.) ectomycorrhizae. *Mycological Progress* **8:** 245-257.

Genney DR, Anderson IC, Alexander IJ. 2006. Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytologist* **170:** 381-390.

Giardina CP, Binkley D, Ryan RG, Fownes JH, Senock RS. 2004. Belowground carbon cycling in a humid tropical forest decreases with fertilization. *Oecologia* **139:** 545-550.

Gibson F, Deacon JW. 1988. Experimental-Study of Establishment of Ectomycorrhizas in Different Regions of Birch Root Systems. *Transactions of the British Mycological Society* **91:** 239-251.

Goodman DM, Trofymow JA. 1998. Distribution of ectomycorrhizas in micro-habitats in mature and old-growth stands of Douglas-fir on southeastern Vancouver Island. *Soil Biology & Biochemistry* **30:** 2127-2138.

Grayston SJ, Campbell CD. 1996. Functional biodiversity of microbial communities in the rhizospheres of hybrid larch (*Larix eurolepis*) and Sitka spruce (*Picea sitchensis*). *Tree Physiology* **16:** 1031-1038.

Grogan P, Baar J, Bruns TD. 2000. Below-ground ectomycorrhizal community structure in a recently burned bishop pine forest. *Journal of Ecology* **88:** 1051-1062.

Guidot A, Debaud JC, Marmeisse R. 2002. Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. *FEMS Microbiology Ecology* **42:** 477-486.

Hackl E, Pfeffer M, Donat C, Bachmann G, Zechmeister-Boltenstern S. 2005. Composition of the microbial communities in the mineral soil under different types of natural forest. *Soil Biology and Biochemistry* **37:** 661-671.

Hamman ST, Burke IC, Stromberger ME. 2007. Relationships between microbial community structure and soil environmental conditions in a recently burned system. *Soil Biology and Biochemistry* **39:** 1703-1711.

Hart SC, DeLuca TH, Newman GS, MacKenzie MD, Boyle SI. 2005. Post-fire vegetative dynamics as drivers of microbial community structrure and function in forest soils. *Forest Ecology and Management* **220:** 166-184.

Heinonsalo J, Jørgensen KS, Haahtela K, Sen R. 2000. Effects of *Pinus sylvestris* root growth and mycorrhizosphere development on bacterial carbon source utilization and hydrocarbon oxidation in forest and petroleum-contaminated soils. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **46:** 451-464.

Heinonsalo J, Jørgensen KS, Sen R. 2001. Microcosm-based analyses of Scots pine seedling growth, ectomycorrhizal fungal community structure and bacterial carbon utilization profiles in boreal forest humus and underlying illuvial mineral horizons. *FEMS Microbiology Ecology* **36:** 73-84.

Hendricks JJ, Mitchell RJ, Kuehn KA, Pecot SD, Sims SE. 2006. Measuring external mycelia production of ectomycorrhizal fungi in the field: the soil matrix matters. *New Phytologist* **171:** 179-186.

Hibbett DS, Gilbert LB, Donoghue MJ. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* **407:** 506-508.

Horton TR, Bruns TD. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* **10:** 1855-1871.

Högberg MN, Högberg P. 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **154:** 791-795.

Högberg P, Bhupinderpal-Singh, Löfvenius MO, Nordgren A. 2009. Partitioning of soil respiration into its autotrophic and heterotrophic components by means of tree-girdling in old boreal spruce forest. *Forest Ecology and Management* **257:** 1764-1767.

Högberg P, Högberg MN, Göttlicher SG, Betson NR, Keel SG, Metcalfe DB, Campbell C, Schindlbacher A, Hurry V, Lundmark T, et al. 2008. High temporal resolution tracing of photosynthate carbon from the tree canopy to forest soil microorganisms. *New Phytologist* **177:** 220-228.

Hutchison LJ. 1990a. Studies on the systematics of ectomycorrhizal fungi in axenic culture. II. The enzymatic degradation of selected carbon and nitrogen compounds. *Canadian Journal of Botany/Revue Canadienne de Botanique* **68:** 1522-1530.

Hutchison LJ. 1990b. Studies on the systematics of ectomycorrhizal fungi in axenic culture. III. Patterns of polyphenol oxidase activity. *Mycologia* **82:** 424-435.

Ice GG, Neary DG, Adams PS. 2004. Effects of wildfire on soils and watershed processes. *Journal of Forestry* **102(6):** 16-20.

Jacobson KM, Miller OK. 1992. Physiological variation between tree-associated populations of *Suillus-granulatus* as determined by *invitro* mycorrhizal synthesis experiments. *Canadian Journal of Botany-Revue Canadienne De Botanique* **70:** 26-31.

Jentschke G, Bonkowski M, Godbold DL, Scheu S. 1995. Soil protozoa and forest tree growth - non-nutritional effects and interaction with mycorrhizae. *Biology and Fertility of Soils* **20:** 263-269.

Jones MD, Durall DM, Tinker PB. 1990. Phosphorus relationships and production of extramatrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. *New Phytologist* **115:** 259-267.

Jonsson L, Dahlberg A, Nilsson M-C, Kårén O, Zackrisson O. 1999. Continuity of ectomycorrhizal fungi in self-regenerating boreal *Pinus sylvestris* forests studied by comparing mycobiont diversity on seedlings and mature trees. *New Phytologist* **142:** 151-162.

Juma NG, Tabatabai MA. 1977. Effects of trace elements on phosphatase activity in soils. *Soil Science Society of America* **1(41):** 343-346.

Juma NG, Tabatabai MA. 1978. Distribution of phosphomonoesterases in soils. *Soil Science* **126:**101-108.

Juma NG, Tabatabai MA. 1988a. Hydrolysis of organic phosphates by corn and soybean roots. *Plant and Soil* **107:**31-38.

Juma NG, Tabatabai MA. 1988b. Phosphatase activity in corn and soybean roots: Conditions for assay and effects of metals. *Plant and Soil 107:*39-47.

Juma NG, Tabatabai MA. 1988c. Comparison of kinetic and thermodynamic parameters of phosphomonoesterases of soils and of corn and soybean roots. *Soil Biology and Biochemistry* **20:**533-539.

Kabir Z, Koide RT. 2002. Effect of autumn and winter mycorrhizal cover crops on soil properties, nutrient uptake and yield of sweet corn in Pennsylvania, USA. *Plant and Soil* **238:** 205-215.

Karst J, Marczak L, Jones MD, Turkington R. 2008. The mutualism-parasitism continuum in ectomycorrhizas: A quantitative assessment using meta-analysis. *Ecology* **89:** 1032-1042.

Kim KY, Jordan D, McDonald GA. 1998. Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. *Biology and Fertility of Soils* **26:** 79-87.

Kirschbaum MUF, Simioni G, Medlyn BE, McMurtrie RE. 2003. On the importance of including soil nutrient feedback effects for predicting ecosystem carbon exchange. *Functional Plant Biology* **30:** 223-237.

Kiss S, Dracan-Bularda M, Radulescu D. 1975. Biological significance of enzymes accumulated in soil. *Advances in Agronomy* **27:**25-87.

Koide RT. 2003. Ectomycorrhizas and retarded decomposition in a *Pinus resinosa* plantation. *New Phytologist* **158:** 401-407.

Koide RT, Kabir Z. 2001. Nutrient economy of red pine is affected by interactions between Pisolithus tinctorius and other forest-floor microbes. *New Phytologist* **150:** 179-188.

Kreutzweiser DP, Hazlett PW, Gunn JM. 2008. Logging impacts on the biogeochemistry of boreal forest soils and nutrient export to aquatic systems: A review. *Environmental Reviews* **16:** 157-179.

Kwari JD, Batey T. 1991. Effect of heating on phosphate sorption and availability in some north-east Nigerian soils. *Journal of Soil Science* **42:** 381-388.

Lamhamedi MS, Bernier PY, Fortin JA. 1992. Growth, nutrition and response to water-stress of *Pinus-pinaster* inoculated with 10 dikaryotic strains of *Pisolithus*-Sp. *Tree Physiology* **10:** 153-167.

Landeweert R, Hoffland E, Finlay RD, Kuyper TW, van Breemen N. 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology & Evolution* **16:** 248-254.

Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E. 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. *Applied and Environmental Microbiology* **69:** 327-333.

Last FT, Mason PA, Wilson JJ. 1983. Fine roots and sheathing mycorrhiza: their formation, function and dynamics. *Plant and Soil* **71:** 9-21.

Leake JR. 2001. Is diversity of ectomycorrhizal fungi important for ecosystem function? *New Phytologist* **152:** 1-3.

Leake JR, Donnelly DP, Boddy L. 2002. Interactions between ectomycorrhizal fungi and saprotrophic fungi in mycorrhizal ecology. *Ecological Studies* **157:** 346-372.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany-Revue Canadienne De Botanique* **82:** 1016-1045.

Leake JR, Read DJ, Wicklow DT, Söderström B. 1997. Mycorrhizal fungi in terrestrial habitats. In: *The Mycota IV. Environmental and microbial relationships.* Heidelberg: Springer-Verlag, 281-301.

Lewis DG, Quirk JP. 1967. Phosphate diffusion in soil and uptake by plants. III - P^{31} movement and uptake by plants as indicated by P^{32}-autoradiography. *Plant and Soil* **26:** 445-454.

Leyval C, Berthelin J. 1993. Rhizodeposition and net release of soluble organic-compounds by pine and beech seedlings inoculated with rhizobacteria and ectomycorrhizal fungi. *Biology and Fertility of Soils* **15:** 259-267.

Lindahl B, Stenlid J, Olsson S, Finlay R. 1999. Translocation of P-32 between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. *New Phytologist* **144:** 183-193.

Linderman RG. 1988. Mycorrhizal interactions with the rhizosphere microflora - the mycorrhizosphere effect. *Phytopathology* **78:** 366-371.

Litton CM, Giardina CP. 2008. Below-ground carbon flux and partitioning: global patterns and response to temperature. *Functional Ecology* **22:** 941-954.

Lundstrom US, van Breemen N, Bain D. 2000. The podzolization process. A review. *Geoderma* **94:** 91-107.

Mackey BG, Keith H, Berry SL, Lindenmayer DB. 2008. *Green Carbon – the role of natural forests in carbon storage Part 1: A green carbon account of Australia's south-eastern Eucalypt forests, and policy implications.* The Australian National University, Canberra: ANU E Press.

Malajczuk N, Hingston FJ. 1981. Ectomycorrhizae associated with jarrah. *Australian Journal of Botany* **29:** 453-462.

De Marco A, Gentile AE, Arena A, De Santo AV. 2005. Organic matter, nutrient content and biological activity in burned and unburned soils of a Mediterranean maquis area of southern Italy. *International Journal of Wildland Fire* **14:** 365-377.

Marx DH. 1981. Variability in ectomycorrhizal development and growth among isolates of *Pisolithus-tinctorius* as affected by source, age, and reisolation. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **11:** 168-174.

Mason PA, Last FT, Pelham J, Ingleby K. 1982. Ecology of some fungi associated with an ageing stand of birches (*Betula pendula* and *B. pubescens*). *Forest Ecology and Management* **4:** 19-39.

McGill WB, Cole CV. 1981. Comparative aspects of cycling of organic C, N, S, and P through soil organic matter. *Geoderma* **26:** 267-286.

Meysselle JP, Gay G, Debaud JC. 1991. Intraspecific genetic-variation of acid-phosphatase-activity in monokaryotic and dikaryotic populations of the ectomycorrhizal fungus *Hebeloma-cylindrosporum. Canadian Journal of Botany-Revue Canadienne De Botanique* **69:** 808-813.

Mogge B, Loferer C, Agerer R, Hutzler P, Hartmann A. 2000. Bacterial community structure and colonization patterns of *Fagus sylvatica* L-ectomycorrhizospheres as determined by fluorescence in situ hybridization and confocal laser scanning microscopy. *Mycorrhiza* **9:** 271-278.

Molina R, Massicotte H, Trappe JM, Allen MF. 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences. In: *Mycorrhizal functioning and integrative plant-fungal process.* New York: Chapman and Hall, 357-423.

Muthukumar T, Udayan K, Rajeshkannan V. 2001. Response of neem (*Azadirachta indica* A. Juss) to indigenous arbuscular mycorrhizal fungi, phosphate-solubilizing and asymbiotic nitrogen-fixing bacteria under tropical nursery conditions. *Biology and Fertility of Soils* **34:** 417-426.

Nannipieri P, Ascher J, Ceccherini T, Landi L, Pietramellara G, Renella G. 2003. Microbial diversity and soil functions. *European Journal of Soil Science* **54:** 655-670.

Neal Jr. JL, Bollen BW, Zak B. 1964. Rhizosphere microflora associated with mycorrhizae of Douglas fir. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **10:** 259-265.

Neary DG, Klopatek CC, DeBano LF, Ffolliott PF. 1999. Fire effects on belowground sustainability: a review and synthesis. *Forest Ecology and Management* **122:** 51-71.

Nilsson LO, Wallander H. 2003. Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New Phytologist* **158:** 409-416.

Norton JM, Smith JL, Firestone MK. 1990. Carbon flow in the rhizosphere of Ponderosa pine-seedlings. *Soil Biology and Biochemistry* **22:** 449-455.

Nurmiaho-Lassila EL, Timonen S, Haahtela K, Sen R. 1997. Bacterial colonization patterns of intact *Pinus sylvestris* mycorrhizospheres in dry pine forest soil: an electron microscopy study. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **43:** 1017-1035.

Olander LP, Vitousek PM. 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry* **49:** 175-190.

Olander LP, Vitousek PM. 2004. Biological and geochemical sinks for phosphorus in soil from a wet tropical forest. *Ecosystems* **7:** 404-419.

Olsson PA, Chalet M, Baath E, Finlay RD, Soderstrom B. 1996. Ectomycorrhizal mycelia reduce bacterial activity in a sandy soil. *FEMS Microbiology Ecology* **21:** 77-86.

Olsson PA, Wallander H. 1998. Interactions between ectomycorrhizal fungi and the bacterial community in soils amended with various primary minerals. *FEMS Microbiology Ecology* **27:** 195-205.

Parfitt RL. 1978. Anion adsoption by soils and soil minerals. *Advances in Agronomy* **30:** 1-50.

Paul EA, Clark FE. 1989. *Soil microbiology and biochemistry.* Toronto: Academica Press.

Perez-Moreno J, Read DJ. 2001. Nutrient transfer from soil nematodes to plants: a direct pathway provided by the mycorrhizal mycelial network. *Plant Cell and Environment* **24:** 1219-1226.

Cade-Menun, B. 1995. Phosphorus forms in podzols on northern Vancouver Island. Ph.D. Thesis, Dept. of Soil Science, University of British Columbia, Vancouver, B.C. 211pp.

Pietikainen J, Fritze H. 1992. Microbial biomass and activity in the humus layer following burning: short-term effects of two different fires. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **23:** 1275-1285.

Pietikäinen J, Fritze H. 1995. Clear-cutting and prescribed burning in coniferous forest: comparison of effects on soil fungal and total microbial biomass, respiration activity and nitrification. *Soil Biology and Biochemistry* **27(1):** 101-109.

Polglase PJ, Attiwill PM, Adams MA. 1992. Nitrogen and phosphorus cycling in relation to stand age of *Eucalyptus regnans* F. Muell III. Labile inorganic and organic P, phosphatase activity and P availability. *Plant and Soil* **142:** 177-185.

Poole EJ, Bending GD, Whipps JM, Read DJ. 2001. Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. *New Phytologist* **151:** 743-751.

Prieto-Fernández A, Acea MJ, Carballas T. 1998. Soil microbial and extractable C and N after wildfire. *Biology and Fertility of Soils* **27:** 132-142.

Probanza A, Mateos JL, Garcia JAL, Ramos B, de Felipe MR, Manero FJG. 2001. Effects of inoculation with PGPR Bacillus and *Pisolithus tinctorius* on *Pinus pinea* L. growth, bacterial rhizosphere colonization, and mycorrhizal infection. *Microbial Ecology* **41:** 140-148.

Raison RJ, Khanna PK, Woods PV. 1985. Transfer of elements to the atmosphere during low intensity prescribed fires in three Australian subalpine eucalypt forests. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **15:** 657-664.

Rambelli A, Marks GC, Kozlowski TT. 1973. The rhizosphere of mycorrhizae. In: *Ectomycorrhizae: Their ecology and physiology.* New York, New York: Academic Press, Inc, 290-349.

Ramirez-Martinez JR. 1968. Organic phosphorus mineralization and phosphatase activity in soils. *Folia Microbiologica (Praha)* **13:**161-174.

Rasanayagam S, Jeffries P. 1992. Production of acid is responsible for antibiosis by some ectomycorrhizal fungi. *Mycological Research* **96:** 971-976.

Read DJ. 1991. Mycorrhizas in ecosystems. *Experientia* **47:** 376-391.

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytologist* **157:** 475-492.

Robichaud PR. 2000. Fire effects on infiltration rates after prescribed fire in Northern Rocky Mountain forests, USA. *Journal of Hydrology* **231-232:** 220-229.

Romanyà J, Khanna PK, Raison RJ. 1994. Effects of slash burning on soil-phosphorus fractions and sorption and desorption of phosphorus. *Forest Ecology and Management* **65:** 89-103.

Rosado SCS, Kropp BR, Piché Y. 1994. Genetics of ectomycorrhizal symbiosis. II. Fungal variability and heritability of ectomycorrhizal traits. *New Phyologist* **126:** 111-117.

Rosling A, Landeweert R, Lindahl BD, Larsson KH, Kuyper TW, Taylor AFS, Finlay RD. 2003. Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytologist* **159:** 775-783.

Rousseau JVD, Sylvia DM, Fox AJ. 1994. Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. *New Phytologist* **128:** 639-644.

Roxburgh SH, Wood SW, Mackey BG, Woldendorp G. 2006. Assessing the carbon sequestration potential of managed forests: a case study from temperate Australia. *Journal of Applied Ecology* **43:** 1149-1159.

Rygiewicz PT, Anderson CP. 1994. Mycorrhizae alter quality and quantity of carbon allocated below ground. *Nature* **369:** 58-60.

Saa A, Trasar-Cepeda MC, Gil-Sotres F, Carballas T. 1993. Changes in Soil Phosphorus and acid phosphatase activity immediately following forest fires. *Soil Biology and Biochemistry* **25(9):** 1223-1230.

Schimel JP, Bennett J. 2004. Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **85:** 591-602.

Schmidt G, Laskowski, M Sr. 1961. Phosphate ester cleavage (Survey). In: Boyer PD et al., eds. *The enzymes*, *2nd ed.*, New York: Academic Press 3-35.

Schlesinger WH. 1997. *Biochemistry: an analysis of global change.* San Diego, California, U.S.A.: Academic Press.

Schreiner RP, Bethlenfalvay GJ. 1995. Mycorrhizal interactions in sustainable agriculture. *Critical Reviews in Biotechnology* **15:** 271-285.

Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT. 2005. Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria. New Phytologist* **168:** 205-216.

Selosse MA, Richard F, He X, Simard SW. 2006. Mycorrhizal networks: des liaisons dangereuses? *Trends in Ecology & Evolution* **21:** 621-628.

Sen R. 1990. Intraspecific variation in two species of *Suillus* from Scots pine (*Pinus sylvestris* L.) forests based on somatic incompatibility and isozyme analyses. *New Phytologist* **114:** 607-616.

Shaw TM, Dighton J, Sanders FE. 1995. Interactions between ectomycorrhizal and saprotrophic fungi on agar and in association with seedlings of Lodgepole pine (*Pinus-contorta*). *Mycological Research* **99:** 159-165.

Shishido M, Massicotte HB, Chanway CP. 1996. Effect of plant growth promoting Bacillus strains on pine and spruce seedling growth and mycorrhizal infection. *Annals of Botany* **77:** 433-441.

Smith NR, Kishchuk BE, Mohn WW. 2008. Effects of wildfire and harvest disturbances on forest soil bacterial communities. *Applied and Environmental Microbiology* **74:** 216-224.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis.* London: Academic Press.

Söderström BE. 1979. Seasonal fluctuations of active fungal biomass in horizons of a podzolized pine-forest soil in central Sweden. *Soil Biology and Biochemistry* **11:** 149-154.

Speir TW, Ross DJ. 1978. Soil phosphatase and sulphatase. In: Burns RG, ed. *Soil enzymes*. New York: Academic Press, 197-250.

Spiers GA, McGill WB. 1979. Effects of phosphorus addition and energy supply on acid phosphatase production and activity in soils. *Soil Biology and Biochemistry* **11(1):** 3-8.

Staddon PL, Ramsey CB, Ostle N, Ineson P, Fitter AH. 2003. Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of C-14. *Science* **300:** 1138-1140.

Staddon WJ, Duchesne LC, Trevors JT. 1998. Impact of clear-cutting and prescribed burning on microbial diversity and community structure in a Jack pine (*Pinus banksiana* Lamb.) clear-cut using Biolog Gram-negative microplates. *World Journal of Microbiology & Biotechnology* **14:** 119-123.

Stevenson FJ, McLaren AD, Peterson GH. 1967. Organic acids in soils. In: *Soil biochemistry.* New York, NY: Marcel Dekker, 119-146.

Ström L, Owen AG, Godbold DL, Jones DL. 2005. Organic acid behaviour in a calcareous soil implications for rhizosphere nutrient cycling. *Soil Biology and Biochemistry* **37:** 2046-2054.

Tabatabai MA. 1994. Soil enzymes. In: *Methods of Soil Analysis, Part 2.* Madison, WI, USA: Soil Science Society of America, 775-833.

Taylor AFS, Martin F, Read DJ. 2000. Fungal diversity in ectomycorrhizal communities of Norway spruce (*Picea abies* (L.) Karst.) and beech (*Fagus sylvatica* L.) along north–south transects in Europe. In Carbon and nitrogen cycling in European forest ecosystems. *Ecological Studies* **142:** 343-365.

Taylor DL, Bruns TD. 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. *Molecular Ecology* **8:** 1837-1850.

Tedersoo L, Koljalg U, Hallenberg N, Larsson KH. 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. *New Phytologist* **159:** 153-165.

Thomson BD, Grove TS, Malajczuk N, Hardy GESJ. 1994. The effectiveness of ectomycorrhizal fungi in increasing the growth of *Eucalyptus globulus* Labill. in relation to root colonization and hyphal development in soil. *New Phytologist* **126:** 517-524.

Tibbett M, Sanders FE, Cairney JWG. 1998a. The effect of temperature and inorganic phosphorus supply on growth and acid phosphatase production in arctic and temperate strains of ectomycorrhizal *Hebeloma* spp. in axenic culture. *Mycological Research* **102:** 129-135.

Tibbett M, Sanders FE, Minto SJ, Dowell M, Cairney JWG. 1998b. Utilization of organic nitrogen by ectomycorrhizal fungi (*Hebeloma spp*.) of arctic and temperate origin. *Mycological Research* **102:** 1525-1532.

Timonen S, Jorgensen KS, Haahtela K, Sen R. 1998. Bacterial community structure at defined locations of *Pinus sylvestris Suillus bovinus* and *Pinus sylvestris Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **44:** 499-513.

Toro M, Azcon R, Barea JM. 1997. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (P-32) and nutrient cycling. *Applied and Environmental Microbiology* **63:** 4408-4412.

Trappe JM, Fogel RD, Marshall JK. 1977. Ecosystematic functions of mycorrhizae. In: *The belowground ecosystem: A synthesis of plant-associated processes.* Fort Collins, Colorado: Colorado State University, 205-226.

Tuininga AR, Dighton J. 2004. Changes in ectomycorrhizal communities and nutrient availability following prescribed burns in two upland pine-oak forests in the New Jersey pine barrens. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **34:** 1755-1765.

Ulery AL, Graham RC, Amrhein C. 1993. Wood-ash composition and soil pH following intense burning. *Soil Science* **156(5):** 358-364.

Uroz S, Calvaruso C, Turpault MP, Pierrat JC, Mustin C, Frey-Klett P. 2007. Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. *Applied and Environmental Microbiology* **73:** 3019-3027.

van Breemen N, Lundstrom US, Jongmans AG. 2000. Do plants drive podzolization via rock-eating mycorrhizal fungi? *Geoderma* **94:** 163-171.

van der Heijden MG, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* **11:** 296-310.

van Wagner CE, Wein RW, MacLean DA. 1983. Fire behavior in northern conifer forests and shrublands. In: *The Role of fire in northern circumpolar ecosystems.* New York: John Wiley & Sons, 65-80.

Vázquez FJ, Acea MJ, Carballas T. 1993. Soil microbial populations after wildfire. *FEMS Microbiology Ecology* **13:** 93-104.

Visser S. 1995. Ectomycorrhizal fungal succession in jack pine stands following wildfire. *New Phytologist* **129:** 289-401.

Wallander H, Fossum A, Rosengren U, Jones H. 2005. Ectomycorrhizal fungal biomass in roots and uptake of P from apatite by *Pinus sylvestris* seedlings growing in forest soil with and without wood ash amendment. *Mycorrhiza* **15:** 143-148.

Wallander H, Nilsson LO, Hagerberg D, Bååth E. 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytologist* **151:** 753-760.

Wallenda T, Kottke I. 1998. Nitrogen deposition and ectomycorrhizas. *New Phytologist* **139:** 169-187.

Wamberg C, Christensen S, Jakobsen I, Muller AK, Sorensen SJ. 2003. The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). *Soil Biology and Biochemistry* **35:** 1349-1357.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH. 2004. Ecological linkages between aboveground and belowground biota. *Science* **304:** 1629-1633.

Whitelaw MA. 2000. Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Advances in Agronomy* **69:** 99-151.

Wood T, Bormann FH, Voigt GK. 1984. Phosphorus cycling in a northern hardwood forest: biological and chemical control. *Science* **223:** 391-393.

Wright DP, Scholes JD, Read DJ, Rolfe SA. 2000. Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. *Plant Cell and Environment* **23:** 39-49.

Wright E, Bollen WB. 1961. Microflora of Douglas-fir forest soil. *Ecology* **42(4):** 825-828.

Yeager CM, Northup DE, Grow CC, Barns SM, Kuske CR. 2005. Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. *Applied and Environmental Microbiology* **71:** 2713-2722.

Zak B, Marks GC, Kozlowski TT. 1973. Classification of ectomycorrhizae. In: *Ectomycorrhizae, their ecology and physiology.* New York: Academic Press, 43-78.

Zhu H, Higginbotham KO, Dancik BP, Navratil S. 1988. Intraspecific genetic-variability of isozymes in the ectomycorrhizal fungus *Suillus-tomentosus. Canadian Journal of Botany-Revue Canadienne De Botanique* **66:** 588-594.

2   A method for linking in situ activities of hydrolytic enzymes to associated organisms in forest soils[1]

2.1   Introduction

Enzymes catalyze soil nutrient transformations. Measurement of soil enzyme activities has, therefore, been recommended as an extremely pertinent method to measure changes in soil quality (Dick, 1992; Visser & Parkinson, 1992), soil recovery from disturbance or stress (Eivazi & Bayan, 1996; Decker *et al.*, 1999) and as the most appropriate indicator of microbial function (Caldwell, 2005). Soil enzymology has been the subject of several excellent reviews (Burns, 1978; Burns & Dick, 2002).

In temperate and boreal forests, many tree species depend upon hydrolytic enzymes released by ectomycorrhizal fungi (EMF) to provide access to organic forms of nitrogen and phosphorus (Abuzinadah *et al.*, 1986; Bending & Read, 1995a,b). In addition, these mycorrhizae support diverse bacterial communities (Mogge *et al.*, 2000), which also have the potential to release extracellular enzymes. There are typically 50 to 100 genotypes of EMF in a forest stand (Byrd *et al.*, 2000; Horton & Bruns, 2001; Twieg *et al.*, 2007). In order to understand the effect of site disturbance on nutrient cycling in forests it is important to know the identity of the functionally active fungi. Recent measurements of enzyme activities on ectomycorrhizal root tips have shown that different fungi appear to be associated with different enzyme profiles (Pritsch *et al.*, 2004; Buée *et al.*, 2007; Courty *et al.*, 2005, 2006, 2007; Rineau& Garbaye, 2009). However, such approaches tell us little about the enzyme activities associated with extramatrical hyphae of these mycorrhizae and their associated hyphospheres. Extramatrical hyphae appear to play a much more important role than mantle hyphae in nutrient remobilization from soil (Perez-Moreno & Read, 2000).

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Phosphorus is second only to N as a limiting nutrient for tree growth in forests (Marschner, 1995). However, unlike nitrogen, which is added to ecosystems through biological fixation and rain deposition, phosphorus must either be mobilized from the soil mineral matrix, or enzymatically recycled from soil organic matter (Burns, 1978; McGill & Cole, 1981; Paul & Clark, 1989; Attiwill & Adams, 1993). Due to the closed nature of the P-cycle and the relative immobility of easily absorbable inorganic phosphorus in mineral soil (Lewis & Quirk, 1967; Farr & Vaidyanathan, 1972; Bhat & Nye, 1973), phosphorus availability in natural soils is low (McGill & Cole, 1981). Biological demand controls the partitioning of soil phosphorus, determining how much is immobilized and how much is adsorbed by soil minerals, even when underlying mineral horizons have high sorptive capacity (Olander & Vitousek, 2005). Losses of inorganic phosphorus from undisturbed forest can be less than $1\ \mu g\ L^{1}\ yr^{-1}$ (Wood *et al.*, 1984); however, losses caused by fire can be significant. More than 50% of the total phosphorus contained in combusted fuel can be lost to the atmosphere during fire (Raison *et al.*, 1985). After fire, more phosphorus can be lost when ash is eroded by wind and rain. When phosphorus is lost from a system, it must be replaced through mobilization of inorganic phosphorus from the mineral substrate or through mineralization of organic phosphorus from soil organic matter.

An important avenue by which tree roots, fungi, and bacteria mineralize phosphorus is through the release of enzymes such as acid phosphatases (Dighton, 1983; Firsching & Claassen, 1996; Aon *et al.*, 2001), which cleave ester-bonded phosphorus from soil organic matter (Speir & Ross, 1978). This biochemical mineralization of phosphorus through the production of phosphatase is strongly controlled by the supply of, and need for, phosphorus by both plants and soil microbes (Spiers & McGill, 1979; McGill & Cole, 1981). Plants and soil microbes maintain low levels of available phosphorus in natural forest soils through preferential uptake of inorganic phosphorus and increased production of phosphatase to access organic forms of phosphorus when inorganic phosphorus becomes limiting (Olander & Vitousek, 2000). Consequently, measurement of available phosphorus may reflect neither the extent nor the rate of actual phosphorus cycling in the ecosystem. Measurement of phosphatase activity, however, may better reflect phosphorus cycling in the forest ecosystem because phosphatase activity correlates with the biological activity driving phosphorus partitioning and uptake.

There are many well-utilized enzyme assays based on colorimetric and fluorimetric substrates that employ rapid microplate techniques, as reviewed by Caldwell (2005). However, these assays all involve removal of soil from the field followed by analysis in the laboratory. Although antiseptic and plasmolytic agents have been used to control soil chemical, physical and biological changes after sampling, changes in enzyme activities are inevitable during the process of handling soil (Tabatabai, 1994). Thus these methods may be considered to report on potential activities only. Although it is now possible to probe for DNA and RNA of specific enzymes in soils (Kelly, 2003; Wellington *et al.*, 2003; Luis *et al.*, 2005), currently there are no genetic probes for acid phosphatase. Furthermore, these molecular approaches are considered to reveal only the potential for these enzymes to be active in soils, not their actual activity.

In their recent review of nitrogen cycling, Schimel & Bennett (2004) argue that increased understanding of soil processes will come only if we study them at much a finer scale than is possible with destructive soil sampling. Methods allowing actual soil and rhizosphere visualization using root boxes, such as those developed by Dinkelaker & Marschner (1992) and Song *et al*. (2000), can begin to address questions at these scales. Our method builds on one of the few field-based approaches to study soil enzyme activities. Grierson & Comerford (2000) used root windows and nitrocellulose membranes to adsorb phosphatase from a soil profile with high spatial resolution. In their approach, enzyme-imprinted membranes were transported from the field to the laboratory where visible images of enzyme activity were developed under standard conditions. This relocation may induce changes in enzyme activity, and the enzyme activity that develops under laboratory conditions may not reflect the actual enzyme activity that would be present under field conditions.

The first objective of this study was to develop a novel method to estimate activities of acid phosphatase *in situ* in forest soil. The method employed a root window inserted into the soil profile. Chromatography paper, treated with a mixture of substrate and colorimetric reagent, was placed directly on the soil surface though the window. Phosphatase activity at the soil surface was indicated by the appearance of coloured products on the imprinting paper. This method enables enzyme activities associated with roots, mycorrhizae, hyphae, and/or bacteria to be visualized directly at fine scales. Our second objective was to test the sensitivity of the

technique to detect the effects of clearcutting and wildfire on soil phosphatase activities in different forest types located in three biogeoclimatic zones of British Columbia; the Interior Cedar-Hemlock, the Interior Douglas-fir and the Engelmann Spruce-Subalpine-fir. Concurrent analyses of soil nutrients at the Interior Cedar-Hemlock sites enabled us to ascertain links between nutrient availability and phosphatase production.

2.2   Methods

2.2.1   Method development

The phosphatase assay was developed and tested in two systems: rhizoboxes in a greenhouse and root windows in the field. The rhizoboxes consisted of two 20 x 20 x 0.3 cm acrylic plates separated by foam weather stripping (1.0 cm wide and 0.5cm thick). The weather stripping was attached to the face of one acrylic plate and aligned along the edges of the plate. The other acrylic plate was placed on top and attached to the first plate along the three sides with weather stripping using duct tape. Soil from under a mature stand of Douglas-fir (*Pseudotsuga menziesii*) at Barriere B.C. was collected, sieved (<1 mm) and used to uniformly fill 20 rhizoboxes. Douglas-fir seeds were germinated on moist filter paper and two-day-old seedlings were planted in each rhizobox. Rhizoboxes were wrapped in thick landscape plastic with the white side exposed and placed vertically in a greenhouse at the University of British Columbia Okanagan in Kelowna, B.C., Canada. After 6 months, rhizoboxes with well-established seedlings were used for initial tests of the imprinting method.

2.2.2   Study sites and root window installation

Two studies quantified imprintable soil phosphatase activity in mature and regenerating forests. One study was located in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) stands in the dry cool Interior Douglas-fir (IDF) biogeoclimatic zone (Lloyd *et al.*, 1990), ranging in elevation from 840 to 1270 m, near Barriere, British Columbia (51°N, 120°W). Three root windows were installed at each of eight sites in May 2004: four mature forest sites (over 100 years-old) and four sites that had been clearcut logged in the summer of 2003. These windows were imprinted in Fall 2004. The other study was located within the wet cold Engelmann Spruce - Subalpine Fir (ESSF) biogeoclimatic zone (Lloyd *et al.*, 1990) at the

Sicamous Creek Silviculture Systems Trial near Sicamous, British Columbia (51° N, 119° W), with plots ranging from 1500 to 1850 m in elevation. These stands consisted primarily of subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) (see Hagerman *et al.*, 1999, for complete description). In 2002, windows were installed at 18 locations; one root window was installed in the centre of each of nine clearcuts (three 0.1 ha, three 1.0 ha, and three 10 ha in size; logged in 1994) and in nine adjacent mature forests (over 175 years-old). The clearcuts had been replanted with Engelmann spruce in 1995 and the windows in the clearcuts were installed within 2 m of the spruce saplings. Enzyme imprints were taken in Fall 2004.

A third study investigated changes in the patterns of imprintable phosphatase in a chronosequence of forests regenerating from stand-replacing wild-fires. This study was located in three variants of the Interior Cedar-Hemlock (ICH) biogeoclimatic zone; the Thompson moist warm, Shuswap moist warm, and Thompson moist cool (Lloyd *et al.*, 1990) in the southern interior of British Columbia, Canada (between 50° and 51° N and 118° and 120° W, and from 550 to 1100 m in elevation. In these biogeoclimatic variants, forests regenerate following stand-replacing fire or clearcut logging to a seral canopy of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), paper birch (*Betula papyrifera* Marsh), and lodgepole pine (*Pinus contorta* var. *latifolia* Doug. Ex Loud.) during the first 100 years. Western redcedar (*Thuja plicata* (Donn. Ex E. Don) Spach) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) gain dominance later in succession. The study comprised three replicate sites of four age classes of post-fire disturbance forest plots: young (5-6 year-old), canopy closure (24-30 year-old), stem exclusion (61-71 year-old), and older (90-103 year-old) (see Table 2.1 for site characteristics). Two of the young sites were generated by clearcut logging followed by broadcast burning, rather than wildfire. The windows for these studies were installed in 2003 and 2004, and imprints were made in Fall 2005.

The root windows were modeled on those of Grierson & Comerford (2000) and consisted of a transparent acrylic panel (77.4 cm wide x 51.6 cm tall x 0.6 cm thick) with a 30 x 30 cm trap door. Each window was supported by two vertical steel rods. Where required, two 5x10x40 cm pieces of lumber were placed horizontally on the outside of the windows to ensure

good contact between windows and the soil face. After each window was installed, a 1-2 cm thickness of soil was backfilled against the inside of the window using soils retained during the digging of the hole. Organic and mineral soils were replaced to match the original depths of the appropriate horizon (Figure 2.1a). All windows were covered with a piece of black plastic, and the pit in front of the window was either backfilled with soil or covered with insulated plywood and canvas to block light from reaching the window. Soil imprinting was carried out no sooner than 5 months after installation.

2.2.3   Acid phosphatase assay

The method of acid phosphatase detection is based on the hydrolysis of α-naphthyl phosphate by phosphatase (EC 3.1.3.2) to release naphthol, which reacts with diazonium salt Fast Red TR to form a stable red precipitate (Dinkelaker & Marschner, 1992). Briefly, freshly prepared substrate (50 mM α-naphthyl phosphate, Sigma N7255 dissolved in 50 mM pH 5.6 citrate buffer) and colour reagent (10 mM Fast Red TR salt, Sigma F2768, prepared in 50 mM pH 5.6 citrate buffer) solutions were mixed at a ratio of 1:10 (v/v) to form the treatment solution. The pH of the buffer was selected based on pH optima determined by Dinkelaker & Marschner (1992). Filter paper (Whatman, Cat. No. 1001 055 for rhizobox tests) or chromatography paper (Whatman, 20 x 20 cm, Cat No. 3030-861 for root windows in the field) was soaked in the treatment solution for approximately 1 min in an acid-washed, glass baking dish, air-dried on aluminum foil at room temperature, and stored in a sealed plastic bag at 4°C. Latex gloves were used at all times when handling the filter or chromatography paper. In preliminary experiments, it was determined that prepared imprinting paper could be stored at 4°C for up to one week without a reduction in colour development.

