LONG-TERM FERTILIZATION EFFECTS ON ECTOMYCORRHIZAL COMMUNITY STRUCTURE AND FINE ROOT LENGTH IN INTERIOR SPRUCE FORESTS

by

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ABSTRACT

Ever-present on the roots of appropriate host trees, ectomycorrhizas are pivotal in the survival and productivity of trees of interior British Columbia. Although ectomycorrhizal (ECM) fungi provide access to nutrients that would otherwise be unavailable to roots, nutrient supply can still limit above ground productivity of trees. Such nutrient constraints have led to the use of fertilization as standard practice for increasing tree yields in some parts of the world. Although much research has been completed on the short-term response of ECM communities to nitrogen enrichment, almost none has focused on responses to long-term fertilization treatments aimed at maximum tree productivity. In our study, the response of ECM fungal communities of three interior spruce (*Picea glauca*) stands in interior British Columbia to 10-15 yr of fertilization was studied. The treatments included (i) unfertilized control, (ii) annual fertilization (kg ha^{-1}); to maintain 1% foliar N (iii) periodic fertilization (kg ha^{-1}); 200N, 100P, 100K, 50 sulphur, 25 magnesium, 1.5 boron applied every 6 years. The study sites were located in three different biogeoclimatic zones: the moist, cold (SBSmc2), the moist cool (SBSmk1) and the wet cool (SBSwk1) variants of the Sub-Boreal Spruce zone of central interior British Columbia. Using morphological and molecular analysis, the identity of the dominant ECM fungal symbionts on the randomly-selected root tips was determined in order to characterize the ECM fungal community. Both ECM fungal diversity and richness differed amongst sites, but did not appear to be affected by fertilization. The relative abundance and frequency of *Tylospora* spp., one of the dominant genera in the community, were lower in plots exposed to annual fertilization at one of the sites. No overall effect on the ECM fungal community was observed in response to fertilization. As the periodic fertilization treatment more closely reflects operational modes of fertilization in these regions, these results suggest that large-scale fertilization could be

established in the central interior of British Columbia without major disturbance to this important component of the soil microbial community.

PREFACE

This study was carried out on long-term research installations established and managed by Rob Brockley, Ministry of Forests and Range. With guidance from my supervisor, Dr. Melanie Jones, I was responsible for the sampling design, implementation, and analysis of the study described in Chapter 2 of this thesis. I was assisted with sample collection in the field, and fine root length and molecular analysis in the lab by research assistants. With guidance from Dr. Jones, I was also responsible for the writing of the entire thesis following this research. This thesis was reviewed by the following members of my supervisory committee, some of which are faculty at the University of British Columbia (Okanagan): Dr. Dan Durall and Dr. Jason Pither, while Dr. Shannon Berch is employed with the Ministry of Environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE	abundance-based coverage estimator
BLAST	Basic Alignment Search Tool
ECM	Ectomycorrhizal
ESSF	Engelmann Spruce-Subalpine Fir
ITS	Internal Transcribed Region
MS	Montane Spruce
MRPP	Multi-response permutation procedure
NCBI	National Centre for Biotechnology Information
NMS	Nonmetric multidimensional scaling
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PRS	Plant root simulator
SBSmc1	Sub-Boreal Spruce biogeoclimatic zone, moist cold
SBSmk1	Sub-Boreal Spruce biogeoclimatic zone, moist cool
SBSwk1	Sub-Boreal Spruce biogeoclimatic zone, wet cool
UNITE	User Friendly Nordic ITS Ectomycorrhizas databases

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For those individuals who strive for a more sustainable future

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Many tree species rely heavily upon a symbiotic relationship formed between their roots and a taxonomically large suite of basidiomycetous and some ascomycetous fungi (Smith and Read 2008). Although benefits attained through ectomycorrhizal (ECM) associations are many (Duchesne *et al.* 1988a, Cumming and Weinstein 1990, Boyle and Hellenbrand 1991, Schier and McQuattie 1996, Van Tichelen *et al.* 1999 Morte *et al.* 2001), the increased access to both inorganic and organic forms of nutrients is one of utmost importance (Hobbie and Hobbie 2008). In return, ECM fungi receive most or all of their carbon from their host plants (Hobbie 2006). Consequently, ECM fungi are responsible for a significant component of forest-soil carbon fluxes and likely contribute to the sequestration of carbon in soil (Högberg *et al.* 2001, Högberg and Högberg 2002, Godbold *et al.* 2006, Hobbie 2006).

Throughout the interior of British Columbia, fertilization studies have confirmed widespread N deficiencies in forests (Brockley 2006, 2010). These nutrient deficiencies, along with British Columbia`s largest mountain pine beetle (*Dendroctonus ponderosae*) epidemic in history, have resulted in serious timber supply shortages, which may threaten the future economic stability of the forestry-dependent communities. Fertilization has been shown to significantly accelerate development of existing immature interior spruce [*Picea glauca* (Moench) Voss and *Picea engelmannii* Parry, or naturally occurring hybrids of these species] stands in interior British Columbia (Brockley 2007a, Brockley and Simpson 2004, Brockley 2010). Although short-term increases in tree growth in response to single fertilizer applications have been observed, with repeated fertilization, long-term growth rate increases are possible (Brockley 2007b). In Sweden, intensive fertilization resulted in decreased rotation periods of Norway spruce (*Picea*

abies) by as much as 20-60 years (Bergh *et al.* 2006). While above ground responses to fertilization have been favourable, there is concern that repeated nutrient additions may affect long-term site productivity through alterations in belowground communities.

The response of ECM fungal communities to fertilization can vary substantially with fertilizer type, method and frequency of application, and the ecosystem being studied (Fransson *et al*. 2000). Decreases (Berch *et al.* 2006), increases (Fransson *et al.* 2000) and no change (Avis *et al.* 2003, Berch *et al.* 2009) in abundances of specific ECM fungi have been observed in response to fertilization. Although ECM fungal species diversity, evenness or richness typically does not change in response to fertilization (Baxter 1999, Berch *et al*. 2006, Wright *et al*. 2009), with some exceptions (Lilleskov *et al.* 2011 references there in), the composition of ECM fungal communities does change (Peter *et al.* 2001, Berch *et al.* 2009, Wright *et al.* 2009). This is important because considerable variation has been seen among ECM fungi in their abilities to acquire nutrients of various forms, their preferences for soil microsites, colonization patterns among different regions on the root, colonization and dispersal from spores, and their ability to tolerate various abiotic factors (Jones *et al*. 2003 and references therein). This functional diversity could potentially influence the overall stability of forest ecosystems and, therefore, any shift in species composition in response to fertilization must be examined carefully.

The role of ectomycorrhizal fungi in nutrient acquisition by trees

Ectomycorrhizal fungi proliferate in boreal and temperate regions where concentrations of inorganic soil nitrogen (N) concentrations are low, but organic N is high (Aerts 2002). In these regions aboveground productivity is limited by N (Nadelhoffer *et al.* 1992). While ECM plants have increased ability to tolerate heavy metals (Cumming and Weinstein 1990, Schier and

McQuattie 1996, Van Tichelen *et al.* 1999), root pathogens (Duchesne *et al.* 1988a) and drought (Boyle and Hellenbrand 1991, Morte *et al.* 2001) the most significant contribution ECM fungi make to their hosts is their facilitation of nutrient acquisition (Smith and Read 2008).

Ectomycorrhizas develop when a fungal mantle forms around fine feeder roots of a host plant and hyphae penetrate the apoplast among cortical (in gymnosperms) and epidermal cells (angiosperms and gymnosperms) leading to the development of a highly branched structure, termed the Hartig net (Peterson *et al.* 2004, Smith and Read 2008). The Hartig net is critical in the functionality of this relationship as it provides the interface where nutrients and carbon are transferred between fungal and root cells (Smith and Read 2008). In addition, exploratory extramatrical (EM) hyphae emanate from the outer mantle and extend into the soil. These hyphae facilitate (i) access to, and utilization of, organic nutrients via enzymatic excretion (Chalot and Bruns 1998, Read and Perez-Moreno 2003), (ii) nutrient uptake from solid mineral substrates through the production and excretion of organic acids (Lapeyrie *et al*. 1991, Wallander 2000, Mahmood *et al.* 2002) and (iii) improvements in the mobilization and uptake of nutrients in soils beyond depleted zones surrounding the roots (Heinrich and Patrick 1986, Bougher *et al*. 1990).

Colonization of plants by ECM fungi facilitates higher uptake of inorganic N including NH^{4+} and NO^{3-} via increased surface area (Chalot *et al.* 2002), and uptake of amino acids and amino sugars and utilization of more recalcitrant forms of N such as protein and chitin, by secreting enzymes (Hobbie and Hobbie 2008). Extracellular hydrolytic and oxidative enzymes, including proteases, phenol oxidases, laccases, peroxidases and chitinases, are released from hyphal tips into the soil. The soil matrix keeps the enzymes in close proximity to the N compounds in question, represses the bacterial activity through the production of antibiotics, and decreases the chances that the

products, including amino acids and oligopeptides, will diffuse away (Hobbie and Hobbie 2008). In a study by Abuzinadah and Read (1986) the ability of *Betula pendula, Picea mariana,* and *Pinus contorta* to utilize protein N in non-mycorrhizal and mycorrhizal states was examined. Approximately 53% of the N provided was readily used by mycorrhizal plants, while non-mycorrhizal plants were, with the exception of *P. sitchensis*, unable to use protein N. Consequently, ECM plants have the ability to explore and exploit nutrient resources that would remain unexplored and untapped by non-mycorrhizal plants.

Phosphorus (P) is required for the synthesis of many vital biological compounds (Kertesz and Mirleau 2004). Ectomycorrhizal fungi increase uptake of both organic (Chalot *et al.* 2002 and references therein) and inorganic (Jones *et al.* 1990) P. For example, *Pinus sylvestris* seedlings colonized with *L. laccata* or *S. bovinus* absorbed approximately 27.8% and 7.4%, respectively, of labelled organic P, compared to only 0.6% in non-mycorrhizal plants (Chalot *et al.* 2002). The ability of these plants to access the more immobile and unavailable forms of P is directly related to the ability of the fungus to secrete the appropriate enzymes (Bending and Read 1995) and to the increased surface area for absorption via extramatrical hyphae (Van Tichelen and Colpaert 2000).

The role of ectomycorrhizal fungi in carbon sequestration

As part of the mutualistic nature of the ECM symbiosis, a considerable amount of carbon is transferred from the host plant to the mycobiont. Approximately 20% of the total net primary productivity of plants such as *Abies amabilis, Pseudotsuga menzsiesii, Pinus sylvestris, Pinus radiata* and *Betula nana* is allocated to these symbionts (Hobbie 2006). Durall *et al.* (1994) found that ECM willow allocated between 3.9 – 11.5% more C belowground than non-

mycorrhizal willow. More than 30% of the soil microbial biomass can be made up of ECM mycelia in Scots pine (*Pinus sylvestris*) forests (Högberg and Högberg 2002), with up to 62% of the total C flux passing through the fungi (Godbold *et al.* 2006). Consequently, any change in biomass as a result of changes in colonization of roots or soil by ECM fungi in response to fertilization would be expected to affect the sequestration of carbon in soil (Högberg *et al*. 2001).

Ectomycorrhizal fungal species show species-specific differences in mycelium production (Deacon and Flemming 1992) and hence, differences in their carbon demand on their host. It is suggested that early stage ECM fungi produce fast growing mycelia and invest in biomass, while later stage ECM fungi demand less C from their host (Deacon and Flemming 1992). For example using Scots pine seedlings grown in a semi-hydroponic medium, *Thelephora terrestris* (an early-stage fungus) developed a sparse mycelium connected with sporulating carpophores, while *Suillus bovinus* (a late-stage fungus) created a dense mycelium (Colpaert *et al.* 1995). The production of mycelium by ECM fungal species is also affected by nutrient status (Colpaert *et al.* 1995): biomass of *Thelephora terrestris* was twice as high under high N compared to low N conditions. As N additions and fertilizers have been shown to, above all, alter ECM fungal community composition and structure (Peter *et al.* 2001, Berch *et al.* 2006, Wright *et al.* 2006, Lilleskov *et al.* 2002), shifts from communities composed of high mycelium-producing fungi to those producing lower amounts of mycelia may have profound effects on the ecosystem's ability to sequester carbon (C).

Nitrogen-deficient ecosystems tend to be highly sensitive to N additions, responding with changes in decomposition rates and C cycling (Allison *et al.* 2008 and references therein). When N availability is high, as it would be following N fertilization, plants are thought to reduce C allocation to these symbionts (Högberg *et al.* 2006). This could result in decreased production of

fungal enzymes or a shift in enzyme profiles as the fungal community changes (Allison *et al.* 2008 and references therein). In a black spruce (*Picea mariana*) forest, N fertilization resulted in a decline in the activity of N-releasing enzymes and an increase in cellulose-degrading enzymes (Allison *et al.* 2008). Similar findings were observed in a study conducted along N-gradients in Alaskan spruce forests (Lilleskov *et al.* 2002a). Consequently N additions could have significant influence over the nature and cycling of the soil organic C pool.

Effects of forest fertilization on tree productivity

Nutrient constraints on above ground forest productivity are widespread (Tamm 1991, Bergh *et al.* 1999, Elser *et al.* 2007) and, as such, fertilization has become standard practice for increasing tree yields in many parts of the world (Tamm 1991). While only a temporary increase in stand and tree growth has been observed with a single application of fertilizer, frequent addition of nutrients produces a large increase in harvest volume and sustained growth in boreal forests (Bergh *et al.* 1999, Tamm *et al.* 1999, Högberg *et al.* 2006, Brockley 2007a). In interior British Columbia, extensive research on the growth responses of above ground productivity in interior spruce and lodgepole pine (*Pinus contorta*) forests to a range of fertilizer treatments has confirmed widespread nutrient deficiencies (Brockley and Simpson 2004, Brockley 2007a).

In a "Maximum Productivity" study initiated in 1992, growth responses of both lodgepole pine and interior spruce forests to various fertilization regimes were examined. In lodgepole pine stands, an increase in stand volume of 19% was observed in response to two applications of N and B (boron), applied periodically (every 6 years), and a more substantial increase of 31% following the periodic addition of N, B and S (Brockley 2007a). Growth of spruce trees responded much more than pine to repeated fertilization. When fertilizer was applied every 6

years, mean stand volumes increased by 75% in response to total additions of 400 kg N ha^{-1} and 3 kg B ha^{-1} and by 128% to additions of N and B at the same rates, but with an additional 100 kg S ha^{-1} (Brockley 2010). Annual applications of these fertilizers resulted in an increase of 277% in spruce forests; however, they were relatively unsuccessful in stimulating growth in the pine stands (Brockley 2007a, 2010b). These results indicate strong deficiencies of N, B and S in spruce forests in central BC. In a Norway spruce stand in Sweden it was estimated that frequent applications of balanced fertilizer could quadruple tree growth rates (Bergh *et al.* 2006). Following 12 years of liquid fertilization of a Norway spruce stand in Sweden, absolute biomass (stem, needles, branches and stump) doubled compared to non-fertilized stands (Iivonen *et al.* 2006). Cumulative N additions of 600-1800 kg N ha^{-1} resulted in a mean increase in above ground tree C stock of 25 kg kg^{-1} of N added compared to control. As such, fertilization may increase C storage in trees, both above and belowground in boreal forests (Iivonen *et al.* 2006; Hyvönen *et al.* 2008). Although responses of above ground productivity have been favourable for both pine and spruce stands, there is concern that repeated nutrient additions may affect long-term site productivity through effects on soil chemistry and microbial communities.

Root length has been deemed a useful measure to characterize environmental effects on root systems (Ostonen *et al.* 2007). Although some studies have reported no effects of N fertilization on root length of forest trees (Ostertag 2001, Sharifi *et al.* 2005), other studies have documented large and persistent changes in fine root development (Majdi and Andersson 2005, Phillips *et al* 2006, Treseder *et al.* 2007, Berch *et al.* 2009). In three Alaskan boreal ecosystems Treseder *et al.* (2007) observed an almost two-fold increase in standing root length in response to N. An increase in fine root production was also observed in a Norway spruce stand (Majdi and Andersson 2005) in response to irrigated fertilized treatments occurring daily for three months

following three years. In the Maximum Productivity Study, fine root length declined in one lodgepole pine stand and increased in an interior spruce stand following 9 years of annual fertilization consisting of 100 – 200 kg N ha^{-1} and 50 – 100 kg N ha^{-1}, respectively, plus other nutrients in balance with foliar N (Berch *et al.* 2009). The variable responses observed among these studies, in part due to type, method and duration of fertilization application, and ecosystem type, makes it challenging to formulate conclusions regarding fertilization impacts on fine root length and as such belowground C allocation.

Effects of forest fertilization on ectomycorrhizal fungal community composition in coniferous forests

Plant biodiversity and ecosystem function have responded negatively to excess N (Vitousek *et al.* 1997). Similarly, declines in ECM diversity, both above- and below-ground, and alterations in communities have also been observed in response to short term fertilization (Lilleskov *et al.* 2011 and references therein). In a white spruce (*Picea glauca* (Moench) Voss) forest, 30 years of N deposition has resulted in declines in species richness and total abundance of ECM fungal sporocarps (Lilleskov *et al.* 2001). At the low N sites 144 species were observed while at the high N sites only six species were observed. Diversity of ECM sporocarps was significantly reduced in response to 1 yr of N additions in a subalpine spruce forest (Peter *et al.* 2001). Wallenda and Kottke (1998) also observed declines in sporocarp production with N deposition. The significance of these results has been in question as sporocarp production is not usually representative of belowground ECM fungal biomass or species composition (Gardes and Brun 1996). However, this does not negate the importance of these results. As genets of many mycorrhizal fungi can be quite small, and turnover rates of individuals can be fast (Dunham

2003), any alterations in dispersal may result in the reduction or elimination of some ECM fungal taxa.

With the advent of polymerase chain reaction (PCR)-based molecular methods, studies have begun to examine belowground responses of ECM fungal communities to N deposition and fertilization. Peter *et al.* (2001) observed no change in ECM fungal richness or diversity in response to two years of N additions. However other studies have observed reduced richness and diversity in response to elevated N (Lilleskov *et al.* 2002a, Avis *et al.* 2003, Berch *et al.* 2006, Lilleskov *et al.* 2008, Jones *et al.* submitted). Following ten years of annually applied fertilizer (totalling 1350 kg N) to lodgepole pine forests in interior British Columbia, ECM fungal richness on roots declined by 40% (Berch *et al.* 2006). Similar responses of species richness were observed with increased N deposition in a white spruce forest in Alaska (Lilleskov *et al.* 2002a). At the low N sites a total of 30 fungal taxa were observed forming ectomycorrhizas, while only six were observed at the high N sites.