Rhizoboxes were used for laboratory testing of the imprint technique. The front plate of the rhizobox was carefully removed to maintain a smooth soil surface and then tightly reattached after an imprinting paper had been placed on the soil surface. Activity of acid phosphatase on the soil surface was localized by the appearance of purple/red spots on the paper and quantified by comparing the colour intensity with a prepared standard curve.

We determined the optimum contact time for maximum activity and the interval required between imprintings to minimize substrate carry-over on the soil surface. Contact

times from 10 to 90 min and intervals from 10 to 60 min between imprintings were tested. Pieces of filter paper treated with only colour reagent (Fast Red TR) were placed alongside the imprinting paper as negative controls. After imprinting, control and imprinting papers were removed, rinsed with deionized water, and air dried. Imprinted phosphatase activity was quantified by comparison with dilution series of acid phosphatase standards (Sigma P3627, from wheat germ) in pH 5.6 citrate buffer (ranging from 0 to 0.35 enzyme units [EU] ml^{-1}) applied to imprinting paper (5 µl each for each standard). The standards were incubated at the same temperature and at the same time that enzyme imprints were made.

2.2.4   Estimates of soil phosphatase activity in forests and clearcuts

As mentioned above, the intensity of colour change of an imprint can be correlated with a standard curve as a semi-quantitative measure of phosphatase activity. Using this approach, studies were conducted in the IDF and ESSF sites that compared the *in situ* soil phosphatase activities of soil profiles in mature forests and in stands regenerating from clearcut logging. An imprinting paper was placed on the soil surface through the trap door of a root window (Figure 2.1b). Standards were applied in 5 µL aliquots to another piece of imprinting paper, which was then placed in a plastic bag and placed on the soil profile beside the other paper. After 1 h, the imprint and standard papers were removed, rinsed with deionized water, wrapped in aluminum foil, and stored on ice in a cooler. After transport to the lab and air drying overnight, both the imprint and standard for the phosphatase assays were scanned (ScanMaker 8700, Microtek, Carson, CA, USA) at 300 dpi.

To estimate phosphatase activity, the entire image was selected for analysis using Image J (freeware, NIH, USA). The area occupied by each standard was selected from the scanned image of the standard paper and the average gray value calculated. A standard curve was generated based on the total EU added per standard spot and the area in pixels occupied by the spot (EU pixel^{-1} vs. gray value).

2.2.5   Fine scale distribution of soil enzyme activity at forest sites along a chronosequence

The intensity and extent of *in situ* soil enzyme activity was characterized across a Douglas-fir/paper birch chronosequence (see Table 2.1 for site characteristics). All imprints

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were taken within a 10-day span and within three days of a rainfall event. Soil profiles contained sufficient soil moisture to immediately wet the imprinting papers when they were applied to the soil profile. Imprinting paper was applied to soil profiles through the trap door of one window at each site and processed as described above for the phosphatase assay. Imprints were then scanned (EPSON Perfection 1660 Photo), imported into Adobe Photoshop Elements (2.0), and saved in EPS format. A PERL script was written to extract spatial and activity information from the EPS files and record the size, maximum intensity, and average intensity of each active area in each scanned image. Total average phosphatase activity was calculated as the average activity for each active area multiplied by the size of the area in pixels.

2.2.6   Nutrient analysis

Soil samples for chemical analysis were collected from the 12 stands located in the ICH comprising the chronosequence of forests regenerating following stand-replacing fire or clearcut logging. The soil sampling, processing, and nutrient analyses have been described by Twieg *et al*. (2009). Briefly, sampling was done in August 2004 during a consistently dry period to minimize any short-term effects that changes in soil moisture might have on soil processes such as nitrogen mineralization. Approximately 1 kg of mineral soil and 300 g of forest floor were removed from eight locations per stand (with the exception of the young, fire-generated stand where there was insufficient forest floor present to sample appropriately). Mineral and forest floor samples were bulked separately at each site, separated into three subsamples, sealed in polyethylene bags, and transported on ice in coolers to the lab.

All soil analyses except for organic phosphorus were performed at the BC Ministry of Forests and Range Analytical Chemistry Laboratory in Victoria, BC. Prior to analysis, mineral soil was first sieved to 2 mm and then air dried. Organic material was air dried, sieved to 4 mm, and then milled. Available phosphorus was determined using the Bray-1 method, while organic phosphorus was estimated as the difference in sulfuric acid-extractable phosphorus between pre and post-ignited soil samples (Olson & Sommers, 1982). Five spectrophotometer readings were averaged for each sample. Mineralizable nitrogen was estimated by subtracting initial available nitrogen from the values of samples incubated in waterlogged, anaerobic conditions for 2 weeks at 30 °C (Waring & Bremner, 1964). Available nitrogen (ammonium and nitrate)

was extracted by shaking for 2 h in 2 N KCL (Bremner, 1996) and the concentration of each was measured on a Technicon AutoAnalyzer II (Seal Analytical, Ltd. , UK). Total carbon and nitrogen were obtained by combustion elemental analysis using a Leco CHN-600 Elemental Analyser (Leco Corp., MI, USA).

2.2.7 Data analysis

The relationship between increasing concentrations of acid phosphatase standards and colour development on the imprinting paper was determined using least-squares regression with a polynomial fitted line using JMP (SAS Institute, USA). Data from the two studies estimating imprintable phosphatase activity as gray values using standard curves (IDF and EFFS studies) were analyzed by one-way ANOVA ($P = 0.05$) using NCSS System Statistical Software (Kaysville, UT, USA). For the study analyzing patterns of phosphatase activity across a chronosequence of forest stands using a database of imprinted phosphatase activity, only phosphatase activity from high activity microsites greater than 15 pixels in size were included in the analysis. Total average imprinted phosphatase for each 20 x 20 cm imprint area was calculated for each root window as the sum of the average activity multiplied by the area in pixels for each high phosphatase area above the size threshold. Total average activity was normalized by log transformation and differences between the four age classes were analyzed by one-way ANOVA ($P = 0.05$; $N = 4$) using JMP (SAS Institute, USA). Significant differences in mean total average activity between age classes were analyzed using Student's t comparison with Bonferroni correction. Soil nutrient data for each stand in the chronosequence was normalized by log transformation and one-way ANOVA was used to test for differences within the organic and mineral layers between age classes. Correlations between chronosequence soil nutrients and phosphatase activity were evaluated by detrended correspondence analysis (DCA) (Hill & Gauch, 1980) using PC-Ord (McCune & Mefford, 2005). The proportion of variance represented by each axis of the DCA ordination was measured as the correlation between the relative Euclidean distances among the ordinated matrix of stand phosphatase activity compared to the relative Euclidean distances in among stands in the original data matrix. A cutoff r-value of 0.198 was used for plotting correlations of soil nutrients with the ordination of the imprinted activity.

2.3   Results

2.3.1   Method development

The application of increasing concentrations of acid phosphatase standards to the imprinting paper resulted in purple-red spots of increasing intensities. Enzyme activity was highly correlated to the colour intensity (Figure 2.2). When applied to rhizoboxes, the imprint paper showed similar colour development, which was interpreted to represent acid phosphatase activity *in situ* (Figure 2.3a). This activity was detected at high resolution: coloured regions had sharp boundaries with little colour development between clearly defined spots. No colour development was observed on control paper applied to rhizoboxes (Figure 2.3b).

As the time of contact increased between the soil surface and the imprinting paper, the intensity of the colour developing on the paper increased, resulting in a decrease in the gray value of the scanned images, with maximum colour development at 60 min. (Figure 2.4). Therefore, a contact time of 1 h was used for acid phosphatase assays in the field. No residues were observed for the acid phosphatase assay when filter paper lacking substrate was applied to rhizoboxes on sequential days following an initial assay (data not shown).

2.3.2   Estimates of soil phosphatase activity in forests and clearcuts

The rates of phosphatase activity present on soil profiles in mature and clearcut forest stands were compared by generating standard curves from phosphatase standard dilution series incubated in the field at the time of imprinting. Imprints taken at root windows installed at the ESSF forests at Sicamous Creek revealed significantly higher phosphatase activities per unit area in forest (24.4 EU m^{-2} h^{-1} +/- 2.1 SEM) than in eight-year-old clearcut locations (6.5 EU m^{-2} h^{-1} +/- 2.2 SEM). By contrast, assays carried out in IDF stands and neighbouring two-year-old clearcuts near Barriere detected no significant differences in phosphatase activities between forest stands (88.1 EU m^{-2} h^{-1} +/- 8.8 SEM) and clearcuts (107.0 EU m^{-2} h^{-1} +/- 11.0 SEM). In the latter case, variability was very high, both among the three imprints at the same site, and among imprints taken at different sites.

2.3.3   Fine-scale distribution of soil enzyme activity at forest sites along a chronosequence

Because enzyme imprints preserve the location and extent of enzyme activity present in the soil profile at the moment the imprint was taken, this method was used to evaluate changes in the pattern of enzyme activity at key stages of stand development using the chronosequence of Douglas-fir/paper birch stands located in the ICH biogeoclimatic zone. Analysis of the fine scale distribution of phosphatase activity across the ICH forest chronosequence revealed a marked change in the spatial distribution of activity at stem exclusion compared to younger sites. At this stage of development, active areas became larger and more numerous, (Figure 2.5a,b). The average total phosphatase activity detected in young (5-6 year-old) and canopy closure (24-30 year-old) stands was lower than that of stem exclusion (61-71 year-old) and older (90-103 year-old) stands, respectively (Figure 2.6).

Four soil nutrients were identified by one-way ANOVA as being present in significantly different concentrations within soil layers between age classes. In every case, the age class that differed was the youngest age class. The C/N ratio in the organic layers of the youngest sites was higher than in other age classes (Table 2.2). These results represented the young sites that were clearcut logged; the young fire site had no organic layer. In the organic layers of the two young clearcuts, the initial ammonium, mineralizable nitrogen, and organic phosphorus were all lower than in the other age classes (Table 2.2). There were no significant differences found for any nutrients in the mineral layer by one-way ANOVA.

Ordination of the 12 chronosequence stands using the sizes and intensities of imprinted phosphatase activity resulted in a separation of the younger and canopy closure stands from the stem exclusion and older stands (Figure 2.7). Axes 1 and 2 accounted for 63% of the variation in the original distance matrix. Five soil nutrients correlated with imprinted phosphatase activity. Young and canopy closure stands primarily separated from older stands along axis 1. This axis was negatively correlated with available phosphorus and positively correlated with initial ammonium in mineral soil. Stem exclusion and older stands separated on axis 1 and axis 2. This separation correlated with higher nitrate and ammonium in mineral layer and higher total percent nitrogen in organic soil. Higher nitrogen levels were also correlated

with lower carbon to nitrogen ratios in the mineral soil in stem exclusion and older stands (Table 2.3).

2.4   Discussion

The *in situ* method described here for non-destructive detection of soil phosphatase activities offers an excellent method for visualizing the exact locations of organic phosphorus cycling activity in the soil profile. This method uses a similar approach for acid phosphatase assays as that used by Dinkelaker & Marschner (1992) and Song *et al.* (2000) in the laboratory. The phosphatase assay developed here is a modification of these earlier approaches combined with a novel root window method used in the field (Grierson & Comerford, 2000).

Installing root windows inevitably results in some initial soil disturbance. In this study soil faces were quite smooth after initial being dug, but a small amount of backfilling was required after the windows were installed. Because soils from each horizon were separated as the holes were dug, it was possible to replace them at the appropriate depths. The disturbance of soil directly against the window would be expected to have an impact on microbial activity for a period after the installation of the windows. However, since the windows were left to stabilize for at least 5 months during the active growing period, allowing roots and observable mycelial fans to grow against the window, there is reason to believe that appropriate microbial communities had re-established at the surface of the soil profile.

We found a close relationship between the concentrations of enzyme standards added to imprinting paper and colour development in the field. Nevertheless, it is best to describe this approach as semi-quantitative, as did Grierson & Comerford (2000). The phosphatase activities from imprinting are significantly correlated with those from traditional root assays, but factors such as the age and diameter of roots influence the degree of agreement between the two methods (MD Jones and PF Grierson, pers. com.). In the field, the degree of contact between the paper and the soil will also influence colour development.

The lower soil phosphatase activities detected on imprints from 8-yr-old ESSF clearcuts compared to mature forests agrees with other research relating to forest age effects on enzyme activities and may indicate a nutrient limitation at early stages of stand development.

Hassett & Zak (2005) observed suppression of soil phosphatase activity, as well as a reduction in total microbial biomass, 9 years after clearcut logging of aspen (*Populus tremuloides* Michx. and *P. grandidentata* Michx.) forest. Kranabetter & Coates (2004) demonstrated that the phosphorus nutrition of hybrid spruce (*Picea glauca* (Moench) Voss x *Picea sitchensis* (Bong.) Carrière) was significantly affected 10 years after clearcut logging; hybrid spruce had lower foliar phosphorus content than spruce in undisturbed forest. This effect was specific to spruce as there was no difference in foliar phosphorus content amongst regenerating Western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) or Western redcedar (*Thuja plicata* Dougl. Ex D. Don), compared to seedlings of the same species in undisturbed forest. In addition, Kranabetter & Coates (2004) found no significant effect of clearcut logging on total soil phosphorus in either the organic or mineral soil of forest 10 years after harvest; however, soil pH was significantly higher in clearcut stands compared to undisturbed forest. Soil pH is negatively correlated with soil phosphatase activity (Dick *et al.*, 1988; Antibus & Linkins III, 1992; Staddon *et al.*, 1999), and both fire and clearcut logging significantly affect pH. Four years after clearcut logging or clearcut logging followed by prescribed burning of jack pine (*Pinus banksiana* Lamb.), Staddon *et al.* (1999) found that undisturbed pine forests had the highest soil phosphatase activity and the lowest soil pH; stands regenerating from clearcut and prescribed burn had the lowest activity and the highest soil pH; and stands that were only clearcut logged had intermediate phosphatase activity and pH values.

　　　　While intermediate to longer term effects of clearcut logging on soil phosphatase activity have been well studied (Adams, 1992; Saa *et al.*, 1993; Eivazi & Bayan, 1996), shorter term effects have received less attention. To our knowledge, no published studies have investigated the effects of clearcut logging on soil phosphatase activity within two years of logging. Therefore, our finding that there was no significant difference in imprintable phosphatase activity between more recently clearcut and mature Douglas-fir forests may be indicative of a general lack of a clearcut logging effect on soil phosphatase activity at this stage of stand development. However, considering the substantial reduction in soil phosphatase observed four to nine years after clearcut logging (Staddon *et al.*, 1999; Hassett & Zak, 2005), the lack of an effect on soil phosphatase within two years of clearcutting could be an indicator

of the time scale on which substantial changes to ecosystem functions may occur. This time frame should be taken into consideration when evaluating the affects of forest management on ecosystem function.

The short and long term effects of fire on soil phosphatase activity have received more attention than the effects of clearcut logging. Several studies have associated fire with reductions in enzyme activity within one year of fire disturbance (Adams, 1992; Saa *et al.*, 1993; Eivazi & Bayan, 1996), and this effect can persist for up to four years (Staddon *et al.*, 1999). Fire may reduce soil enzyme activity by sterilizing soil, denaturing enzymes, altering soil structure, reducing microbial biomass, and increasing inorganic phosphorus (Speir & Ross, 1978; Adams, 1992; Eivazi & Bayan, 1996). Fire-induced increases in soil inorganic phosphorus are considered especially important in reducing soil phosphatase activity. Polglase *et al.* (1992) found that concentrations of inorganic phosphorus increased to 34 μg g^{-1} soil immediately after burning of *Eucalyptus regnans* forest; however, they decreased to 2.3 μg g^{-1} soil by 16 years after fire and remained constant as stands aged to 250 y. In that study, soil phosphatase rose substantially as regenerating *Eucalyptus regnans* forests reached 20 y, reaching a maximum activity when stands reached 80 y, and declining in 250-yr-old stands, described as "overmature". Our results from the ICH chronosequence are consistent with this finding; imprintable soil phosphatase activity rose significantly in Douglas-fir/paper birch stands at stem exclusion (61-71yr) and declined in older stands (90-103yr). Because *Eucalyptus regnans* forests regenerate very rapidly, reaching canopy closure within as few as five years, a 20 years *Eucalyptus regnans* stand is at a similar stage of stand development to the 61-71 years Douglas-fir/paper birch stands in our study. Therefore the lower soil phosphatase activity observed in 5 years stands of mountain ash is analogous to the lower imprintable phosphatase activity seen in Douglas-fir/paper birch stands just reaching canopy closure. This highlights the importance of comparing stands at similar stages of development, regardless of their ages.

Because production of phosphatase is tightly controlled by biological demand, phosphatase production is expected to be lower when easily assimilated phosphorus is more abundant. Imprinting detected lower soil phosphatase activities at younger and canopy closure stands, and as expected, this lower activity was negatively correlated with inorganic available

phosphorus. However, the levels of available phosphorus, as measured by standard soil tests, were not significantly different between age classes, demonstrating that measuring soil enzyme activity is an important addition to standard assays of soil nutrients. Because organic phosphorus is mineralized by fire, there is a fertilization effect after wild-fire or broadcast burning. While the increase in measurable available phosphorus is short-lived, this fertilization effect can be long lasting. In a fertilization study, western hemlock (*Tsuga heterophylla* (Raf) Sarge) stands that received a one-time phosphorus treatment had a sustained growth response 15 years after fertilization (Blevins *et al*., 2006). A phosphorus fertilization effect, which would cause available nitrogen to be rapidly taken up, might account for correlation between broader soil carbon to nitrogen ratio with the lower phosphatase activity of young and canopy closure stands,. Correlations between higher ammonium and nitrate, and higher phosphatase activity in soils of the stem exclusion and older stands, may indicate that the high phosphatase activity at these sites was not mobilizing enough phosphorus to allow for the complete uptake of available nitrogen from these soils.

Many factors that affect enzyme activity, such as pH, moisture, and temperature, will vary at fine scales across individual root windows. For example, in another study where 60 root windows were installed across 16 Douglas-fir-dominated sites exposed to various types of disturbance, pH was found to vary as much as 0.1 units across one window and as much as 1.3 units between windows on the same site (S. Dong, pers. comm.). Temperature, as measured by a non-contact infrared thermometer, varied as much as 3.2°C across one window and readings ranged from 0.5 to 18°C across all measurements at all windows. In addition, various phenolic and humic compounds that can bind to proteins and inhibit enzymatic activities may be heterogeneously distributed in forest soil. Although this variation might be seen as a disadvantage compared to measuring bulk soil enzyme activities in the laboratory, it can also be seen as a major advantage of the method. This method detects actual enzyme activity as it occurs in the field in soil microsites. This fine-scale variation in environmental conditions is lost during typical soil sampling. Thus, this is exactly the type of approach that will allow us to study soils at the scales suggested by Schimel & Bennett (2004) and hence, deepen our understanding of soil processes. By extending published methods for studying phosphatase *in*

situ, combining this new assay with a root window approach, and integrating this approach with computer analysis, this study demonstrates new approaches to the fine scale study of soil ecology in the field.

This approach will complement approaches that probe for specific genes (Kelly, 2003; Wellington *et al.*, 2003; Luis *et al.*, 2005) by providing a mechanism for correlating the presence of organisms with directly measured activity, rather than the potential activity measured by DNA or RNA. For example, this approach can be used to determine the proportion of activity associated with actively growing white roots and their rhizospheres versus suberized brown roots and their rhizospheres. The method can be used to test for functional diversity of ectomycorrhizal fungi on different root tips or in visible mycelial mats. All of these structures are visible in images taken with a high-resolution camera. Furthermore, by using molecular techniques such as DGGE or T-RFLP (Terminal Restriction Fragment Length Polymorphism), soil fungal and prokaryotic communities associated with 'hot spots' of enzyme activity can be described.

2.5   Figures and tables

Figure 2.1 Root window based on Grierson & Comerford (2000) (a) immediately after being installed in a mature Douglas-fir stand (b) with treated imprint paper in place. Imprint typically placed below upper litter layer, spanning the organic (litter, fragmentation/fermentation, humic) and upper mineral layers (Ah/Ae and upper B layers).
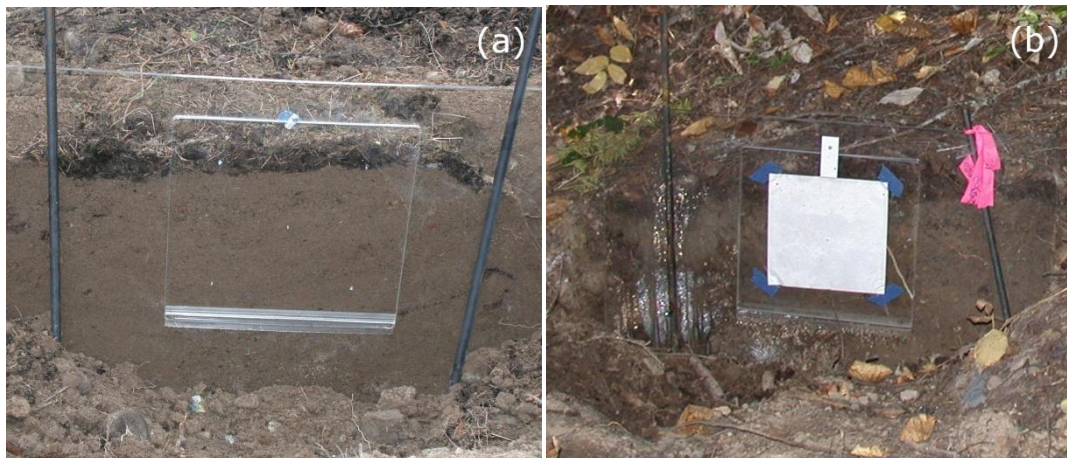
Figure 2.2 Representative standard curve showing the relationship between the concentrations of standard (acid phosphomonoesterase from wheat germ) applied in 5 μL aliquots and color intensity on chromatography paper treated with α-naphthyl phosphate and Fast Red TR and quantified as the average gray values of pixels in spots on the scanned image.
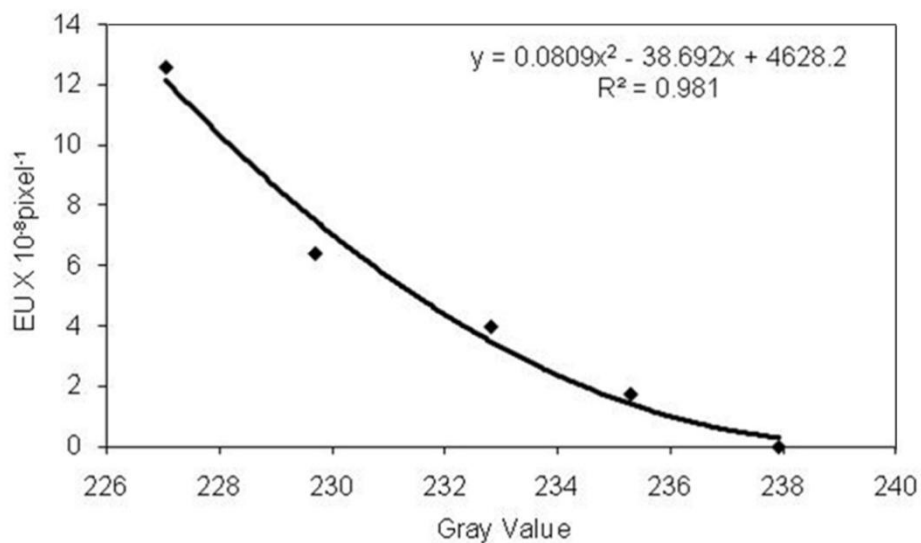
Figure 2.3 Scanned images of (a) phosphatase imprint activity on filter paper treated with α-naphthyl phosphate and Fast Red TR and applied to a rhizobox of greenhouse-grown Douglas-fir seedlings; and (b) control filter paper with Fast Red TR only and applied to the same rhizobox.
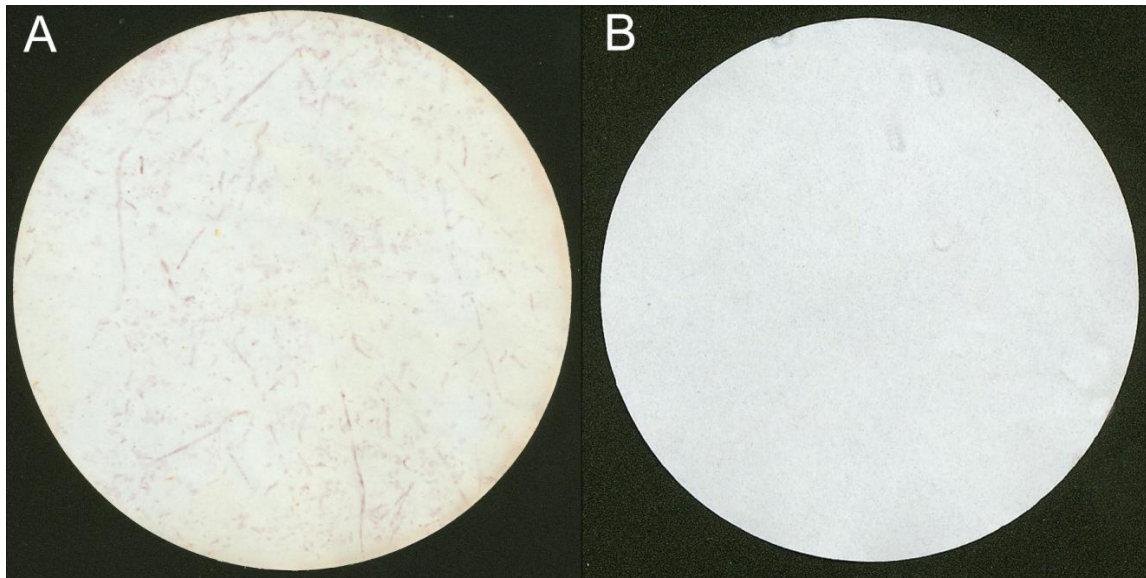
Figure 2.4 Colour development, expressed as gray value, of imprints from assays for acid phosphatase, with increasing time of contact with rhizobox soil (means from two trials).
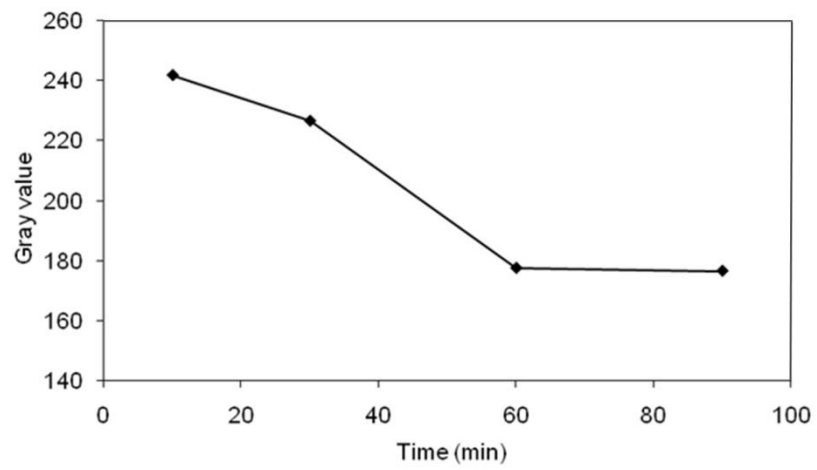
Figure 2.5 Frequencies of high phosphatase areas of different sizes. Phosphatase activity derived from scanned 20 x 20 cm imprints taken from a Douglas-fir/birch chronosequence of four ages: young (5-6 years-old), canopy closure (24-30 years-old), stem exclusion (61-71 years-old), and older (90-103 years-old) in the southern interior of British Columbia, Canada. (a) Frequency of smaller (<50 pixels) and larger (>50 pixels) high phosphatase areas. High phosphatase microsites include at least 15 coloured pixels (minimum diameter of approximately 1 mm) (b) Frequency distribution of larger (>50 pixels) high phosphatase areas.
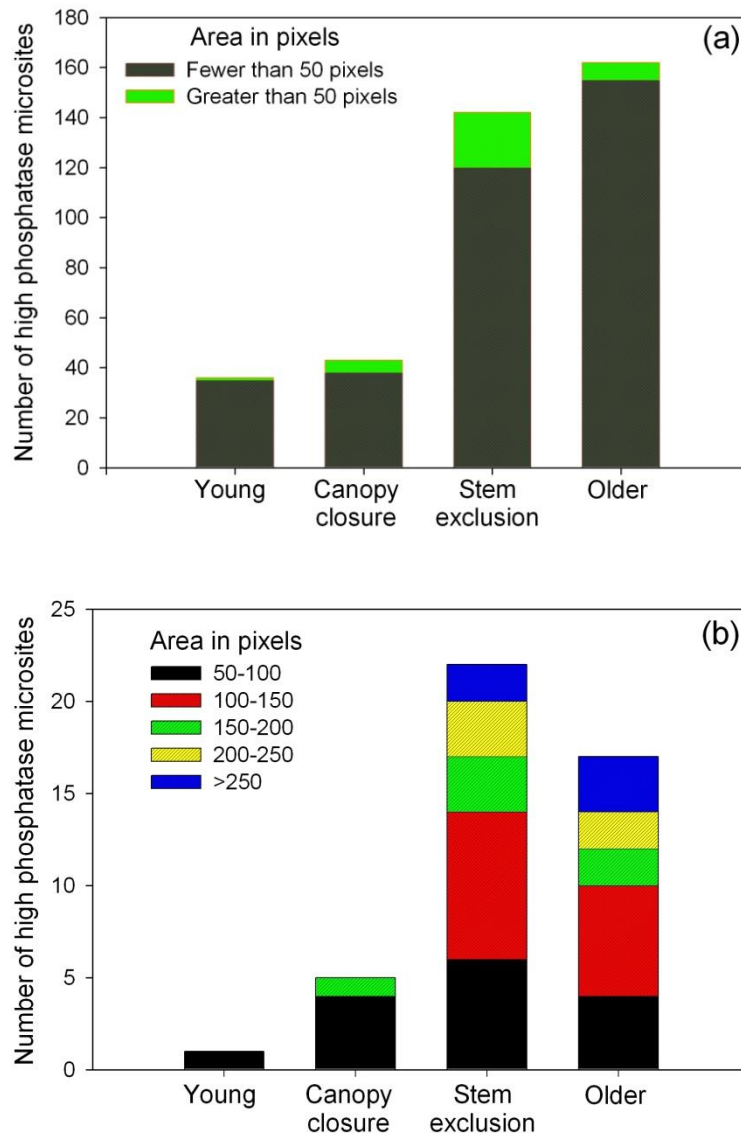
Figure 2.6 Average total phosphatase activity of scanned soil phosphatase imprints for each forest age class in a chronosequence of Douglas-fir/paper birch stands in the southern interior of British Columbia, Canada. Total activity was calculated as the number of coloured pixels in each active area larger than 15 pixels in size multiplied by the mean colour intensity of that area. Data were log transformed and analyzed by one way ANOVA (N=3). Different letters indicate differences in mean activity values between age classes (*P*=0.05).
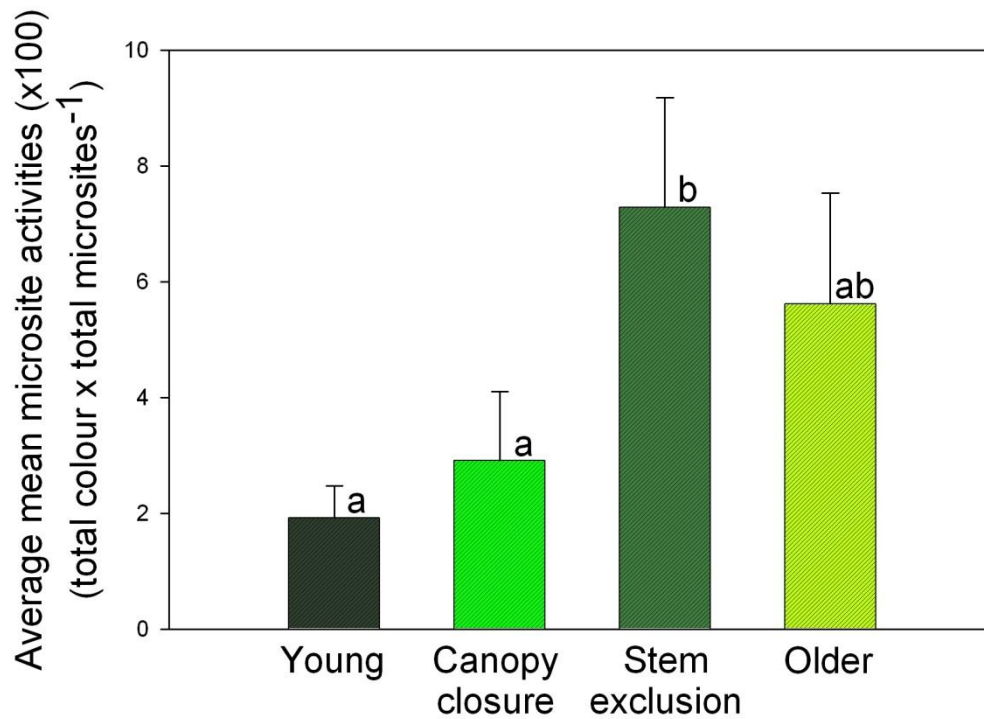
Figure 2.7 Detrended correspondence analysis of imprinted phosphatase activity with soil nutrients for each forest age class in a chronosequence of Douglas-fir/paper birch stands in the southern interior of British Columbia, Canada. Proportion of variance explained: Axis 1 = 0.434; Axis 2 = 0.323. Nutrients with correlations (r) greater than 0.2 are represented as vectors. Mineral nutrients: initial nitrate = NO3i_M; initial ammonium = NH4i_M; C/N ratio = C/N_M; available phosphorus = Pa_M. Organic nutrient: total percent nitrogen = N%t_O.
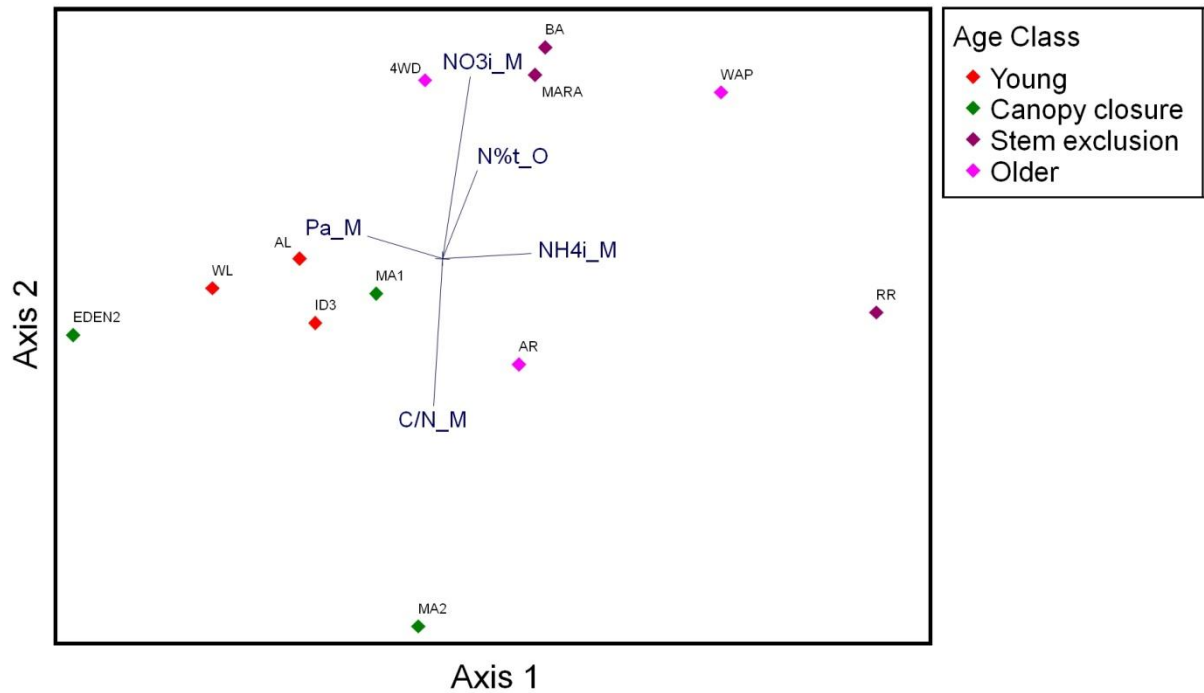
Table 2.1 Site locations and characteristics of a chronosequence of Douglas-fir/paper birch stands in the southern interior of British Columbia, Canada. Interior Cedar-Hemlock biogeoclimatic variants are defined in Lloyd *et al.* (1990). Table modified from Twieg *et al*. (2007). Used with permission.

Age class (Site code)	Age (yr)	Stand initiation type	ICH variant	Elevation (m)	Latitude/ longitude	Crown closure (%)	Soil texture[a]	Tree species composition (%)[b]		
								Douglas-fir	Paper birch	Other ECM broadleaf spp.
Young (AL)	6	clearcut	mw2	600	N 50º 32' 43" W 118º 52' 49"	45	SL	25	50	7.5
Young (IDA3)	5	wildfire	mw2	650	N 50º 40' 00" W 119º 18' 57"	35	SL	7.5	80	2.5
Young (WL)	5	clearcut	mw3	700	N 50º 53' 51" W 119º 16' 27"	50	fSL	10	70	2.5
Canopy closure (ED2)	30	wildfire	mk2	1000	N 50º 44' 12" W 119º 22' 17"	85	SiL	35	45	5
Canopy closure (MA1)	24	wildfire	mw3	930	N 50° 55' 20" W 118° 50' 41"	70	SL	40	45	10
Canopy closure (MA2)	24	wildfire	mw3	975	N 50° 55' 3" W 118° 50' 56"	70	SL	45	40	7.5
Stem exclusion (BA)	63	wildfire	mw2	700	N 50º 34' 03" W 118º 50' 50"	85	SL	45	50	0
Stem exclusion (MARA)	71	wildfire	mw2	600	N 50º 39' 28" W 119º 03' 49"	80	SL	45	50	0
Stem exclusion (RR)	61	wildfire	mw2	800	N 50º 41' 55" W 118º 46' 07"	75	SL	40	60	0
Older (4WD)	103	wildfire	mw2	550	N 50º 36' 47" W 118º 50" 26"	90	SL	50	45	0
Older (ACR)	98	wildfire	mw2	600	N 50º 37' 25" W 118º 46' 06"	80	LS	55	30	0
Older (WAP)	90	wildfire	mw2	650	N 50° 45' 1" W 118° 34' 12"	80	SL	50	40	0

[a] SiL, silty loam; SL, sandy loam; SCL, sandy clay loam; LS, loamy sand; f, fine; vf, very fine.

[b] Tree species compositional percentages were estimated as each species' or group's proportion of the total estimated canopy cover.

Table 2.2 Mean soil nutrients by stand age (N = 3; standard error of mean in parentheses). Values expressed as mg kg^{-1} soil, except C/N ratio. Letters following means indicate significant differences as determined by one-way ANOVA. Means followed by the same letter were not significantly different by multiple comparisons. (This data is a subset of the data published by Twieg *et al*. 2009)

Stand age class	C/N ratio	Initial ammonium	Initial nitrate	Mineralizable ammonium	Mineralizable nitrate	Organic P	Available P
Mineral soil layer							
Young	28.46 (3.75)	1.89 (0.19)	0.31 (0.01)	18.78 (3.14)	0.91 (0.07)	N/A	183.25 (29.64)
Canopy closure	32.53 (3.09)	2.28 (0.38)	0.20 (0.08)	11.64 (2.44)	1.16 (0.12)	N/A	115.53 (14.56)
Stem exclusion	24.31 (1.22)	2.60 (0.48)	0.36 (0.08)	17.61 (7.45)	0.86 (0.05)	N/A	134.19 (48.24)
Older	26.22 (1.45)	1.99 (0.38)	0.36 (0.01)	14.52 (2.46)	0.76 (0.10)	N/A	169.73 (31.01)
Organic soil layer							
Young	54.38 (1.80)a	7.59 (0.28)b	0.32 (0.32)	144.75 (27.12)b	1.21 (0.84)	483.34 (71.31)b	111.20 (2.34)
Canopy closure	36.90 (2.59)b	24.01 (2.28)a	0.86 (0.29)	412.63 (42.40)a	2.78 (0.67)	912.20 (126.86)a	140.99 (13.81)
Stem exclusion	35.87 (4.08)b	19.40 (5.20)a	0.88 (0.47)	346.39 (69.62)a	2.29 (0.01)	801.83 (58.31)a	109.11 (22.23)
Older	35.14 (1.82)b	22.81 (3.66)a	0.77 (0.40)	351.50 (57.50)a	2.33 (0.08)	906.80 (58.95)a	106.47 (14.96)

Table 2.3 Pearson and Kendall correlations of nutrients with ordination axes of detrended correspondence analysis of imprinted phosphatase activity with soil nutrients for each forest age class in a chronosequence of Douglas-fir/paper birch stands in the southern interior of British Columbia, Canada.

Nutrient parameter	Nutrient code	Axis 1			Axis 2		
		r^a	r-sq	tau[b]	r	r-sq	tau
Mineral soil layer							
Available phosphorus	Pa_M	-0.433	0.187	-0.152	0.239	0.057	0.242
Initial nitrate	NO3i_M	0.267	0.071	0.485	0.674	0.455	0.212
Initial ammonium	NH4i_M	0.469	0.220	0.333	0.122	0.015	0.242
C/Ni ratio	C/N_M	-0.148	0.022	-0.152	-0.605	0.366	-0.364
Organic soil layer							
Total %nitrogen	N%t_O	0.291	0.085	0.303	0.470	0.221	0.273

[a] Pearson's *r* expresses the linear relationships between ordination scores and individual variables.
[b] Kenall's tau expresses the rank relationships between ordination scores and individual variables.

2.6   Bibliography

Aon MA, Cabello MN, Sarena DE, Colaneri AC, Franco MG, Burgos JL, Cortassa S. 2001. I. Spatio-temporal patterns of soil microbial and enzymatic activities in an agricultural soil. *Applied Soil Ecology* **18:** 239-254.

Abuzinadah RA, Finlay RD, Read DJ. 1986. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants II. Utilization of protein by mycorrhizal plants of *Pinus-Contorta*. *New Phytologist* **103:** 495-506.

Adams MA. 1992. Phosphatase activity and phosphorus fractions in Karri (*Eucalyptus diversicolor* F. Muell.) forest soils. *Biology and Fertility of Soils* **14:** 200-204.

Antibus RK, Linkins III AE. 1992. Effects of liming a red pine forest floor on mycorrhizal numbers and mycorrhizal and soil acid phosphatase activities. *Soil Biology and Biochemistry* **24:** 479-487.

Attiwill PM, Adams MA. 1993. Tansley Review No. 50. Nutrient cycling in forests. *New Phytologist* **124:** 561-582.

Bending GD, Read DJ. 1995a. The structure and function of the vegetative mycelium of ectomycorrhizal plants. VI. Activities of nutrient mobilizing enzymes in birch litter colonized by *Paxillus involutus* (Fr.) Fr. *New Phytologist* **130:** 411-417.

Bending GD, Read DJ. 1995b. The structure and function of the vegetative mycelium of ectomycorrhizal plants.V. Foraging behaviour and translocation of nutrients from exploited litter. *New Phytologist* **130:** 401-409.

Bhat KKS, Nye PH. 1973. Diffusion of phosphate to plant roots in soil I. Quantitative autoradiography of the depletion zone. *Plant and Soil* **38:** 161-175.

Blevins LL, Prescott CE, Van Niejenhuis A. 2006. The roles of nitrogen and phosphorus in increasing productivity of western hemlock and western redcedar plantation on northern Vancouver Island. Forest Ecology and Management 234: 116-122.

Bremner JM. 1996. Nitrogen availability indexes. In: Black CA ed. *Methods of soil analysis, part 2, Agronomy 9*. Wisconsin, USA: American Society of Agronomy, Inc, 1324-1345.

Buée M, Courty PE, Mignot D, Garbaye J. 2007. Soil niche effect on species diversity and catabolic activities in an ectomycorrhizal fungal community. *Soil Biology and Biochemistry* **39:** 1947-1955.

Burns RG. 1978. *Soil Enzymes.* New York: Academic Press.

Burns RG, Dick RP. 2002. *Enzymes in the environment: Activity, ecology and applications.* New York: Marcel Dekker.

Byrd KB, Parker VT, Vogler DR, Cullings KW. 2000. The influence of clear-cutting on ectomycorrhizal fungus diversity in a lodgepole pine (*Pinus contorta*) stand, Yellowstone National Park, Wyoming, and Gallatin National Forest, Montana. *Canadian Journal of Botany* **78:** 149-156.

Caldwell BA. 2005. Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia-International Journal of Soil Biology* **49:** 637-644.

Courty P-E, Bréda N, Garbaye J. 2007. Relation between oak tree phenology and the secretion of organic matter degrading enzymes by Lactarius quietus ectomycorrhizas before and during bud break. *Soil Biology and Biochemistry* **39:** 1655-1663.

Courty PE, Pouysegur R, Buée M, Garbaye J. 2006. Laccase and phosphatase activities of the dominant ectomycorrhizal types in a lowland oak forest. *Soil Biology & Biochemistry* **38:** 1219-1222.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167:** 309-319.

Decker KLM, Boerner REJ, Morris SJ. 1999. Scale-dependent patterns of soil enzyme activity in a forested landscape. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **29:** 232-241.

Dick RP. 1992. A Review - Long-term effects of agricultural systems on soil biochemical and microbial parameters. *Agriculture Ecosystems & Environment* **40:** 25-36.

Dick RP, Rasmussen PE, Kerle EA. 1988. Influence of long-term residue management on soil enzyme-activities in relation to soil chemical-properties of a wheat-fallow system. *Biology and Fertility of Soils* **6:** 159-164.

Dighton J. 1983. Phosphatase production by mycorrhizal fungi. *Plant and Soil* **71:** 455-462.

Dinkelaker B, Marschner H. 1992. *In vivo* demonstration of acid phosphatase activity in the rhizosphere of soil-grown plants. *Plant and Soil* **144:** 199-205.

Eivazi F, Bayan MR. 1996. Effects of long-term prescribed burning on the activity of select soil enzymes in an oak-hickory forest. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **26:** 1799-1804.

Farr E, Vaidyanathan LV. 1972. The supply of nutrient ions by diffusion to plant roots in soil IV. Direct measurement of changes in labile phosphate content in soil near absorbing roots. *Plant and Soil* **37:** 609-616.

Firsching BM, Claassen N. 1996. Root phosphatase activity and soil organic phosphorus utilization by Norway spruce [Picea abies (L.) Karst.]. *Soil Biology and Biochemistry* **28:** 1417-1424.

Grierson PF, Comerford NB. 2000. Non-destructive measurement of acid phosphatase activity in the rhizosphere using nitrocellulose membranes and image analysis. *Plant and Soil* **218:** 49-57.

Hagerman SM, Jones MD, Bradfield GE, Gillespie M, Durall DM. 1999. Effects of clear-cut logging on the diversity and persistence of ectomycorrhizae at a subalpine forest. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **29:** 124-134.

Hassett JE, Zak DR. 2005. Aspen harvest intensity decreases microbial biomass, extracellular enzyme activity, and soil nitrogen cycling. *Soil Science Society of America Journal* **69:** 227-235.

Hill MO, Gauch HG. 1980. Detrended correspondence analysis: an improved ordination technique. *Vegetatio* **42:** 47-58.

Horton TR, Bruns TD. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* **10:** 1855-1871.

Kelly JJ. 2003. Molecular techniques for the analysis of soil microbial processes: Functional gene analysis and the utility of DNA microarrays. *Soil Science* **168:** 597-605.

Kranabetter JM, Coats KD. 2004. Ten-year postharvest effects of silviculture systems on soil-resource availability and conifer nutrition in a northern temperate forest. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **34:** 800-809.

Lewis DG, Quirk JP. 1967. Phosphate diffusion in soil and uptake by plants. III - P^{31} movement and uptake by plants as indicated by P^{32}-autoradiography. *Plant and Soil* **26:** 445-454.

Lloyd D, Angrove K, Hope G, Thompson C. 1990. *A guide to site identification and interpretation for the Kamloops forest region.* Victoria, B.C.: Research Branch, B.C. Ministry of Forests.

Luis P, Kellner H, Martin F, Buscot F. 2005. A molecular method to evaluate basidiomycete laccase gene expression in forest soils. *Geoderma* **128:** 18-27.

Marschner H. 1995. Mineral Nutrition of Higher Plants. Sand Diego, California, U.S.A.: Academic Press Ltd.

McCune B, Mefford J. 2005. *PC-Ord for Windows: multivariate analysis of ecological data version 5.* Gleneden Beach, Oregon, U.S.A.: MJM Software Design.

McGill WB, Cole CV. 1981. Comparative aspects of cycling of organic C, N, S, and P through soil organic matter. *Geoderma* **26:** 267-286.

Mogge B, Loferer C, Agerer R, Hutzler P, Hartmann A. 2000. Bacterial community structure and colonization patterns of *Fagus sylvatica* L-ectomycorrhizospheres as determined by fluorescence in situ hybridization and confocal laser scanning microscopy. *Mycorrhiza* **9:** 271-278.

Olander LP, Vitousek PM. 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry* **49:** 175-190.

Olander LP, Vitousek PM. 2005. Short-term controls over inorganic phosphorus during soil and ecosystem development. *Soil Biology and Biochemistry* **37:** 651-659.

Olson SR, Sommers LE. 1982. Phosphorus. In: Miller RH, Keeney DR, eds. *Methods of soil analysis, part 2: chemical and microbiological properties*. Madison, WI, USA: American Society of Agronomy, Inc, 403-427.

Paul EA, Clark FE. 1989. *Soil microbiology and biochemistry.* Toronto: Academica Press.

Perez-Moreno J, Read DJ. 2000. Mobilization and transfer of nutrients from litter to tree seedlings via the vegetative mycelium of ectomycorrhizal plants. *New Phytologist* **145:** 301-309.

Polglase PJ, Attiwill PM, Adams MA. 1992. Nitrogen and phosphorus cycling in relation to stand age of *Eucalyptus regnans* F. Muell III. Labile inorganic and organic P, phosphatase activity and P availability. *Plant and Soil* **142:** 177-185.

Pritsch K, Raidl S, Marksteiner E, Blaschke H, Agerer R, Schloter M, Hartmann A. 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* **58:** 233-241.

Raison RJ, Khanna PK, Woods PV. 1985. Transfer of elements to the atmosphere during low intensity prescribed fires in three Australian subalpine eucalypt forests. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **15:** 657-664.

Rineau F, Garbaye J. 2009. Does forest liming impact the enzymatic profiles of ectomycorrhizal communities through specialized fungal symbionts? *Mycorrhiza* **19:** 493-500.

Saa A, Trasar-Cepeda MC, Gil-Sotres F, Carballas T. 1993. Changes in Soil Phosphorus and acid phosphatase activity immediately following forest fires. *Soil Biology and Biochemistry* **25(9):** 1223-1230.

Schimel JP, Bennett J. 2004. Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **85:** 591-602.

Song Y, Li X, Feng G, Zhang F, Christie P. 2000. Rapid assessment of acid phosphatase activity in mycorrhizosphere and in arbuscular mycorrhizal fungal hyphae. *Chinese Science Bulletin* **45:** 1187-1191.

Speir TW, Ross DJ. 1978. Soil phosphatase and sulphatase. In: Burns RG, ed. *Soil Enzymes.* New York: Academic Press, 197-245.

Spiers GA, McGill WB. 1979. Effects of phosphorus addition and energy supply on acid phosphatase production and activity in soils. *Soil Biology and Biochemistry* **11(1):** 3-8.

Staddon WJ, Duchesne LC, Trevors JT. 1999. The role of microbial indicators of soil quality in ecological forest management. *Forestry Chronicle* **75:** 81-86.

Tabatabai MA. 1994. Soil Enzymes. In: Weaver RW, Angle JS, Bottomley PS, eds. *Methods of soil analysis. Part 2, Microbiological and biochemical properties.* Soil Science Society of America, Inc., 775-833.

Twieg BD, Durall DM, Simard SW. 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* **176:** 437-447.

Twieg BD, Durall DM, Simard SW, Jones MD. 2009. Influence of soil nutrients on ectomycorrhizal communities in a chronosequence of mixed temperate forests. *Mycorrhiza* **19:** 305-316.

Visser S, Parkinson D. 1992. Soil biological criteria as indicators of soil quality: soil microorganisms. *American Journal of Alternative Agriculture* **7:** 33-37.

Waring SA, Bremner JM. 1964. Ammonium production in soil under waterlogged conditions as an index of nitrogen availability. *Nature* **201**: 951-952.

Wellington EMH, Berry A, Krsek M. 2003. Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. *Current Opinion in Microbiolgy* **6:** 295-301.

Wood T, Bormann FH, Voigt GK. 1984. Phosphorus cycling in a northern hardwood forest: biological and chemical control. *Science* **223:** 391-393.

3   Mapping ectomycorrhizal fungi to fine-scale soil phosphatase activity in regenerating forests[2]

3.1   Introduction

Conventionally, ectomycorrhizal fungi (EMF) have been considered to be a group of soil microbes primarily responsible for increasing nutrient uptake by plants. Now, with increased appreciation of their substantial biomass in forest soils (Högberg & Högberg, 2002; Wallander *et al.*, 2001), dominant contribution to soil organic carbon (Godbold *et al.*, 2006), and ability to secrete extracellular hydrolytic enzymes (Luis *et al.*, 2005; Bending & Read, 1995b), EMF are increasingly viewed as playing a major role in nutrient cycling in forest ecosystems (Schimel & Bennett, 2004; Talbot *et al.*, 2008; van der Heijden *et al.*, 2008). In addition to their well-studied role in the mineralization of organic nitrogen, EMF are also associated with the mobilization of organic phosphorus in microcosms through their release of phosphatases (Bending & Read, 1995a,b; Brandes *et al.*, 1998; Zadworny *et al.*, 2008) and with extraction of inorganic phosphorus in the field (Wallander *et al.*, 2005). Most of our information on the importance of EMF in nutrient cycling is based on activities associated with either mantle tissues from field-collected ectomycorrhizae (Pritsch *et al.*, 2004; Courty *et al.*, 2005; Cullings *et al.*, 2008) or EMF hyphae in pure culture under artificial conditions (Alvarez *et al.*, 2005; Nygren & Rosling, 2009). While wide variation in phosphatase activities has been demonstrated between and within EMF species present as mycorrhizae in forest soils (Courty *et al.*, 2005), activities also vary between the mantle and extramatrical hyphae (those hyphae that extend beyond the ectomycorrhizal mantle) of strains of the same EMF species (Timonen *et al.*, 1998). It is enzyme activity associated with these extramatrical hyphae that likely plays the primary role in nutrient release (Bending & Read, 1995b), yet currently, the enzymatic activities associated with EMF hyphae *in situ* in the field remain almost entirely uncharacterized.

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A major challenge in studying processes in forest soils is that physical and biological features vary at scales below which sampling typically takes place, yet an understanding of this soil heterogeneity is needed in order to develop accurate models of biogeochemical cycles to predict impacts of climate change and other perturbations on soil processes (Jones *et al.*, 2005; Hobbie & Hobbie, 2008; Litton & Giardina, 2008). Typical methodologies treat soil as a homogeneous medium, where bulk soil samples are assumed to represent whole-soil processes, yet measurements of soil enzyme activities at a fine scale have demonstrated that measurements on bulk soil do not accurately reflect soil enzymatic capacity (Allison, 2006; Dorodnikov *et al.*, 2009). Furthermore, studying enzyme activities associated with extramatrical hyphae in intact soils is extremely difficult because of hyphal fragility. *In situ* assays have the potential to overcome some of these limitations by studying soil enzyme activities under ambient field conditions, with minimal disturbance to microbial communities, and at fine scales (Wallenstein & Weintraub, 2008). For example, in a previous study (Chapter 2) a fine-scale soil imprinting approach was used to investigate phosphatase enzyme activity *in situ* in the Interior Cedar-Hemlock biogeoclimatic zone of southern British Columbia. This method revealed a shift in the physical distribution of phosphatase activity measured in young, mixed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) - paper birch (*Betula papyrifera* Marsh) stands compared to older stands (Dong *et al.*, 2007). Soils in stands less than 61 years-old were shown to be characterized by many, small (< 1 mm) areas of phosphatase activity, while stands that were 61 years and older showed fewer, larger areas of activity (Dong *et al.*, 2007). Such results suggest a change in the types and distribution of organisms responsible for the phosphatase activity, clues that would not have been observed with conventional sampling and assay methods.

A possible explanation for the shift in distribution of phosphatase activity was suggested by a parallel study using the same replicated chronosequence of mixed birch - Douglas-fir stands. Not only did Twieg *et al.* (2007) find that the EMF community present on root tips changed at the same stand age as the shift in the pattern of phosphatase activity, the prevalence of specific EMF taxa was also associated with the amount of available phosphorus in the soil of these stands (Twieg *et al.*, 2009). A correlation between the EMF community

structure present on root tips and a change in soil enzyme activity was also observed in *Quercus rubra* forests (Lucas & Casper, 2008). There, nitrogen deposition resulted in a shift in EMF community composition that correlated with a decrease in the activity of lignin degrading enzymes. However, in a mixed pine/oak forest (*Pinus rigida*, *Pinus echinata*, *Quercus velutina*), the increased biomass of EMF hyphae in soil resulting from small additions of N-containing organic compounds was not correlated with a change in soil enzyme activity (Lucas *et al.*, 2007). These contrasting results demonstrate that the relationship between EMF and soil enzyme activity may vary depending on whether ectomycorrhizae or extramatrical hyphae are considered.

The study reported here determines whether the shift in fine-scale soil phosphatase activity observed in older birch - Douglas-fir forests (Dong *et al.*, 2007) was related to a change in the structure of the EMF community occurring as hyphae in the soil, as suggested by the data of Twieg *et al.* (2007) on ectomycorrhizal root tips. To test this hypothesis, a new method was developed that correlated the composition of fungal DNA to the pattern of phosphatase activity in soil. This method applied an enzyme imprint method (Grierson & Comerford, 2000; Dong *et al.*, 2007) to reveal fine-scale phosphatase activity of soil profiles *in situ* and then used the imprints to guide immediate, millimeter-scale soil sampling of the soil profile. Fungal DNA was characterized using Terminal Restriction Fragment Length Polymorphisms (TRFLPs). EMF signatures were identified using a library of EMF DNA collected during the previous studies at these stands (Twieg *et al.*, 2007, 2009). My study addresses three questions. (i) Do different general fungal or EMF communities associate with different levels of fine-scale soil phosphatase activity in these stands? (ii) If so, does this association between general fungal or EMF communities and fine-scale soil phosphatase activity change with stand age, as would be predicted by the altered spatial distribution of soil phosphatase activity and the change in EMF community on root tips observed previously? (iii) Are particular EMF taxa associated with *in situ* phosphatase activity? In addition, we evaluated the applicability of this technique in the context of the landscape-scale process of forest regeneration and assessed the scale at which general fungal and EMF community differences could be detected.

3.2 Methods

3.2.1 Study sites

In the summers of 2004 and 2005, root windows were installed in 12 forest stands: three replicate stands for each of four stand-age classes. Replicate stands were separated by an average distance of 30 km (range: 0.33 to 79 km). The forests were mixed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), paper birch (*Betula papyrifera* Marsh) stands located in the moist, warm (ICHmw2, ICHmw3) or moist, cool (ICHmk2) variants of the Interior Cedar-Hemlock (ICH) biogeoclimatic zone (Lloyd *et al.*, 1990) of southern interior British Columbia, Canada. The four stand age classes represented a chronosequence of stand development (Oliver & Larson, 1996). The youngest stands were in the stand initiation stage, when Douglas-fir and paper birch establish concurrently (5–6 year-old). The 24-30 year-old stands were in the canopy closure stage, when Douglas-fir and paper birch compete intensely for resources. The 61-71 year-old stands were in the stem exclusion stage, when birch starts to senesce, creating canopy gaps. The oldest stands were in the stand re-initiation stage, when Douglas-fir dominates over remaining paper birch (90–103 year-old). Two of the three stands in the youngest age class were the result of recent clear-cutting followed by broadcast burning and tree planting while the other was generated by stand-replacing wildfire (Youngest treatment). Despite the different site histories of the stands in the Youngest treatment, a previous study had shown that the overall diversity and relative abundance of EMF was not significantly different between to the two initiation types (Twieg *et al.*, 2007); however, four EMF taxa found on young clear-cut sites were also found only in stands of the oldest age class. Soil nutrients were also largely similar between initiation types, with no significant difference in organic or available phosphorus, or mineralizable ammonium or nitrate found (Twieg *et al.*, 2009). Canopy closure stands had been salvage logged and replanted to Douglas fir after stand-replacing wildfires (Canopy Closure treatment). The two older age classes had regenerated naturally to Douglas fir and paper birch after stand-replacing wildfires that (Stem Exclusion and Oldest treatments). Soils were fine textured silty loam, sandy loam, or loamy sands (See Table 3.1 for site descriptions)

Root-windows consisted of transparent acrylic panels (77 x 52 x 0.6 cm), each with a 30 x 30 cm trap door (Grierson & Comerford, 2000) (Figure 3.1a). Two of these were installed at each stand (24 total) following the protocol of Dong *et al.* (2007). These root-windows were dug vertically into the soil profile, such that the top of the trap door was at the soil surface (Figure 3.1a), and anchored in place with two 1.27 cm-diameter iron bars. During excavation, care was taken to reserve soil from each soil layer. Any gaps between the excavated soil profile and the window surface were filled with soil from the appropriate soil layer. Windows were oriented perpendicular to the slope, midway between paired birch and Douglas-fir trees, at locations chosen to minimize the presence of tree roots of other tree species. After installation, plywood panels were placed against the windows for protection, and the holes in front of the windows were refilled with soil to ensure that temperature regimes experienced by the soil profiles behind the windows were the same as the surrounding soil.

3.2.2 Sampling

In October 2006, a minimum of one year after installation, imprinting and targeted soil sampling took place at one root window per stand. Phosphatase-reactive imprinting sheets were treated by soaking 20 cm X 20 cm sheets of chromatography paper (Whatman, 20 x 20 cm, Cat No. 3030-861) for 1 min in a 1:10 (v/v) mixture of 50 mM α-naphthyl phosphate (Sigma N7255) and 10 mM Fast Red TR (Sigma F2768) (Dinkelaker & Marschner, 1992; Dong *et al.*, 2007). Each reagent was prepared in 50 mM pH 5.6 citrate buffer. The sheets were air-dried, wrapped in aluminum foil, and stored at 4 ºC until use. In this method acid phosphatase detection is based on the release of naphthol by phosphomonoesterase through the hydrolysis of α-naphthyl phosphate. The released naphthol reacted with the diazonium salt (Fast Red TR) to form a stable red precipitate on the imprinting sheet.

A sheet of Mylar™ plastic was used as an alignment and sampling guide. The Mylar™ sheet was cut to exactly fit the dimensions of the opening in the window and was taped to the inside of the trap door Figure 3.1b). The interface between the organic (litter, fermentation, and humus zone) and mineral layers was then marked onto the Mylar™ with a felt tip pen. Each treated imprinting sheet was then taped to the side of the Mylar™ facing the soil profile and was applied directly to an intact soil profile by closing the trap door (Figure 3.1c). Imprints were

incubated in place for one hour. After incubation, the Mylar™ sheet, with imprint attached, was removed from the window and rinsed briefly with dH_2O (Figure 3.1d). On the imprint, dark pink areas of at least 5 mm in diameter were considered areas of high phosphatase activity (Figure 3.2a) and were marked for sampling using a felt tip pen (Figure 3.2b). Low phosphatase activity sample sites were marked in the centers of the largest areas with no colour change. Where present in sufficient numbers, three high phosphatase and three low phosphatase sampling sites were chosen, at random, from amongst the marked locations in both the organic and mineral layers. To guide sample collection, a 5 mm-diameter, hammer-driven, leather punch was used to punch holes in the Mylar™ sheet at these chosen locations (Figure 3.2c). The sheet was then attached to a frame made of foam core poster board, which fit exactly inside the opening (Figure 3.2d). The frame reinforced the Mylar™ sheet and ensured that the Mylar™ sheet was replaced against the soil profile in exactly its previous location (Figure 3.3). Microsamples of soil of approximately 0.25 g (fresh weight) were collected from the chosen locations using sterile forceps, transferred into 1 ml microcentrifuge tubes, and kept on dry ice until transferred to -80 °C in the lab. Between six and 12 microsamples were collected per window.

3.2.3   Microsample TRFLP fingerprint construction

DNA was extracted from soil samples using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) following the supplied protocol. Fungal DNA was amplified by polymerase chain reaction (PCR) using a nested protocol. The first amplification used primer set NSA3/NLC2 (Martin & Rygiewicz, 2005) in 25 µl reactions (12.5 µl Fermentas Master Mix, 9.5 µl Fluka nuclease-free water, 10 pmol of each primer, and approximately 1 ng DNA) using the following thermocycler settings: 8 min at 95 ºC, 35 cycles of 30 sec at 95 ºC, 40 sec at 66 ºC, 40 sec at 72 ºC, 15 min at 72 ºC. All reactions were cleaned using a Mo Bio UltraClean[TM] PCR Clean-up DNA Purification Kit. The NSA3/NLC2 products were then re-amplified using fluorescently labeled VIC-ITS1F/NED-ITS4 (Applied Biosystems, USA) (Gardes & Bruns, 1993) in 25 µl PCR reactions consisting of: 12.5 µl Fermentas Master Mix, 11 µl Fluka nuclease-free water, 5 pmol of each primer, and approximately 0.5 ng DNA. The thermocycler settings for the second amplification were 5 min at 94 ºC, 35 cycles of 1 min at 94 ºC, 50 sec at 55 ºC, 50 sec at 72 ºC, and an

72

extension of 15 min at 72 ºC. Terminal restriction fragment length polymorphisms (TRFLPs) were created by restriction digestion of the labeled products with *Hin*f1 and *Hae*III endonucleases (New England Biolabs, USA) in separate reactions, following manufacturer's instructions. Terminal restriction fragment lengths were determined using an Applied Biosystems PRISM 377 automated sequencer (Nucleic Acid Protein Service Unit, University of British Columbia Vancouver, Canada) running a custom designed 1200 base pair ladder (Applied Biosystems, USA). Sequencer data files were processed using PeakScanner Version 1.0 (Applied Biosystems, USA) and EXCEL (Microsoft Corp., USA). Fluorescence peak intensities in each sample were normalized by the total fluorescence intensity of each sample.

3.2.4 Construction of TRFLP signature library of ectomycorrhizal fungi

In order to identify EMF TRFLP signatures within the general fungal TRFLP fingerprints, a reference library was constructed of TRFLP signatures of EMF found on birch and Douglas-fir roots at these same stands over a two-year collection period (Twieg *et al.*, 2007). The EMF taxa included in the library were selected from root-tip DNA that had yielded high quality sequences with at least 98% agreement over 500 base pairs to a named (genus or species) sequence originating from voucher specimens in the GenBank and UNITE databases (Twieg *et al*., 2007). The PCR products originally used for sequence verification of these taxa in the Twieg *et al.* (2007) study were re-amplified using fluorescently-labeled primers VIC-ITS1F and NED-ITS4 and digested with *Hinf*1 and *Hae*III, as described above.

For some PCR products, more than four different labeled fragments were produced when the fluorescent PCR products were digested. In these cases the original PCR product was cloned (pGEM-T Easy Vector System, Promega, USA). For each cloning reaction, at least five white colonies were picked and re-amplified using an unlabeled ITS1F/ITS4 primer set and the PCR protocol described above. These PCR products were then sequenced and the taxon identity verified by matching the aligned sequence with the NCBI database using the BLASTn algorithm (Altschul *et al.*, 1997). Clones that matched the original taxon identity by at least 97% were then re-processed for TRFLP analysis as described above.

3.2.5 Identification of EMF signatures within the TRFLP fingerprints

The TRAMPR program (Fitzjohn & Dickie, 2007) was used to identify EMF TRFLP signatures in the soil microsamples (TRAMP options: accept.error = 1.5, min.comb = 3). PeakScanner output files were loaded into TRAMPR using custom-written software (Fitzjohn, pers. comm.). An EMF TRFLP signature was counted as present if three of its four restriction fragments (VIC-*Hinf*1, NED-*Hinf*1, VIC-*Hae*III, and NED-*Hae*III) were observed in the microsample TRFLP fingerprint with an intensity of greater than 70 fluorescence units. Because partial signatures were used for identification, the TRAMPR option Group = Strict was used to identify partial signatures that matched more than one library EMF TRFLP signature.

3.2.6 Data analyses

Assemblages of EMF TRFLP signatures were constructed by placing the signatures identified in each microsample into 17 groups based on genera. All the TRFLP fingerprints and EMF assemblages obtained from the microsamples were then grouped *a priori* by phosphatase activity status (low, high), soil layer (organic, mineral), root window, or stand age-class, depending on the scale being examined. The null hypothesis of no difference between groups was tested using the multi-response permutation procedure (MRPP) (Mielke Jr & Berry, 2001) in PC-Ord (McCune & Mefford, 2005). For example, for each root window, fingerprints produced from microsamples taken from areas of high phosphatase activity were grouped together within each soil layer and compared to the fingerprints from microsamples taken from areas of low phosphatase activity for that soil layer (two to three fingerprints per group). MRPP is a nonparametric procedure that generates a measure of within-group agreement or effect size (A) and a test statistic that quantifies between-group difference (T). When inter-group heterogeneity is greater than expected by chance, then $T < 0$. A value of 1.0 for A indicates perfect intra-group similarity, with $A \approx 0.4$ considered an indication of good similarity (McCune & Grace, 2002). All MRPP were calculated using rank transformed, squared Euclidean distance matrices and group weighting of n/sum(n).

The grouping of root windows by forest age class was tested for robustness by comparing the average MRPP T and A statistics for four possible combinations of three of the four age-class groupings (C_4^3) against the average of 50 randomly constructed combinations

where each grouping contained sites from multiple age classes using a two-tailed Welch's *t*-test in JMP (JMP 7.0.1, SAS Institute Inc., 2007). Because the replicate stands for each age class were largely interspersed across the landscape, the effect of the geographic distance between stands was compared to the effect of age class detected by fine-scale sampling. (In other words, were stands of different age classes that were close to each other were more similar than they were to a more distant replicate stand?) The effect of geographic distance on the apparent between-group difference (*T*) was evaluated using Mantel's Asymptotic Approximation (Mantel, 1967) in PC-Ord. The effect of geographical distance between each pair of microsamples within a root window was also tested using the Mantel test, using the cluster analysis procedure in PC-Ord to generate a Euclidean distance matrix for TRFLP fingerprints and the assemblages of EMF TRFLP signatures present in each microsample in each root window.

Ectomycorrhizal TRFLP signatures were grouped by genera, and groups exhibiting strong spatial structure in relation to soil phosphatase activity were identified using Indicator Species Analysis (Dufrêne & Legendre, 1997) with 4999 Monte Carlo randomizations in PC-Ord. The number of occurrences of EMF signatures in fingerprints from high phosphatase microsites was contrasted with the number from low phosphatase microsites within each soil layer for each age class using Student's *t*-test in JMP. The frequency of *Tomentella* spp*.* and *Cortinarius* spp., in high verses low phosphatase microsites was evaluated by Correspondence Analysis in JMP.

3.3 Results

Of the 100 DNA samples from EMF on root-tips, 66 produced the four unique enzyme digest end products required for inclusion in the TRFLP database (Appendix B Table B-1). TRAMPR software detected partial signatures for 63 of these 66 EMF TRFLP signatures in the general fungal fingerprints of the soil microsamples. The three signatures present in the library that were not identified in the samples were *Atheliaceae* 1, *Lactarius* 1, *Russula* 1. Ectomycorrhizal signatures from the database were detected in 111 out of the 135 microsamples. The maximum number of EMF signatures detected in any sample was 37, with an average of 11 taxa detected per microsample.

3.3.1 Differences in fungal DNA signatures associated with fine-scale differences in phosphatase activity

The frequency of EMF signatures in imprint-guided microsamples differed between areas of high and low *in situ* phosphatase activity, and this relationship changed with age class and soil layer (Figure 3.4). On average, EMF signatures were detected more often in low than high phosphatase microsites in both the organic (Student's *t*-test; *P* = 0.05) and mineral (*P* = 0.08) layers of the Stem Exclusion age class and in the mineral layer of the Oldest age class (*P* = 0.04). By contrast, in the organic layers of the Oldest age class, more EMF signatures tended to be detected in high phosphatase microsites (Student's *t*-test; *P* = 0.06).

The most frequently detected EMF signatures across all stands were of *Cortinarius* spp. and *Tomentella* spp., and, as with the overall number of different EMF signatures, these signatures were generally associated with low rather than high phosphatase microsites. Signatures of *Cortinarius* spp. and *Tomentella* spp. were more frequent in low phosphatase microsites of the mineral layers of all age classes and in the organic layers of the younger three age classes (Chi square test; *P* < 0.01). When the EMF signatures were evaluated by Indicator Species Analysis, signatures of eight EMF species or genera appeared to associate with microsites with specific phosphatase activities in the older three age classes (Table 3.2). The signatures of *Amphinema byssoides* and *Xerocomus ferrugineus* were associated with high phosphatase microsites, while signatures of *Cortinarius* spp*., Hebeloma* spp., *Inocybe* spp*., Rhizopogon rudus*, *Thelephora* spp., and *Tomentella* spp. tended to be associated with low phosphatase microsites (Table 3.2). No EMF genera specifically were indicative of either low or high phosphatase activities in the Youngest age class.

Fine-scale structuring with respect to phosphatase activity was also detected within most windows using general fungal fingerprints or assemblages of EMF signatures. When microsamples from each soil layer of each window were grouped by phosphatase activity and analyzed by MRPP, assemblages of EMF signatures differed between high and low phosphatase microsites in the mineral layers of two windows (Table 3.3). When general fungal TRFLP fingerprints were considered, differences were found between high and low phosphatase

microsites in the mineral layers of six of the 12 windows and in the organic layer of one additional window (Table 3.3).

3.3.2 Differences in fungal DNA signatures associated with landscape-scale differences in forest age class

When the general fungal TRFLP fingerprints or EMF TRFLP signatures were grouped by forest age class, there was a significant difference between age classes (MRPP; $P < 0.001$ for both general fungal and EMF TRFLP signatures). The stand age effect for the general fungal fingerprints was relatively small ($A = 0.11$; $T = -22.04$; group size 31 to 35), but was found to be robust when compared to random groupings of root windows. The average T and A scores for groups comprised of windows from the same age class were significantly different from the average of the scores of groups with randomly chosen root windows (two-tailed Welch's t-test; $P = 0.01$ for T and p = 0.04 for A). This result verifies that groups of windows of the same age class were more uniform (e.g. larger A values) and more distinct from each other (e.g. smaller T values) than groups of randomly assigned windows. Furthermore, using Mantel's asymptotic approximation, no significant correlation was found between the physical distance between windows and the similarity of the TRFLP fingerprints between windows (as expressed by the MRPP T statistic), indicating that the stand age effect was greater than the effect of distance between windows. Compared to general fungal fingerprints, an even stronger forest age class effect was detected when EMF signatures were grouped by forest age ($A = 0.20$; group size = 23 to 32; $T = -16.94$; $P < 0.001$). As was found with the general fungal fingerprints, there was no significant correlation between the distance between windows and the similarity between EMF signatures grouped by window.

3.3.3 Differences in fungal DNA signatures associated with soil layer

While fine-scale soil sampling detected the landscape-scale effect of stand age, the detection of the effect of soil layer (organic vs. mineral) on fungal community structure was less clear. No significant differences were found when microsamples from organic soil were compared to mineral microsamples of the three replicate windows of an age class. However, at the finer scale within each window, general fungal fingerprints demonstrated spatial autocorrelation by soil layer within five of 12 windows, while EMF TRFLP signatures were

significantly correlated with soil layer within only two windows (See Appendix B, Table B-2). No differentiation by soil layer was found in any of the Youngest stands.

3.4   Discussion

One of the unique and significant findings of this study was that the frequency of EMF TRFLP signatures in high phosphatase microsites was lower than in low phosphatase microsites of almost all of the forest age classes. Several testable hypotheses follow from this observation. Because of the molecular method used, we cannot assume that the amount of EMF hyphal biomass was correlated with the number of EMF signatures. If EMF biomass was lower in microsites with high phosphatase activities than those with low activities, however, this result would be consistent with negative interactions between EMF and saprotrophs in high-resource microsites. Negative interactions between saprotrophic fungi and EMF have been demonstrated in microcosms (Lindahl *et al.*, 1999, 2001; Leake *et al.*, 2001) and implied by some (Cairney & Meharg, 2002 and references therein; Buée *et al.*, 2007; Lindahl *et al.*, 2007), but not all (Wallander *et al.*, 2006) field studies. In the presence of a saprotrophic fungus, especially one with access to a large supply of carbon, EMF hyphae appear to direct less carbon to mycelial fronts colonizing organic-rich patches and are less competitive in retaining mineral nutrients (Leake *et al.*, 2001; Lindahl *et al.*, 2001). Although it was not possible to measure the concentrations of nutrients in the microsamples without compromising DNA extraction, high-phosphatase microsites would be expected to be areas of enhanced microbial activity associated with the breakdown of organic matter (Olander & Vitousek, 2000, 2005; Sinsabaugh *et al.*, 1991, 1993).