Differences in responses observed among studies may be due to differences in duration, frequency and intensity of fertilizer application. For example, at the time of sampling, the spruce plots studied by Berch *et al.* (2009) had received 800 kg N ha^{-1} in the periodic plots and 1400 kg N ha^{-1} in the annual plots. Similarly total N levels received following 100 years of pre-industrial N deposition were ~1000 kg N ha^{-1} in the study conducted by Peter *et al.* (2001). However other studies show much lower N depositions of ~20 kg N ha^{-1} in the higher areas and ~1 kg N ha^{-1} in the lower ends (Lilleskov *et al.* 2001, 2002a, 2011). Both the lodgepole pine study by Berch *et al.* (2006), which had significant changes in community structure and the interior spruce study by Berch *et al.* (2009), which had very little change in community structure, had similar type of fertilizer, method of application and amount of N added. As such the different community

responses to N additions may not always be explained by the amount of N received. In such cases, differences may be attributable to tree species and/or age of stands. My study was conducted using the same fertilization regime as Berch *et al.* (2009) and shared the same site.

Although there is much variation in the response of ECM fungal species richness and diversity to N deposition and fertilization, changes in species composition and structure are surprisingly consistent across studies (Karen and Nylund 1997, Avis *et al.* 2003, Berch *et al.* 2006, Berch *et al.* 2009, Lilleskov *et al.* 2001, 2002a, 2005, 2008, 2011, Peter *et al.* 2001). Along a gradient of N deposition in a white spruce forest in Alaska, *Piloderma* spp., *Amphinema byssoides* and *Cortinarius* spp. dominated among the low-N sites (Lilleskov *et al.* 2002a), whereas at the high-N sites, these ECM fungi disappeared completely and were replaced by *Lactarius theiogalus*, *Paxillus involutus*, *Tylospora fibrillose*, *Tomentella sublilacina* and *Thelephora terristris*. In a 10-year fertilization study conducted on lodgepole pine, increased N led to significant reductions or eliminations of *Russula* sp., *Suillus* sp., *Piloderma* sp. and *Cenococcum* sp., increased abundance of *Wilcoxina* sp. and no change in *Amphinema* sp. (Berch *et al.* 2006). Results obtained from a reanalysis of a study conducted on red spruce in north-eastern U.S.A. (Lilleskov *et al.* 2008) indicates that with increased stand N availability the relative abundance of both *Piloderma* spp. and *Cenococcum geophilum* declined, while an increase was observed in other smooth mantled types. After reviewing this literature, Lilleskov *et al*. (2011) concluded that *Cortinarius*, *Suillus*, *Tricholoma* and *Piloderma* mycorrhizas typically decrease in abundance following N additions, whereas *Thelephora*, *Laccaria* and *Lactarius* generally increase in abundance. Both *Russula* and *Cenococcum* show mixed responses.

Similarly, when shifts in ECM fungal communities are analyzed with respect to functional changes, patterns in responses to N enrichment emerge. Based on the changes in abundance of

ECM fungal genera mentioned above, communities appear to be shifting from fungi that are "nitrophobic", that is, specialized for N uptake under N limiting nutrient conditions, to those that are "nitrophillic", that is, adapted for uptake of other nutrients and/or adapted to N-rich environments (Lilleskov *et al.* 2011). In attempting to determine effects of N deposition and fertilization on ecosystems as a whole, the functionality of these communities, that is their ability to utilize and take up various forms of nutrients, decrease pathogen resistance and uptake water, and the influence N additions has on those functions, will need to be addressed.

Effects of fertilization on soil chemistry in temperate coniferous forests

Two major aspects of long-term fertilization that could affect forest productivity are acidification of soils and the depletion of base cations such as Ca^{2+}, Mg^{2+} and K^{+} (Högberg *et al.* 2006, Ring *et al.* 2011). In the Maximum Productivity Study, a general decrease in soil pH was observed following a range of N-based fertilizer applications in British Columbia pine and spruce forests (Harrison 2011). Furthermore, with increasing N application rates, there was a significant decrease in exchangeable Mg^{2+} and Ca^{2+}. In Fennoscandian boreal forest, Högberg *et al.* (2006) also observed 70% decreases in exchangeable base cations (Ca^{2+}, Mg^{2+}, K^{+}) in response to high N treatments in a 30-year N-loading experiment. Reduction of soil pH and the concommitent replacement of base cations adsorbed to soil particles with hydrogen (H^{+}) ions increases cation leaching. In addition, as plant and microbial N demand decreases, nitrate (NO_3^{-}), which is highly mobile, tends to leach quickly down the soil profile, removing base cations as it goes. This loss of essential plant nutrients can result in decreased plant productivity (Fenn *et al.* 2006). Such changes in soil solution chemistry can affect the ECM fungal community directly via changes in pH or base cation concentrations (Rineau and Garbaye 2009) or indirectly through effects on vigour and mortality rates of their hosts (Fenn *et al.* 2006 and references therein).

Effects of fertilization on soil mesofauna

Decomposition of dead organic matter is enhanced through feeding activities (Seastedt 1984) of intermediate-sized animals (0.2 mm to 10 mm) including nematodes, Acari and Collembolans (Lindberg *et al.* 2004) referred to as soil mesofauna. In the context of this thesis, the most important role of mesofauna is their grazing of fungal hyphae, thereby regulating population sizes and succession of fungi (Newell 1984, Larsen *et al.* 2008 and references therein) and affecting plant-fungal interactions (Gange 2000). Fertilization significantly affects mesofaunal densities in both spruce (Lindberg and Persson 2004, Berch *et al.* 2009) and pine forest stands (Berch *et al.* 2006) though responses are not consistent across all types of mesofauna. In long-term fertilization studies, Acari (mites) densities, more specifically the Prostigmatid and Oribatid groups, increased significantly in an interior spruce forest (Berch *et al.* 2009) and decreased marginally in a Norway spruce stand (Lindberg and Persson 2004). Small, if any, increases in soil mesofauna were observed in response to moderate (~50-100 N kg ha^{-1} yr^{-1}) rates of N addition, while higher (~100-200 N kg ha^{-1} yr^{-1}) rates resulted in significant declines in some mesofauna components (Berch *et al.* 2006). As mesofauna communities are highly influential in fungal community dynamics, fertilization could affect ECM communities indirectly via effects on mesofauna communities.

Research overview and objectives

Given the paucity of information about belowground effects of fertilization in interior spruce and lodgepole pine stands in British Columbia, a large interdisciplinary NSERC Strategic Project was initiated in 2007 at previously-established, long-term fertilization research study sites in central British Columbia. This Maximum Productivity Study was established between 1992 and

1999 with the purpose of comparing the effects of various fertilization treatments on timber and non-timber resources (Brockley and Simpson 2004). Originally it was composed of six lodgepole pine and three spruce stands. Each study site comprised 18 plots: 3 replicate plots for each of six fertilizer treatments, including a control. Soon after establishment one of the pine sites was abandoned, while two others were abandoned following an attack by the mountain pine beetle. As part of the collaborative NSERC study the effects of two fertilizer treatments on (i) supply of soluble inorganic nutrients (Dan Harrison and Doug Maynard, University of Victoria and Pacific Forestry Centre)**,** (ii) quality and quantity of litter and soil organic matter (Lori Ann Phillips and Melanie Jones, UBC Okanagan), (iii) physiology and taxonomy of ectomycorrhizas in pine stands (Roland Treu, Valerie Ward, Shannon Berch and Melanie Jones) and (iv) nitrification by soil bacteria and archaea (Sophie Wertz and Sue Grayston, UBC Vancouver) were compared to the control treatment. The purpose of the research described in this thesis was to assess whether, at the Maximum Productivity spruce sites, fertilization had influenced:

(1) fine root length of spruce in the upper 10 cm of soil;

(2) species diversity and richness of ECM fungi colonizing spruce fine roots;

(3) relative abundance and frequency of the dominant ECM fungal genera on spruce roots;

(4) ECM fungal community structure through direct or indirect effects of changes in soil chemistry.

Based on previous research at one of these sites (Berch *et al.* 2009) and others, I predicted that, compared to unfertilized control plots, (i) total fine root length would increase in fertilized treatments; (ii) species diversity and richness of ECM fungi colonizing spruce roots would be

lower in fertilized treatments and (iii) the relative ECM fungal abundance and frequencies of some of the dominant genera including *Cortinarius* and *Piloderma* would be lower in response to fertilization treatments. Furthermore, I expected that there would be a significant relationship between ECM community structure and soil chemistry.

Chapter 2 of this thesis is the main data chapter, which provides a detailed description of the study sites, methods for fine root length analysis and ECM fungal community characterization, statistical analysis, results and discussion. Chapter 3 provides a more general discussion of this research including a summary and comparison of my results with those of other members of the collaborative team. It also contains a discussion regarding the implications of long-term fertilization on plant productivity, soil chemistry and soil C dynamics, assumptions and limitations of the research, and suggestions for future research.

CHAPTER 2: FERTILIZATION EFFECTS ON THE ECTOMYCORRHIZAL COMMUNITY STRUCTURE AND FINE ROOT LENGTH IN INTERIOR SPRUCE FORESTS

Synopsis

Due to nutrient constraints on aboveground forest productivity for many forest ecosystems (Tamm 1991, Bergh *et al*. 1999, Elser *et al*. 2007), fertilization has become standard practice for increasing tree yields in many parts of the world (Tamm 1991). While only a temporary increase in stand and tree growth has been observed with a single application of fertilizer, frequent addition of nutrients produces a large increase in harvest volume and sustained growth in boreal forests (Tamm 1991, Bergh *et al*. 1999, Tamm *et al*. 1999). In order to assess effects of removing nutrient constraints on above ground productivity in both lodgepole pine (*Pinus contorta*) and interior spruce (*Picea glauca* [Moench] Voss and *Picea engelmannii* Parry, or naturally occurring hybrids of these species) stands, the former British Columbia Ministry of Forests and Range began to establish a small system of "maximum productivity" field installations in 1992 (Brockley and Simpson 2004). The eight study sites, with harvest origins between 1992 and 1999, included five lodgepole pine and three interior spruce stands located within three major biogeoclimatic zones: Engelmann Spruce-Subalpine Fir (ESSF), Montane Spruce (MS) and Sub-Boreal Spruce (SBS) zones. The Maximum Productivity Study has provided information necessary for justifying future investments in large-scale fertilization. For example, fertilization has been proven to be effective in accelerating tree and stand development in existing immature stands (Brockley 2006, Fisher and Binkley 2000). However little is known regarding the responses of the belowground ecosystems to fertilization treatments.

Many tree species rely heavily upon a symbiotic relationship formed with ectomycorrhizal (ECM) fungi in order to enhance nutrient acquisition (Smith and Read 2008). In turn, ECM fungi benefit by receiving photosynthetically-derived carbon from their host plants (Hobbie 2006). Approximately 20% of the total net primary productivity of plants such as *Abies amabilis, Pseudotsuga menziesii, Pinus sylvestris, Pinus radiata* and *Betula nana* is allocated to these symbionts (Hobbie 2006) and more than 30% of the soil microbial biomass can be made up of ECM mycelia in *P. sylvestris* (Norway spruce) forests (Högberg and Högberg 2002). Consequently, ECM fungi are responsible for a significant component of carbon fluxes in forest soils and likely contribute to the sequestration of carbon in soil (Högberg *et al*. 2001, Högberg and Högberg 2002, Godbold *et al*. 2006, Hobbie 2006). Therefore, understanding the long-term implications of forest fertilization on ECM fungal communities is fundamental to intensive forest management.

Fertilization influences ECM fungal community composition and structure (Baum and Makeschin 2000, Peter *et al.* 2001, Nilsson and Wallander 2003, Berch *et al.* 2006, 2009, Wright *et al.* 2009). A reduction in species richness, percent root length colonized, mycelium production and total sporocarp biomass (Berch *et al*. 2006, Baum and Makeschin 2000, Nilsson and Wallander 2003) has been observed in response to fertilization. Shifts in ECM fungal community structure have also been observed, including a reduction and or elimination of some of the dominant ECM fungal taxa (Berch *et al*. 2006, Wright *et al*. 2009). Although research has demonstrated a response of ECM fungal communities to fertilization (Baum and Makeshcin 2000, Berch *et al*. 2006, Wright *et al*. 2009) many fertilization studies consider responses to a single application of fertilizer and/or are over a short duration. The focus of my thesis is to

address the potential impacts of repeated long-term fertilization on ECM communities in interior spruce forests.

Characterizing the ECM community (i.e., the identity and relative numbers of ectomycorrhizas formed by different ECM fungi) is challenging. Firstly ECM fungi are difficult to identify to species based solely on the morphology of their ectomycorrhizas. Using molecular approaches alone is also prone to error because DNA of some taxa is difficult to amplify using general fungal-specific primers. Most studies use a combination of morphological and molecular approaches to develop an accurate representation of the community. A typical approach is to separate and count ectomycorrhizas based on morphological characters and then to use molecular approaches (typically direct sequencing) to identify the taxa. The ability to identify a fungus to species is limited by the quality of information in on-line databases; it is better for some fungal taxa than others. The second challenge is that ECM fungi are distributed in a highly patchy manner in soil (Lilleskov *et al*. 2004). This is due both to preferential proliferation of some species in soils with distinct physical and chemical properties, as well as to varying dispersal methods and genet sizes (Lilleskov 2011 and references therein). This pre-existing spatial heterogeneity can have a significant impact on the ability to detect any changes in species occurrence or relative abundance as a result of an imposed treatment such as fertilization.

In order to better understand how ECM fungal communities and fine root length in interior spruce forests respond to repeated fertilization, I sampled roots from three replicate plots of three treatments at each of three spruce sites from the Maximum Productivity Study. Over the last 10-15 years, plots in the periodic treatments were fertilized with N and other nutrients every six years, while the annual treatments were fertilized annually with formulations designed to maintain foliar N concentrations at 1.3%, with other nutrients in balance with N. Control plots

received no fertilization. In addition to my research on ectomycorrhizal communities, Dan Harrison, an M.Sc. student at the University of Victoria, examined chemical responses to fertilization of the mineral soil using ion-exchange membrane plant root simulator (PRS) probes and traditional soil and foliar analysis. Overall, he found that increased frequency of fertilization was associated with an increase in soil and foliar N; soil, tree and total ecosystem C; soil acidification; and a decrease in soil and foliar Ca (Harrison 2011). Based on these changes in soil chemistry, previous research at one of these sites, and other studies on forest fertilization, I predicted that (i) total fine root length would increase in fertilized treatments compared to the control treatment; (ii) ECM fungal OTU diversity and richness of ECM fungi on colonized roots would be lower in the fertilized treatment; (iii) the relative abundance and frequency of some of the dominant ECM fungal genera, especially *Cortinarius* spp. and *Piloderma* spp., would be lower in fertilized plots than control plots; and (iv) the change in community structure with fertilization would correlate with specific changes in soil chemistry.

Methods

Study description

This research was conducted at three interior spruce (*Picea glauca* x *engelmannii*) sites in central interior British Columbia. The Crow Creek (Site 1) site is located approximately 60 km southeast of Houston, BC [latitude 54° 20', longitude 126° 17'], within the moist cold subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSmc2; www.for.gov.bc.ca/hre/becweb/). Soils are well-drained loams and clay loams with approximately 25% gravels in the upper horizons (Brockley and Simpson 2004 and references therein). Soils are classified as Orthic Dystric Brunisols (Brockley and Simpson 2004). In 1985, the previous stand was clear-cut

harvested and broadcast burned; the site was replanted in the spring of 1986. During study establishment in 1994, all treatment plots were thinned to 1100 stems per hectare (Brockley and Simpson 2004). Upon commencement of my study in August 2008, the stand was 24 years old (Brockley and Simpson 2004).

Located approximately 88 km northwest of Prince George, B.C [latitude 54°, longitude 122° 53'] the Hand Lake (Site 2) site is located within the Mossvale variant of the moist cool subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSmk1; www.for.gov.bc.ca/hre/becweb/). The soil originated from morainal parent material and has a fine loamy texture (Brockley and Simpson 2004). Within the top 20 cm, few coarse fragments are present, with up to 25% coarse fragments composed of gravels and cobbles with depth. Soils are mostly Orthic Dystric Brunisols, with some Gleyed Dystric Brunisols where there is evidence of mottling at depth (Brockley and Simpson 2004 and references therein). In 1985 the previous mature stand was clear-cut harvested and, in 1986, the cutblock was broadcast burned and replanted. During plot establishment in 1999, all treatment plots were thinned to a uniform density of 1100 stems per hectare. Upon commencement of my study in August 2008, the stand was 24 years old (Brockley and Simpson 2004).

The Lodi Lake site (Site 3) is located approximately 40 km southeast of Hixon, BC [latitude 53° 22', longitude 122° 06'] and is situated within the wet cool subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSwk1; www.for.gov.bc.ca/hre/becweb/). Originating from morainal parent material on an east-facing mid-slope (<5%), these soils are moderately well drained and relatively stone free (Brockley and Simpson 2004 and references therein). Soils in all plots are classified as Eluviated Dystric Brunisols (Brockley and Simpson 2004 and references therein). In 1985, the previously existing stand was clear-cut harvested and broadcast burned. The stand

was replanted in 1987. During site establishment in 1995, all treatment plots were thinned to 1100 stems per hectare. Upon commencement of this study in August 2008, the stand was 23 years old (Brockley and Simpson 2004).

Experimental design

Two fertilizer treatments and an unfertilized control were replicated three times for a total of nine 0.164 ha (36.24/45.30 m) treatment plots at each of the three sites. For the periodic treatments, fertilizer was applied every 6 years at a rate of 200 kg N ha^{-1}, 100 kg P ha^{-1}, 100 kg K ha^{-1}, 50 kg S ha^{-1}, 25 kg Mg ha^{-1}, 1.5 kg B ha^{-1}. The most recent application prior to sampling was in 2007 for Site 1, 2005 for Site 2, and 2008 for Site 3 (Table 2.1). The annual treatments were fertilized at rates formulated to maintain foliar N concentrations at 1.3%, with other nutrients in balance with N. The rate of N applied in the annual treatment ranged from 50 to 75 kg ha^{-1}, depending on the year (Table 2.1). No fertilization was applied to the control treatment. Adjacent plots were separated by at least 5 m. Fertilization was performed by hand soon after spring snowmelt (Table 2.1). At both Crow Creek (Fig A.1; Site 1) and Hand Lake (Fig A.2; Site 2) treatment plots were arranged in a complete randomized experimental design while Lodi Lake (Fig A.3; Site 3) was arranged in a complete randomized block experimental design.

Table 2.1 Description of fertilizer treatments (kg ha^{-1}) follow Brockley and Sanborn (2009).