A negative correlation between EMF biomass and phosphatase activities would also result if EMF suppressed other microbes that secrete phosphatases. Although several studies have shown that bacteria associated with ectomycorrhizal root-tips demonstrate an enhanced capacity to solubilize inorganic phosphorus when compared to bacteria from bulk soil (Frey-Klett *et al.*, 2005; Calvaruso *et al.*, 2007; Uroz *et al.*, 2007), this potential synergy may not be seen near EMF hyphae. My recent results indicate that EMF hyphae can suppress the occurrence of actinobacteria with higher phosphatase production capacities compared to

actinobacteria from bulk soil (Chapter 4). If suppression of bacteria with higher phosphatase activity correlated with lower bacterially mediated decomposition rates as well, then the presence of EMF hyphae could result in decreased decomposition as observed by Gadgil & Gadgil (1971).

In the TRFLP-based approach used in the current study, an EMF taxon was counted only once in each microsample, regardless of the relative biomass or number of individuals of that taxon. Therefore, we cannot discount the possibility that lower numbers of EMF signatures were associated with increased EMF biomass of those few species. The general pattern of lower frequency of EMF in high phosphatase microsites did not appear to result from monopolization of high phosphatase microsites by specialized EMF because there were only two, infrequent, EMF TRFLP signatures that were strongly associated with these microsites. The results could, however, be explained by priority effects. In this model, the first species to establish in a site dominates that location, excluding other species (Alford & Wilbur, 1985; Shorrocks & Bingley, 1984). This effect has been demonstrated for colonization of root tips by EMF (Parladé & Alvarez, 1993; Kennedy *et al.*, 2007, 2009), and it would not be surprising if it also occurred in nutrient-rich patches of soil. Ectomycorrhizal fungi rapidly colonize microsites containing partially decomposed organic material; translocate substantial amounts of newly acquired carbon to other parts of the mycelium; and release phosphatases and other enzymes (Leake *et al.*, 2001; Bending & Read, 1995b). This model would explain the results without invoking interactions with saprotrophs, and this would be consistent with the observations of Lindahl *et al.* (2007). Lindahl *et al.* (2007) demonstrated that saprotrophic TRFLP signatures were primarily confined to the upper litter layers in a boreal pine forest, with EMF TRFLP signatures dominating in the lower organic and mineral layers. In our study, the more particulate, upper litter layers were not sampled because there was typically not enough contact between the litter and the imprinting paper to create a spot at least 5 mm in diameter. However in the fermentation, humus, and mineral soil layers sampled in my study, EMF hyphae would be expected to be more prevalent than saprotrophic fungi and to be the dominant type of fungus encountered in resource-rich microsites.

The notable exception to the general relationship between EMF frequency and fine-scale phosphatase activities was a significant reduction in the number of EMF TRFLP signatures found in low phosphatase microsites in the organic layers of the Oldest stands. The organic layer of the Oldest stands had the narrowest C:N ratio of all the age classes (Twieg *et al.*, 2009). According to our first model, where negative saprotroph – EMF interactions explain the result, the reduced number of EMF signatures in low phosphatase microsites of the organic layer of the Oldest stand age class could be indicative of a stage in stand development where saprotrophic fungi no longer have access to sufficient carbon resources to exclude EMF from phosphorus-rich microsites. These results demonstrate the development of contrasting distribution patterns of EMF hyphae with relation to soil phosphatase between the organic and mineral soil layers as forests age.

Of the EMF TRFLP signature groups that could be used as indicators of microsite phosphatase status, most were indicative of low phosphatase activities. One of these was *Rhizopogon rudus*., which demonstrated low phosphatase activities associated with ectomycorrhizal mantle tissue when compared to two other common types of ectomycorrhizae (*Lactarius torminosus* and *Russula* sp.) from these sites using a root tip assay (Twieg *et al.*, 2009). In the mineral layer, the only signature indicative of high phosphatase microsites was *Xerocomus ferrugineus*, and this signature only acted as an indicator of high phosphatase activity in the Canopy Closure age class. This result corroborates the findings of Twieg *et al.* (2007), which identified *Xerocomus* spp. on root tips only in this stand age-class. Although phosphatase excretion cannot be attributed to *X. ferrugineus*, these results are consistent with earlier findings that *Xerocomus* spp. mycorrhizae maintain elevated phosphatase activities in their mycorrhizospheres and are able to acquire and store more phosphorus compared to mycorrhizae of other EMF species (Kottke *et al.*, 1998; Pritsch *et al.*, 2004; Rineau & Garbaye, 2009). Similarly, the signature of *Amphinema byssoides* was the only EMF that was indicative of high phosphatase microsites in the organic layer, and only acted as an indicator in the Oldest age class. *Amphinema* sporocarps and mycorrhizae are associated with coarse woody debris micro-habitats; especially with well rotted birch logs (Goodman & Trofymow, 1998; Tedersoo *et al.*, 2003, 2008). Therefore, its association with phosphatase active microsites in the organic

layer may be connected to the decomposition of coarse woody debris of birch on the Oldest stands.

It is important to emphasize that while TRFLP signatures of EMF from our site-specific library were identified within general fungal TRFLP fingerprints in each microsample, these identifications cannot be considered absolute. Only three of four identifying fragments of an EMF TRFLP signature were required for a taxon to be identified, and there are known issues in using TRFLP to identify EMF in soil (reviewed by Anderson & Cairney, 2004). Not only can the amplification of spore DNA and/or differential amplification efficiencies between EMF species bias the outcome of any PCR reaction (Avis *et al.*, 2006), there is also the possibility that a TRFLP signature ascribed to an EMF taxon may also be shared by a saprotroph or microfungus. Despite these limitations, TRFLP has proven useful for detecting differences in the distribution of EMF species present in soil as hyphae (Dickie *et al.*, 2002, 2009; Koide *et al.*, 2005) and was effective in this study for detecting differences in the frequency of EMF TRFLP signatures with relation to the phosphatase status of soil microsites. Our results demonstrate that fine-scale sampling of 20 cm X 20 cm areas of soil profile can detect differences in fungal assemblages associated with landscape-scale (tens to hundreds of kilometers) changes in vegetation. While the effect of stand age was detected with general fungal community fingerprints, it was detected even more strongly when EMF TRFLP signatures were considered, indicating that EMF are more strongly affected by stand age and are better indicators of forest development than other fungi.

While it is important to emphasize that this fine-scale sampling protocol was not designed to characterize the entire composition or distribution of fungal communities in these stands, these results make an important contribution to the expansion of fine-scale molecular methods to large-scale ecological questions, such as recommended by Schimel & Bennett (2004). Most previous studies using molecular methods to identify fungal hyphae in soil have focused on characterizing communities present within soil cores taken from homogenous stands and did not address landscape-scale questions (Dickie *et al.*, 2002; Koide *et al.*, 2005; Genney *et al.*, 2006). However, our findings are supported by recent work by Dickie *et al.* (2009) where TRFLP was used to compare fungal communities in soils of forests exposed to different

harvesting regimes. In that study, EMF community signatures were also found to be highly diverse at a local scale, but relatively similar between replicate stands.

A high level of diversity and heterogeneous distribution at fine spatial scales is characteristic of EMF, as both mycorrhizae (Rosling *et al.*, 2003; Lilleskov *et al.*, 2004; Izzo *et al.*, 2005; Pickles *et al*., 2010) and as hyphae (Dickie *et al.*, 2002; Landeweert *et al.*, 2003; Genney *et al.*, 2006). The community composition of EMF mycorrhizae can be highly variable at the scale of 5-20 cm (Rosling *et al.*, 2003; Gebhardt *et al.*, 2009), with a complete change in EMF species composition as mycorrhizae seen at scales as small as 50 cm (Tedersoo *et al.*, 2003). This may explain why differences in the general fungal community fingerprints or assemblages of EMF signatures were detected at the scale of a 20 cm X 20 cm window, even with only three microsamples from each phosphatase activity level per soil layer, but similar differences were not detected when data were combined across sites. The finding that the assemblages of EMF TRFLP signatures differed in relation to soil phosphatase activity at a fine scale rather than at a larger scale agrees with the results of Courty *et al.* (2005), who found that enzyme activities associated with the mycorrhizae of single EMF species were quite consistent at the centimeter scale, but varied significantly at the meter scale. The stratification of EMF hyphae by soil layer has also been well established (Landeweert *et al.*, 2003; Koide *et al.*, 2005; Genney *et al.*, 2006). Given the success of the microsampling technique in detecting the large-scale effect of stand age, it would be expected that differences in assemblage composition between soil layers would be detected as well. But when the organic microsamples were compared to the mineral microsamples from the three replicate windows of an age class, a soil layer effect was seen in only one age class. However, differences between organic and mineral layers emerged when fingerprints or EMF signatures were examined at a finer scale within each window. Despite limitations due to the irregular sampling pattern imposed by sampling for locations of high and low phosphatase across the soil profile, stratification of the soil fungal community by soil layer was revealed in all age classes except the Youngest. An area of further research will be to explore whether stratification of the EMF community in forest soil changes after stand replacing fire or clear-cutting.

By demonstrating significant differences in the frequency of EMF TRFLP signatures with respect to the phosphatase status of soil microsites across a chronosequence of forest stands, this study takes an important step toward characterizing the functional role of EMF in nutrient mobilization in the field. A number of hypotheses have been generated about interactions between EMF and other soil microorganisms to explain these unique results. These hypotheses could now be tested using newly-available high-throughput sequencing approaches. The new approach of coupling an *in situ* imprint method for localizing phosphatase with immediate, millimeter-scale soil sampling was capable of detecting the fine-scale effect of microsite phosphatase status as well as the landscape-scale effect of forest age class on the structure of general fungal and EMF communities. By connecting EMF associated with a fine-scale soil process to the ecosystem process of forest regeneration, this study helps elucidate one component of the belowground interactions that may be involved in fine-scale soil spatial heterogeneity. This understanding is essential for the modeling of fine-scale soil processes and for determining how these processes affect ecosystems at a landscape-scale (Schimel & Bennett, 2004; Jones *et al.*, 2005; Hobbie & Hobbie, 2008).

3.5　Figures and tables

Figure 3.1 Imprinting soil enzyme activity using a root window to access the soil profile. (a) acrylic root window installed with trap door open; (b) Enzyme imprinting paper attached to Mylar™ plastic backing sheet, then sheet aligned with edges of trap door; (c) Trap door closed, pressing imprinting paper against soil profile; (d) Imprinted paper with attached backing removed for processing.
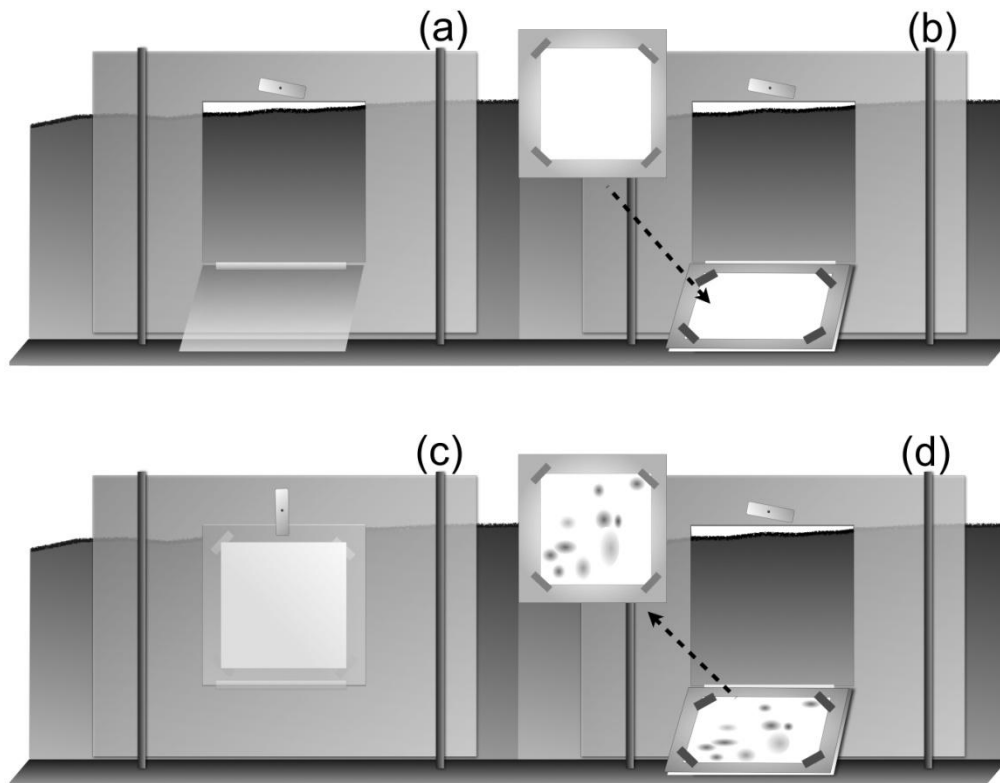
Figure 3.2 Processing of imprint and plastic guide for fine-scale soil enzyme sampling. (a) Enzyme imprint with attached plastic backing; (b) Areas of high and low enzyme activity marked for selection; (c) Holes punched through backing at selected locations; (d) Plastic backing attached to reinforcing poster board frame (imprinting paper removed).
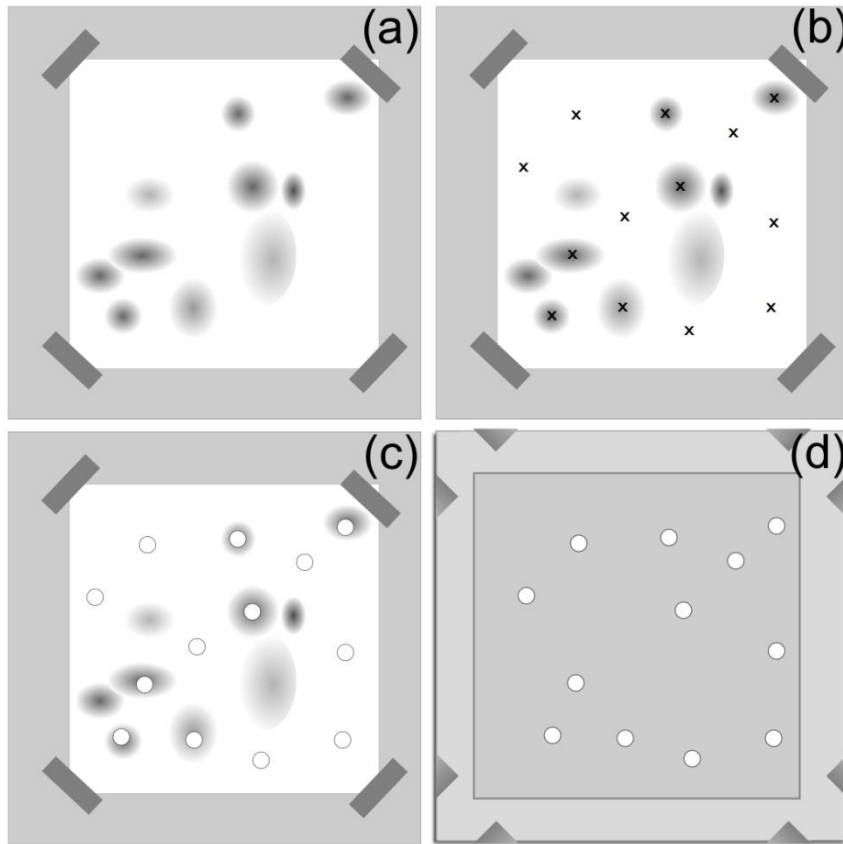
Figure 3.3 Fine-scale soil enzyme sampling. Punched plastic sheet attached to reinforcing frame replaced in the trap door opening. Small soil samples taken with forceps either through the punched holes or locations marked through holes for sampling after the guide is removed.
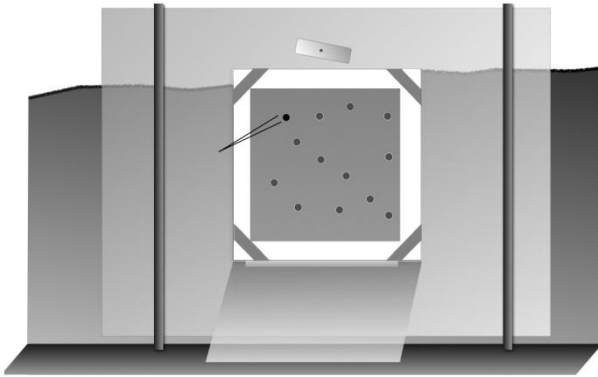
Figure 3.4 Frequency of ectomycorrhizal fungal TRFLP signatures for three replicate windows of each forest age class, grouped by microsite phosphatase status within soil layers (organic or mineral). High phosphatase microsites are indicated by black bars, and low phosphatase microsites by grey bars. The four forest age classes are indicated as Youngest (YG), Canopy Closure (CC), Stem Exclusion (SE), Oldest (OD). Significant differences in the average frequency of EMF signatures between high and low phosphatase microsites within each age class (Student's t-test) are indicated (** for $P \le 0.05$; * for $P \le 0.1$). Error bars represent one SE of the mean.
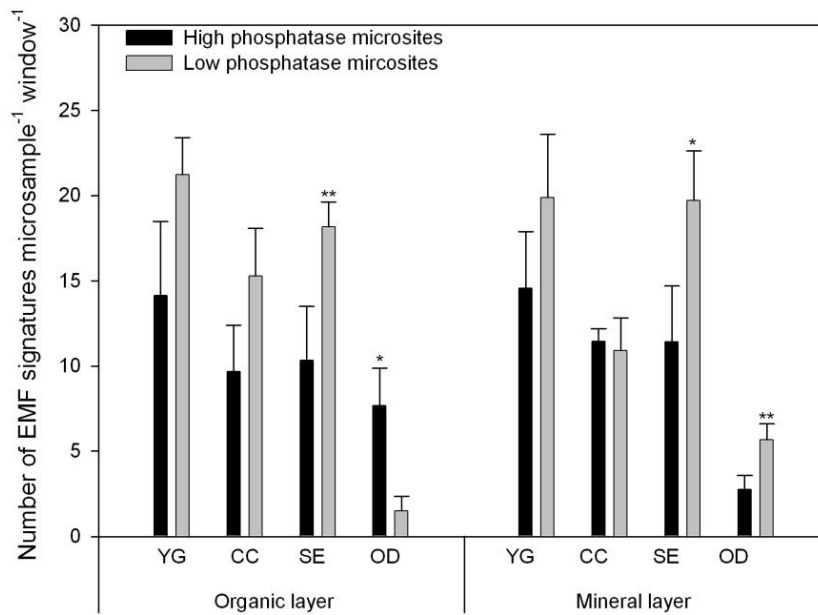
Table 3.1 Site locations and characteristics of a chronosequence of Douglas-fir/paper birch stands in the southern interior of British Columbia, Canada. Interior Cedar-Hemlock biogeoclimatic variants are defined in Lloyd *et al.* (1990). Table modified from Twieg *et al.* (2007). Used with permission.

Age class (Site code)	Age (yr)	Stand initiation type	ICH variant	Elevation (m)	Latitude/ longitude	Crown closure (%)	Soil texture[a]	Tree species composition (%)[b]		
								Douglas-fir	Paper birch	Other ECM broadleaf spp.
Young (AL)	6	clearcut	mw2	600	N 50° 32' 43" W 118° 52' 49"	45	SL	25	50	7.5
Young (IDA3)	5	wildfire	mw2	650	N 50° 40' 00" W 119° 18' 57"	35	SL	7.5	80	2.5
Young (WL)	5	clearcut	mw3	700	N 50° 53' 51" W 119° 16' 27"	50	fSL	10	70	2.5
Canopy closure (ED2)	30	wildfire	mk2	1000	N 50° 44' 12" W 119° 22' 17"	85	SiL	35	45	5
Canopy closure (MA1)	24	wildfire	mw3	930	N 50° 55' 20" W 118° 50' 41"	70	SL	40	45	10
Canopy closure (MA2)	24	wildfire	mw3	975	N 50° 55' 3" W 118° 50' 56"	70	SL	45	40	7.5
Stem exclusion (BA)	63	wildfire	mw2	700	N 50° 34' 03" W 118° 50' 50"	85	SL	45	50	0
Stem exclusion (MARA)	71	wildfire	mw2	600	N 50° 39' 28" W 119° 03' 49"	80	SL	45	50	0
Stem exclusion (RR)	61	wildfire	mw2	800	N 50° 41' 55" W 118° 46' 07"	75	SL	40	60	0
Older (4WD)	103	wildfire	mw2	550	N 50° 36' 47" W 118° 50" 26"	90	SL	50	45	0
Older (ACR)	98	wildfire	mw2	600	N 50° 37' 25" W 118° 46' 06"	80	LS	55	30	0
Older (WAP)	90	wildfire	mw2	650	N 50° 45' 1" W 118° 34' 12"	80	SL	50	40	0

[a] SiL, silty loam; SL, sandy loam; SCL, sandy clay loam; LS, loamy sand; f, fine; vf, very fine.

[b] Tree species compositional percentages were estimated as each species' or group's proportion of the total estimated canopy cover.

Table 3.2 Ectomycorrhizal fungal TRFLP signature groups that correlated with microsite phosphatase status by indicator species analysis using Monte Carlo randomization. $P \leq 0.05$ indicates significant correlation between microsite phosphatase status and an ectomycorrhizal fungal TRFLP signature.

Age class	Stand age (yrs)	Soil layer	Microsite phosphatase	Genus or species group	P
Youngest[a]	5-6				
Canopy closure	24-30	Mineral	Low	*Cortinarius* spp.	0.08
			Low	*Hebeloma* spp.	0.10
			Low	*Inocybe*	0.03
			High	*Xerocomus ferrugineus*	0.03
Stem exclusion	61-71	Organic	Low	*Thelephora* spp.	0.04
			Low	*Tomentella* spp.	0.02
		Mineral	Low	*Rhizopogon rudus*	0.10
Oldest	90-103	Organic	High	*Amphinema byssoides*	0.04

[a] No ectomycorrhizal fungal TRFLP signature groups were correlated with microsite phosphatase status in the Youngest age class

Table 3.3 Comparison of general fungal TRFLP fingerprints or assemblages of ectomycorrhizal fungal (EMF) TRFLP signatures between high and low phosphatase microsites using non-parametric MRPP analysis. Groups of two to three microsamples were compared using rank transformed, squared Euclidean distances. $P \leq 0.05$ indicates that the composition of the general fungal TRFLP fingerprints or assemblages of EMF signatures differed between high and low phosphatase microsites within a soil layer of a window.

Age class	Stand age (yrs)	Window ID[a]	Soil layer	General fungal TRFLP fingerprints			EMF TRFLP signatures		
				MRPP			MRPP		
				T[b]	*A*[c]	*P*	*T*	*A*	*P*
Youngest	5-6	IDA	Mineral	-2.79	0.27	0.02	-	-	-
Canopy closure	24-30	ED2	Organic	-2.46	0.31	0.03	-	-	-
		MA2 MA1	Mineral	-1.76	0.28	0.05	-2.62	0.38	0.02
Stem exclusion	61-71	SL	Mineral	-1.61	0.14	0.09	-2.27	0.15	0.02
		RR	Mineral	-1.95	0.19	0.04	-	-	-
Older	90-103	ACR	Mineral	-2.46	0.11	0.03	-	-	-
		WAP	Mineral	-1.14	0.14	0.01	-	-	-

[a] Windows are named according to the forest stands in which they were located, using an abbreviation of the original naming convention developed for the stands by Twieg et al. (2007).
[b] When inter-group heterogeneity is greater than expected by chance, $T < 0$.
[c] When within-group agreement is strong, $A \approx 0.4$ (McCune & Grace, 2002).

3.6   Bibliography

Alford RA, Wilbur HM. 1985. Priority effects in experimental pond communities: competition between *Bufo* and *Rana. Ecology* **66:** 1097-1105.

Allison SD. 2006. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. *Biogeochemistry* **81:** 361-373.

Altschul SF, Madden TL, Zhang Z, Miller W, Lipman CJ. 1997. Gapped BLAST and PSI-BLAST – a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

Alvarez M, Godoy R, Heyser W, Härtel S. 2005. Anatomical-physiological determination of surface bound phosphatase activity in ectomycorrhizae of *Nothofagus obliqua. Soil Biology & Biochemistry* **37:** 125-132.

Anderson IC, Cairney JW. 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* **6:** 769-779.

Avis PG, Dickie IA, Mueller GM. 2006. A 'dirty' business: testing the limitations of terminal restriction fragment length polymorphism (TRFLP) analysis of soil fungi. *Molecular Ecology* **15:** 873-882.

Bending GD, Read DJ. 1995a. The structure and function of the vegetative mycelium of ectomycorrhizal plants. VI. Activities of nutrient mobilizing enzymes in birch litter colonized by *Paxillus involutus* (Fr.) Fr. *New Phytologist* **130:** 411-417.

Bending GD, Read DJ. 1995b. The structure and function of the vegetative mycelium of ectomycorrhizal plants.V. Foraging behaviour and translocation of nutrients from exploited litter. *New Phytologist* **130:** 401-409.

Brandes B, Godbold DL, Kuhn AJ, Jentschke G. 1998. Nitrogen and phosphorus acquisition by the mycelium of the ectomycorrhizal fungus *Paxillus involutus* and its effect on host nutrition. *New Phytologist* **140:** 735-743.

Buée M, Courty PE, Mignot D, Garbaye J. 2007. Soil niche effect on species diversity and catabolic activities in an ectomycorrhizal fungal community. *Soil Biology and Biochemistry* **39:** 1947-1955.

Calvaruso C, Turpault MP, Leclerc E, Frey-Klett P. 2007. Impact of ectomycorrhizosphere on the functional diversity of soil bacterial and fungal communities from a forest stand in relation to nutrient mobilization processes. *Microbial Ecology* **54:** 567-577.

Cairney JWG, Meharg AA. 2002. Interactions between ectomycorrhizal fungi and soil saprotrophs: implications for decomposition of organic matter in soils and degradation of organic pollutants in the rhizosphere. *Canadian Journal of Botany/Revue Canadienne de Botanique* **80:** 803-809.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167:** 309-319.

Cullings K, Ishkhanova G, Henson J. 2008. Defoliation effects on enzyme activities of the ectomycorrhizal fungus *Suillus granulatus* in a *Pinus contorta* (lodgepole pine) stand in Yellowstone National Park. *Oecologia* **158:** 77-83.

Dickie IA, Richardson SJ, Wiser SK. 2009. Ectomycorrhizal fungal communities and soil chemistry in harvested and unharvested temperate *Nothofagus* rainforests. *Canadian Journal of Forest Research* **39:** 1069-1079.

Dickie IA, Xu B, Koide RT. 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist* **156:** 527-535.

Dinkelaker B, Marschner H. 1992. *In vivo* demonstration of acid phosphatase activity in the rhizosphere of soil-grown plants. *Plant and Soil* **144:** 199-205.

Dong S, Brooks D, Jones MD, Grayston SJ. 2007. A method for linking *in situ* activities of hydrolytic enzymes to associated organisms in forest soils. *Soil Biology and Biochemistry* **39:** 2414-2419.

Dorodnikov M, Blagodatskaya E, Blagodatsky S, Marhan S, Fangmeier A, Kuzyakov Y. 2009. Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. *Global Change Biology* **15:** 1603-1614.

Dufrêne M, Legendre P. 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecological Monographs* **67:** 345-366.

Fitzjohn RG, Dickie IA. 2007. TRAMPR: An R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. *Molecular Ecology Notes* **7:** 583-587.

Frey-Klett P, Chavatte M, Clausse ML, Courrier S, Le Roux C, Raaijmakers J, Martinotti MG, Pierrat JC, Garbaye J. 2005. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. *New Phytologist* **165:** 317-328.

Gadgil RL, Gadgil PD. 1971. Mycorrhia and litter decomposition. *Nature* **233:** 133.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113-118.

Gebhardt S, Wöllecke J, Münzenberger B, Hüttl RF. 2009. Microscale spatial distribution patterns of red oak (*Quercus rubra L*.) ectomycorrhizae. *Mycological Progress* **8:** 245-257.

Genney DR, Anderson IC, Alexander IJ. 2006. Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytologist* **170:** 381-390.

Godbold DL, Hoosbeek MR, Lukac M, Cotrufo MF, Janssens IA, Ceulemans R, Polle A, Velthorst EJ, Scarascia-Mugnozza G, De Angelis P, *et al.* 2006. Mycorrhizal hyphal turnover as a dominant process for carbon input into soil organic matter. *Plant and Soil* **281:** 15-24.

Goodman DM, Trofymow JA. 1998. Distribution of ectomycorrhizas in micro-habitats in mature and old-growth stands of Douglas-fir on southeastern Vancouver Island. *Soil Biology & Biochemistry* **30:** 2127-2138.

Grierson PF, Comerford NB. 2000. Non-destructive measurement of acid phosphatase activity in the rhizosphere using nitrocellulose membranes and image analysis. *Plant and Soil* **218:** 49-57.

Hobbie EA, Hobbie JE. 2008. Natural abundance of ^{15}N in nitrogen-limited forests and tundra can estimate nitrogen cycling through mycorrhizal fungi: a review. *Ecosystems* **11:** 815-830.

Högberg MN, Högberg P. 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **154:** 791-795.

Izzo A, Agbowo J, Bruns TD. 2005. Detection of plot-level changes in ectomycorrhizal communities across years in an old-growth mixed-conifer forest. *New Phytologist* **166:** 619-630.

Jones DL, Healey JR, Willett WB, Farrar JF, Hodge A. 2005. Dissolved organic nitrogen uptake by plants - an important N uptake pathway? *Soil Biology & Biochemistry* **37:** 413-423.

Kennedy PG, Hortal S, Bergemann SE, Bruns TD. 2007. Competitive interactions among three ectomycorrhizal fungi and their relation to host plant performance. *Ecology* **95:** 1338-1345.

Kennedy PG, Peay KB, Bruns TD. 2009. Root tip competition among ectomycorrhizal fungi: Are priority effects a rule or an exception? *Ecology* **90(8):** 2098-2107.

Koide RT, Xu B, Sharda J, Lekberg Y, Ostiguy N. 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* **165:** 305-316.

Kottke I, Qian XM, Pritsch K, Haug I, Oberwinkler F. 1998. *Xerocomus badius – Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. *Mycorrhiza* **7:** 267-275.

Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E. 2003. Molecular identification of ectomycorrhizal mycelium in soil horizons. *Applied and Environmental Microbiology* **69:** 327-333.

Leake JR, Donnelly DP, Saunders EM, Boddy L, Read DJ. 2001. Rates and quantities of carbon flux to ectomycorrhizal mycelium following C-14 pulse labeling of *Pinus sylvestris* seedlings: effects of litter patches and interaction with a wood-decomposer fungus. *Tree Physiology* **21:** 71-82.

Lilleskov EA, Bruns TD, Horton TR, Taylor D, Grogan P. 2004. Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities. *FEMS Microbiology Ecology* **49:** 319-332.

Lindahl B, Stenlid J, Finlay R. 2001. Effects of resource availability on mycelial interactions and ^{32}P transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. *FEMS Microbiology Ecology* **38:** 43-52.

Lindahl B, Stenlid J, Olsson S, Finlay R. 1999. Translocation of P-32 between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. *New Phytologist* **144:** 183-193.

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD. 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist* **173:** 611-620.

Litton CM, Giardina CP. 2008. Below-ground carbon flux and partitioning: global patterns and response to temperature. *Functional Ecology* **22:** 941-954.

Lloyd D, Angrove K, Hope G, Thompson C. 1990. *A guide to site identification and interpretation for the Kamloops forest region.* Victoria, B.C.: Research Branch, B.C. Ministry of Forests.

Lucas RW, Casper BB. 2008. Ectomycorrhizal community and extracellular enzyme activity following simulated atmospheric N deposition. *Soil Biology & Biochemistry* **40:** 1662-1669.

Lucas RW, Casper BB, Jackson JK, Balser TC. 2007. Soil microbial communities and extracellular enzyme activity in the New Jersey Pinelands. *Soil Biology and Biochemistry* **39:** 2508-2519.

Luis P, Kellner H, Martin F, Buscot F. 2005. A molecular method to evaluate basidiomycete laccase gene expression in forest soils. *Geoderma* **128:** 18-27.

Mantel N. 1967. The detection of disease clustering and generalized regression approach. *Cancer Research* **27:** 77-89.

Martin KJ, Rygiewicz PT. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology* **5:** 28.

McCune B, Grace JB. 2002. *Analysis of ecological communities.* Gleneden Beach, Oregon, U.S.A.: MjM Software Design.

McCune B, Mefford J. 2005. *PC-Ord for Windows: multivariate analysis of ecological data version 5.* Gleneden Beach, Oregon, U.S.A.: MJM Software Design.

Mielke Jr PW, Berry KJ. 2001. *Permutation methods: a distance function approach.* New York, USA: Springer.

Nygren CMR, Rosling A. 2009. Localisation of phosphomonoesterase activity in ectomycorrhizal fungi grown on different phosphorus sources. *Mycorrhiza* **19:** 197-204.

Olander LP, Vitousek PM. 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry* **49:** 175-190.

Olander LP, Vitousek PM. 2005. Short-term controls over inorganic phosphorus during soil and ecosystem development. *Soil Biology and Biochemistry* **37:** 651-659.

Oliver CD, Larson BC. 1996. *Forest stand dynamics.* New York, NY, USA: Wiley & Sons, Inc.

Parladé J, Alvarez IF. 1993. Coinoculation of aseptically grown Douglas fir with pairs of ectomycorrhizal fungi. *Mycorrhiza* **3:** 93-96.

Pickles BJ, Genney DR, Potts JM, Lennon JJ, Anderson IC, Alexander IJ. 2010. Spatial and temporal ecology of Scots pine ectomycorrhizas. *New Phytologist* (Early Online). doi: 10.1111/j.1469-8137.2010.03204.x

Pritsch K, Raidl S, Marksteiner E, Blaschke H, Agerer R, Schloter M, Hartmann A. 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* **58:** 233-241.

Rineau F, Garbaye J. 2009. Does forest liming impact the enzymatic profiles of ectomycorrhizal communities through specialized fungal symbionts? *Mycorrhiza* **19:** 493-500.

Rosling A, Landeweert R, Lindahl BD, Larsson KH, Kuyper TW, Taylor AFS, Finlay RD. 2003. Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytologist* **159:** 775-783.

Schimel JP, Bennett J. 2004. Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **85:** 591-602.

Shorrocks B, Bingley M. 1994. Priority effects and species coexistence: experiments with fungal-breeding *Drosophila*. *Journal of Animal Ecology* 799-806.

Sinsabaugh RL, Antibus RK, Linkins AE. 1991. An Enzymatic Approach to the Analysis of Microbial Activity during Plant Litter Decomposition. *Agriculture Ecosystems & Environment* **34:** 43-54.

Sinsabaugh RL, Antibus RK, Linkins AE, McClaugherty CA, Rayburn L, Repert D, Weiland T. 1993. Wood Decomposition - Nitrogen and Phosphorus Dynamics in Relation to Extracellular Enzyme-Activity. *Ecology* **74:** 1586-1593.

Talbot JM, Allison SD, Treseder KK. 2008. Decomposers in disguise: mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. *Functional Ecology* **22:** 955-963.

Tedersoo L, Koljalg U, Hallenberg N, Larsson KH. 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. *New Phytologist* **159:** 153-165.

Tedersoo L, Suvi T, Jairus T, Kõljalg U. 2008. Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of *Picea abies* and *Betula pendula*. *Environmental Microbiology* **10:** 1189-1201.

Timonen S, Jorgensen KS, Haahtela K, Sen R. 1998. Bacterial community structure at defined locations of *Pinus sylvestris Suillus bovinus* and *Pinus sylvestris Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. *Canadian Journal of Microbiology* **44:** 499-513.

Twieg BD, Durall DM, Simard SW. 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* **176:** 437-447.

Twieg BD, Durall DM, Simard SW, Jones MD. 2009. Influence of soil nutrients on ectomycorrhizal communities in a chronosequence of mixed temperate forests. *Mycorrhiza* **19:** 305-316.

Uroz S, Calvaruso C, Turpault MP, Pierrat JC, Mustin C, Frey-Klett P. 2007. Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. *Applied and Environmental Microbiology* **73:** 3019-3027.

van der Heijden MG, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* **11:** 296-310.

Wallander H, Fossum A, Rosengren U, Jones H. 2005. Ectomycorrhizal fungal biomass in roots and uptake of P from apatite by *Pinus sylvestris* seedlings growing in forest soil with and without wood ash amendment. *Mycorrhiza* **15:** 143-148.

Wallander H, Lindahl BD, Nilsson LO. 2006. Limited transfer of nitrogen between wood decomposing and ectomycorrhizal mycelia when studied in the field. *Mycorrhiza* **16:** 213-217.

Wallander H, Nilsson LO, Hagerberg D, Bååth E. 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytologist* **151:** 753-760.

Wallenstein MD, Weintraub MN. 2008. Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. *Soil Biology and Biochemistry* **40:** 2098-2106.

Zadworny M, Smolinski D, Idzikowska K, Werner A. 2007. Ultrastructural and cytochemical aspects of the interaction between the ectomycorrhizal fungus *Laccaria laccata* and the saprotrophic fungi*, Trichoderma harzianum* and *T. virens*. *Biocontrol Science and Technology* **17:** 921-932.

4 Ectomycorrhizal hyphae interact with soil bacteria to reduce organic phosphorus mobilization[3]

4.1 Introduction

The hyphae of ectomycorrhizal fungi (EMF) are the most poorly understood and studied component of the ectomycorrhizal symbiosis (Staddon *et al.*, 2003; Cairney, 2005; Anderson & Cairney, 2007). EMF hyphae are microscopic and intermingled with those of closely related saprotrophic fungi (Hibbett *et al.*, 2000). However, hyphae act as the main nutrient-absorbing interface between EMF and the soil (Smith & Read, 1997), encompassing a large absorptive area ranging from 3–600 m cm^3 of soil (reviewed by Leake *et al.*, 2004) and accounting for at least a third of the total microbial biomass in boreal forest soil (Högberg & Högberg, 2002). Read and Perez-Moreno (2003) have estimated that 15% of the phosphorus (P) supplied to trees is mobilized by EMF hyphae. Mycorrhizal hyphae not only absorb P from the soil solution, but also extract inorganic P by actively weathering soil minerals (Cumming & Weinstein, 1990; van Breemen *et al.*, 2000; Wallander, 2000b, 2000a; Wallander & Hagerberg, 2004; Wallander & Pallon., 2005) and mobilize P from organic compounds through the production of phosphatases (Leake *et al.*, 1997; Smith & Read, 1997; Perez-Moreno & Read, 2001; Koide, 2003; Read & Perez-Moreno, 2003; Read *et al.*, 2004). Therefore, hyphal activity has the potential to influence forest productivity.