Crow Creek (Site 1)

Date	Annual	Periodic
May 5, 1995	100N, 100P, 100K, 50S, 25Mg, 1.5B	200N, 100P, 100K, 50S, 25Mg, 1.5B
May 13, 1996	100N, 100P, 100K, 50S, 25Mg	
May 22, 1997	50N, 50P, 50K, 100Mg, 50S, 1.5B	
June 15, 1998	50N, 50P, 50K, 50Mg, 49S, 1.5B	
May 17, 1999	50N	
May 12, 2000	100N, 50K, 63S, 32Mg	
May 13-14, 2001	100N, 10Fe, 3Cu, 2S, 2Zn	200N, 100P. 100K, 50S, 25Mg, 1.5B
June 4, 2002	50N, 1.5B	
May 20, 2003	100N, 50S	
May 17, 2004	100N, 50P, 50K, 3S, 1.5B, 5Cu, 10Fe, 3 Zn	
May 18, 2005	75N, 50S	
May 16, 2006	50N, 50P, 50K, 52S, 25Mg, 5Cu, 10Fe, 3Zn	
May 30-31, 2007	75N, 50P, 50K, 50S, 25Mg, 1.5B	200N, 100P, 100K, 50S, 25Mg, 1.5B
June, 2008	75N, 50S	
May 28, 2009	50N, 50P, 50K, 1.5B	
Total	**1125N, 500P, 550K, 519S, 282Mg, 10.5B, 13Cu, 30Fe, 8Zn**	**600N, 300P, 300K, 150S, 75Mg, 4.5B**

Hand Lake (Site 2)

Date	Annual	Periodic
May 18-19, 2000	100N, 100P, 100K, 50S, 25Mg, 1.5B	200N, 100P, 100K, 50S, 25Mg, 1.5B
May 23, 2001	100N, 100P, 100K, 50S, 25Mg	
May 29, 2002	50N, 50P, 50K, 100Mg, 50S, 1.5B	
May 16, 2004	50N, 50P, 50K, 50Mg, 49S, 1.5B	
May 17, 1999	50N	
May 20-21, 2006	100N, 100P, 100K, 100Mg, 62S, 1.5B	200N, 100P. 100K, 50S, 25Mg, 1.5B
June 12, 2007	100N, 50P, 50K, 63S, 32Mg	
June, 2008	50N, 1.5B	
May 18-19, 2000	100N, 100P, 100K, 50S, 25Mg, 1.5B	
TOTAL	**675N, 300P, 300K, 380S, 182Mg, 6b, 10Cu, 20Fe, 6Zn**	**400N, 200P, 200K, 100S, 50Mg, 3B**

Lodi Lake (Site 3)

Date	Annual	Periodic
May 7-8, 1996	100N, 100P, 100K, 50S, 25Mg, 1.5B	200N, 100P, 100K, 50S, 25Mg, 1.5B
May 21, 1997	50N, 50P, 50K, 50S, 100Mg	
May 5, 1998	50N, 50P, 50K, 100Mg, 50S, 1.5B	
May 20, 1999	50N	
May 16, 2000	100N, 50K, 63S, 32Mg	
May 16, 2001	100N	
May 30-31, 2002	50N, 1.5B	200N, 100P. 100K, 50S, 25Mg, 1.5B
May16, 2003	50N, 50S	
May 13, 2004	75N, 50P, 50K, 52S, 25Mg, 5Cu, 10Fe, 3Zn, 1.5B	
May 13, 2005	75N, 50S	
May 9, 2006	50N, 50P, 50K, 52S1.5B, 5Cu, 10Fe, 3 Zn, 25Mg	
May 22, 2007	75N, 50S, 50K, 50S, 25Mg	
May 30-31, 2008	75N, 50S	200N, 100P, 100K, 50S, 25Mg, 1.5B
May 25, 2009	50N, 50P, 50K, 50S, 25Mg, 1.5B	
Total	**950N, 400P, 450K, 517S, 282Mg, 9B, 10Cu, 20Fe, 6Zn**	**600N, 300P, 300K, 150S, 75Mg, 4.5B**

Note: Nutrient application rate (kg ha^{-1}) represented by numbers preceding each nutrient symbol. Nutrient abbreviations: N = nitrogen, P = phosphorus, K = potassium, S = sulphur, Mg = magnesium, B = boron, Fe = iron, Zn = zinc, Cu = copper.

Soil sampling and processing

From August 30 to September 4, 2008, seven randomly-located 10 cm X 10 cm X 10 cm soil samples, including both forest floor and mineral soil, were collected from the three replicate plots for each fertilization regime treatment from each site (3 sites X 3 fertilizer treatments X 3 replicate plots X 7 samples = 189). Samples were then divided into four sub-samples by quartering. Each sub-sample was used for a different measurement: fine root length, ECM community, soil chemistry, and archael and bacterial assessment. Subsamples for fine root length and for ECM characterization were weighed and then frozen at -80 °C until processing was initiated in January 2009. Analysis of soil chemistry and description of the archael community was completed by others.

Fine root analysis

Upon removal from the freezer, samples were soaked in water for approximately 15-30 minutes and then gently washed over a 1 mm sieve to remove excess soil. Using a stereomicroscope, all fine spruce roots in samples were removed from the residual forest floor, soil and coarse woody debris, and set aside for immediate estimation of root length. A fine root was defined as any root with a diameter < 2 mm. Root length was estimated on a 0.4 X 0.4 cm grid following the line intersect method (modified from Tennant 1975). As roots were easily observed, the basic fuschsin solution used to dye fine roots was not used. Fine root length estimation was performed separately on mineral soil and forests floor.

Characterization of ectomycorrhizas

For characterization of ectomycorrhizas, soil samples were thawed, soaked in tap water, gently shaken, and then washed over a 2 mm sieve. All roots, as well as rotting wood and clumps of organic material potentially containing colonized roots, were picked out, cut into 1 cm pieces and spread out in a 20 cm X 20 cm X 5 cm dish in water and swirled to distribute.

Using a random number table, 40 root pieces or clumps of forest floor were selected using a numbered 1 cm grid located under the glass dish. The ECM root or branched system found in the left top quadrant of dish The ECM root tip located closest to the left or top of the root or branched system, located in the left top section of the dish was selected and cleaned. This resulted in a sample of 40 independent ECM root tips per soil sample. Each tip or system was examined in detail under dissecting and compound microscopes and described according to Agerer (1987-2002) and Goodman *et al*. (1996). Specifically, a combination of morphological features such as colour and texture of the whole mycorrhiza, and anatomical features such as size and pattern of hyphae in the mantle, diameter and ornamentation of extramatrical hyphae and mycelia strand structures, presence or absence of anastomosis and/or clamps and presence/absence of cystidia were used to group the ectomycorrhizas into morphotypes. Two of the most active-looking tips from each morphotype were stored in dry Eppendorf tubes and frozen at -80 °C (Lee *et al*. 2007) for molecular analyses.

Molecular methods and identification of ECM fungi

Four hundred and forty nine tips, each representing one morphotype per soil sample were subjected to DNA extraction and PCR amplification. DNA was extracted using the REDExtract-N-Amp Plant PCR Kit, (Sigma, St. Louis, Missouri, USA). The manufacturer's protocol was

followed with the exception that only 1 µl of the extraction and dilution solutions was added to each tip rather than 100 µl. The highly variable internal transcribed spacer (ITS) region of the fungal ribosomal RNA gene was used for identification of fungal operational taxonomic units (OTUs) (Goodman *et al*. 1996-2002, Martin and Rygiewicz 2005). Thermocycler conditions for PCR amplification of the entire ITS using primers ITS1F and ITS4 (Gardes and Bruns 1993, Tedersoo *et al*. 2003) were as follows: 3 minutes at 94 °C, 35 cycles of 1 minute at 94 °C, 1 minute at 50 °C, 1 minute at 72 °C and 10 minute incubation at 72 °C. Success of amplification was assessed using a 1% agarose gel, stained with SYBRsafe (Invitrogen, Carlsbad, CA). Reactions displaying clear single bands were cleaned of excess primers and nucleotides using ExoSAP-IT kit (Fisher Scientific, Ottawa). When no or multiple bands were observed, PCR was repeated on genomic DNA diluted by 1/10. When multiple or no PCR products were observed after dilution, the second tip from that morphotype and sample was extracted.

Amplicons were sequenced using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) on an ABI3130xl capillary sequencer at the Fragment Analysis and DNA Sequence Service at University of British Columbia's Okanagan campus. Alignment and correction of sequences were performed in Sequencher 4.2 (GeneCodes, Ann Arbor, MI, USA). Each sequence was then BLASTed (Basic Alignment Search Tool; Altschul et al. 1997) against National Centre for Biotechnology Information (NCBI) and User Friendly Nordic ITS Ectomycorrhizas (UNITE) databases. The species name of the accessioned sequence was applied to the sample sequence if at least 97% of base pairs (bp) were identical over a sequence length of at least 450 bp. Next, all sample sequences BLASTed to the same genus or family were aligned using ClustalX (Thompson *et al*. 1997), pairwise sequence similarities calculated, and phylogenetic trees constructed by the neighbour-joining method using PHYLIP 3.69

(Felsenstein 1989). Those samples whose sequences that matched database sequences at 85% to 97% or that matched sequence lengths between 250 bp and 450 bp were named based on the taxonomic affiliations of the BLAST hits in the databases and with the morphological descriptions. If amplification was not successful from either saved tip, that morphotype from that sample was named at the genus level based on morphological data and sequence data from similar morphotypes from other samples.

Data analysis

Ectomycorrhizal fungal diversity, richness and evenness measures were determined per plot (combined data from all tips from all samples) using EstimateS (Colwell 1994-2011), using fungal operational taxonomic unit (OTU) as an equivalent of species. The Shannon diversity index was selected for description of taxonomic diversity per plot due to its sensitivity to rarer OTU's. Relative abundance was calculated per plot as the number of root tips colonized by a fungus (OTU) divided by the total number of ECM root tips identified in all samples from that plot. Frequency was calculated as the number of soil samples (out of seven) in which an OTU occurred in a given plot. Due to high patchiness in the ECM communities, sample-based rarefaction curves were executed without replacement using Mao Tau, as recommended by Mao and Lindsay (2003) to compare observed species richness among treatment plots at each site. As the dataset comprised a few dominant and many rare species, abundance-based coverage estimator (ACE) was used to estimate total species richness per plot for each treatment at each site (Colwell 1994-2011).

Treatment effects on ECM fungal diversity, species richness, species evenness, relative abundance and frequency, as well as on fine root length, were assessed for each site separately

by a one-factor analysis of variance (ANOVA) using JMP 8.0.2 (SAS Institute Inc. 2009), with fertilizer treatment as the explanatory variable. Plots were used as replicates, resulting in an n = 3. An α value of less than 0.05 was considered to indicate a significant difference among fertilizer treatments while $0.05 < \alpha < 0.10$ was considered to indicate an effect worth mentioning. Normality of the data was tested using the Shapiro-Wilk's goodness of fit test, and the Bartlett test was used to assess homogeneity of variance (Bartlett and Kendall 1946). When an ANOVA detected a significant difference, a Tukey's honestly significant difference (HSD) post-hoc test was used to determine differences among individual treatment means. When assumptions of normality and homogeneity of variance could not be met, square root transformations or log transformations were used. Where transformations did not improve distribution or variance, a non-parametric Wilcoxon test was performed to compare means among treatments.

Indicator species analysis and ordinations of the ECM fungal community were conducted in PC-ORD, version 6 (McCune and Mefford, 1995-2011). As ECM fungal communities are highly spatially heterogeneous and my data did not conform to either a linear or unimodal distribution (McCune and Grace 2002), non-metric multidimensional scaling (NMS) ordinations were used to visually compare ECM fungal community structure for the nine plots at each site. Bray-Curtis similarity index was selected as the distance measure. Dimensionality was determined based on reduced stress levels, zero instability and interpretability of results (McCune and Grace 2002). Following Clarke's rule of thumb (Clarke 1993), dimensions resulting in stress levels below 5 (excellent representation, no risk of misinterpretation) and between 5 and 10 (good with no real chance of drawing false conclusions) were selected. To evaluate whether NMS was extracting stronger axes than expected by chance, a Monte Carlo randomization test was used. Based on these criteria, a dimensionality of three was selected for each site. As a non-Euclidean distance

measure was used in the ordinations, a multi-response permutation procedure (MRPP) was used to test for treatment effects ($\alpha = 0.05$) on ECM community structure (McCune and Grace 2002). Soil chemistry data provided by Harrison (2011) were used in the joint plot ordinations to assess any chemical influence on the ECM fungal community structure. These included NH_4^+-N, NO_3^--N, Mg, K, P, Fe, Mn, Ca, Cu, Zn, B and S as measured in the soil solution by PRS probes.

Results

Fine root length

Effect of fertilization on fine root length was minor. At each site, no fertilization treatment effect was detected on root length density in forest floor (Fig 2.1a; $P > 0.2$). At Site 3 only, an affect of fertilization on root length density in mineral soil was detected ($F = 4.90$; $P = 0.05$), with the lowest mean root density observed in the annual fertilization treatment (Fig 2.1b). At the other two sites, fertilization did not affect root length density in mineral soil ($P > 0.1$).

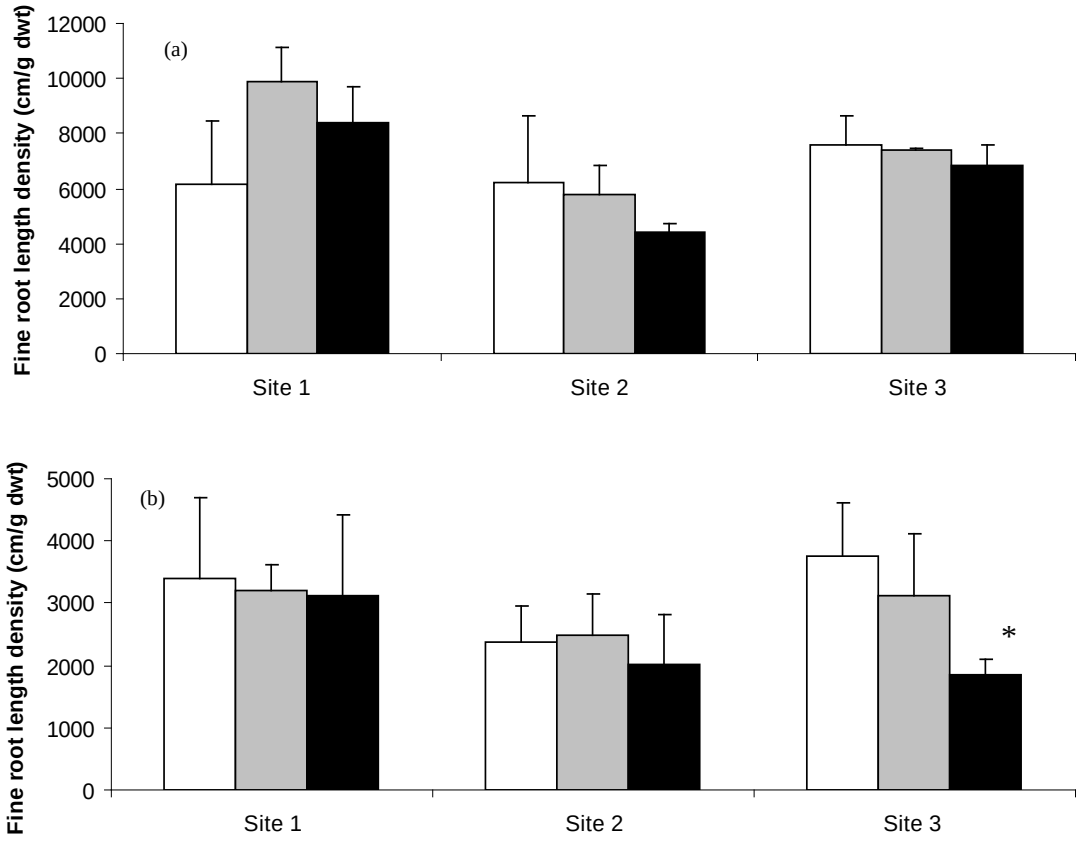

Figure 2.1 Mean (n = 3) fine root lengths per g of oven-dry (a) forest floor and (b) mineral soil across three interior spruce sites in control (white bars), periodic (gray bars) and annual (black bars) fertilization treatments. Error bars represent 1 SE of the mean.

Taxonomic characterization of ectomycorrhizal fungi from colonized root tips

A total of 871 morphotype subsamples (2 tips per subsample) were collected across all three sites for molecular analysis; DNA extraction was attempted from 449 (53%) of this total. A total of 359 tips were from the first tip and 89 were from the second tip. DNA was successfully extracted from 229 tips, 200 from the first and 29 from the second tip per morphotype per sample. Of the successfully extracted samples, 103 (24%) sequences from the first tip and 5 (2%) from the second, were of sufficient quality and length to place into OTUs or species. Genotype classification of the 121 (27%) tips, 97 from the first and 24 from the second, producing shorter length sequences and/or ones of poorer quality were determined individually

via clustering with the previously identified sequences and in conjunction with the morphological identification. Accession numbers for best match and abundance values per treatment for each site are provided in Table S1. Due to the low sequence success, and time and budget constraints, the remaining tips were not sequenced.

There was considerable amount of overlap in the taxa of ECM fungi detected among treatments at each site. At Site 1, 23 ECM fungal OTUs were observed; 12 of these were found in plots of all three treatments, 7 were found solely in the control plots, 2 in the periodic plots and 2 in the annual plots. Again at Site 2, 23 ECM fungal OTUs were observed; 16 of these were observed across all three treatments, 3 were found only in the control plots, 2 in the periodic plots and 2 in the annual plots. A total of 18 ECM fungal OTUs were detected at Site 3; 7 OTU's were observed across all three treatments, 3 were exclusively found in the control plots, 7 in the periodic plots and 1 in the annual plots. A total of 15 OTU's were common to all three sites.