There is considerable evidence that EMF influence the taxonomic composition of communities of soil bacteria, although this effect can vary between mycorrhizae of different EMF species and between different root-tips colonized with the same EMF species (Burke *et al.*, 2008; Kataoka *et al.*, 2008). In intact mycorrhizospheres of *Pinus sylvestris-Paxillus involutus* and *Pinus sylvestris-Suillus bovinus*, bacteria formed relatively sparse micro-colonies on hyphal

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[3] A version of this chapter will be submitted for publication. **Brooks D, Chan R, Starks ER, Grayston SJ, Jones MD. 2010.** Ectomycorrhizal hyphae interact with soil bacteria to reduce organic phosphorus mobilization.

strands and mycorrrhizae, but aggregated to form biofilms on extending hyphal tips (Nurmiaho-Lassila *et al.*, 1997). Furthermore, the bacterial numbers near extramatrical hyphae foraging in forest soil are three to four times higher than numbers near mycorrhizal roots (Timonen & Hurek, 2006). The proportion of Proteobacteria in the bacterial communities associated with hyphae, ectomycorrhizal root tips, and non-mycorrhizal short roots of pine is much higher than in bulk soil (Timonen & Hurek, 2006); however, Gram-positive bacteria can be most prevalent near hyphal fans (Timonen *et al.*, 1998). Gram-positive bacteria accounted for 80% of the bacteria isolated from the hyphae of ectomycorrhizal pine seedlings growing on dry forest humus compared to 40% near mycorrhizae and 50% in bulk forest soil (Timonen *et al.*, 1998), indicating that spore-forming, Gram-positive bacteria can be an important component of the bacterial community associated with EMF hyphae in forest soil.

The physiological characteristics of bacterial communities are also influenced by their proximity to EMF, and this effect can vary depending on the portion of the symbiosis (ectomycorrhiza, extramatrical mycelia, or hyphal fan) with which the bacteria are associated. For example, bacterial communities associated with EMF hyphae utilize fungal sugars such as trehalose and mannitol as energy sources more effectively than bacterial communities originating from bulk soil (Frey *et al.*, 1997; Timonen *et al.*, 1998; Duponnois & Kisa, 2006). Furthermore, Gram-positive bacteria isolated from fine hyphae demonstrated an enhanced ability to utilize chitin in the form of *N*-acetyl glucosamine than bacteria from other compartments of the symbiosis or from bulk soil (Timonen *et al.*, 1998). Bacteria isolated from ectomycorrhizosphere soil and the surface of ectomycorrhizal roots can have enhanced capacities for inorganic phosphorus mobilization (e.g. produce more mineral destabilizing organic acids) (Calvaruso *et al.*, 2007; Uroz *et al.*, 2007).

The effect of the interaction between bacteria and EMF on nutrient mobilization is unclear, especially when the interactions occur in the extramatrical part of the association. Enhanced phosphorus mobilization by bacteria in the mycorrhizosphere has been proposed as a possible mechanism for positive synergy between bacteria and EMF (Timonen & Hurek, 2006; Calvaruso *et al.*, 2007; Uroz *et al.*, 2007). However, while bacterial communities near mycorrhizae may be structured for enhanced mobilization of inorganic phosphorus, there is no

clear evidence that EMF select for particular bacterial species near mycorrhizal root tips. The enhanced mobilization of inorganic phosphorus by bacteria within and adjacent to mycorrhizal root tips may result from general competitive interactions between bacterial strains as they are excluded from resources by EMF. According to de Boer (2005), the hyphal growth habit of some Actinobacteria, which are commonly encountered near ectomycorrhizal hyphae, would make them the strongest competitors to hyphal strand-forming fungi because these bacteria can physically access the same nutrient resources in soil. Soil bacteria can also interact negatively with fungal hyphae, degrading fungal cell walls through the production of *N*-acetyl glucosaminidase (NAGase or chitinase) (Höppener-Ogawa *et al.*, 2007; Bhattacharya *et al.*, 2007; Fritsche *et al.*, 2008; Xiao *et al.*, 2009). Bacterial NAGase production is also stimulated by both chitin addition and hyphal growth (de Boer *et al.*, 1999, 2001), indicating that soil bacteria have the potential to mobilize nitrogen from fungal cell wall material and, possibly, directly from growing hyphae.

Ectomycorrhizal fungal hyphae preferentially penetrate and explore nutrient-poor soil and sand compared to the hyphae of saprotrophic fungi (Wallander *et al.*, 2001; Nilsson & Wallander, 2003). Therefore, field studies using sand-filled mesh bags enable investigation of the bacterial community associated with EMF hyphae *in situ.* Korkama *et al*. (2007) used sandbags that allowed ingrowth of hyphae while excluding roots to access the bacterial community associated with the EMF hyphae of different clones of Norway spruce. More Gram-positive bacteria were associated with the hyphae of fast-growing ectomycorrhizal spruce compared to slower growing spruce. However, the functional characteristics of bacteria associated with hyphae trapped using ingrowth sandbags has not been investigated.

In this study, six stands of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and paper birch (*Betula papyrifera* Marsh) in the southern interior of British Columbia, Canada, which had regenerated naturally after stand-replacing wildfire or clear cutting and ranging in age from 6 to 101 years, were used to test the effectiveness of sand-filled mesh bags for allowing or excluding the ingrowth of hyphae. This was tested using quantitative real-time PCR (qPCR) of fungal ribosomal ITS DNA. Bags were also incubated at an additional 60-yr-old stand). The enzyme activities of bacteria isolated from hyphae ingrowth bags were compared with the

activities of bacteria isolated from hyphae exclusion bags. This study addressed two hypotheses and predictions. *i*) If bacteria interact synergistically with EMF hyphae to mobilize phosphorus from organic matter, extracellular phosphatase production is expected to be higher in bacteria from bags containing hyphae compared to bacteria from bags excluding hyphae. *ii*) If bacteria interact opportunistically with hyphae by mobilizing nitrogen from chitin in fungal cell walls, bacterial NAGase production would provide a benefit to nearby bacteria, selecting for higher potential NAGase activity in bacteria isolated from bags containing hyphae versus bacteria from bags excluding hyphae.

4.2 Methods

4.2.1 Site description

The study was conducted in the southern interior of British Columbia at a series of forest stands selected for similar site characteristics. The sites ranged from 600 to 1000 m elevation in two moist, cool variants (ICHmk2 and ICHmk3) of the Interior Cedar-Hemlock (ICH) biogeoclimatic zone (Lloyd *et al.*, 1990) on sandy or silty loam soils. While classified as a moist, cool region, the period June through September is typically warm and dry. Mean daily temperatures range from -4.9 °C in January to 17.9 °C in July. The average annual precipitation is 628.3 mm, which falls predominately as rain during May to July (http://www.climate.weatheroffice.ec.gc.ca; accessed January 10, 2010). These forests had regenerated to Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and paper birch (*Betula papyrifera* Marsh) after a stand-replacing wildfire or after clearcut logging. These sites have been described by Twieg *et al.* (2007) and labeled in that study as: 19MR, ED1, MA1, MA2, RR, BBP, and MARA. Six of these stands, ranging in age from 6 to 101 year-old (19MR, ED1, MA1, MA2, RR, and BBP), were used to test the effectiveness of sand-filled mesh bags for hyphae ingrowth or exclusion by quantitative real-time PCR (qPCR) of ribosomal ITS DNA regions. One stand (MARA) was used for the detailed bacterial isolation and enzymatic characterization. This 60 year-old stand was selected for bacterial isolation because a previous study on these sites had demonstrated that soil phosphatase activity was highest at this stage of stand development (Dong *et al.*, 2007).

4.2.2 Bag construction and placement

Three types of bags were used: hyphae-ingrowth (35 μm mesh, six replicates), hyphae-exclusion (0.5 μm mesh, six replicates), and total exclusion (sand bacterial control: Ziploc™ plastic bags, two replicates). Nylon mesh fabric (Plastoc Industries, City, UK) was cut to 10 cm x 5 cm, partially sealed using a hot melt glue gun, filled with No. 3 silica sand, and then completely sealed. The same substrate was placed in the Ziploc™ bags.

In June 2007, bags were placed horizontally between the organic and mineral layers within one 50 m x 50 m plot at each of the seven forest stands. One ingrowth and one exclusion bag were placed between six randomly-selected pairs of Douglas-fir and paper birch trees within each plot. Two locations at each plot also received a total exclusion bag. Typically, hyphae ingrowth bags are incubated *in situ* for extended periods (1 to 2 years) to maximize fungal biomass (both live and dead) for analysis (Nilsson & Wallander, 2003, Nilsson *et al.*, 2005, 2007; Korkama *et al.*, 2007). The goal of this study, however, was to enrich for bacteria influenced by freshly expanding hyphal fans and to minimize the presence and influence of dead hyphae in the bags. Therefore, we intended to incubate the bags *in situ* for the shortest period of time for hyphal penetration. Because the summer of 2007 was quite dry and hot, the bags were incubated until the fall rains commenced in early October and were collected shortly thereafter. After removal from the soil, each bag was immediately sealed in a larger plastic Ziploc™ bag and placed on ice for up to 48 hours until refrigerated at 4 °C. Bags were refrigerated for two weeks and then frozen at -10 °C to select for spore-forming and cold-tolerant bacteria.

4.2.3 Evaluation of mesh effectiveness

The effectiveness of the mesh treatments for allowing or excluding hyphal growth was verified in the bags from all seven stands, either by direct observation of hyphae using a modification of the filter-extraction method of Hanssen (1974) or by qPCR of ribosomal ITS DNA. For qPCR, DNA was extracted from sand substrate of ingrowth and bags from 19MR, ED1, MA1, MA2, RR, BBP using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA) following the supplied protocol. Extractions were made from three replicate bags of each bag type from each site. PCR reactions were performed using the general fungal primers ITS1F/ITS4 (Gardes &

Bruns, 1993) in 96-well plates with a StepOne Plus PCR system (Applied Biosystems, USA). All reactions had a final volume of 20 µl, including 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 5.6 µl nuclease-free water, 6 pmol of each primer, and 30 nmol of template DNA. PCR cycling conditions were 10 minutes at 94 ºC followed by 35 cycles of 94 ºC for 80 seconds, 56 ºC for 50 seconds and an extension at 72 ºC for 80 seconds. Each reaction plate included triplicates of each sample and a five-point set of 10-fold dilution standards. Melting curve analysis of PCR products was performed to ensure amplification specificity, and amplification curves were inspected to insure that each apparently positive sample had produced a sigmoidal plot (Reid *et al.*, 2002) so that a cycle threshold (CT) value could be assigned to each PCR reaction (Alexandersen *et al.*, 2001; Oleksiewicz *et al.*, 2001; Reid et al., 2003). A CT value of 20 was selected as the positive/negative cut-off for the detection threshold (the cycle at which the target sequence was detected) (Reid *et al.*, 2003).

For the 60 year-old stand (MARA) used for bacterial isolation, the effectiveness of the mesh treatments was verified using a hyphae extraction method. The sand substrate of ingrowth and exclusion bags was homogenized (1 g frozen sand in 50 ml dH$_2$O in a blender for 10 seconds), and the suspensions pipetted onto 2 µm Millipore™ filters. To visualize hyphae, while still in the filter holder, filters were covered with Coomassie Brilliant Blue stain for five minutes and then rinsed with dH$_2$O.

4.2.4   Bacterial isolation

Frozen sand (1 g) from each bag was suspended in 50 ml sterile Winogradsky's salt solution (0.4 g K$_2$HPO$_4$; 0.13 g MgSO$_4$ · 7H$_2$O; 0.13 g NaCl; 0.0025 g MnSO$_4$ ·7H$_2$O; 0.5 g NH$_4$NO$_3$; 1000 mL sterile, dH$_2$O; pH 7.2). To select for slower growing bacteria adapted to low nutrient soil conditions, suspensions were diluted to 10^{-4} with sterile dH$_2$O, and 100 µl aliquots plated onto 1% tryptic soy broth (TSB) agar with filter-sterilized, 50 mg L^{-1} cyclohexamide incorporated into the media to inhibit fungal growth. Plates were incubated in the dark at room temperature (approx. 21 °C) for seven days. Sixteen to 20 bacterial colonies were randomly selected from each plate. The resulting 276 potential isolates were re-streaked three times and then inoculated into sterilized screwtop plastic tubes containing 50 ml of 10 % sterilized TSB liquid medium with cyclohexamide.

4.2.5 Bacterial clone library

The non-cultured bacterial community was investigated by sequencing 384 cloned PCR products amplified from a single composite sample comprised of 1 g sand from each bag of all three treatments (ingrowth, exclusion, and Ziploc™ bags; 14 bags total), incubated at the MARA site. DNA was extracted from the composite sample using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA) following the supplied protocol. The general bacterial 16S rRNA gene primers 63F (5'-AGG CCT AAC ACA TGC AAG TC-3') and 1087R (5'-CTC GTT GCG GGA CTT ACC CC-3') (Nunan *et al.*, 2005) (Integrated DNA Technologies, USA) were used for the PCR amplification. All PCR reactions were performed in a final volume of 25 µl containing 12.5 µl of PCR Master Mix, 2x (Promega, USA), 9.5 µL of nuclease-free water, 5 pg of each primer, and 10 to 30 ng template DNA. PCR cycling conditions were: one cycle of 15 min at 95 °C followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension time of 10 min at 72 °C (MJMini Personal Thermal Cycler, BioRad, USA). PCR products were visualized by ethidium bromide staining on a 1% (w/v) agarose gel using UV radiation (EpiChemi II Darkroom, UVP Labs, USA) and were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Cleaned products were transformed using the pGEM-T Easy vector (Promega Corp.) according to the manufacturer's instructions and sent to BC Cancer Agency Genome Sciences Center (Vancouver, Canada) for robotic cloning and sequencing.

4.2.6 Bacterial identification

The purity and identity of each isolate was determined by sequencing using the general bacterial 16S rRNA gene primers and PCR protocol described above for the general clone library. PCR products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sequenced at the Nucleic Acid Protein Service Unit (NAPS Unit, University of British Columbia, Vancouver, Canada) with a PRISM 377 sequencer (Applied Biosystems, USA) and BigDye v3.1 Terminator Chemistry (Applied Biosystems, USA).

Isolate and clone library bacterial sequences were aligned using ClustalX2 (Larkin *et al.*, 2007) and identified to genus or species using the naïve Bayesian classifier (Wang *et al.*, 2007) available through the Ribosomal Database Project, Center for Microbial Ecology, Michigan State University, USA (Cole *et al.*, 2007). A database taxon was accepted as an isolate or clone

identity if the sequence had 97% or greater similarity and aligned over at least 450 base pairs. Bacterial isolates identified by 16S rDNA sequence were either grouped by genus; classified as "No ID" if the phylogeny was not available in the RDP database; or labeled as "Unknown" if the isolate could not be amplified by PCR. Sequences for clones and isolates have been accessioned in GenBank (GU300156-GU300723).

4.2.7 Growth curves and enzyme assays

Growth curves were constructed for each isolate. Isolates were inoculated into sterilized, screwtop plastic tubes containing 50 ml of 1% TSB liquid medium with cyclohexamide. The optical density of each isolate suspension was measured daily with a standard spectrophotometer (Spectra Max 340, Molecular Devices, USA) at 600 nm (OD_{600}) until no further change in OD_{600} was observed. Growth rate was calculated for each isolate by subtracting the initial OD_{600} reading from the maximum reading and dividing by the time (in days) required to reach the maximum reading.

Each isolate was assayed for the production of acid phosphomonoesterase and N-actyl glucosaminidase (NAGase) using a modification of a fluorometric method (Findlay *et al.*, 2001; Marx *et al.*, 2001). The optical density of each isolate suspension was measured daily with a standard spectrophotometer (Spectra Max 340, Molecular Devices, USA) at 600 nm (OD_{600}) until the isolate suspension reached an OD_{600} of 0.1 ± 0.05 units. Isolate suspensions were then centrifuged at 3,500 rpm for 45 min. The supernatant was then decanted and bacterial pellets resuspended in sterilized sodium acetate buffer (50 mM, pH 5.0) to an OD_{600} of 0.1 ± 0.05 units. Methylumbelliferyl (MUF)-phosphate and MUF-*N*-acetyl-β-glucosaminide were used as model substrates for phosphatase and NAGase, respectively. Isolate suspension reactions, standards and blanks were replicated eight times on each microplate. Isolate suspension reaction wells received 200 μl isolate suspension plus 50 μl of 200 μM of the model substrate. Two types of standards were used: quench standard wells contained 50 μl of 10 μM methylumbelliferone plus 200 μl isolate suspension as a quench standard; activity reference standard wells contained or 50 μl of 10 μM methylumbelliferone plus 200 μl of actetate buffer. Blank wells contained only acetate buffer. Based on preliminary experimentation, the minimum incubation time needed for reproducible microplate readings was two hours for the phosphatase assay

and three hours for the NAGase assay. Microplates were incubated in the dark and then reactions were stopped by adding 20 µL of 0.5 M NaOH to each well. Fluorescence was measured using a microplate fluorometer (CytoFluor II, PerSeptive Biosystems, USA) with 365 nm excitation and 450 nm emission filters. Phosphatase and NAGase activities were calculated as nmol of substrate converted per hour per initial unit OD_{600}. Background luminescence of the reference standard was subtracted from the total activity and the quench standard was used to adjust total activity for any signal quenching that resulted from fluorescence occlusion by the presence of bacterial cells in the reaction.

4.2.8   Data analysis

All statistical tests were performed using JMP 7.0.1 (SAS Institute, USA). The qPCR results testing the effectiveness of the mesh size for the six replicate ingrowth and exclusion bags from each of the six replicate forest stands were analyzed using a nominal logistic fit Log Likelihood model (DF=6) and the effect of bag type was determined by effect likelihood ratio tests testing for log odds of fungal DNA presence/absence. Fungal DNA presence was defined as DNA amplification requiring less than 20 PCR cycles. Simpson's diversity (D) was calculated according to Simpson (1964) for all identified genera cultured from the six replicate ingrowth and exclusion bags incubated at the MARA site. Seven isolates not identified to genus were excluded from the Simpson's diversity calculation. The diversity indices of ingrowth and exclusion bags were compared by one-way ANOVA after normalization by square root transformation. The enzyme activity was averaged amongst the isolates from each bag, then tested for differences between mesh treatments by one-way ANOVA, after all assumptions of the test were met. The relationship between isolate growth rate and isolate phosphatase and NAGase production were analyzed using linear regression, as was the relationship between Simpson diversity and average total phosphatase and NAGase activities of all isolates from each bag.

Bacterial isolates from hyphae ingrowth and hyphae exclusion bags were placed into five phylogenetic groups for comparison of their enzyme activities between treatments. The four genera represented by more than ten isolates, from hyphae exclusion and hyphae ingrowth bags, were included as named groups, while less frequent genera, (represented by

ten or fewer isolates), were grouped together as "Other" to form a fifth group. Student's *t* test (or Welch's ANOVA if standard deviations were not similar) was used to test for treatment effects on phosphatase, NAGase and growth rates of each group of isolates. Differences in the number of isolates belonging to each group between hyphae ingrowth and exclusion bags was tested for by contingency analysis using Fisher's Exact Test.

4.3   Results

4.3.1   Hyphal colonization of bags

Quantitative PCR verified that the 0.5 µm mesh of the hyphae-exclusion bags was effective in reducing hyphal colonization compared to the 35 µm mesh of the hyphae-ingrowth bags. Fungal DNA was detected in a higher proportion of ingrowth bags than exclusion bags when bags from six sites were evaluated (Likelihood Ratio Test for Bag type effect: *P*= 0.002) (Table 4.1; see Appendix C Table C-4 for whole model results and parameter estimates). However, DNA extractions from the sand substrate often resulted in low DNA yields. The presence of hyphae in the ingrowth bags from the MARA site was confirmed by Coomassie Brilliant Blue staining.

4.3.2   Bacterial community composition

Bacterial isolate diversity was higher among the exclusion bags (Simpson's D = 2.4 +/- 0.27 SEM) than the ingrowth bags (Simpson's D =1.67 +/- 0.2 SEM) (ANOVA: *P*=0.05). Of the two Ziploc® bags used to assess the effect of the initial population of the sand, one had the lowest diversity of all the sandbags (Simpson's D = 1.2), while the other had diversity similar to hyphae exclusion bags (Simpson's D = 2.5).

The clone library created from DNA extracted from composited samples from ingrowth, exclusion, and Ziploc® bags indicated that the overall bacterial community amplified by PCR was dominated by Proteobacteria (68.2%) and Actinobacteria (16.4%) (Table 4.2). The isolation method used successfully enriched for Actinobacteria (74.3%), with a reduced dominance by Proteobacteria (19.6%). However, all phyla representing 5% or more of the clones were represented among the isolates, with the exception of the *Acidobacteriales* (Table 4.2). *Streptomyces* accounted for 30.2% of the isolated Actinobacteria genera, followed by

Arthrobacter (23.4%), *Microbacterium* (7.8%), and *Rhodococcus* (7.8%) (Appendix C Table C-1). Phyllobacterium isolates were the most frequently isolated of the Proteobacteria, accounting for 35.2% of Proteobacteria (Appendix C Table C-1).

To evaluate the effect of hyphal ingrowth on nearby soil bacteria, the isolates from hyphae ingrowth and hyphae exclusion bags were grouped by genus. Genus groups consisted of genera represented by more than ten isolates from each of these two bag types: *Streptomyces* group (62 isolates) *Arthrobacter* group (32 isolates), *Phyllobacterium* group (19 isolates), and *Microbacterium* group 14 isolates) (Table 4.3Table 4.3). Other bacterial genera (isolated ten or fewer times from each bag type) were grouped together as "Other" genera (102 isolates) (Table 4.3; See Appendix C Table C-2 for a list of all genera included in the Other group). The number of isolates belonging to each genus group (*Streptomyces* group, *Arthrobacter* group, *Phyllobacteria* group, *Microbacterium* group and Other group) differed between the hyphae ingrowth and exclusion bags. Genera represented by ten or fewer isolates (Other group) were more commonly isolated from the hyphae exclusion bags (Fisher's Exact Test: $P < 0.0001$), while bacteria in the *Streptomyces* and *Phyllobacterium* groups were more commonly isolated from hyphae ingrowth bags (Fisher's Exact Test: *Streptomyces* group $P=0.001$, *Phyllobacterium* group $P=0.001$) (Figure 4.1a,b).

4.3.3 Enzyme activities

The modified microplate assay used to determine enzyme activities of bacterial isolates using live cell suspensions detected differences in enzyme production amongst bacterial isolates and amongst isolate assemblages from bags with different mesh sizes. The modified NAGase microplate assay detected NAGase in all *Streptomyces* isolates and in a wide range of other bacteria (Table 4.4). Average extracellular phosphatase activity of isolates from ingrowth bags was significantly lower than the activity of isolates from exclusion bags, while average extracellular NAGase production was not different between isolates from the two types of bags (Figure 4.2). No difference was found between the average growth rates of the bacterial assemblages from hyphae ingrowth compared to exclusion bags. Regression analysis revealed no relationship between isolate growth rate and enzyme production. There was, however, a significant positive relationship between the phosphatase activities of the

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assemblages originating from each bag and the Simpson's diversity indices ($P < 0.01$; $r^2 = 0.54$) (Figure 4.3).

Enzyme activities of the genus groups also differed by bag type. The mean phosphatase activities of isolates in the *Streptomyces* group from ingrowth bags was lower than the mean activities of isolates from exclusion bags (Student's *t:* Streptomyces group $P=0.01$), with the tendency for a similar pattern in the Other group ($P=0.09$). The reverse tendency was observed for isolates in the *Microbacterium* group (Student's *t*: $P=0.10$) (Figure 4.1c,d). The mean NAGase activities of isolates of the *Arthrobacter* and *Phyllobacterium* groups from hyphae ingrowth bags was higher than that of isolates from exclusion bags (Student's *t*: *Arthrobacter* group $P=0.04$, *Phyllobacterium* group $P=0.01$) (Figure 4.1e,f). On average, isolates in the *Arthrobacter* and *Microbacterium* groups originating from hyphae ingrowth bags grew more slowly than those from exclusion bags (Student's *t*: *Arthrobacter* group $P=0.001$, *Microbacterium* group $P=0.06$; Appendix C Table C-3), while isolates in the *Phyllobacterium* and *Streptomyces* groups from ingrowth bags grew more quickly (Student's *t: Phyllobacterium* group $P=0.03$, *Streptomyces* group $P=0.05$).

4.4   Discussion

4.4.1   Effectiveness of method

This study is the first to assess hyphal ingrowth into sand-filled mesh bags by qPCR. While 5 µm mesh generally excluded colonization, hyphae successfully colonized sand in bags of 35 µm mesh. Previous studies utilizing sand-filled mesh bags to select for EMF hyphae have assessed hyphal ingrowth visually and by phospholipid fatty acid (PLFA) profiling (Wallander *et al.*, 2001, 2003, Nilsson & Wallander, 2003, Nilsson *et al.*, 2005, 2007; Korkama *et al.*, 2007). This is also the first use of sand-filled mesh bags to characterize the cultural components of the bacterial community associated with fungal hyphae growing in a forest soil. The short incubation time used was intended to minimize dead hyphae in the bags in order to assess, as much as possible, the effects of living hyphae on nearby soil bacteria. Instead of isolating bacteria directly from hyphae extracted from ingrowth bags and then from uncolonized sand from hyphae exclusion bags, bacteria were isolated from random subsamples from each

sandbag type. In this way, the isolated assemblages should better reflect the general effects of hyphae on nearby soil bacteria. However, the sand was subsampled without considering the extent of hyphal colonization in any subsample. Because colonization was sparse and patchy, isolates from hyphae ingrowth bags were not necessarily closely associated with hyphae. However, the technique proved effective at revealing differences in average phosphatase activities of the cultured isolates from the hyphae ingrowth and hyphae exclusion treatments, as well as relative enzyme activities of specific bacterial groups.

4.4.2   Bacterial communities associated with fungi

Even though the taxonomic composition of the bacterial assemblages differed between bag types, it appears that soil bacteria could readily migrate into both types of bags as both contained greater numbers and more diverse bacteria than the initial population found in the sand (incubated in plastic bags). Over half of the bacterial genera were common to bags of both mesh sizes, and it was the bags with the smallest mesh size that supported the highest number of genera. Because the isolation methods used selected for slower-growing, predominantly Gram-positive bacteria, the prevalence of these bacteria in the sand-bag environment was assessed using a clone library generated from a composited sample from all bag types. Interestingly, the amplicons were dominated by sequences identified as Proteobacteria, followed by the Actinobacteria. This proportion of Proteobacteria is similar to that reported by Chow et al. (2002) from rhizosphere soil of Lodgepole pine (*Pinus contorta*) seedlings sampled from the British Columbia (BC) Ministry of Forests Long-Term Soil Productivity installations in central BC, Canada, where Proteobacteria accounted for 55% of the clone library. However, in that study, Acidobacteria were the next most prevalent group (19%), and Actinobacteria accounted for only 3% of the clones. A small percentage of Actinobacteria is typical of the rhizosphere of grasses and forbs, where they make up 1 to 3% of the rhizosphere bacterial community, whereas Proteobacteria typically account for 27 to 66% (see review by Bueé *et al*. 2009). However bacterial communities in bulk forest soil and forest humus are often characterized by a larger proportion of Actinobacteria. Axelrood *et al.* (2002) isolated bacteria from bulk soil samples taken from the same sites that Chow *et al.* (2002) used for their study characterizing seedling rhizosphere bacteria by cloning and found that Proteobacteria made up

33.5% of the bacteria isolated from bulk soil samples, while 23.4% of the isolates were Actinobacteria. Similarly, in a high throughput sequencing project investigating the bacterial community present in rhizosphere and mineral soils in an oak forest, Uroz *et al.* (2010) found that Proteobacteria accounted for 36.4% of the sequences obtained, followed by 24.8% identified as Acidobacteria, and 11.8% as Actinobacteria, with no differences between bulk soil and rhizosphere samples for these groups. However, while no difference in the proportions of Proteobacteria and Actinobacteria between bulk and rhizosphere soil were found in that study, a larger proportion of Proteobacteria is more typical of rhizosphere than bulk soil.

Ectomycorrhizal hyphae can extend the bacterial community characteristic of the rhizosphere into the soil environment. Timonen et al. (2006) isolated bacteria from the rhizosphere of *Pinus sylvestris* and the mycorrhizospheres of *Pinus sylvestris-Suillus bovinus* and found a larger proportion of Proteobacteria than Actinobacteria associated with external hyphae and the rhizosphere on non-mycorrhizal *Pinus* roots than with either mycorrhizae or uncolonized soil. The large proportion of Proteobacteria sequences identified in the clone library obtained from the composited sample is more consistent with rhizosphere than bulk soil bacteria communities, as might result from EMF hyphae creating habitats conducive to rhizosphere bacteria in soil.

4.4.3   Bacterial enzymatic activities

The modified microplate enzyme assay rapidly and repeatably detected bacterial isolate enzyme production. This allowed prompt screening of bacterial isolates with high levels of replication for comparison of bacterial communities between treatments. Similar enzyme microplate assays have been used to compare community enzyme activity of biofilms growing on tiles (Findlay *et al.*, 2001) and gravel (Findlay *et al.*, 2003). In this study, the assay was modified somewhat from the approach used by Findlay et al. (2001). Instead of assaying all cells from a given surface area (Findlay *et al.*, 2001, 2003), a concentration of cells sufficient to generate a minimum fluorometer OD_{600} reading was used. Because actual cell counts were not determined and cell morphology can affect optical density, it would have been inappropriate to directly compare the enzyme production amongst isolates using this method. By standardizing both the initial concentration of all isolates to the same OD_{600} and length of the assay, the

average activities of the assemblages of isolates from each bag can be compared. By assaying isolates individually and then averaging their activities within a bag, the modified microplate assay demonstrated significant differences in bacterial enzyme activity between hyphae ingrowth and exclusion bags. These groupings of isolates from each bag were considered to be community proxies.

Isolates originating from ingrowth bags were associated with less extracellular phosphatase on average than isolates from exclusion bags. This reduction in phosphatase activity was largely driven by isolates belonging to *Streptomyces*; the most highly represented genus. Despite the lower phosphatase activities of isolates from ingrowth bags, these bags supported higher numbers of *Streptomyces* than exclusion bags, and *Streptomyces* isolates from ingrowth bags grew faster than those from exclusion bags. These results indicate that *Streptomyces* populations with lower enzymatic activities were stimulated by the presence of EMF hyphae. These results support previous findings by Olsson *et al.* (1996) and Olsson & Wallander (1998), where the presence of EMF hyphae reduced bacterial metabolic activities as, estimated by thymidine incorporation, in a sandy soil while not reducing bacterial biomass.

One mechanism for the stimulation of *Streptomyces* growth by EMF hyphae may be the production of organic acids such as citrate and oxalate by these fungi (Unestam & Sun, 1995). Ectomycorrhizal hyphae can increase the concentration of citric acid in soil solution ten-fold (Olsson & Wallander, 1998). Both citric and oxalic acids form calcium salts that adhere to hyphae growing through sand patches, but not on hyphae growing through patches of organic matter (Wallander *et al.*, 2005). While secretion of oxalic acid by EMF fungi increases the rate of soil weathering and enhances the availability of phosphorus for host uptake (Griffiths *et al.*, 1994; Landeweert *et al.*, 2001; Rosling *et al.*, 2004), these acids do not accumulate in forest soil. It is hypothesized that these low energy organic acids are utilized as a carbon source by soil bacteria. In a study of microorganisms associated with oxalate-encrusted fungal mats of *Hysterangium crassum* (Tul. et Tul.) associated with Douglas-fir, nearly half of the bacteria isolated could utilize oxalate as a carbon source; and one third of those bacteria were identified as *Streptomyces* (Knutson & Hutchins, 1980).

Bacterial NAGase production can be stimulated by both chitin addition and hyphal growth (de Boer *et al.*, 1999, 2001). Bacteria isolated from fine hyphae preferentially utilize *N*-acetyl glucosamine (Timonen *et al.*, 1998). Therefore, it is probable that soil bacteria with enhanced NAGase production would readily utilize carbon or nitrogen from fungal cell wall material, gaining a competitive advantage in hyphae ingrowth bags. However, there was no increase in NAGase production by *Streptomyces* isolates from hyphae ingrowth bags compared to those from exclusion bags. This is in spite of the larger numbers and faster growth rates of isolates from ingrowth bags compared exclusion bags. This finding is counter to expectations, especially given predictions by de Boer *et al*. (2005) that *Streptomyces* would be strong competitors with strand-forming fungi, and to other studies demonstrating anti-fungal activities of *Streptomyces* (Axelrood *et al.*, 1996; Becker *et al*., 1999; Jayasinghe and Parkinson, 2008). The results here suggest that enhanced capabilities for the utilization of hyphal cell wall material as a carbon or nitrogen source did not contribute to the promotion of *Streptomyces* in hyphae ingrowth bags.

Enhanced NAGase activity was mainly seen amongst isolates from the *Arthrobacter* and *Phyllobacterium* groups originating from ingrowth bags. *Arthobacter* are common bacteria frequently isolated from forest soil (Axelrood *et al*., 2002) and humus (Elo *et al*., 2000). However the enhanced NAGase activity of *Arthrobacter* isolates from hyphae ingrowth bags did not correspond with other indicators of fitness for these bacteria as there was neither an increase in the number of isolates nor faster growth rates of these isolates compared to *Arthrobacter* isolates from exclusion bags. *Phyllobacterium* isolated from root tissues of pine can promote the growth of pine (Chanway *et al.*, 1994) and canola (Bertrand *et al*., 2001). Elo *et al*. (2000) isolated *Phyllobacterium sp*. capable of fixing nitrogen from the rhizosphere of birch seedlings. Recent additions to the genus have been often been capable of fixing nitrogen and some may be capable of nodulating *Trifolium* and *Lupinus* (Valverde *et al*., 2005; Mantelin *et al*., 2006). *Phyllobacterium sp.* were the most numerous Gram-negative isolates in this study, and the higher NAGase activity of Phyllobacterium isolates from hyphae ingrowth bags corresponded with both increased numbers and faster growth rates for these isolates compared to *Phyllobacterium* isolates from hyphae exclusion, indicating increased fitness of

Phyllobacterium with potentially enhanced capacity to utilize fungal hyphae as a substrate for growth.

4.4.4   Bacterial-fungal interactions

The reduction in bacterial community diversity associated with fungal hyphae indicates that some selection pressure is affecting bacterial community structure. It has been demonstrated that addition of "refractory" carbon sources to aquatic bacterial communities result in a reduction in bacterial diversity due to selection of more specialized bacterial guilds (Foreman & Covert, 2003; Sinsabaugh & Foreman, 2003). The lower bacterial taxonomic diversity found in ingrowth bags may indicate the utilization of a more labile carbon source by more generalist bacteria. Bacteria associated with mycorrhizae readily utilized fungal sugars as carbon sources in a microcosm study using mycorrhizal pine seedlings; bacteria isolated from *Suillus bovinus* mycorrhizospheres favoured mannitol, while bacteria isolated from *Paxillus involutus* favoured fructose (Timonen *et al.*, 1998). In a nursery study, fluorescent pseudomands isolated from Douglas-fir seedlings mycorrhizal with *Laccaria bicolor* preferentially utilized trehalose (Frey *et al.*, 1997). Trehalose has is also required for the promotion of hyphal growth by the mycorrhizal helper bacterium *Pseudomonas monteilii* strain HR13 (Duponnois & Kisa, 2006).

An alternate hypothesis would be that *Streptomyces* selected for, and promoted, the ingrowth of fungal hyphae. *Streptomyces* can promote hyphal growth (Becker *et al.*, 1999; Schrey *et al.*, 2005; Riedlinger *et al.*, 2006), as well as suppress it (Axelrood *et al.*, 1996; Becker *et al.*, 1999; Jayasinghe & Parkinson, 2008). *Streptomyces,* under phosphorus starvation, release secondary metabolites such as antibiotics (Martín, 1977) and hyphal growth factors such as axofuran (Riedlinger *et al.*, 2006). Therefore, it is possible that *Streptomyces* with lower phosphatase production capacities may stimulate hyphal growth in order to take advantage of hyphal phosphatase production as a strategy for phosphorus acquisition in the low nutrient sandbag environment. Because EMF also produce antibacterial compounds that preferentially inhibit Gram-negative bacteria compared to Gram-positive bacteria (Garrido 1982), the production of antibiotic compounds by EMF could select against less resistant soil bacteria,

increasing the prevalence of gram-positive Actinobacteria while reducing general bacterial diversity in the vicinity of EMF hyphae.

4.4.5   Conclusions

This study is the first to use mesh-filled sand bags to demonstrate changes in bacterial community composition in the presence of EMF at the genus level and to study the effects of fungal-bacterial interaction on microbial community functioning. This study complements other studies that have demonstrated functional diversity of bacteria near mycorrhizal roots compared to bacteria from adjacent bulk soil; bacteria near mycorrhizal roots were better solubilizers of inorganic phosphorus compared to bacteria originating from bulk soil (Calvaruso *et al.*, 2007; Uroz *et al.*, 2007). These earlier studies were conducted in an oak forest where soil samples originating from outside the immediate vicinity of mycorrhizal roots were described as bulk soil. However, since forest soil is typically highly ramified with EMF hyphae, it is reasonable to assume that the bulk soil samples in these studies were influenced by hyphae. Therefore, the lower inorganic phosphorus mobilization by bacteria originating from hyphae-influenced bulk soil in these previous studies, taken together with the lower organic phosphorus mobilization by bacteria isolated from ingrowth sandbags in this study, provides further support for the hypothesis that EMF hyphae select for soil bacteria with reduced nutrient mobilization capabilities.