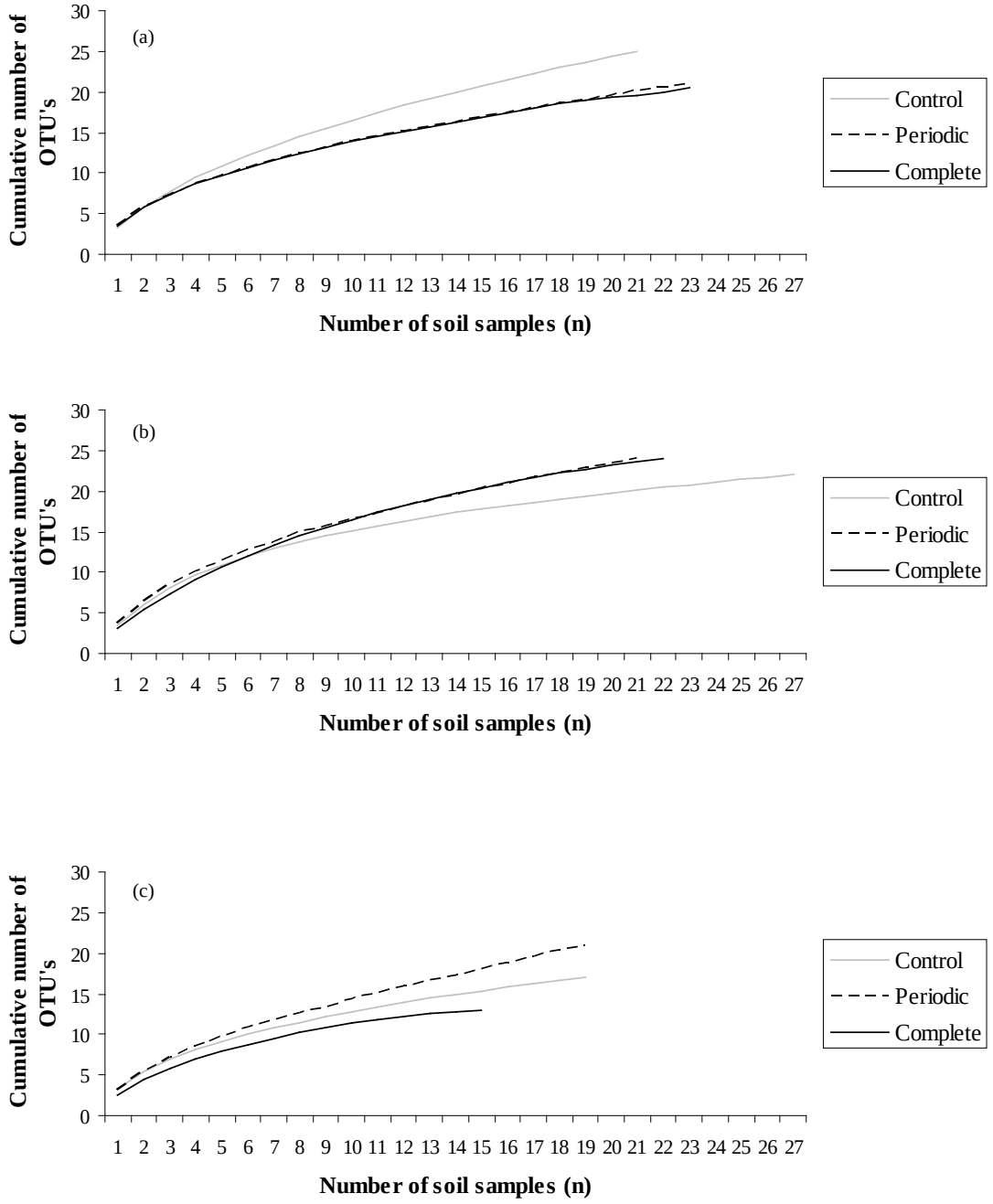

Figure 2.2 Mao Tau species rarefaction curves without replacement; (a) Site 1, (b) Site 2 and (c) Site 3. Fertilization treatments include; Control (light-gray), Periodic (dashed) and Annual (black).

Richness and diversity of ectomycorrhizal fungi from colonized root tips

Sample based rarefaction curves for each treatment at each site did not reach asymptotes although the slopes of the curves were not steep (Fig 2.2). Nevertheless, this indicates that sampling was not sufficient to account for all fungal OTUs present at each treatment for each site. Rarefied observed richness of ECM fungal OTUs (Mao Tau) per plot (combined tips from all samples) did not differ among treatments at Site 1 and Site 2 ($P > 0.8$); however, OTU richness tended to be marginally lower in annually fertilized plots at Site 3 ($F = 3.96$; $P = 0.08$; Fig 2.3a). Estimated total species richness also tended to be lower at Site 3 in response to annual fertilization compared to both control and periodic treatments (ACE; Table 2.2).

Whether based on the relative abundance (calculated as the number of root tips colonized by a fungal OTU in a sample divided by the total number of ECM fungal root tips identified in that sample) (Fig 2.3b and c), or frequency (number of samples in which an OTU occurred in a given plot) of occurrence per plot (data not shown), significant differences in ECM fungal Shannon diversity or evenness per plot were not detected among fertilization treatments at any site ($P > 0.8$).

Table 2.2 Mean abundance-based coverage (ACE) estimator of ECM fungal OTU richness per plot in response to fertilizer application (n = 3).

	Control	Periodic	Annual	SE	*F*	P value
Site 1	7.922	8.25	8.79	0.91	0.23	0.81
Site 2	9.77	9.74	10.12	0.99	0.06	0.94
Site 3	9.12	8.20	5.36	0.85	3.63	0.09

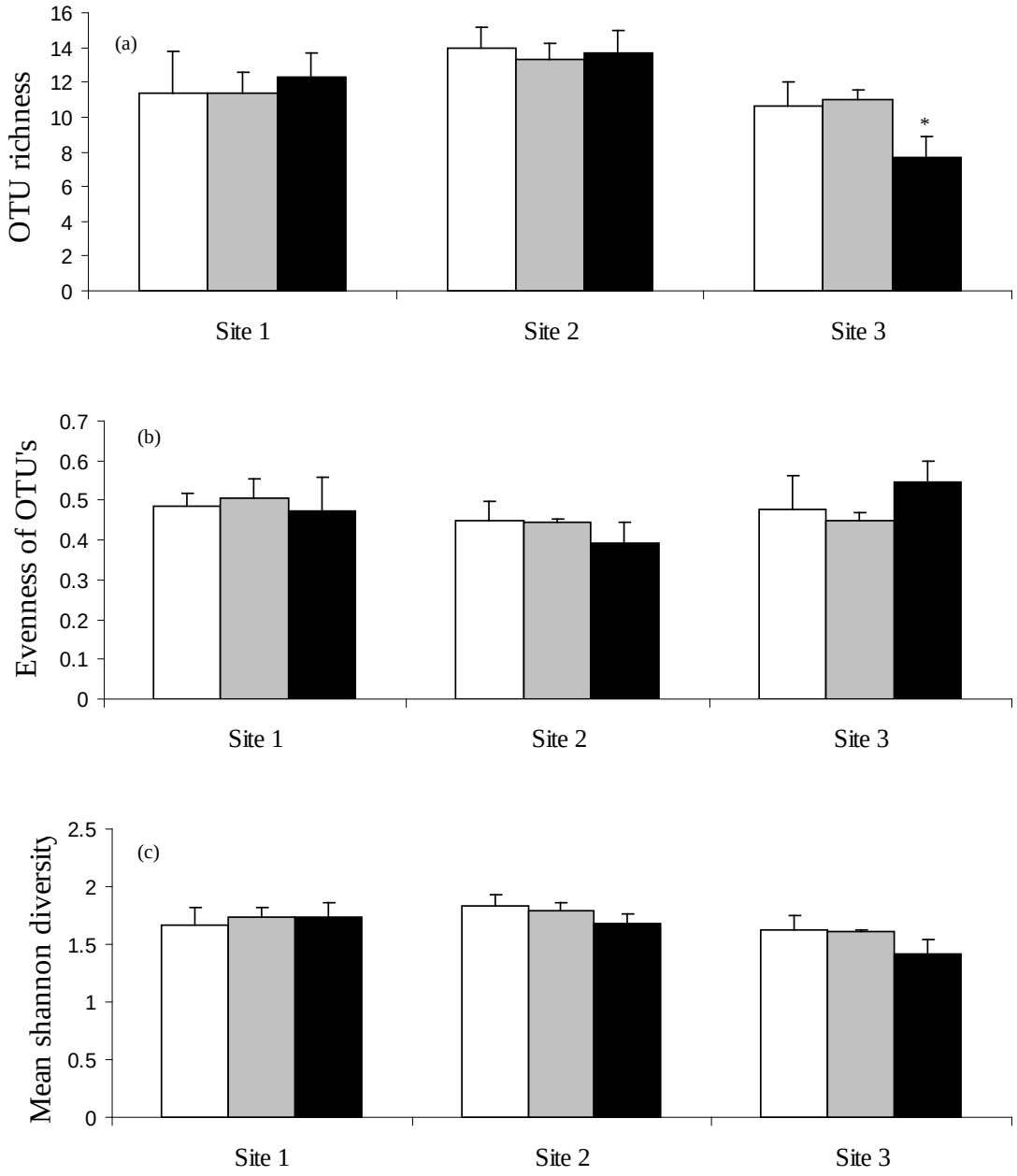

Figure 2.3 (a) Estimated total richness (ACE), (b) evenness and (c) Shannon diversity of ectomycorrhizal fungal OTUs (based on relative abundance) per plot (n = 3) at three spruce sites in response to no (light gray bars), periodic (dark gray bars) or annual (black bars) fertilization treatments. *difference among treatments at this site at p = 0.08. Error bars represent 1 SE of the mean.