The increased number of *Streptomyces* isolated from ingrowth bags and the enhanced growth rate, but lower enzyme activities of these isolates, indicates that fungal hyphae can have a positive effect on populations of soil *Streptomyces*, while at the same time, structuring these potentially competitive bacteria for decreased extracellular enzyme production. This selection may function at the bacterial strain level, selecting for individuals that differ in their capacity to produce extra-cellular enzymes. Growth curves conducted with high and low phosphatase-producing isolates indicated that, on average, bacteria with higher extra-cellular phosphatase production were not faster growing than low phosphatase producers. If interactions between fungal hyphae and bulk soil bacteria result in the selection of strains within the community that differ in their functional capabilities, then comparison of community composition by standard molecular methods may not reveal the underlying functional structure

of the bacterial community. This potential for the existence of cryptic functional structure highlights the need for continued efforts to not only bring soil bacteria into cultivation, but to continue to develop methods to quickly and effectively screen large numbers of environmental isolates. This study investigated the slower growing components of the bacterial community and found that even within this component of the community, the bacteria associated with fungal hyphae varied significantly from that in the absence of hyphae. Further research is needed to determine the selection mechanism involved in the changes in bacterial community structure and function associated with EMF hyphae. It is important to understand the role of these slower-growing, primarily Gram-positive, bacteria in the nutrient dynamics of forest soils because they are abundant and represent potential competitors to EMF for phosphorus and nitrogen (de Boer *et al.*, 2005).

4.5   Figures and tables

Figure 4.1 Proportions and enzyme activities of 210 bacterial isolates from hyphae ingrowth and hyphae exclusion bags incubated in the organic soil layer of a mixed Douglas-fir and birch stand (MARA) grouped by genus: exclusion bag isolates (left) and ingrowth bag isolates (right). (a,b) Proportions of each genus group calculated for each bag type as the number of isolates belonging to a genus group divided by the total number isolates originating from the bag type. Differences in the number of isolates in a genus group between mesh sizes were tested by contingency analysis using Fisher's Exact Test (** P <0.05). (c,d) Proportions of phosphatase activity and (e,f) proportions of NAGase activity for each genus group. Proportions of enzyme activity were calculated for each bag type as the sum of the enzyme activity of isolates belonging to a genus group divided by the total phosphatase activity by isolates originating from the same bag type. Differences in enzyme activity (nmol substrate converted hour^{-1} initial unit OD$_{600}$$^{-1}$) within genus groups between mesh sizes were tested by Student's t or Welch ANOVA when standard deviations differed (** P <0.05; * P<0.10) (See Appendix C Table C-3 for mean values and SEM).

Figure 4.2 Box plots of enzyme activities, as measured by microplate assay, averaged among all isolates originating from replicate hyphae ingrowth and exclusion bags incubated in the organic soil layer of a mixed Douglas-fir and birch stand (MARA). Differences among treatments, as detected by ANOVA at α = 0.05, are indicated by different letters. Enzyme activity was calculated as nmol substrate converted $hour^{-1}$ initial unit OD_{600}^{-1}. (n=6).

Figure 4.3 Average phosphatase activity per bag, for assemblages of bacteria isolated from hyphae exclusion bags (solid circles), Ziploc® total exclusion bags (open circles), and hyphae ingrowth bags (solid triangles) incubated in the organic soil layer of a mixed Douglas-fir and birch stand (MARA), plotted against Simpson's diversity index (D) of bacteria isolated from each bag. ($P < 0.01$; $r^2 = 0.54$). Phosphatase activity was calculated as the sum of nmol substrate converted hour^{-1} initial unit $OD_{600}$$^{-1}$ by all isolates from each bag.
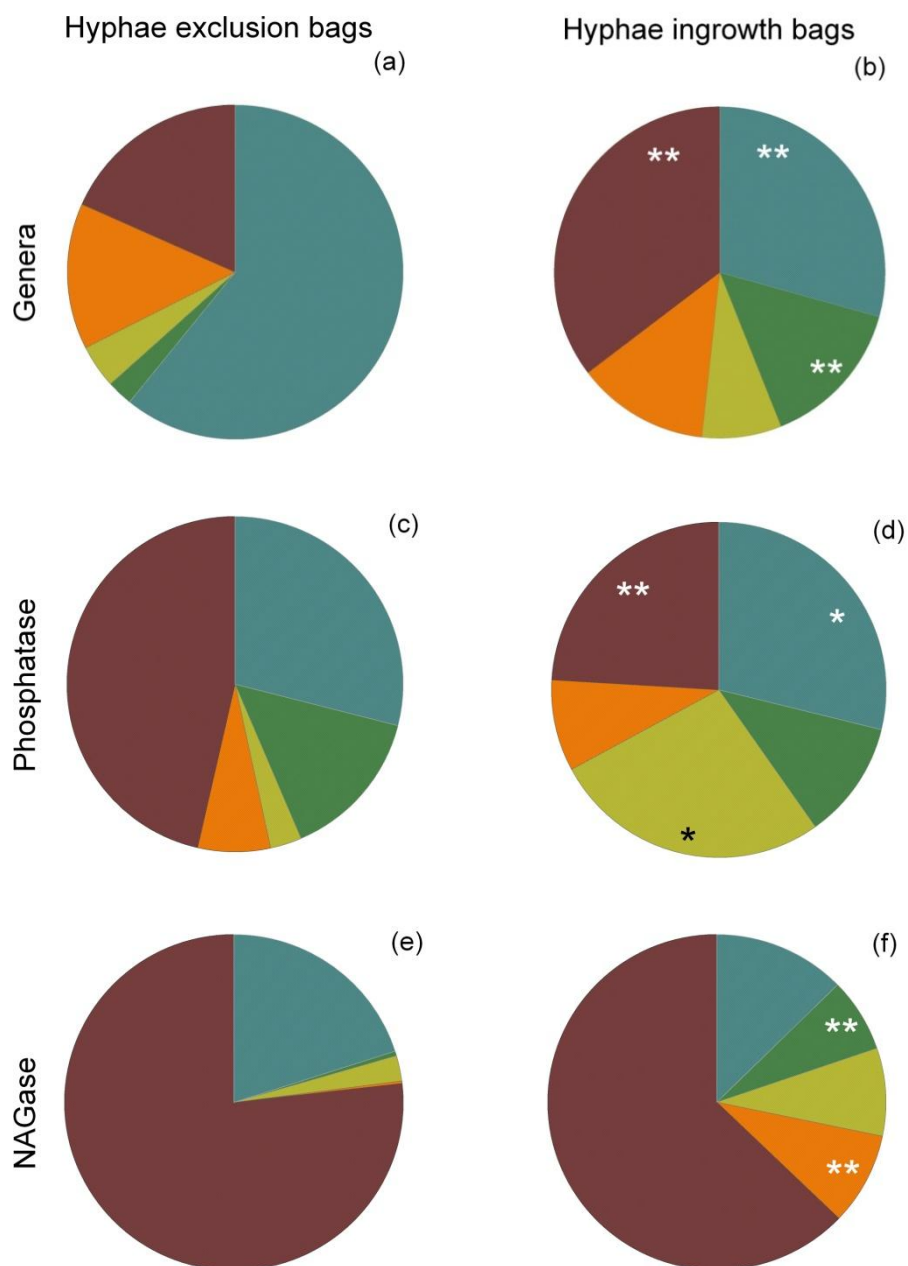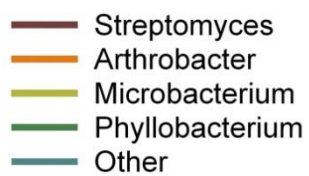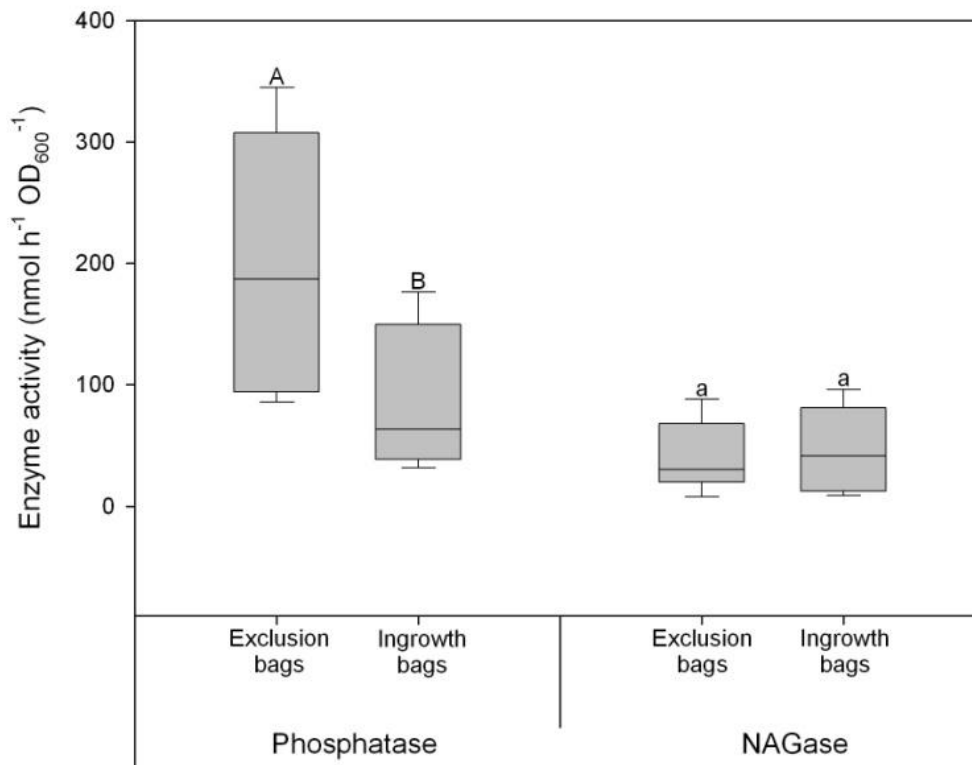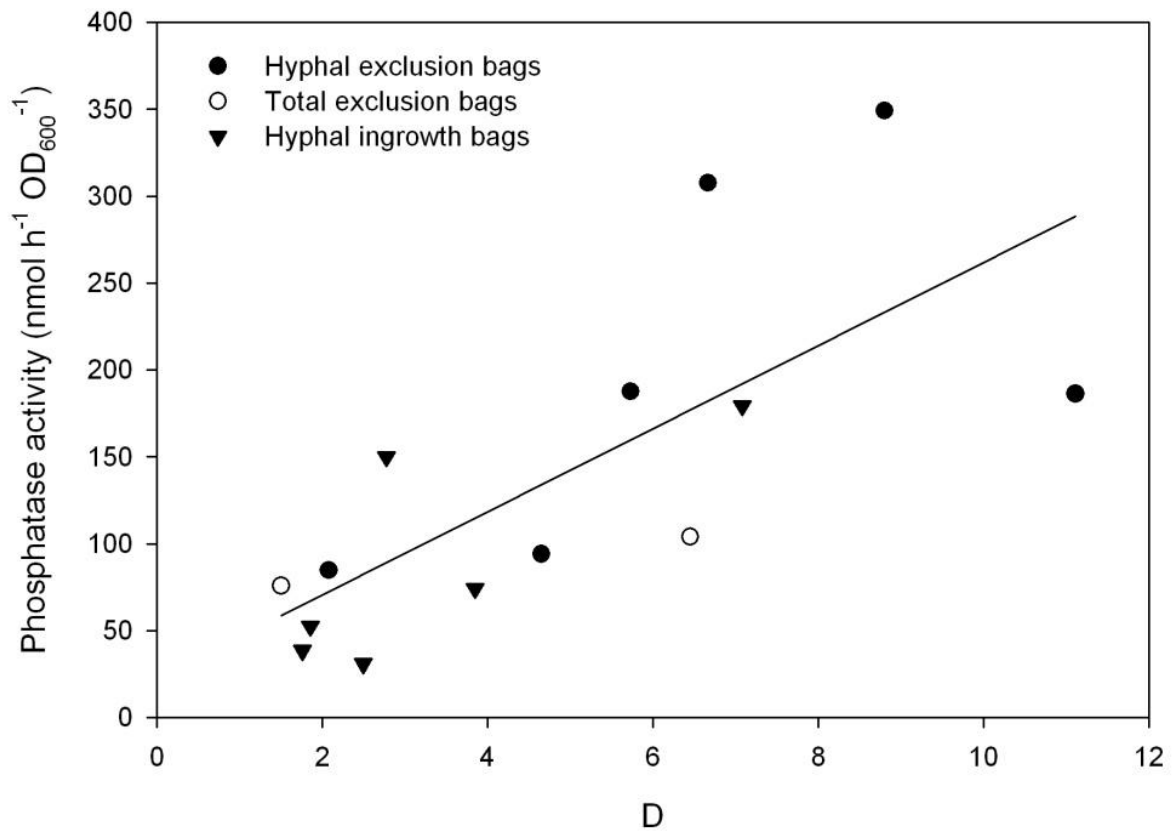
Table 4.1 qPCR results for replicate DNA extractions from three hyphae ingrowth and three exclusion bags incubated in the organic layers of six mixed Douglas-fir and birch forests regenerating from stand replacing wildfire in the southern interior of British Columbia. Extractions with detectable fungal DNA amplification by fewer than 20 PCR cycles are indicated by "+". Samples without significant amplification with 20 PCR cycles are indicated by "-".

| Stand ID[a] | Stand age (yrs)[b] | Bag replicate | Fungal DNA amplification | |
			Hyphae ingrowth bags	Hyphae exclusion bags
19MR	6	1	+	-
		2	-	-
		3	-	-
ED1	30	1	+	-
		2	+	-
		3	+	-
MA1	24	1	-	+
		2	+	-
		3	+	-
MA2	24	1	-	-
		2	+	-
		3	-	-
RR	61	1	-	-
		2	+	-
		3	+	-
BBP	101	1	-	+
		2	-	-
		3	+	-

[a] Stand identification codes as defined by Twieg *et al.* (2007).
[b] These forest stands have been previously described by Twieg et al. (2007).

Table 4.2 Percent occurrence of bacteria taxa from sand bags incubated in the organic soil layer of a mixed Douglas-fir and birch stand (MARA) identified from either cloned PCR products (299 identified clones from a composited sample from all bags) or bacterial isolation (276 identified isolates, cultured separately from each bag).

Phylogeny (Phylum / Class)	Number of clones	Clones %	Number of isolates	Isolates %
Acidobacteria total	**18**	**6.0**	**0**	**0**
Acidobacteria	18		0	
Actinobacteria total	**49**	**16.4**	**205**	**74.3**
Actinobacteria	49		205	
Bacteroidetes total	**27**	**9.0**	**4**	**1.5**
Flavobacteria	1		0	
Sphingobacteria	26		4	
Firmicutes total	**0**	**0**	**6**	**2.2**
Bacilli	0		6	
Gemmatimonadetes total	**1**	**0.3**	**0**	**0**
Gemmatimonadetes	1		0	
Proteobacteria total	**204**	**68.2**	**54**	**19.6**
α-proteobacteria	117		30	
β-proteobacteria	58		23	
γ -proteobacteria	23		1	
δ -proteobacteria	6		0	
Grand Total	**299**		**276**	

Table 4.3 Genus groupings for 229 isolated bacteria from hyphae ingrowth and hyphae exclusion sand bags incubated in the organic layer of a mixed Douglas-fir and birch stand (MARA).

Genus grouping	Isolates in group	Proportion of isolates (%)
Arthrobacter group	32	14.0
Microbacterium group	14	6.1
Phyllobacterium group	19	8.3
Streptomyces group	62	27.1
Other group[a]	102	44.5

[a] See Appendix C Table C-2 for phylogenenic affiliations of isolates grouped as "Other" .

Table 4.4 Percent of isolates of the most commonly isolated genera expressing *N*-actyl glucosaminidase (NAGase) from hyphae ingrowth, hyphae exclusion and Ziploc® sand bags incubated in the organic layer of a mixed Douglas-fir and birch stand (MARA). (n.a. indicates no isolates of that genus obtained from the treatment.)

Genus	Ziploc® bag isolates (%)	Hyphae exclusion bag isolates (%)	Hyphae ingrowth bag isolates (%)
Arthrobacter	29.4	46.9	60.0
Microbacterium	50.0	88.9	90.9
Phyllobacterium	n.a.	94.7	100.0
Streptomyces	n.a.	100.0	100.0
Other isolates	66.7	74.8	81.8
Average %	50.0	77.9	88.8

[a] See Appendix C Table C-1 for phylogenenic affiliations of all identified isolates .

4.6   Bibliography

Anderson IC, Cairney JWG. 2007. Ectomycorrhizal fungi: exploring the mycelial frontier. *FEMS Microbiology Reviews* **31:** 388-406.

Axelrood PE, Chow ML, Arnold CS, Lu K, McDermott JM, Davies J. 2002. Cultivation-dependent characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **48:** 643-654.

Axelrood PE, Clarke AM, Radley R, Zemcov SJ. 1996. Douglas-fir root-associated microorganisms with inhibitory activity towards fungal plant pathogens and human bacterial pathogens. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **42:** 690-700.

Becker DM, Bagley ST, Podila GK. 1999. Effects of mycorrhizal-associated streptomycetes on growth of *Laccaria bicolor, Cenococcum geophilum*, and *Armillaria* species and on gene expression in *Laccaria bicolor. Mycologia* **91:** 33-40.

Bertrand H, Nalin R, Bally R, Cleyet-Marel J-C. 2001. Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (*Brassica napus*). *Biology and Fertility of Soils* **33:** 152-156.

Bhattacharya D, Nagpure A, Gupta RK. 2007. Bacterial chitinases: properties and potential. *Critical Reviews in Biotechnology* **27:** 21-28.

de Boer W, Folman LB, Summerbell RC, Boddy L. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews* **29:** 795-811.

de Boer W, Gerards S, Gunnewiek KPJA, Modderman R. 1999. Response of the chitinolytic microbial community to chitin amendments of dune soils. *Biology and Fertility of Soils* **29:** 170-177.

de Boer W, Gunnewiek PJ, Kowalchuk GA, van Veen JA. 2001. Growth of chitinolytic dune soil ß-subclass Proteobacteria in response to invading fungal hyphae. *Applied and Environmental Microbiology* **67:** 3358-3362.

Brierley EDR, Wood M. 2001. Heterotrophic nitrification in an acid forest soil: isolation and characterisation of a nitrifying bacterium. *Soil Biology & Biochemistry* **33:** 1403-1409.

Buée M, De Boer W, Martin F, van Overbeek L, Jukevitch E. 2009. The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of the their structuring factors. *Plant and Soil* **321:** 189-212.

Burke DJ, Dunham SM, Kretzer AM. 2008. Molecular analysis of bacterial communities associated with the roots of Douglas fir (*Pseudotsuga menziesii*) colonized by different ectomycorrhizal fungi. *FEMS Microbiology Ecology* **65:** 299-309.

Cairney JWG. 2005. Basidiomycete mycelia in forest soils: dimensions, dynamics and roles in nutrient distribution. *Mycological Research* **109:** 7-20.

Calvaruso C, Turpault MP, Leclerc E, Frey-Klett P. 2007. Impact of ectomycorrhizosphere on the functional diversity of soil bacterial and fungal communities from a forest stand in relation to nutrient mobilization processes. *Microbial Ecology* **54:** 567-577.

Chanway CP, Shishido M., Holl FB. 1994. Root-endophytic and rhizosphere plant growth-promoting rhizobacteria for conifer seedlings. In: Ryder, ed. *Improving Plant Productivity with Rhizosphere Bacteria.* Glen Osmond, Australia: CSIRO Division of Soils, 72-74.

Chow ML, Radomski CC, McDermott JM, Davies J, Axelrood PE. 2002. Molecular characterization of bacterial diversity in Lodgepole pine (Pinus contorta) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. *FEMS Microbiol Ecol* **42:** 347-357.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedge JM. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35 (Database issue):** D169-D172; doi: 10.1093/nar/gkl889.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167:** 309-319.

Cumming JR, Weinstein LH. 1990. Utilization of $AlPO_4$ as a phosphorus source by ectomycorrhizal *Pinus-rigida* Mill. seedlings. *New Phytologist* **116:** 99-106.

Duponnois R, Kisa M. 2006. The possible role of trehalose in the mycorrhiza helper bacterium effect. *Canadian Journal of Botany/Revue Canadienne de Botanique* **84:** 1005.

Elo S, Maunuksela L, Salkinoja-Salonen M, Smolander A, Haahtela K. 2000. Humus bacteria of Norway spruce stands: plant growth promoting properties and birch, red fescue and alder colonizing capacity. *FEMS Microbiology Ecology* **31:** 143-152.

Findlay S, Quinn JM, Hickey CW, Burrell G, Downes M. 2001. Effects of land use and riparian flowpath on delivery of dissolved organic carbon to streams. *Limnology and Oceanography* **46:** 345-355.

Findlay SEG, Sinsabaugh RL, Sobczak WV, Hoostal M. 2003. Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. *Limnology and Oceanography* **48:** 1608-1617.

Foreman CM, Covert JS. 2003. Linkages between dissolved organic matter composition and bacterial community structure. In: Findlay SEG, Sinsabaugh RL, eds. *Aquatic ecosystems: Interactivity of dissolved organic matter.* USA: Academic Press, 343-362..

Frey P, Frey-Klett P, Garbaye J, Berge O, Heulin T. 1997. Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with the Douglas fir *Laccaria bicolo*r mycorrhizosphere. *Applied and Environmental Microbiology* **63:** 1852-1860.

Fritsche K, de Boer W, Gerards S, van den Berg M, van Veen JA, Leveau JHJ. 2008. Identification and characterization of genes underlying chitinolysis in *Collimonas fungivorans* Ter331. *FEMS Microbiology Ecology* **66:** 123-135.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113-118.

Garrido N, Becerra J, Marticorena C, Oehrens E, Silva M, Horak E. 1982. Antibiotic properties of ectomycorrhizae and saprophytic fungi growing on Pinus radiata D. Don I. *Mycopathologia* **77:** 93-98.

Griffiths RP, Baham JE, Caldwell BA. 1994. Soil solution chemistry of ectomycorrhizal mats in forest soil. *Soil Biology and Biochemistry* **26(3):** 331-337.

Hanssen JF. 1974. Evaluation of hyphal lengths and fungal biomass in soil by a membrane filter technique. *OIKOS* **25:** 102-107.

Hibbett DS, Gilbert LB, Donoghue MJ. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* **407:** 506-508.

Högberg MN, Högberg P. 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **154:** 791-795.

Höppener-Ogawa S, Leveau JH, Smant W, van Veen JA, de Boer W. 2007. Specific detection and real-time PCR quantification of potentially mycophagous bacteria belonging to the genus *Collimonas* in different soil ecosystems. *Applied and Environmental Microbiology* **73:** 4191-4197.

Jayasinghe BATD, Parkinson D. 2008. Actinomycetes as antagonists of litter decomposer fungi. *Applied Soil Ecology* **38:** 109-118.

Kataoka R, Taniguchi T, Ooshima H, Futai K. 2008. Comparison of the bacterial communities established on the mycorrhizae formed on *Pinus thunbergii* root tips by eight species of fungi. *Plant and Soil* **304:** 267-275.

Knutson DM, Hutchins AS. 1980. The association of calcium oxalate-utilizing *Streptomyces* with conifer ectomycorrhizae. *Antonie van Leeuwenhoek* **46:** 611-619.

Koide RT. 2003. Ectomycorrhizas and retarded decomposition in a *Pinus resinosa* plantation. *New Phytologist* **158:** 401-407.

Korkama T, Fritze H, Pakkanen A, Pennanen T. 2007. Interactions between extraradical ectomycorrhizal mycelia, microbes associated with the mycelia and growth rate of Norway spruce (*Picea abies*) clones. *New Phytologist* **173:** 798-807.

Landeweert R, Hoffland E, Finlay RD, Kuyper TW, van Breemen N. 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology & Evolution* **16:** 248-254.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23:** 2947-2948.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany-Revue Canadienne De Botanique* **82:** 1016-1045.

Leake JR, Read DJ, Wicklow DT, Söderström B. 1997. Mycorrhizal fungi in terrestrial habitats. In: *The Mycota IV. Environmental and microbial relationships.* Heidelberg: Springer-Verlag, 281-301.

Leyval C, Reid CPP. 1991. Utilization of microbial siderophores by mycorrhizal and non-mycorrhizal pine roots. *New Phytologist* **119:** 93-98.

Lloyd D, Angrove K, Hope G, Thompson C. 1990. *A guide to site identification and interpretation for the Kamloops forest region.* Victoria, B.C.: Research Branch, B.C. Ministry of Forests.

Mantelin S, Saux MF, Zakhia F, Béna G, Bonneau S, Jeder H, de Lajudie P, Cleyet-Marel JC. 2006. Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassicacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **56:** 827-839.

Martín JF. 1977. Control of antibiotic synthesis by phosphate. In: *Advances in Biochemical Engineering.* Berlin/Heidelberg: Springer, 105-127.

Marx MC, Wood M, Jarvis SC. 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. *Soil Biology and Biochemistry* **33:** 1633-1640.

Nilsson LO, Bååth E, Falkengren-Grerup U, Wallander H. 2007. Growth of ectomycorrhizal mycelia and composition of soil microbial communities in oak forest soils along a nitrogen deposition gradient. *Oecologia* **153:** 375-384.

Nilsson LO, Giesler R, Baath E, Wallander H. 2005. Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. *New Phytologist* **165:** 613-622.

Nilsson LO, Wallander H. 2003. Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New Phytologist* **158:** 409-416.

Nunan N, Daniell TJ, Singh BK, Papert A, McNicol JW, Prosser JI. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. *Applied and Environmental Microbiology* **71:** 6784-6792.

Nurmiaho-Lassila EL, Timonen S, Haahtela K, Sen R. 1997. Bacterial colonization patterns of intact *Pinus sylvestris* mycorrhizospheres in dry pine forest soil: an electron microscopy study. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **43:** 1017-1035.

Olsson PA, Chalet M, Baath E, Finlay RD, Soderstrom B. 1996. Ectomycorrhizal mycelia reduce bacterial activity in a sandy soil. *FEMS Microbiology Ecology* **21:** 77-86.

Olsson PA, Wallander H. 1998. Interactions between ectomycorrhizal fungi and the bacterial community in soils amended with various primary minerals. *FEMS Microbiology Ecology* **27:** 195-205.

Perez-Moreno J, Read DJ. 2001. Nutrient transfer from soil nematodes to plants: a direct pathway provided by the mycorrhizal mycelial network. *Pant, Cell and Environment* **24:** 1219-1226.

Read DJ, Leake JR, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Canadian Journal of Botany-Revue Canadienne De Botanique* **82:** 1243-1263.

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytologist* **157:** 475-492.

Riedlinger J, Schrey SD, Tarkka MT, Hampp R, Kapur M, Fiedler HP. 2006. Auxofuran, a novel metabolite that stimulates the growth of fly agaric, is produced by the mycorrhiza helper bacterium *Streptomyces* strain AcH 505. *Applied and Environmental Microbiology* **72:** 3550-3557.

Rosling A, Lindahl BD, Taylor AFS, Finlay RD. 2004. Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. *FEMS Microbiol Ecol* **47:** 31-37.

Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT. 2005. Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria. New Phytologist* **168:** 205-216.

Simpson GG. 1964. Species density of North American recent mammals. *Systematic Zoology* **13(2):** 57-73.

Sinsabaugh RL, Foreman CM. 2003. Integrative dissolved organic matter metabolism and microbial diversity: An overview of conceptual models. In: Findlay SEG, Sinsabaugh RL, eds. *Aquatic ecosystems: Interactivity of dissolved organic matter.* USA: Academic Press, 425-454.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis.* London: Academic Press.

Staddon PL, Ramsey CB, Ostle N, Ineson P, Fitter AH. 2003. Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of C-14. *Science* **300:** 1138-1140.

Timonen S, Hurek T. 2006. Characterization of culturable bacterial populations associating with *Pinus sylvestris--Suillus bovinus* mycorrhizospheres. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **52:** 769-778.

Timonen S, Jorgensen KS, Haahtela K, Sen R. 1998. Bacterial community structure at defined locations of *Pinus sylvestris Suillus bovinus* and *Pinus sylvestris Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **44:** 499-513.

Twieg BD, Durall DM, Simard SW. 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* **176:** 437-447.

Unestam T, Sun YP. 1995. Extramatrical structures of hydrophobic and hydrophilic ectomycorrhizal fungi. *Mycorrhiza* **5:** 301-311.

Uroz S, Bueé M, Murat C, Frey-Klett P, Martin F. 2010. Pyrosequencing reveals a contrasted bacterial diversity between oak rhizosphere and surrounding soil. *Environmental Microbiology Reports, .*

Uroz S, Calvaruso C, Turpault MP, Pierrat JC, Mustin C, Frey-Klett P. 2007. Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. *Applied and Environmental Microbiology* **73:** 3019-3027.

Valverde A, Velázquez E, Fernández-Santos F, Vizcaíno N, Rivas R, Mateos PF, Martínez-Molina E, Igual JM, Willems A. 2005. *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. *International Journal of Systematic and Evolutionary Microbiology* **55:** 1985-1989.

van Breemen N, Lundstrom US, Jongmans AG. 2000. Do plants drive podzolization via rock-eating mycorrhizal fungi? *Geoderma* **94:** 163-171.

Wallander H. 2000a. Uptake of P from apatite by *Pinus sylvestris* seedlings colonised by different ectomycorrhizal fungi. *Plant and Soil* **218:** 249-256.

Wallander H. 2000b. Use of strontium isotopes and foliar K content to estimate weathering of biotite induced by pine seedlings colonised by ectomycorrhizal fungi from two different soils. *Plant and Soil* **222:** 215-229.

Wallander H, Hagerberg D. 2004. Do ectomycorrhizal fungi have a significant role in weathering of minerals in forest soil? *Symbiosis* **37:** 249-257.

Wallander H, Nilsson LO, Hagerberg D, Bååth E. 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytologist* **151:** 753-760.

Wallander H, Nilsson LO, Hagerberg D, Rosengren U. 2003. Direct estimates of C : N ratios of ectomycorrhizal mycelia collected from Norway spruce forest soils. *Soil Biology & Biochemistry* **35:** 997-999.

Wallander H, Pallon J. 2005. Temporal changes in the elemental composition of Rhizopogon rhizomorphs during colonization of patches with fresh organic matter or acid-washed sand. *Mycologia* **97:** 295-303.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73(16):** 5261-5267.

Xiao L, Xie CC, Cai J, Lin ZJ, Chen YH. 2009. Identification and characterization of a chitinase-produced bacillus showing significant antifungal activity. *Current Opinion in Microbiology* **58:** 528-533.

5   Discussion

I have investigated how ectomycorrhizal fungi may act as gatekeepers of forest productivity by controlling access to organic phosphorus as forests age after disturbance by stand replacing wildfire and after clearcut logging followed by broadcast burning. I developed new techniques to explore the functioning of ectomycorrhizal fungal hyphae in forest soil, taking an important step in the study of soil ecology at a fine scale in the field. The studies presented here focused on characterizing the contributions of ectomycorrhizal hyphae to an important nutrient mobilization process in forest soil. In Chapter 2 I visualized and quantified patterns of organic phosphorus mobilization (soil phosphatase activity) at a fine scale, *in situ*, and determined how these patterns changed in a chronosequence of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and paper birch (*Betula papyrifera* Marsh) in the southern interior of British Columbia, Canada. In Chapter 3 I associated ectomycorrhizal fungi with these patterns of organic phosphorus mobilization and determined that this relationship changed along the chronosequence. In Chapter 4 I characterized the effect of ectomycorrhizal hyphae on the bacterial community structure and found that phosphatase activities of nearby forest soil bacteria were reduced in the presence of EMF hyphae.

Because many economically important tree species depend on nutrients provided by root symbiotic ectomycorrhizal fungi (EMF) (Smith & Read, 1997; Read *et al.*, 2004), considerable effort has been directed toward understanding the structure and function of these fungal communities. Now, with increased appreciation of their substantial biomass in forest soils (Högberg & Högberg, 2002; Wallander *et al.*, 2001), dominant contribution to soil organic carbon (Godbold *et al.*, 2006), and ability to secrete extracellular hydrolytic enzymes (Luis *et al.*, 2005; Bending & Read, 1995), EMF are increasingly viewed as playing a major role in nutrient cycling in forest ecosystems (Schimel & Bennett, 2004; Talbot *et al.*, 2008; van der Heijden *et al.*, 2008). Many studies have surveyed EMF species present on root tips as a proxy for the relative abundance and importance of these species in forest ecosystems (reviewed by Horton & Bruns, 2001), and enzyme assays conducted on ectomycorrhizal root tips also have been used to approximate the contributions of EMF to nutrient mobilization in these systems

(Pritsch *et al.*, 2004; Courty *et al.*, 2005; Cullings *et al.*, 2008). Nevertheless, it is the extramatrical mycelia, in particular the hyphae, that are the primary interface between the fungus and soil nutrients, with an estimated 15% of the phosphorus supplied to trees mobilized by hyphae (Read & Perez-Moreno, 2003). Despite the common use of EMF species on root tips as a proxy for EMF community structure, the distribution and prevalence of these species on root tips does not accurately reflect the distribution of hyphae in soil (Koide *et al.*, 2005; Genney *et al.*, 2006; Gebhardt *et al.*, 2009). Furthermore, it is not known whether enzyme activities observed on ectomycorrhizal root tips are an accurate indication of activities near hyphal fans, which are interacting directly with organic and mineral substrates. By developing a method for visualizing and quantifying fine-scale soil phosphatase activity *in situ* (Chapter 2), I have provided an important tool for investigating the enzyme activities associated with hyphae growing in natural soil.

Ectomycorrhizal fungal communities change after disturbances such as wildfire and clear-cut logging, and there has been an ongoing effort to determine to what extent these changes translate into changes in nutrient mobilization by EMF. While EMF community structure may be affected by the changes in fungal inoculum present in soil after a stand-replacing event, it has been proposed that changes in EMF community structure may also be driven by the proliferation of fungi best suited to the changed soil conditions after disturbance (Jones *et al.*, 2003). EMF that colonize seedlings growing in recently disturbed areas appear to be better adapted to soil conditions in those areas. The fungi on these seedlings will persist on the seedlings after they are transplanted to disturbed areas, while EMF characteristic of undisturbed forest do not persist on seedlings transplanted to disturbed areas (Kranabetter & Friesen, 2002). How these changes in community structure relate to changes in function, such as nutrient mobilization to host trees, is not clear. While the species composition of EMF on root tips varies depending on whether the root tips are growing in high or low nutrient substrate (Tedersoo *et al.*, 2003, 2008), enzymatic activities of individual EMF species also vary depending on the substrate (Conn & Dighton, 2000; Tedersoo *et al.*, 2003); complicating the task of mapping changes in EMF community to changes in function (Jones *et al*. 2010). By developing a new soil sampling method that connects EMF species present in soil with locations

of active nutrient mobilization (Chapter 3), I provide the first evidence of changes in the distributions of EMF hyphae with respect to a nutrient mobilization process as forests age.

EMF hyphae exist in a complex soil environment where they interact with other EMF as well as saprotrophic fungi and bacteria to gain access to organic nutrients. Because EMF hyphae are microscopic, fragile, and often intermingled with the hyphae of closely related saprotrophic fungi (Hibbett *et al.*, 2000), it has been a challenge to study the functions and interactions of EMF hyphae in the field. Ectomycorrhizal hyphae proliferate in patches of organic matter present in soil microcosms (Perez-Moreno & Read, 2000, 2001), and phosphatase enzymes are primarily located where fine hyphae interact with organic substrates (Timonen *et al.*, 1998). However, EMF hyphae can be displaced from organic substrates by saprotrophic hyphae (Lindahl *et al.*, 1999; Leake *et al.*, 2002), and saprotrophic hyphae can be displaced by saprotrophic bacteria (Jayasinghe & Parkinson, 2008). Antagonistic interactions between saprotrophic bacteria, especially the Streptomycetes and soil fungi have been well documented (Friedman *et al.*, 1989; Axelrood *et al.*, 1996; Maier *et al.*, 2004; Jayasinghe & Parkinson, 2008); however, numerous saprotrophic bacteria are also capable of promoting the growth of EMF hyphae, including a *Streptomyces* sp. (Schrey *et al.*, 2005). While the bacterial community structure near hyphae growing in soil is significantly different than the community found in uncolonized soil (Timonen & Hurek, 2006), it is unclear to what extent bacteria located near hyphae affect nutrient mobilization by EMF. By extending an *in situ* technique to trap forest soil bacteria and bacteria associated with EMF hyphae and comparing the enzyme activities of these bacteria with a modified microplate enzyme assay (Chapter 4), I am the first to demonstrate significant functional and structural differences between these two bacterial communities, especially amongst the potentially competitive *Streptomyces* spp.

5.1   Main findings

In Chapter 2 I visualized soil phosphatase activity at a fine scale, *in situ*, using a novel enzyme imprinting technique and characterized rates and patterns of this nutrient mobilizing process using image analysis. I demonstrated changes in both the quantity and pattern of phosphatase activity in a chronosequence of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco)

and paper birch (*Betula papyrifera* Marsh) in the southern interior of British Columbia, Canada, spanning several stages of stand development. In particular, I demonstrated a significant increase in phosphatase activity and increase in the average size of areas of phosphatase activity as forests reached the stem exclusion stage of stand development.

In Chapter 3 I expanded the imprint method developed in Chapter 2 to map fine scale phosphatase activity to fungal DNA present in soil. By using phosphatase imprints to guide the collection of small soil samples, I found a distinct shift in the distribution of molecular signatures of EMF species with relation to fine scale phosphatase activity at both the stem exclusion and post-stem exclusion stages of stand development. In the younger, initiation and canopy closure stands, where the pattern of phosphatase activity was characterized by a low number of small areas of enzyme activity, EMF species present in soil were more uniformly distributed in the soil profile than in the older, stem exclusion and post-stem exclusion stands. In those older stands, soil phosphatase activity occurred in larger patches, and in most cases, there were fewer EMF species present in these patches of phosphatase activity than in soil areas with lower activity. The exception to this pattern was in the organic layers of the oldest stands where EMF species were largely missing from soil areas with lower phosphatase activity, and the few species identified were present in high activity patches. Only two, infrequent EMF species were found more often in high phosphatase patches than lower activity areas, while six of the most commonly encountered EMF species were more frequent in areas of lower phosphatase activity.

In Chapter 4 I characterized the effects of EMF hyphae on the enzyme activities of nearby soil bacteria and demonstrated that bacteria isolated from sand colonized by EMF hyphae had lower phosphatase activities than bacteria isolated from uncolonized sand. I found that EMF hyphae not only changed the community structure of nearby soil bacteria by reducing diversity, but that this lower diversity was correlated with lower bacterial phosphatase activities. I also found that potentially antagonistic *Streptomyces* spp. were more prevalent in colonized sand and were faster growing that *Streptomyces* spp. isolated from uncolonized sand. However, this promotion of *Streptomyces* spp. in colonized sand did not correlate with indicators of enhanced capacity to compete with fungi: the *N*-acetyl glucosaminadase (NAGase)

activities of these isolates, which would have provided access to the nitrogen and carbon from fungal cell walls, was not enhanced compared to these activities in *Streptomyces* spp. isolated from uncolonized sand. The only frequently encountered genus to be more prevalent in colonized sand and to both grow faster and demonstrate enhanced NAGase activity when isolated from colonized sand, was *Phyllobacterium*; a known plant growth-promoting bacterial genus commonly found in the rhizosphere.

5.2   Strengths and weaknesses

In Chapter 2 I extended published methods for studying phosphatase *in situ*, combined this new assay with a root window approach, and used a custom written program to generate a spatial database of phosphatase activity; creating a new approach to the fine-scale study of soil ecology in the field. Schimel & Bennett (2004) argue that increased understanding of soil processes will come only if we study them at much a finer scale than is possible with destructive soil sampling. While the enzyme imprinting method was non-destructive, the installation of root windows resulted in some initial soil disturbance. I was able to dig soil faces that were quite smooth, but they still required a small amount soil replacement behind the window after installation. Because the soil horizons were kept separate as the holes were dug, I was able to replace soil horizons to the appropriate depths behind the windows. However, the soil directly against the window was certainly disturbed, and this disturbance would be expected to have an impact on microbial activity for a period after the installation of the windows. Nevertheless, by leaving the windows in place for at least five months during the active growing period, I feel confident that appropriate microbial communities had re-established at the surface of the soil profile.

The close relationship between the concentrations of standard enzyme added to imprinting paper and colour development in the field allowed the construction of standard curves for the estimation of enzyme activities *in situ*. However, while imprinted phosphatase activity has been found to be significantly correlated with those from traditional root assays, factors such as the age and diameter of can roots influence the degree of agreement between the two methods (Jones & Grierson, unpublished). Therefore, it is best to describe this

approach as semi-quantitative. In addition, both the degree of contact between the paper and the soil, and the soil moisture content at the time of imprinting could also influence colour development. Soil pH and temperature also affect enzyme activity and these factors can vary at a fine scale even within the soil profile of individual root windows. For example, in the windows installed across a chronosequence of Douglas fir/paper birch forests, pH varied as much as 0.1 unit across one window and as much as 1.3 units between windows on the same site (Dong & Jones, unpublished). Moreover, the temperature, as measured by a non-contact infrared thermometer, varied as much as 3.2 °C across one window and ranged from 0.5 to 18 °C across all measurements (Dong & Jones, unpublished). So while imprinted enzyme activity was highly variable between forests, the major advantage of enzyme imprinting is that it detected actual enzyme activity as it occurred in the field in soil microsites, and it is this fine-scale variation in environmental conditions that is typically lost during soil sampling.

While the stands used for the chronosequence were carefully selected to be as similar as possible in soil and stand characteristics, there were still important differences in their regeneration history that need to be considered when interpreting the changes in phosphatase activity seen in the older stands. The stands at the canopy closure stage of development had regenerated from wildfire after being salvaged logged and replanted. Amongst the youngest stands, one site had been generated by wildfire and subsequently salvage logged and replanted. The other two sites were generated by clear-cut logging followed by burning and replanting. In contrast, the stands at the stem exclusion and post stem exclusion stages of development had regenerated naturally from stand-replacing wildfires, with dead stems left in place. Therefore, the initial coarse woody debris present on the older sites may have been quite different than on the younger sites, changing the initial resources available to saprotrophic fungi in these stands. Using traditional soil enzyme assays, other research has shown a significant increase in phosphatase activity at the canopy closure stage compared to initiating stands, with a subsequent tendency to decrease in stem exclusion stands of mountain ash (*Eucalyptus regnans* F. Muell III.) (Polglase *et al.*, 1992). In contrast, the canopy closure stands in our study had significantly lower imprintable phosphatase activity compared to stem exclusion stands. The relationship between imprintable phosphatase activity and traditional soil enzyme assays

needs further testing, and further studies will be needed to determine whether differences in imprintable phosphatase activity are consistent with stages of stand development and type of stand initiation event among different types of forest.

The study in Chapter 3 is the first study to link EMF to an active soil process *in situ*. Our results demonstrated that fine-scale sampling within the area imprinted for enzyme activity (20 cm x 20 cm areas of soil profile) could detect differences in fungal assemblages associated with landscape-scale (many kilometers) changes in vegetation as well as fine-scale differences between soil microsites. Because previous studies using molecular methods to identify fungal hyphae in soil used soil cores taken from homogenous stands and did not address landscape-scale questions (Dickie *et al.*, 2002; Koide *et al.*, 2005; Genney *et al.*, 2006), our results made an important contribution to the expansion of fine-scale molecular methods to large-scale ecological questions. However, as this was the first study to apply this sort of fine-scale method to a landscape-scale process, the sampling intensity needed to characterize differences in the fine-scale distribution of hyphae in soil with stand age in replicate stands replicated across the southern interior of British Columbia was entirely unknown when the study was initiated. While some significant differences were detected, the data obtained was highly variable and the expected differences in EMF community between organic and mineral soil layers were not detected. Future studies are needed to further refine the sampling intensity and appropriate spatial scales for the application of this fine-scale, imprint guided microsampling technique.

The molecular method used to identify EMF in soil (Terminal Restriction Fragment Length Polymorphism or TRFLP) was chosen because it was appropriate for characterizing the large number of samples needed for a landscape scale analysis, being both rapid and inexpensive. An additional benefit was access to DNA previously extracted from ectomycorrhizal root tips taken from these stands at two different sampling dates by Twieg *et al*. (2007) for the development of a site-specific library of EMF TRFLP signatures. I developed this library for the identification of EMF signatures in the soil microsamples; however, the library was not comprehensive because extracted root tip DNA representing 33% of the unique phylotypes present on the study sites failed to generate the type of molecular signature required for the TRFLP signature library. Given the limitations of the library used to EMF

identification, TRFLP proved effective for identifying differences in the EMF DNA from high phosphatase soil patches compared to low activity soil areas. However, because this technique provides no information about the total EMF biomass present or the biomass of any individual EMF species, these results cannot be used to infer a difference in the amount of EMF hyphae present in these microsites. Furthermore, because the molecular method used was based on ribosomal DNA, which can be long-lived in soil, rather than on much shorter-lived mRNA, it is likely that DNA from fungal necromass as well as from some fungal spores was amplified along with DNA from living fungal hyphae. While it is unclear what proportion of EMF species was identified from living hyphae, necromass, or spores, the distribution of each of these EMF components in soil is indicative of fungal growth and activities in soil over time.

In Chapter 4 I extended two previously developed techniques to address new questions. To my knowledge, I was the first to use sand-filled mesh bags to characterize the effects of fungal hyphae on the enzyme activities of forest soil bacteria. The microplate enzyme assay I modified to quantify bacterial isolate enzyme activity proved to be both rapid and repeatable. In addition, by using isolation methods that selected for cold-tolerant, slow-growing bacteria, I characterized a segment of the bacterial community that is highly characteristic of forest soils (Elo *et al.*, 2000; Axelrood *et al.*, 2002; Chow *et al.*, 2002) and made a significant contribution to the understanding of the effects of EMF hyphae on their potential competitors, the Streptomycetes. While isolating bacteria from the sand-filled mesh bags allowed us to measure important nutrient mobilization activities by these bacteria, these isolates represent only a small portion of soil bacteria present in the sand bags. It is well known that only a small proportion of soil bacteria can be readily isolated. The Acidobacteria in particular, are quite prevalent in forest soil (Axelrood *et al.*, 2002; Uroz *et al.*, 2010), but are difficult to isolate (Janssen *et al.*, 2002). None were isolated in our study. Our isolation techniques favored the selection of Actinobacteria, and this bacterial group usually accounts for a relatively small proportion of the bacteria present in soils of conifer forests (Elo *et al.*, 2000; Axelrood *et al.*, 2002; Chow *et al.*, 2002). However, in the clone library I generated, representing the PCR-amplifiable bacterial community present in the bags, PCR products representing Actinobacteria made up a large fraction of the products generated from the sand

bags. In addition there were more Gram-negative and fewer Gram-positive bacteria in the clone library from the sand than would be expected in forest soil, suggesting that the bacterial community in the mesh bags likely differed from that in the adjacent soil. However, a clone library generated from uncolonized forest soil from the study site would have provided more information regarding the composition of the bacterial community available for migration into the bags.

The sand-filled mesh bags effectively allowed us to access segments of the soil bacterial community that had been influenced by hyphal ingrowth. However, sand is an artificial substrate and the behavior of EMF hyphae changes when growing through sand (Olsson & Wallander, 1998; Wallander *et al.*, 2005). In addition, because I wanted to assess the effects of living hyphae on nearby soil bacteria, the bags were made as small as possible and were incubated *in situ* for a reasonably short time period (five months). While the short incubation time minimized the extent of hyphal necromass in the bags, hyphal ingrowth was also sparse and patchy. Ingrowth was difficult to verify, either visually on the bags used for bacterial isolation or by qPCR. The small size of the bags compensated for the short incubation time by limiting the volume of substrate that the hyphae had to penetrate; however, it also resulted in a very limited amount of substrate for DNA extraction, bacterial isolation, and verification of hyphal ingrowth and exclusion. Because our goal was to assess the effect of EMF hyphae on nearby soil bacteria, I took blind, random samples of substrate from each sand bag for bacterial isolation without regard to the relative level of hyphal colonization of the substrate. In addition to assessing the effects of hyphae on soil bacteria, additional information about the structure and function of the bacterial communities in the hyphosphere could have been gained by extracting hyphae from hyphae-ingrowth bags and isolating bacteria directly from the hyphosphere. This additional investigation was not possible in our study due to the small size of the mesh bags used and labor intensive nature of bacterial isolation and characterization. Nevertheless, in future studies I recommend that hyphosphere bacteria also be extracted and characterized.

5.3   Interpretation and future directions

These studies suggest that EMF affect, and are affected by, the mobilization of organic phosphorus. Patterns of soil phosphatase changed from smaller to larger areas of activity in the same stand age when changes in EMF community composition had been observed to stabilize (Twieg *et al.*, 2007). The highest level of imprintable phosphatase activity also occurred at the stand age where guided microsampling found significantly fewer EMF TRFLP signatures in high phosphatase microsites compared to low phosphatase microsites. The paucity of EMF species in low phosphatase areas in the organic layers of the oldest stands also correlated with the slightly lower phosphatase activity observed in these stands. The EMF hyphae growing on older stands also influenced nearby soil bacterial communities by reducing their phosphatase activities. However, I faced several challenges in extrapolating from these results.

First, the histories of the stands in the chronosequence were not identical; stem exclusion and older stands had not been salvage-logged and replanted after stand-replacing wild-fire. Therefore, the significant increase in phosphatase and the increase in the number of larger, high phosphatase patches seen in the stem exclusion and older sites may have resulted from these differences in site history, as well as by stand age. For example, if stems left on site were providing a significant source of carbon for saprotrophic fungi at stem exclusion and in the older stands, saprotrophic fungal activities may have accounted for the change in the pattern of phosphatase. Because the outcomes of competitive interactions between saprotrophic fungi and EMF are influenced by the carbon resources available to the competitors (Lindahl *et al.*, 2001), then additional carbon for saprotrophs from decomposing boles may allow these fungi to exclude EMF from high phosphorus resources. To address this issue, smaller root windows, which could be inserted with less soil disturbance, could be used. These could be developed into a low cost, rapid system for assessing changes in soil function with various site factors including site history and stand age. By developing a database of "natural" patterns of activity for stands with the same history it would be possible track and model the effects of disturbance and modify harvesting to better simulate natural disturbances.

Second, the lack of biomass information for the EMF species sampled from high and low phosphatase patches limits our ability to interpret the functional role of EMF with relation

to phosphatase activity. In particular, it does not allow us to determine whether the higher frequency of EMF species in low compared to high phosphatase areas is indicative of a suppression of phosphatase by these species or is a consequence of the proliferation of EMF species that may have encountered a high phosphorus resource first. It is also possible that EMF are excluded from high phosphorus resources by saprotrophic fungi, but this interaction would likely be largely restricted to the organic layer where saprotrophic fungi proliferate. Therefore, to explore the fungal dynamics that may control access to organic phosphorus in organic soil layers, a high-throughput sequencing method should be used to characterize both the EMF and saprotrophic fungi present in soil microsites. In the mineral layer, it is possible that EMF are excluded from resources by saprotrophic bacteria, but known fungal antagonists, such as the Streptomycetes are strictly aerobic and would be less abundant in the lower mineral layers. Rather than being excluded, the more likely explanation for the greater number of EMF species in low phosphatase areas is that EMF hyphae proliferate around areas of phosphatase activity in order to absorb phosphorus mobilized by both bacterial and fungal phosphatase production. However, physiological barriers may exist in the mineral layer that could result in the inability of fungal hyphae to penetrate high resource microsites. The higher respiration that results from increased microbial activity can result in lower oxygen tensions in high resource areas in soil (Kremen *et al.*, 2005); possibly leading to exclusion of the strictly aerobic fungi from these hot spots. This sort of interaction could contribute to fine scale soil structures that support soil fertility by harnessing the ability of bacteria to function across a broad range of oxygen tensions and capturing the resources they release for tree growth.

Third, by conducting enzyme assays on bacteria isolated from sand bags, I have only characterized the effects of hyphae on a small proportion of the soil bacterial community. Also, hyphae exhibit different characteristics when growing through sand than through soil (Wallander *et al.*, 2005), and these differences may affect the nearby bacterial community structure. Using forest soil as the ingrowth substrate would give a more realistic picture of the effect of hyphae on the soil community. However, while a sand substrate selects for EMF hyphae, both saprotrophic and EMF hyphae may tend to explore a soil substrate. Current efforts are underway to investigate the interaction of EMF hyphae with particular substrates by

first enclosing the substrates in sand-filled mesh containers before incubating them on site (L. Phillips, pers. comm.). A similar approach might be to use natural soil as the substrate for ingrowth and exclusion bags and place these bags in sand-filled mesh containers for incubation. However, because of the large number of bacteria in natural soils, characterizing changes in the bacterial community using isolation methods may not be practical. Instead, changes in the soil bacterial community could be tracked by molecular methods; characterizing the 16S rDNA, along with other functional genes (reviewed by Kelly, 2003) as indicators of changes in community structure and function. Furthermore, the effects of hyphae on bacterial enzyme activities could be further investigated by conducting a transplantation study using bacterial isolates from hyphae ingrowth and exclusion bags. Isolates from ingrowth bags could be inoculated into microcosms containing non-mycorrhizal seedlings, while isolates from exclusion bags could be inoculated into microcosms with mycorrhizal seedlings. Bacteria could then be re-isolated from the two microcosm types and tested for enzyme activity to determine if the differences in enzyme activity were maintained after exposure (or loss of exposure) to EMF hyphae.

5.4   Overall significance

This research provides important scientific information addressing a serious gap in our knowledge of forest ecosystem functioning and the sustainability of forest resource use. B.C.'s Forest Practices Act (http://www.for.gov.bc.ca/code/), Canada's National Forest Strategy (http://www.ccfm.org/english/coreproducts-nscf.asp), and Canada's Biodiversity Strategy (http://www.eman-rese.ca/eman/reports/publications/rt_biostrat/intro.html) all emphasise the importance of maintaining forest biological diversity, the ecological integrity of forests, and ensuring sustainable use of forest resources. The studies presented here connect EMF associated with a fine-scale soil process to the ecosystem process of forest regeneration, helping to elucidate one component of the below-ground interactions that may be in involved in fine-scale soil spatial heterogeneity. This understanding is essential for the understanding of fine-scale soil processes and for determining how these processes affect ecosystems at a landscape scale.

These were the first studies to demonstrate differences in the function of EMF communities present as hyphae in the soil of regenerating forests at key stages of stand development. The shift from EMF hyphae in the organic forest floor layer being associated with low phosphatase activity at most stages of stand development to being associated with high phosphatase activity in the oldest forest stands may have important management implications. This change in EMF hyphal distribution may indicate a more active role for EMF in acquisition of organic phosphorus in older stands and could indicate a functional maturity of the EMF fungal community. If this, in turn, was associated with fruiting of some of these fungi, such as those requiring decaying logs to form their reproductive structures, then maintaining these older stands in the landscape would be particularly important for renewal of the EMF spore bank to inoculate regenerating stands.

The methods developed here have potential for establishing indicators of natural regeneration and for monitoring forest soil function. By revealing a shift in EMF function with stand age, the fine-scale sampling method has provided evidence of a critical stage in forest stand development, at the stem exclusion stage, that is closely linked with tree nutrient acquisition. By monitoring when this shift in EMF function occurs in managed stands, compared to naturally regenerating stands, we can better assess and tailor management methods that simulate natural disturbance and enhance healthy regeneration.

5.5   Bibliography

Axelrood PE, Chow ML, Arnold CS, Lu K, McDermott JM, Davies J. 2002. Cultivation-dependent characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **48:** 643-654.

Axelrood PE, Clarke AM, Radley R, Zemcov SJ. 1996. Douglas-fir root-associated microorganisms with inhibitory activity towards fungal plant pathogens and human bacterial pathogens. *Canadian Journal of Forest Research/Revue Canadienne de Recherche Forestiere* **42:** 690-700.

Bearden BN, Peterson L. 2000. Influence of arbuscular mycorrhizal fungi on soil structure and aggregate stability of a vertisol. *Plant and Soil* **218:** 173-183.

Bending GD, Read DJ. 1995. The structure and function of the vegetative mycelium of ectomycorrhizal plants.V. Foraging behaviour and translocation of nutrients from exploited litter. *New Phytologist* **130:** 401-409.

Bossuyt H, Denef K, Six J, Frey SD, Merckx R, Paustian K. 2001. Influence of microbial populations and residue quality on aggregate stability. *Applied Soil Ecology* **16:** 195-208.

Chow ML, Radomski CC, McDermott JM, Davies J, Axelrood PE. 2002. Molecular characterization of bacterial diversity in Lodgepole pine (Pinus contorta) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. *FEMS Microbiol Ecol* **42:** 347-357.

Conn C, Dighton J. 2000. Litter quality influences on decomposition, ectomycorrhizal community structure and mycorrhizal root surface acid phosphatase activity. *Soil Biology & Biochemistry* **32:** 489-496.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167:** 309-319.

Cullings K, Ishkhanova G, Henson J. 2008. Defoliation effects on enzyme activities of the ectomycorrhizal fungus Suillus granulatus in a Pinus contorta (lodgepole pine) stand in Yellowstone National Park. *Oecologia* **158:** 77-83.

Dickie IA, Xu B, Koide RT. 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist* **156:** 527-535.

Dorodnikov M, Blagodatskaya E, Blagodatsky S, Marhan S, Fangmeier A, Kuzyakov Y. 2009. Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. *Global Change Biology* **15:** 1603-1614.

Elo S, Maunuksela L, Salkinoja-Salonen M, Smolander A, Haahtela K. 2000. Humus bacteria of Norway spruce stands: plant growth promoting properties and birch, red fescue and alder colonizing capacity. *FEMS Microbiology Ecology* **31:** 143-152.

Fansler SJ, Smith JL, Bolton Jr. H, Bailey VL. 2005. Distribution of two C cycle enzymes in soil aggregates of a prairie chronosequence. *Biology and Fertility of Soils* **42:** 17-23.

Friedman J, Hutchins A, Li CY, Perry DA. 1989. Actinomycetes inducing phytotoxic or fungistatic activity in a Douglas-fir forest and in an adjacent area of repeated regeneration failure in Southwestern Oregon. *Biologia Plantarum* **31(6):** 487-495.

Gartzia-Bengoetxea N, González-Arias A, Merino A, Martínez de Arano I. 2009. Soil organic matter in soil physical fractions in adjacent semi-natural and cultivated stands in temperate Atlantic forests. *Soil Biology and Biochemistry* **41:** 1674-1683.

Gebhardt S, Wöllecke J, Münzenberger B, Hüttl RF. 2009. Microscale spatial distribution patterns of red oak (Quercus rubra L.) ectomycorrhizae. *Mycological Progress* **8:** 245-257.

Genney DR, Anderson IC, Alexander IJ. 2006. Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytologist* **170:** 381-390.

Godbold DL, Hoosbeek MR, Lukac M, Cotrufo MF, Janssens IA, Ceulemans R, Polle A, Velthorst EJ, Scarascia-Mugnozza G, De Angelis P, et al. 2006. Mycorrhizal hyphal turnover as a dominant process for carbon input into soil organic matter. *Plant and Soil* **281:** 15-24.

Guggenberger G, Elliott ET, Frey SD, Six J, Paustian K. 1999. Microbial contributions in the aggregations of a cultivated grassland soil amended with starch. *Soil Biology and Biochemistry* **31:** 407-419.

Hibbett DS, Gilbert LB, Donoghue MJ. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* **407:** 506-508.

Horton TR, Bruns TD. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* **10:** 1855-1871.

Högberg MN, Högberg P. 2002. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **154:** 791-795.

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia. Applied and Environmental Microbiology* **68:** 2391-2396.

Jasinska E, Wetzel H, Baumgartl T, Horn R. 2006. Heterogeneity of physico-chemical properties in structured soils and its consequences. *Pedosphere* **16:** 284-296.

Jayasinghe BATD, Parkinson D. 2008. Actinomycetes as antagonists of litter decomposer fungi. *Applied Soil Ecology* **38:** 109-118.

Jones MD, Durall DM, Cairney JWG. 2003. Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging. *New Phytologist* **157:** 399-422.

Jones MD, Twied BD, Ward V, Barker J, Durall DM, Simard SW. 2010. Functional complementarity of Douglas-fir ectomycorrhizas for extracellular enzyme activity after wildfire or clearcut logging. *Functional Ecology (In press).*

Kelly JJ. 2003. Molecular techniques for the analysis of soil microbial processes: Functional gene analysis and the utility of DNA microarrays. *Soil Science* **168:** 597-605.

Koide RT, Xu B, Sharda J, Lekberg Y, Ostiguy N. 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* **165:** 305-316.

Kranabetter JM, Friesen J. 2002. Ectomycorrhizal community structure on western hemlock (Tsuga heterophylla) seedlings transplanted from forests into openings. *Canadian Journal of Botany/Revue Canadienne de Botanique* **80:** 861-868.

Kremen A, Bear J, Shavit U, Shaviv A. 2005. Model demonstrating the potential for coupled nitrification denitrification in soil aggregates. *Environ Sci Technol* **39:** 4180-4188.

Leake JR, Donnelly DP, Boddy L. 2002. Interactions between ectomycorrhizal fungi and saprotrophic fungi in mycorrhizal ecology. *Ecological Studies* **157:** 346-372.

Lindahl B, Stenlid J, Finlay R. 2001. Effects of resource availability on mycelial interactions and 32P transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. *FEMS Microbiology Ecology* **38:** 43-52.

Lindahl B, Stenlid J, Olsson S, Finlay R. 1999. Translocation of P-32 between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. *New Phytologist* **144:** 183-193.

Luis P, Kellner H, Martin F, Buscot F. 2005. A molecular method to evaluate basidiomycete laccase gene expression in forest soils. *Geoderma* **128:** 18-27.

Maier A, Riedlinger J, Fiedler HP, Hampp R. 2004. Actinomycetales bacteria from a spruce stand: characterization and effects on growth of root symbiotic and plant parasitic soil fungi in dual culture. *Mycological Progress* **3:** 129-136.

Olsson PA, Wallander H. 1998. Interactions between ectomycorrhizal fungi and the bacterial community in soils amended with various primary minerals. *FEMS Microbiology Ecology* **27:** 195-205.

Perez-Moreno J, Read DJ. 2000. Mobilization and transfer of nutrients from litter to tree seedlings via the vegetative mycelium of ectomycorrhizal plants. *New Phytologist* **145:** 301-309.

Perez-Moreno J, Read DJ. 2001. Nutrient transfer from soil nematodes to plants: a direct pathway provided by the mycorrhizal mycelial network. *Plant Cell and Environment* **24:** 1219-1226.

Polglase PJ, Attiwill PM, Adams MA. 1992. Nitrogen and phosphorus cycling in relation to stand age of *Eucalyptus regnans* F. Muell III. Labile inorganic and organic P, phosphatase activity and P availability. *Plant and Soil* **142:** 177-185.

Pritsch K, Raidl S, Marksteiner E, Blaschke H, Agerer R, Schloter M, Hartmann A. 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* **58:** 233-241.

Read DJ, Leake JR, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Canadian Journal of Botany-Revue Canadienne De Botanique* **82:** 1243-1263.

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytologist* **157:** 475-492.

Schimel JP, Bennett J. 2004. Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **85:** 591-602.

Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT. 2005. Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria. New Phytologist* **168:** 205-216.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis.* London: Academic Press.

Talbot JM, Allison SD, Treseder KK. 2008. Decomposers in disguise: mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. *Functional Ecology* **22:** 955-963.

Tedersoo L, Koljalg U, Hallenberg N, Larsson KH. 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. *New Phytologist* **159:** 153-165.

Tedersoo L, Suvi T, Jairus T, Kõljalg U. 2008. Forest microsite effects on community composition of ectomycorrhizal fungi on seedlings of Picea abies and Betula pendula. *Environmental Microbiology* **10:** 1189-1201.

Timonen S, Hurek T. 2006. Characterization of culturable bacterial populations associating with *Pinus sylvestris--Suillus bovinus* mycorrhizospheres. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **52:** 769-778.

Timonen S, Jorgensen KS, Haahtela K, Sen R. 1998. Bacterial community structure at defined locations of *Pinus sylvestris Suillus bovinus* and *Pinus sylvestris Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. *Canadian Journal of Microbiology/Revue Canadienne de Microbiologie* **44:** 499-513.

Twieg BD, Durall DM, Simard SW. 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* **176:** 437-447.

Uroz S, Bueé M, Murat C, Frey-Klett P, Martin F. 2010. Pyrosequencing reveals a contrasted bacterial diversity between oak rhizosphere and surrounding soil. *Environmental Microbiology Reports, .*

van der Heijden MG, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* **11:** 296-310.

Wallander H, Fossum A, Rosengren U, Jones H. 2005. Ectomycorrhizal fungal biomass in roots and uptake of P from apatite by *Pinus sylvestris* seedlings growing in forest soil with and without wood ash amendment. *Mycorrhiza* **15:** 143-148.

Wallander H, Nilsson LO, Hagerberg D, Bååth E. 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytologist* **151:** 753-760.

Appendices

Appendix A   Imprinting soil enzyme activity[4]

A-1 Introduction

In the methods presented here, root windows are used to access relatively undisturbed soil profiles for imprinting of enzyme activity. Nitrocellulose membranes or cellulose sheets, with or without imbedded substrates, are applied to the soil face to produce imprints of enzyme activity. Depending on the method used, information obtained from the imprints can be used to correlate enzyme activities with visual features on high resolution images of the soil face, or to guide sampling of soils for molecular analysis of soil microbial communities in patches differing in enzyme activity levels.

A-2   Sampling: window installation

(Grierson & Comerford, 2000; Smit *et al*., 2000)

The sampling window consists of a transparent and acrylic sheet e.g., Plexiglas (5 mm thick) inset with a hinged window held in place by a small latch (Figure A-1). Windows may vary in size depending on the purpose, but smaller windows tend to maintain better contact with the soil. In the design of Grierson & Comerford (2000), hinges are covered by a flexible rubber seal on the inside of the window, which prevents any soil particles falling into the hinge and becoming trapped when the window is open and preventing window re-closure. The sampling window has an internal measurement area of 20 cm X 20 cm. A small hole (0.5 mm i.d.) is drilled into each corner and in the centre of the measurement area to assist in alignment of any images of enzyme activity and photos of the root system or soil surface that may require subsequent analysis.

A-2.1   Apparatus

1.   Plexiglas or other transparent glass or plastic, 5 mm thick

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[4] A version of this appendix has been submitted for publication. **Jones MD, Brooks D, Courty P-E, Garbaye J, Grierson PF and Pritsch K. 2009.** Methods for linking enzyme activities with fine-scale soil microsites.

2. Two hinges, non-corrosive

3. Rubber adhesive for hinge seal

4. Steel plate approximately 4 cm wider than the Plexiglas plate, 3-5 mm thick

5. Hammer and shovel

6. Vertical steel rods, 1 m long x 1 cm diameter

7. Wooden pegs, 50 cm length, four per window

8. Wooden wedges

9. Insulation block e.g. Styrodur, Styrofoam (or plywood and fibreglass insulation as an alternative)

10. Black plastic

11. Aluminum screening, chicken wire, or hardware cloth (optional)

A-2.2 Procedure

Installation of the window follows the general recommendations of Smit et al. (2000).

1. Hammer the steel plate at a specific angle to the soil surface, usually at an angle of 90°, although the angle may vary depending on the soil depth, the plant species and the soil type that are the focus of the study.

2. Excavate all the soil away from the plate, to the depth of plate installation. Soil from the excavated pit should be separated by depth in case backfilling is required and stored well away from the window to prevent contamination. Construction of a ramp on the side of the pit opposite the plate to facilitate access and observation of the pit may also make working in the pit more comfortable.

3. Remove the steel plate and smooth the surface. Where there are large roots or stones, these may be trimmed and the stones removed and the voids filled with the soil from the appropriate depth.

4. While Smit *et al*. (2000) recommend heating of the Plexiglas window in a warm oven prior to installation to produce slight concavity as this enhances overall soil-window contact on installation, in this instance the hinged sampling portion of the window may become distorted. Consequently, we do not recommend this step. Instead, push the window firmly against the surface, and hammer two vertical steel rods in

about 10 cm either side of the hinged sampling window (Figure A-1). Additional wooden pegs with wedges will further improve contact between the window and the soil surface (see Smit *et al*. 2000 and Figure A-1 for details).

5.   Cover the window with a layer of insulation and black plastic to protect against light and temperature fluctuations. This can comprise a piece of rigid insulation, such as Styrofoam or Styrodur, or a piece of plywood covered with fiberglass insulation. In either case, the insulation should be protected with aluminum screening or some heavier kind of metal mesh to protect it from attack if you are working in areas where small mammals might use it as nesting material. Alternatively, the root-window pit can be backfilled to the original soil grade and re-excavated as needed, particularly in the period where the window is being left to stabilize across seasons. Backfilling the pit is also more resistant to disturbance by animals and weather.

Comments: Installing root windows inevitably results in some initial soil disturbance and this would be expected to have an impact on enzyme activity. Windows should be left undisturbed for a period of time to allow the soil to stabilize and for roots and microbial communities to establish or re-equilibrate against the window. The time required could range from weeks to years depending on the question being asked, the time of year, and the environmental conditions in the area. Consideration should also be given to the degree of backfilling of original soil behind the window that is required. This aspect is of less importance if the objective is experimental and different areas of the soil interface have been manipulated e.g., backfilling different parts of the window with soils of variable texture, nutrient content, organic matter content or pH. The integrity of the window should be maintained at all times by preventing disturbance or walking on the ground behind the window.

A-3   Preparation of imprints

(Dinkelaker & Marschner, 1992; Grierson & Comerford, 2000; Dong *et al*., 2007)

Imprinting assays have been developed for four hydrolytic enzymes to date: acid phosphatase (monoesterase), leucine aminopeptidase, chitinase and β-glucosidase. Detection of acid phosphatase is based on the hydrolysis of α-naphthyl phosphate by phosphomonoesterase to release naphthol, which reacts with the diazonium salt Fast Red TR to

form a stable red precipitate (Dinkelaker & Marschner, 1992). The pH of the buffer recommended below was selected based on pH optima determined by Dinkelaker & Marschner (1992) and confirmed with subsequent testing by Grierson & Comerford (unpublished data). The detection of amino peptidase is based on the hydrolysis of L-leucyl 2-naphthylamide to release naphthol, which reacts with Fast Blue BB Salt hemi (zinc chloride) salt to form an orange-red colour (Humble *et al.*, 1977; Reymond & Wahler, 2002). Fast Blue is more effective than Fast Red for this assay (Dong *et al.*, 2007).

Numerous 4-methylumbelliferone (4-MUB) -linked substrate analogues are now available. Methods to quantify enzyme activities based on the release of fluorescent 4-MUB have been developed for small soil samples and root tips (Hoppe, 1983; Sinsabaugh *et al.*, 1991; Pritsch *et al.*, 2004; Courty *et al.*, 2005). Two types of enzyme activities, chitinase and β-glucosidase, have been studied on soil imprints using these substrates (Dong *et al.*, 2007). Chitinase activities are based on the hydrolysis of MU-N-acetyl-β-glucosaminide. The detection of β-glucosidase is based on the hydrolysis of 4-methylumbelliferyl-β-glucopyranoside dehydrate.

These in situ enzyme assays use either cellulose sheets or nitrocellulose membranes, which are treated with a mixture of substrate and chromogenic reagent, or with a fluorogenic substrate analogue, either before or after being placed directly against the soil profile. Enzyme activity at the soil surface is indicated by the appearance of spots of coloured or fluorescent product on the membranes. To obtain imprints with sufficient resolution to allow correlations of activity with visible soil features at a pixel-by-pixel scale, nitrocellulose membranes must be used. However, nitrocellulose membranes are more expensive than chromatography paper, and consequently the advantages of increased resolution need to be weighed against the higher costs, especially if the experiment involves repeated sampling of many root windows. If resolution at the mm scale is sufficient, then cellulose sheets can be used. Chromatography or filter papers can be imbedded with substrate prior to imprinting for colour development *in situ* against the soil face, or enzymes can be bound with high spatial precision to nitrocellulose membranes, with exposure to substrates occurring later under lab conditions.

A-4 Imprinting of acid phosphomonoesterase activity – Method 1: using imprints of nitrocellulose membranes developed under standard lab conditions

(Dinkelaker & Marschner, 1992; Grierson & Comerford, 2000)

This method is made semi-quantitative by comparing the intensity of colour development on the membranes with colour development of standards applied to known areas of membrane using a slot blot apparatus.

A-4.1 Apparatus

1. Nitrocellulose membranes (0.45µm)

2. Latex or nitrile gloves

3. Plastic squeeze bottle filled with sterile, deionized water

4. Plastic squeeze bottle filled with 70% ethanol

5. Capillary tubing (<0.5 mm diameter)

6. Plastic or glass tray, 30 x 40 cm

7. Flat tip forceps, transported in ethanol

8. Cling wrap plastic

9. Aluminum foil

10. Insulated container and ice

11. Large, flat-bottomed glass dish (23 cm x 23 cm x 8 cm)

12. High resolution digital camera with tripod

13. Microfiltration apparatus (e.g., Bio-Dot SF Microfiltration Apparatus, Bio-Rad)

14. Scanner

A-4.2 Reagents

Prepare all reagents except buffer on the day of use.

1. Citrate buffer, 0.05 mole L-1: Dissolve 6.48 g citrate trisodium anhydrous salt (Sigma C3674, $C_6H_5O_7Na_3$) in 500 mL deionized water and adjust pH to 5.6 with 1 mole L-1 HCl.

2. Substrate solution, 0.05 mol L-1: Dissolve 0.34 g α-naphthyl phosphate disodium salt (Sigma N7255) in 25 mL of 0.05 mole L-1 citrate buffer (pH 5.6).

3. Colourimetric reagent solution, 10 mole L-1: Dissolve 0.64 g Fast Red TR salt (Sigma F2768) in 250 mL of 50 mM citrate buffer (pH 5.6).

A-4.3 Standards

1. Phosphatase stock solution: Make up stock solution of acid phosphatase from acid phosphatase wheatgerm extract (Sigma P3627) by dissolving 0.036 g in 250 mL of 0.05 mole L-1citrate buffer, pH 5.6

2. Working standards: Prepare working standards according to Table A-1.

A-4.4 Procedure – in the field

1. Remove the backing from a 22 cm x 22 cm pre-cut piece of nitrocellulose membrane and spray with sterile water in the glass dish until the membrane is saturated. Cut one corner of the membrane to assist with subsequent orientation of the image.

2. Open the window and spray the inside with 70% ethanol and wipe dry with clean tissues. Place the membrane on the inside of the open window, using the etched lines on the Plexiglas or perspex to align the centre of the membrane with the centre of the window. Close the window, cover with aluminum foil. As good contact between the membrane and the soil surface is paramount, additional pressure can be applied to the outside of the window either by hand or by using a wedge. Contact should be maintained for 10 minutes. As binding of the enzymes to nitrocellulose membranes is theoretically instantaneous, longer time periods should not be necessary; however, some assessment should be undertaken to optimize timing for the experimental conditions.

3. Prior to removing the membrane, pass a fine capillary tube through each of the small holes to mark the corners and centre of the membrane. These holes will be used as (x, y) coordinates for subsequent image analysis. Open the window and remove the membrane using sterile forceps, wrap in plastic and then aluminum foil, and transport from the field to the laboratory on ice.

4. Take a digital photograph through the Plexiglas window to record roots, hyphae and other features on the soil surface. Images should capture the positioning of the

corner and centre holes marked on the window and be at least 300 dpi in resolution if quantitative analysis is required.

A-4.5   Procedure – in the lab

1. Prepare the reagents and mix 1 part freshly prepared substrate solution with 10 parts colourimetric reagent solution (1:10 v/v).

2. According to the procedures outlined for the microfiltration unit, load three 0.5 mL replicates of each working standard to individual slots, under vacuum, on to a membrane cut to fit the set area. Cut one corner of the standard membrane in order to recognize the top and bottom edges of the membrane once it is developed.

3. Rinse the imprint membrane used in A-4.4 and allow to air dry at room temperature prior to development. This helps the proteins bind more strongly to the membrane and prevents loss of target material during subsequent washes.

4. After loading the standards membrane, place it with the imprint membrane in a large glass or Pyrex tray and develop in the substrate solution in the dark for 1-2 hours at 37 oC. The exact development time is not crucial. However, the standard membranes should be left in the solution until the imprints are clear and, critically, for the same time as the membranes from the root windows.

5. To stop the reaction, remove both membranes from the solution and rinse with distilled water to remove excess substrate.

6. Air-dry both imprint and standard membranes on clean tissue before scanning and analyzing (see section A-8.2). The acid phosphatase-induced colour change on the membranes is stable for many months after the imprints have been air-dried.

Comments: Any nitrocellulose membrane used should have good wet-ability, high strength and excellent bonding capacity for proteins. Great care should be taken when handling the treated paper to prevent contamination from finger. Wear gloves and use forceps at all times.

Prior to development, imprints may be stable for up to 12 hours, but this period is dependent on field conditions. We recommend that imprints be developed as soon as possible after collection. The intensity of the colour imprint is dependent on the duration of the developing time. A period of between 1 and 4 hours is generally sufficient. Longer time periods

usually result in saturation of the membrane, and some background discolouration. The total developing time is not crucial if the standard membrane is left to develop for the same period of time as the imprint from the roots. This is advantageous, as it also allows comparison between different sampling events. However, the indicator reaction may be enhanced by increasing the concentration of the dye in the developing solution (this should also decrease the developing time) (Gundlach & Mühlhausen, 1980). All reactions should be kept in darkness by covering with foil.

Small areas of yellow discolouration occasionally appear on imprints, and are probably the result of a reaction between the Fast Red dye and an unidentified compound, present either due to damage to the roots or cell leakage. However, because the colour is distinct from the acid phosphatase reaction, the two reactions can be separated without interfering with analysis of the phosphatase imprint.

A-5 Imprinting of acid phosphomonoesterase activity – Method 2: using imprints of pre-soaked cellulose sheets developed under field conditions

(Dinkelaker & Marschner, 1992; Dong *et al.*, 2007)

Although the following method is less spatially precise than Method 1, calibration strips can again be incubated alongside the treated paper. These can then be used to estimate the range of enzyme activities being detected under the conditions at each window and to determine those that are in the linear range of colour intensity. Compared to Method 1, higher concentrations of enzyme standard must be used because smaller volumes are applied to each spot on the strip. It is generally not practical to use a slot blot apparatus in the field, so the size of the spots of the standards should be constrained by impregnating the perimeter of defined areas with some hydrophobic substance.

A-5.1 Apparatus

1. Chromatography paper (Whatman, 20 x 20 cm, Cat No. 3030-861)
2. Large, flat-bottomed glass dish (23 cm x 23 cm x 8 cm)
3. Acid bath
4. Latex or nitrile gloves

5.	Insulated container for holding ice

6.	Aluminum foil

7.	Flat tip forceps

8.	Cling wrap plastic

9.	High resolution digital camera with tripod

10.	Pipettor

11.	Small plastic bags with zip closures

## A-5.2	Reagents

As described in A-4.2

## A-5.3	Standards

1.	Phosphatase stock solution: Make up stock solution of acid phosphatase from acid phosphatase wheatgerm extract (Sigma P3627) by dissolving 0.036 g in 2.5 mL of 0.05 mole L-1citrate buffer, pH 5.6.

2.	Create a series of standards ranging from 0.08 to 33 X 10-4 katal per 5 µL aliquot by using the dilutions in Table A-1, but using volumes 100-fold lower. For example, to create Standard solution A, add 20 µL of phosphatase stock to 80 µL of citrate buffer.

## A-5.4	Procedure – before going to the field

1.	In the lab, mix 1 part freshly prepared substrate solution with 10 parts colourimetric reagent solution (1:10 v/v). Immediately soak chromatography paper in the solution, one sheet at a time, for approximately 1 minute in an acid-washed, flat-bottomed, glass dish. Air-dry treated papers in the dark on aluminum foil at room temperature.

2.	When dry, store in a sealed plastic bag at 4°C for up to one week.

3.	Cut strips of pre-soaked papers into 2 cm by 5 cm pieces for use as calibration strips. Use a stencil to define the perimeters of spots to which the standards will be applied to the strip in the field (circles of 3 mm diameter are appropriate). Apply an inert hydrophobic material, such as lanolin or melted parafin around the perimeter of the stencils. Prepare sufficient spots for receiving three replicates of eight to ten standards.

A-5.5 Procedure – in the field

1. Prepare the calibration strips in the field immediately before making an enzyme imprint by pipetting a 5 µl aliquot of each working standard as a discrete spot along a strip of treated enzyme imprint paper. Once the solutions have been absorbed, seal the calibration strip in a plastic bag.

2. Create soil enzyme imprints by following step 2 in section A-4.4, but with the paper left in place for 60 min. Maintain firm contact between the paper and the soil surface. Place the calibration strip in its plastic bag against the soil profile next to the treated imprint paper and incubate along with the treated paper.

3. Prior to removing the paper, take a high-resolution image of the paper in place. If following step 5, use a tripod-mounted camera and leave it in place.

4. Remove paper and rinse with distilled water to remove any attached soil particles. Wrap washed imprints and calibration strips in aluminum foil and maintain on ice until they can be air-dried in the dark (no later than the end of the day).

5. A photograph can be taken with the window open, using the tripod-mounted camera without having disturbed it from step 3 if the researcher wishes to record very fine structures, such as individual hyphae.

A-5.6 Procedure – upon return to the lab

Scan the dried imprint and calibration strips at a resolution of at least 300 dpi.

Comments: For the imprinting to be successful using chromatography paper, sufficient moisture must be present in the exposed soil face to allow water to be drawn into the treated paper. In cold soils, the time required for imprinting may be longer than 60 min. The concentrations of the standards may also need to be varied to match the activities in specific soils. Some preliminary tests should be done under field conditions in order to optimize for specific conditions. The acid phosphatase-induced colour change on the treated paper is stable for weeks after the imprints have been air-dried.

A-6   Imprinting of leucine aminopeptidase activity

(Humble *et al*., 1977; Reymond and Wahler, 2002; Dong *et al*., 2007)

Areas of aminopeptidase activity present on the soil profile are indicated by an orange-red colour on the treated paper.

A-6.1   Apparatus

As per A-5.1 plus:

1.   Fine-mist sprayer or atomizer

2.   Infrared light (150 W)

A-6.2   Reagents

1.   Substrate solution, 0.02 mol L-1: Dissolve 2.928 g L-leucine 2-naphthylamide hydrochloride (Sigma L0376) into 500 mL 90% ethanol (Fisher A962-4).

2.   Colorimetric indicator solution, 0.0024 mol L-1: Dissolve 0.8 g Fast Blue BB (Sigma F3378) Salt hemi (zinc chloride) salt in 400 mL deionized water. (This reagent solution should be used within 1 hour after preparation.).

A-6.3   Standards

1.   Make up stock solution by mixing 0.1 g of fungal protease/peptidase complex of Aspergillus oryzae (Sigma P6110) with 1.0 mL deionized water.

2.   Use Table A-2 to construct a series of standards.

A-6.4   Procedure – before going to the field

Follow steps 1-3 for phosphatase imprinting in section A-5.4, except that the chromatography paper should be soaked in full strength substrate solution only.

A-6.5   Procedure – in the field

1.   Follow steps 1-5 in section A-5.5, with the following exceptions. The cellulose sheet needs to be in contact with the soil face for a shorter time than for phosphatase imprints, approximately 30 min.

2.   That evening, spray each imprint and calibration strip with freshly prepared colour reagent using a fine mist. An atomizer is recommended.

3.   Expose the imprints and strips to a 150 W heating lamp for 60 sec or to a 750 W lamp for 30 sec to prevent non-specific coloring.

4.   Dry at room temperature overnight, then store in aluminum foil in a plastic zip bag.

A-6.6 Procedure – upon return to the lab

Scan the imprint and calibration strips at a resolution of at least 300 dpi.

Comments: This assay results in relatively high levels of background colour; however, this method is still useful for visually associating enzyme activity with specific plant structures or for targeted sampling of microbial communities in the soil.

Care must be taken in applying the colourimetric indicator solution as the substrate does not bind strongly to the chromatography paper and will migrate in the paper if the imprint becomes saturated by large droplets of solution. However, once the imprint is dry, the induced colour change on the imprint is stable for weeks.

See additional comments under phosphatase methods above.

A-7 Imprinting of chitinase or β-glucosidase activity

(Hoppe, 1983; Pritsch et al., 2004; Dong et al., 2007)

Areas of chitinase or β-glucosidase activity present on the soil profile are indicated by fluorescence on the treated paper.

A-7.1 Apparatus

As per section A-5.1, plus a gel documentation system

A-7.2 Reagents

1. Chitinase fluorometric indicator solution, 0.005 moles L-1: Dissolve 1.04 g 4-methylumbelliferyl N acetyl-β-D-glucosaminide dehydrate (Sigma M2133) into 500 mL 2-methoxylethanol (Sigma M5378). This solution should be prepared fresh just before use.

2. β-glucosidase fluorometric indicator solution, 0.005 moles L-1: Dissolve 0.85 g 4-methylumbelliferyl β-D-glucoside (Sigma M3633) into 500 mL 2-methoxylethanol (Sigma M5378). This solution should be prepared fresh just before use.

A-7.3 Standards

1. Make a standard stock of 0.025 moles L-1 by dissolving 44.05 mg 4-methylumbelliferone (Sigma M1381) in 100 mL 2-methoxylethanol (Sigma M5378). This solution should be kept in a dark bottle wrapped in aluminum foil in a -20 $^{\circ}$C freezer.

2. Place 0, 31.25, 62.5, 125 and 250 µl of standard stock solution into 1.5 mL Eppendorf micro-centrifuge tubes and dilute to 1 mL with 2-methoxyethanol (Sigma M5378) to create standard concentrations of 0, 0.78, 0.156, 0.313, and 0.625 µmoles L-1.

A-7.4 Procedure – before going to the field

Follow steps 1-3 in section A-5.4, except that the chromatography papers should be soaked in full strength substrate solution only.

A-7.5 Procedure – in the field

Follow steps 1-5 in section A-5.5, except that the treated cellulose sheet needs to be in contact with the soil face for a shorter time than for phosphatase imprints, approximately 30 min.

A-7.6 Procedure – upon return to the lab

Capture images of air-dried imprints using a gel documentation system. Optimize the F-stop, exposure time, and binning settings under UV transillumination conditions without a filter.

Comments: MUB-based imprinting results in a detectable fluorescent residue on the soil profile for up to 8 days afterwards. Therefore, allow at least a 10-day interval before any 4-MUB-based tests are repeated on the same area of a soil profile. See additional comments under phosphatase methods above.

A-8 Correlating activities with observable soil structures

The approaches described below integrate the area of colour reaction with the amount of enzyme loaded onto the standards membrane to create a standard curve. The relative enzyme activity detected in specific regions of the imprint is determined using a histogram analysis of colour intensity and calibrating the colour intensity in each pixel with the standard curve.

A-8.1 Apparatus

Image processing program such as Adobe Photoshop or GIMP.

A-8.2 Procedure

1. Scan the imprints and standards at high resolution (at least 300 dpi) and format as .tiff files.

2. Prepare the imprint for analysis by using an image analysis program such as Adobe Photoshop or GIMP. First, sharpen the image (under Filter menu). Second, remove unwanted background colour either by selecting the Magic Wand in Adobe Photoshop. Alternatively, remove any artifacts ("speckles") from the imprint by setting the minimum spot diameter to a level greater than the diameter of the speckles. Then, detect spots using a "spot detection" wizard. To do this, use the normal settings and then select a Gaussian distribution to remove any streaks or discolouration. A 3D-viewer in software can also help to identify artifacts that can be filtered from an image. Finally, use filters to remove any background discolouration or soil staining.

3. If imprints were marked with capillary tubes, crop the image to remove the edges of the imprints, using the x, y coordinates marked by the capillary tube and adjust the size to 700 dpi x 700 dpi.

4. To align the imprint image with an image of the soil taken through the Plexiglas, the soil image first needs to be sized to exactly match the size of the imprint image. Crop the soil window image using the five holes as a guide for the corner and centre points. The window images should then be resized to 700 dpi x 700 dpi i.e., the same size as the imprint. Go to step 6.

5. Alternatively, to align an imprint image with an image of the soil taken with the window open (in which case the capillary tubes cannot be used to align the images):

    a. Open the photo of the paper in place in the window in Adobe Photoshop.

    b. Select the polygon lasso tool.

    c. On a new layer, trace the outline of the paper visible in the photo.

    d. Cut the outlined area out of the layer.

    e. Open the photo of the soil taken with the window open.

    f. Copy the modified layer of the photo of the paper in place onto the photo of the soil as a new layer.

    g. Select the polygon lasso tool and trace on the photo layer the outline of the soil visible through the modified layer.

h. Cut the outlined area out of the photo layer.

i. Paste this cutout using "Paste as new image." This new image will be of the exact imprint area of the soil profile.

j. Adjust the dimensions of the new image to match the dimensions of the scanned imprint.

k. Rotate the image of the scanned imprint as needed to ensure that the imprint is in the original orientation to the soil profile.

l. Copy the scanned imprint image and paste it into the image of the soil profile as a new layer.

6. Adjust the image to gray-scale and 0-256 colours.

7. Filters and other tools can then be applied to the soil image to identify key features of interest, such as roots, fungi or microbial hotspots. To confer an attribute value, such as root type, to the image, it is preferable to re-colour the image by selecting desired pixels and changing to the same colour to create a root type category. In the case of roots, a number of categories might be used in the classification; for example, long white roots, bifurcated root tips, long brown laterals, mycorrhizal roots, root clusters, "dead" roots and so on. Categories should be reasonably easy to identify and have some functional significance. Where there are numerous images, resizing, sharpening and optimizing can be undertaken to a large extent by automated batch actions in a range of software that process images automatically with parameters you set.

8. To estimate activities associated with visible soil features of interest on the soil profile, first outline the feature with the lasso tool. Use the outlined area(s) to cut the scanned imprint layer. Paste the selected areas of the imprint using 'Paste as new image'. These can be evaluated as described under 'Calculations'.

A-8.3   Calculations

1. To calibrate the imprint, first develop the standard curve by regressing the intensity of colour development on slot-blot membranes or calibration strips against the enzyme activities of the standards. To do this for the slot-blot apparatus, select the

core interior of the area developed for each working standard by using the marquee tool in Adobe Photoshop set at a standard size (e.g., 60 x 6 pixels). For the calibration strips, select a consistent core area appropriate for the size of spot used. Record the mean colour intensity per total number of pixels and then plot colour intensity (0-256 in gray-scale) against enzyme units or katal/pixel. A range of enzyme activities should be tested in order to determine the linear range of activity and to span the colour intensities found on the imprints (for example, see Grierson & Comerford, 2000). At very low activities, an exponential curve should be fitted.

2. It is relatively simple to develop algorithms to analyze the image files for the correspondence of enzyme activity with observable soil structures or certain root classes as long as the images have been geo-referenced. Similarly, the enzyme imprints can be re-classed according to user-defined categories related to known enzyme activities calculated from the standard curves (see above) or to calculate the percentage occurrence of a feature of interest with a specific class of enzyme activity. This is analogous to overlaying the enzyme imprint over the root image. To calibrate the intensity of colour development in a region of the imprint with enzyme activities, select an image-defined area for integration, as described in step 8, and use the histogram function in software such as NIH Image or Adobe Photoshop to obtain a frequency distribution of the number of pixels at each intensity. Then using the standard curve, sum the product of the number of pixels reporting a particular grayscale value (0-256) by the enzyme concentration correlating with that grayscale value. For example, Grierson & Comerford (2000) classed the phosphatase imprints into five categories of phosphatase activity, ranging from no activity (colour value 0) to high intensity of activity (range 22.44 x 10-7 E.U./pixel to 83.5 x 10-7 E.U./pixel), and the roots into lateral roots and root clusters.

Comments: There are many commercial software packages currently available that are likely suitable for the quantification of 2-D enzyme datasets, many of them developed to analyze gel-data (e.g., PDQuest, Progenesis). Similarly, image analysis software used routinely in GIS applications (IDRISI, ArcGIS, ENVI) is also useful for analyzing the spatial distribution of data

captured on membranes and papers. Choice of software must take into consideration how many images will be analyzed, the relative cost of the software and the customer support. Researchers interested in quantifying enzyme data from imprints are advised to trial a number of programs. When used in conjunction with software designed for spatial analysis, this procedure should be useful in assessing the relationship of enzyme activity and root class (e.g., white, brown, lateral, mycorrhizal) and to make quantitative estimates of relative enzyme activity associated with root surface area, degree of mycorrhizal infection or microbial community composition. However, the preparation of root images suitable for spatial analysis can be time-consuming. Likewise, there are some logistic difficulties applying the method to a field situation. For example, both root and phosphatase imprints must be the exact same dimensions with sufficient coordinates to align the images. The development of software and programs to simplify the process of analyzing the image data is on-going, and will improve the accuracy and speed of the image analysis.

A-9   Use of imprints for guiding soil sampling for microbial community analysis

Colourimetric soil enzyme imprinting provides a unique opportunity to investigate the microbial community associated with in situ enzyme activity at millimeter-scale resolution. Fine scale soil sampling can proceed directly after development of the imprint by using a frame to maintain alignment between the imprint and a transparent sheet used as a template. Holes punched in the transparency align with the enzyme activities detected by the imprinting and, hence, can be used to target sampling of roots or soil.

A-9.1   Apparatus

1. Foam-core poster board cut to fit window trap-door opening
2. Mylar™ plastic sheets
3. Easy-release paper tape (masking tape)
4. Fine permanent marking pens
5. Hole-punch tool (hammer-driven leather punch)
6. Toothpicks
7. Forceps

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8.   Ethanol for sterilizing forceps

9.   Sterile microcentrifuge tubes

10.   Dry ice

11.   Container to hold dry ice

12.   Materials for imprinting as described in earlier sections

A-9.2 Procedure

1. Cut a transparent sheet of Mylar™ to exactly fit into the opening of the root window. Use a piece of foam core poster board cut to fit exactly into the trap-door to stabilize the transparency in the trap-door opening. First cut the foam core poster board precisely to size. Then cut out the center leaving a frame 6 cm wide and tape the transparency to this.

2. Before creating an imprint, fit the frame and transparent sheet into the opening against the soil profile. If relevant to the sampling scheme, trace any visible soil features of interest, such as the interface between organic and mineral soil horizons, fungal mats, or plant roots onto the transparency with a fine permanent marker. Remove the transparency and frame from the window, and remove the transparency from the frame.

3. Open the trap door, clean and dry the soil side surface, tape the cellulose sheet to the door, and close the trap door. Follow the instructions for the particular enzyme assay in earlier sections of this chapter.

4. At the end of the assay, place the transparency, soil side up, on top of the cellulose sheet while the sheet is still attached to the trap door aligning the transparency precisely with the edges of the trap door (Figure A-2a). Mark selected areas of colour development on the transparency with permanent marker. The types of areas marked will depend on the question being asked. For example, some researchers may be interested only in those in proximity of roots; others may be interested in activities only in certain soil horizons.

5. Remove the transparency from the trap door and punch holes through the transparency at locations of interest using a sharp, hammer-driven leather punch.

6. Reattach the transparency onto the frame exactly as it was when the soil features were originally marked and replace it in the opening of the root window (Figure A-2b). (Since the frame is the same size and shape as the trap door, the punched holes in the transparency are now aligned with the soil features that originally produced the color change on the imprint.) Insert toothpicks through the holes in the transparency to mark the locations for targeted soil sampling, and remove the transparency and frame (Figure A-2c).

7. Collect soil samples with sterile forceps (Figure A-2d), and place them in sterile microcentrifuge tubes. Keep on dry ice during transport to the lab.

Comments: Soil fungal and prokaryotic communities associated with hot spots of soil enzyme activity can be investigated and described by combining targeted soil sampling with molecular techniques. DNA can be extracted from small (less than 0.25 g), targeted soil samples using standard DNA extraction kits (e.g. PowerSoil™ DNA Isolation Kit, MoBio). Genes of interest can be amplified by PCR, and the PCR products can be rapidly and economically sequenced by robotic clone sequencing. On-line databases (e.g. NCBI, RDP, GreenGenes) expedite the process of identifying and categorizing sequences obtained through high-throughput sequencing methods, providing in-depth information about the presence and/or activity of functional genes and microbial species that co-occur with in situ soil enzyme activity.

A-10   Figures and tables

Figure A-1 Root window installed.

Figure A-2 Sampling soil microbial community guided by enzyme imprints. (a) Preparing to mark coloured imprint areas onto the transparency; (b) Marking sampling areas through holes punched in transparency, (c) Removing transparency, (d) Sampling marked soil profile.
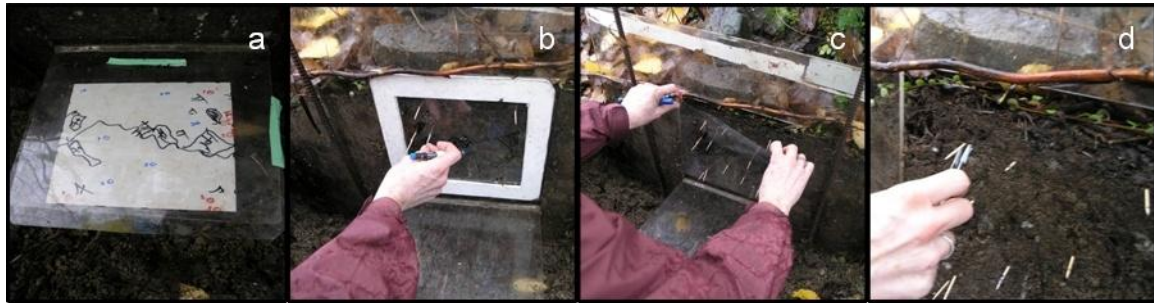
Table A-1 Phosphatase and buffer additions for standard curve using a Bio-Dot SF Microfiltration Apparatus.

Standard solution	Phosphatase activity (Enzyme Units x 10^{-4})	Phosphatase activity (katal x 10^{-4})	Aliquot of phosphatase stock (mL)		Citrate buffer addition (mL)
A	2.0	33.33	2		8.0
B	1.5	25.00	1.5		8.5
C	1.0	16.67	1		9.0
D	0.67	11.17	2	x A	4.0
E	0.5	8.33	1	x A	3.0
F	0.33	5.50	2	x B	4.0
G	0.2	3.33	1	x A	7.0
H	0.15	2.50	1.5	x C	8.5
I	0.1	1.67	1	x C	9.0
J	0.05	0.83	1	x G	4.0
K	0.033	0.55	2	x I	4.0
L	0.02	0.33	1	x G	9.0
M	0.015	0.25	1.5	x I	8.5
N	0.01	0.17	1	x I	9.0
O	0.005	0.08	1	x L	3.0

* one katal will hydrolyze 1.0 mole of p-nitrophenyl phosphate per second at 37°C

Table A-2 Protease and water additions for standard curve for aminopeptidase imprinting.

Standard solution	Minimum* activity per 5 µL (Enzyme Units x 10^{-3})	Minimum activity (katal x 10^{-9})†	Aliquot of protease stock (mL)	Deionized water (mL)
A	250	4.17	0.5	0
B	150	2.50	0.3	0.2
C	75	1.25	0.25 x B	0.25
D	37.5	0.625	0.25 x C	0.25
E	18.75	0.313	0.25 x D	0.25

*There are $\geq$ 500 EU per g.

† One katal will hydrolyze 1 mole of L-leucine-p-nitroanilide per second.

A-11   Bibliography

Dinkelaker, B, and Marschner, H. 1992. *In vivo* demonstration of acid-phosphatase-activity in the rhizosphere of soil-grown plants. *Plant and Soil* **144:**199-205.

Dong, S, Brooks, D, Jones, MD, and Grayston, SJ. 2007. A method for linking *in situ* activities of hydrolytic enzymes to associated organisms in forest soils. *Soil Biology and Biochemistry* **39:**2414-2419.

Grierson, PF, and Comerford, NB. 2000. Non-destructive measurement of acid phosphatase activity in the rhizosphere using nitrocellulose membranes and image analysis. *Plant and Soil* **218:**49-57.

Gundlach, G, Mühlhausen, B. 1980. Coupling of 1-naphthol with fast-red-tr .1. Studies on the optimization of a continuous determination of acid-phosphatase. *Journal of Clinical Chemistry and Clinical Biochemistry* **18(10):** 603-610.

Hoppe, HG. 1983. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Marine Ecology Progress Series* **11:**299–308.

Humble, MW, King, A, and Phillips, I. 1977. Api zym - simple rapid system for detection of bacterial enzymes. *Journal of Clinical Pathology* **30:**275-277.

Pritsch, K, Raidl, S, Marksteiner, E, Blaschke, H, Agerer, R, Schloter, M, and Hartmann, A. 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* **58:**233-241.

Reymond, JL, and Wahler, D. 2002. Substrate arrays as enzyme fingerprinting tools. *Chemistry Biochemistry* **3:**701-708.

Smit, AL, George, E, and Groenwold, J. 2000. Root observations and measurements at (transparent) interfaces with soil. In: AL Smit, AG Bengough, C Engels, M Van Noordwijk, S Pellerin and SC Van de Geijn (eds.) *Root methods. A handbook*. Springer, Berlin. 235-271.

Appendix B   Chapter 3 supplementary material

Table B-1 TRFLP signature library generated from ectomycorrhizal fungal (EMF) PCR products. TRFLP signatures were produced by nested PCR (ITS1/ITS4 primer set) followed by digestion with *Hae*III and *Hinf*1 endonucleases.

Genus or family[a]	BLAST identity[b]	*Hae*III[c]		*Hinf*I	
		ITS1F	ITS4	ITS1F	ITS4
Amphinema	*Amphinema byssoides*	92.27	603.05	68.59	334.23
Atheliaceae	*Atheliaceae* 1	735.94	730.24	387.34	72.85
Cortinarius	*Cortinarius* 1	443.91	190.95	482.45	306.65
	Cortinarius 5	446.88	197.18	316.79	313.84
	Cortinarius 6	445.10	198.02	320.08	314.16
	Cortinarius 9	282.11	438.11	394.52	321.41
	Cortinarius 12	234.26	439.32	400.59	322.73
	Cortinarius armillatus[d]	130.10	171.27	312.41	265.74
	Cortinarius armillatus[d]	220.33	220.57	319.67	307.92
	Cortinarius balaustinus	503.39	175.53	394.94	277.53
	Cortinarius cf.	445.17	189.42	322.78	305.20
	Cortinarius gentiles	150.36	155.86	330.43	264.81
	Cortinarius hemitrichus	442.15	190.40	321.27	304.27
	Cortinarius renidens	131.07	455.59	318.41	264.37
	Cortinarius subsertipes	157.54	188.83	319.01	303.45
	Cortinarius talus	226.59	194.24	390.52	317.00
Hebeloma	*Hebeloma collariatum*	93.79	452.30	393.28	330.83
	Hebeloma leucosarx	279.67	451.24	228.09	330.85
Inocybe	*Inocybe* 1	627.90	96.27	173.91	327.05
	Inocybe 2	781.88	775.97	167.19	343.65
	Inocybe 9	97.37	159.30	313.68	325.70
	Inocybe 5a	695.13	688.73	370.41	307.46
Laccaria	*Laccaria* 1	730.15	724.73	392.18	320.86
Lactarius	*Lactarius* 1	506.39	254.22	382.26	371.62
	Lactarius pubescens	510.84	252.33	383.96	367.08
	Lactarius rubrilacteus	508.17	262.21	382.61	380.64
	Lactarius scrobiculatus[d]	506.05	269.62	381.83	387.57
	Lactarius scrobiculatus[d]	440.00	188.31	317.84	302.89
	Lactarius torminosus	502.95	260.82	378.82	378.58
Meliniomyces	*Meliniomyces bicolor*	117.75	447.09	403.17	261.90
Phallales	*Phallales* 1	426.19	217.33	340.28	313.92

Genus or family[a]	BLAST identity[b]	*Hae*III[c]		*Hinf*I	
		ITS1F	ITS4	ITS1F	ITS4
Piloderma	*Piloderma fallax*	93.45	486.26	348.08	153.45
	Piloderma sp.	93.42	486.82	347.47	153.99
Rhizopogon	*Rhizopogon rudus*	481.10	218.67	178.18	194.77
Russula	*Russula* 1	486.78	242.85	356.51	369.22
	Russula 3	460.92	251.23	348.10	357.86
	Russula 5	471.50	197.00	341.91	69.43
	Russula 6	471.48	196.96	341.82	69.34
	Russula 7[d]	449.40	192.33	320.40	313.92
	Russula 7[d]	470.33	196.87	265.80	66.14
	Russula aeruginea	167.09	196.02	352.26	71.67
	Russula roseipes	740.78	736.38	339.17	382.96
	Russula velenovskyi	167.93	560.81	345.22	379.37
	Russulaceae 1	95.95	584.82	339.45	214.25
Sebacinaceae	*Sebacinaceae* 4	94.41	496.33	72.11	316.08
Thelephora	*Thelephora terrestris*	91.30	218.82	354.71	241.86
	Thelephoraceae 2	481.01	218.67	178.18	194.80
Tomentella	*Tomentella* 1	164.49	533.35	351.90	191.94
	Tomentella 2[d]	387.90	217.80	354.43	194.61
	Tomentella 2[d]	387.78	217.72	354.37	194.63
	Tomentella 3	480.26	217.97	178.09	337.17
	Tomentella 4	91.69	528.93	349.88	233.25
	Tomentella 5	93.07	218.80	353.77	191.44
	Tomentella 6	93.09	218.80	353.63	191.33
	Tomentella 7	168.45	211.66	356.91	194.54
	Tomentella 8	478.82	218.37	351.20	191.43
	Tomentella 9	479.23	218.47	174.27	191.21
	Tomentella 11	165.91	217.95	353.66	336.85
	Tomentella 12	479.67	218.58	177.01	194.71
	Tomentella 13	479.75	217.97	177.11	237.35
	Tomentella ramosissima	168.29	219.83	355.88	194.86
Tricholoma	*Tricholoma flavovirens*	737.81	731.51	386.21	221.87
	Tricholoma scalpaturatum	393.33	169.59	344.44	356.26
Xerocomus	*Xerocomus ferrugineus*	102.93	202.73	71.78	381.31

[a] Ectomycorrhizal fungal TRFLP signatures were grouped by family or genus to create assemblages for MRPP analysis.

[b] PCR products not identified to species are numbered as in Twieg *et al.*, 2007.

[c] PCR products from Twieg *et al.* 2007.

[d] Entries with identical BLAST identities were PCR products with similar sequences that were identified as the same phylotype but produced different sized TRFLPs.

Table B-2 Fine-scale analysis using Mantel tests to compare the locations of microsamples in organic or mineral layers of each root window with their general fungal TRFLP fingerprints or assemblages of ectomycorrhizal fungal (EMF) TRFLP signatures. Mantel's r was computed using asymptotic approximation; distance matrices of 9 x 9 to 12 x 12 elements contained pairwise physical distances in cm and cluster analysis Euclidean distances between microsample TRFLP fingerprints or assemblages of EMF TRFLP signatures. $P \leq 0.5$ indicates significant spatial structuring.

Age class	Stand age (yrs)	General fungal TRFLP fingerprints		EMF TRFLP signatures	
		Mantel's standardized statistic (r)	*P*	Mantel's standardized statistic (r)	*P*
Youngest[a]	5-6				
Canopy closure	24-30	0.31	0.06	0.31	0.01
Stem exclusion	61-71	0.42	<0.01	0.31	0.02
Older	90-103	0.42	<0.01		

[a] No spatial structure was detected in any of the root windows of the Youngest age class.

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Appendix C Chapter 4 supplementary material

Table C-1 Phylogenies of 269 identified bacteria isolated from hyphae ingrowth, hyphae exclusion and Ziploc® control sand bags incubated in the organic layer of a mixed Douglas-fir and birch stand (MARA) regenerating from stand replacing wildfire in the southern interior of British Columbia as determined by the Ribosomal Database Project.

Isolate phylogeny	Hyphae exclusion bags	Hyphae ingrowth bags	Ziploc® plastic bags	Total number of isolates
Actinobacteria (total)	**(83)**	**(86)**	**(36)**	**(205)**
Actinobacteria				
Actinobacteridae				
Actinomycetales				
Corynebacterineae				
Nocardiaceae				
Rhodococcus		9	7	16
Williamsiaceae				
Williamsia			2	2
Micrococcineae				
Cellulomonadaceae				
Cellulomonas	3			3
Intrasporangiaceae				
Janibacter			5	5
Microbacteriaceae				
Agreia	3	7		10
Curtobacterium	1		1	2
Frigoribacterium	1	1		2
Leifsonia	9	1		10
Microbacterium	5	9	2	16
Plantibacter	4			4
Salinibacterium		1		1
Subtercola	4	1	3	8
Micrococcaceae				
Acaricomes	1			1
Arthrobacter	17	15	16	48
Propionibacterineae				
Nocardioidaceae				
Aeromicrobium	9			9
Nocardioides	4			4
Pseudonocardineae				
Pseudonocardiaceae				

Isolate phylogeny	Hyphae exclusion bags	Hyphae ingrowth bags	Ziploc® plastic bags	Total number of isolates
Amycolatopsis	1	1		2
Streptomycineae				
Streptomycetaceae				
Streptomyces	21	41		62
Bacteroidetes (total)	**(2)**	**(2)**		**(4)**
Sphingobacteria				
Sphingobacteriales				
Sphingobacteriaceae				
Pedobacter	1	1		2
Sphingobacterium	1	1		2
Firmicutes (total)	**3**	**2**	**1**	**6**
Bacilli				
Bacillales				
Bacillaceae				
Bacillus	1	2		3
Paenibacillaceae				
Paenibacillus	2		1	3
Proteobacteria (total)	**(27)**	**(24)**	**(3)**	**(54)**
α-proteobacteria				
Caulobacterales				
Caulobacteraceae				
Brevundimonas	1	1	1	3
Caulobacter		1		1
Rhizobiales				
Methylobacteriaceae				
Methylobacterium		1		1
Phyllobacteriaceae				
Mesorhizobium	1			1
Phyllobacterium	3	16		19
Sphingomonadales				
Sphingomonadaceae				
Sphingomonas	4		1	5
β-proteobacteria				
Burkholderiales				
Comamonadaceae				
Curvibacter	4			4
Polaromonas	2			2

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Isolate phylogeny	Hyphae exclusion bags	Hyphae ingrowth bags	Ziploc® plastic bags	Total number of isolates
Rhodoferax	2			2
Variovorax		2		2
Incertae sedis 5				
Methylibium	1			1
Oxalobacteraceae				
Collimonas	2	3	1	6
Janthinobacterium	5			5
Massilia	1			1
γ-proteobacteria				
Xanthomonadales				
Xanthomonadaceae				
Dyella	1			1

Table C-2 Phylogenies of 102 bacteria grouped as "Other" isolated from hyphae ingrowth and hyphae exclusion sand bags incubated in the organic layer of a mixed Douglas-fir and birch stand (MARA).

Isolate phylogeny	Hyphae exclusion bags	Hyphae ingrowth bags	Total isolates
Actinobacteria			
Actinomycetales			
Cellulomonadaceae			
Cellulomonas	3	0	3
Microbacteriaceae			
Agreia	3	7	10
Curtobacterium	1	0	1
Frigoribacterium	1	1	2
Leifsonia	9	1	10
Plantibacter	4	0	4
Salinibacterium	0	1	1
Subtercola	4	1	5
Micrococcaceae			
Acaricomes	1	0	1
Nocardiaceae			
Rhodococcus	0	9	9
Nocardioidaceae			
Aeromicrobium	9	0	9
Nocardioides	4	0	4
Pseudonocardiaceae			
Amycolatopsis	1	1	2
Bacteroidetes			
Sphingobacteriales			
Sphingobacteriaceae			
Pedobacter	1	1	2
Sphingobacterium	1	1	2
Bacilli			
Bacillales			
Bacillaceae			
Bacillus	1	2	3
Paenibacillaceae			
Paenibacillus	2	0	2
Proteobacteria			
α-proteobacteria			
Caulobacteraceae			

Isolate phylogeny	Hyphae exclusion bags	Hyphae ingrowth bags	Total isolates
Brevundimona	1	1	2
Caulobacter	0	1	1
Rhizobiales			
Mythylobacteriaceae			
Methylobacterium	0	1	1
Phyllobacteriaceae			
Mezorhizobium	1	0	1
Sphingomonadales			
Sphingomonadaceae			
Sphingomonas	4	0	4
β-proteobacteria			
Burkholderiales			
Comamonadaceae			
Curvibacter	4	0	4
Polaromonas	2	0	2
Rhodoferax	2	0	2
Vaiovorax	0	2	2
Incertae sedis 5			
Methylibium	1	0	1
Oxalobacteraceae			
Collimonas	2	3	5
Janthinobacter	5	0	5
Massilia	1	0	1
γ-proteobacteria			
Xanthomonadales			
Xanthomonadaceae			
Dyella	1	0	1

Table C-3 Enzyme activities and growth rates for 210 isolates from hyphae ingrowth and hyphae exclusion bags incubated in the organic soil layer of a mixed Douglas-fir and birch stand (MARA) grouped by genus. Differences in enzyme activity (nmol substrate converted hour^{-1} initial unit OD_{600}^{-1}) and growth rates (initial OD_{600} reading subtracted from the maximum reading divided by the time in days required to reach the maximum reading) within genus groups between mesh sizes were tested by Student's *t* or Welch ANOVA when standard deviations differed (** *P* <0.05; * *P*<0.10).

Assay	Genus Group	Hyphae exclusion bags		Hyphae ingrowth bags	
		Average	SEM	Average	SEM
Phosphatase	Streptomyces	340.5	92.5	97.1 **	30.9
	Arthrobacter	51.2	12.0	35.5	9.9
	Microbacterium	22.1	13.3	108.5 *	59.8
	Phyllobacterium	107.0	56.6	46.0	12.2
	Other	212.6	67.0	116.3 *	28.8
NAGase	Streptomyces	120.5	41.2	99.8	16.4
	Arthrobacter	0.4	0.2	14.1 **	7.2
	Microbacterium	3.8	2.8	13.5	11.6
	Phyllobacterium	0.7	0.6	11.3 **	4.2
	Other	31.5	13.7	20.1	11.9
Growth[a]	Streptomyces	1.3	0.3	2.0 **	0.3
	Arthrobacter	7.6	0.5	5.7 **	0.5
	Microbacterium	5.9	1.5	2.9 *	0.5
	Phyllobacterium	0.8	0.4	2.3 **	0.3
	Other	3.13	0.3	2.6	0.3

[a] Growth rate values are expressed as (growth rate x 100) for table clarity

Table C-4 Nominal Logistic Fit for fungal DNA presence/absence qPCR results for replicate DNA extractions from three replicate hyphae ingrowth and three exclusion bags incubated in the organic layers of six mixed Douglas-fir and birch forests regenerating from stand replacing wildfire in the southern interior of British Columbia.

Whole model test

Model	LogLikelihood	DF	ChiSquare	Prob>ChiSquare
Difference	6.300635	6	12.60127	0.0498
Full	16.613875			

Parameter estimates

Term	Estimate	Std. Error	ChiSquare	Pro>ChiSquare
Intercept	1.04860961	0.4809278	4.75	0.0292
Bag type[Exlcusion]	1.30984271	0.4934357	7.05	0.0079
Site[19M]	1.09573262	1.0674864	1.05	0.3047
Site[BBN]	-0.047123	0.9337076	0.00	0.9597
Site[EDN]	-1.0486096	0.9461664	1.23	0.2677
Site[MA1]	-1.0486096	0.9461664	1.23	0.2677
Site[MA2]	1.09573262	1.0674864	1.05	0.3047

For log odds of absence/presence

Effect likelihood ratio tests

Source	N parm	DF	L-R ChiSquare	Prob>ChiSquare
Bag type	1	1	9.49759211	0.0021
Site	5	5	4.06082293	0.5407