Species composition and structure of ectomycorrhizal fungal communities

Overall six ECM fungal genera dominated (meaning > 5% of ectomycorrhizas identified per plot) samples collected from the spruce stands: *Cenococcum*, *Amphinema*, *Cortinarius*, *Lactarius*, *Piloderma* and *Tylospora.* In general, there appeared to be no consistent response of these dominant mycorrhizas to fertilization across sites (Fig 2.4, Fig 2.5). Furthermore, *Tylospora* was the only fungus whose mycorrhizas varied in relative abundance with fertilization at any one site ($F = 9.0453$; $P = 0.02$ at Site 3; Fig 2.4f), where its relative abundance was lower in annually fertilized plots than in control plots. Indicator species analysis revealed the presence of *Tylospora* sp1 mycorrhizas to be marginally correlated with the control treatment at Site 3 (Monte Carlo test; $P = 0.10$) and *Cortinarius* sp1 (Monte Carlo; $P = 0.06$) and *Piloderma* sp1 ($P = 0.06$) with the control treatment at S1. No effect of fertilization on the frequency of occurrence of dominant types of ectomycorrhizas in soil samples was detected, with the exception of *Tylospora* mycorrhizas (Fig 2.5f). At Site 3 only, *Tylospora* mycorrhizas were encountered in about one-third of the samples in the annually fertilized plots compared to control plots ($F = 5.5813$; $P = 0.04$).

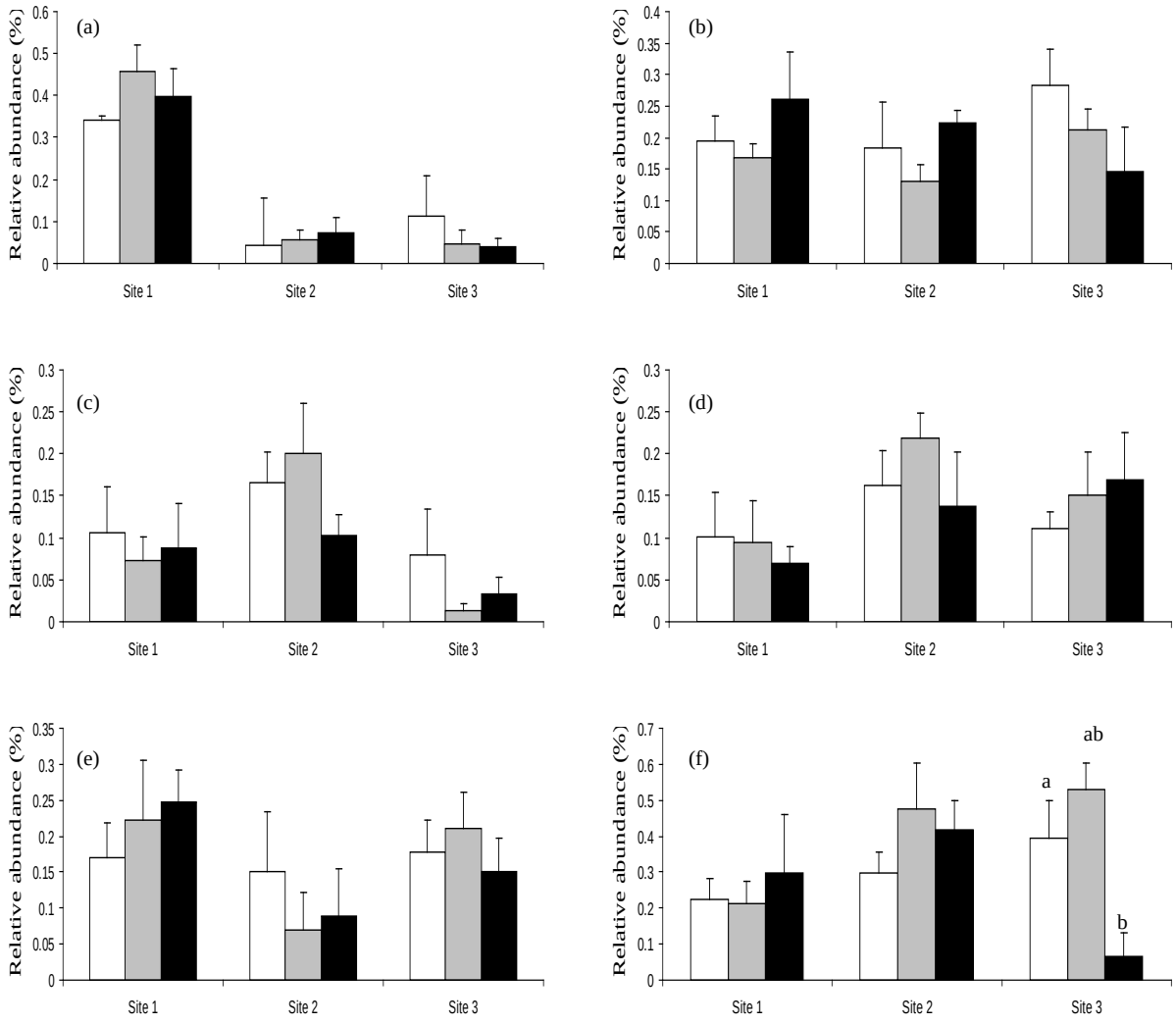

Figure 2.4 Mean relative abundance (the proportion of spruce mycorrhizas) per plot of (a) *Amphinema* (b) *Cenococcum* (c) *Cortinarius* (d) *Lactarius* (e) *Piloderma* and (f) *Tylospora* mycorrhizas in the control (white bars), periodic (gray bars) and annual (black bars) fertilization treatments (n = 3 plots per site). Different letters above bars indicate significant differences in relative abundance of the dominant ECM fungi between treatments (P = 0.02). Error bars represent 1 SE of the mean.

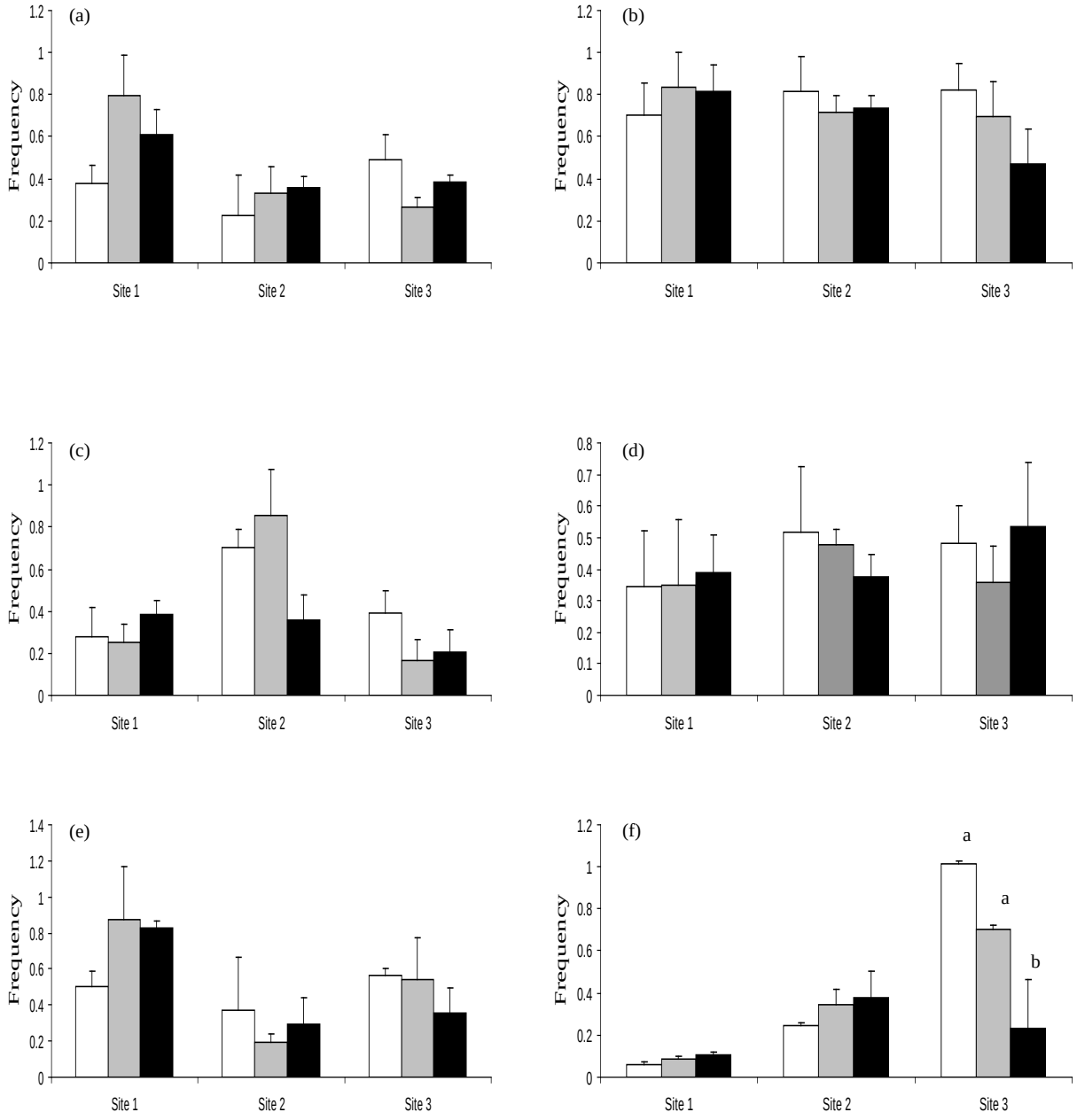

Figure 2.5 Frequency (number of times in which an OTU occurred) per plot, of (a) *Amphinema* (b) *Cenococcum* (c) *Cortinarius* (d) *Lactarius* (e) *Piloderma* and (f) *Tylospora* mycorrhizas on the root tips of spruce, in the control (white bars), periodic (gray bars) and annual (black bars) treatments. Different letters above bars indicate significant differences in frequency of the dominant ECM fungi between treatment means ($p = 0.04$). Error bars represent 1 SE of the mean.

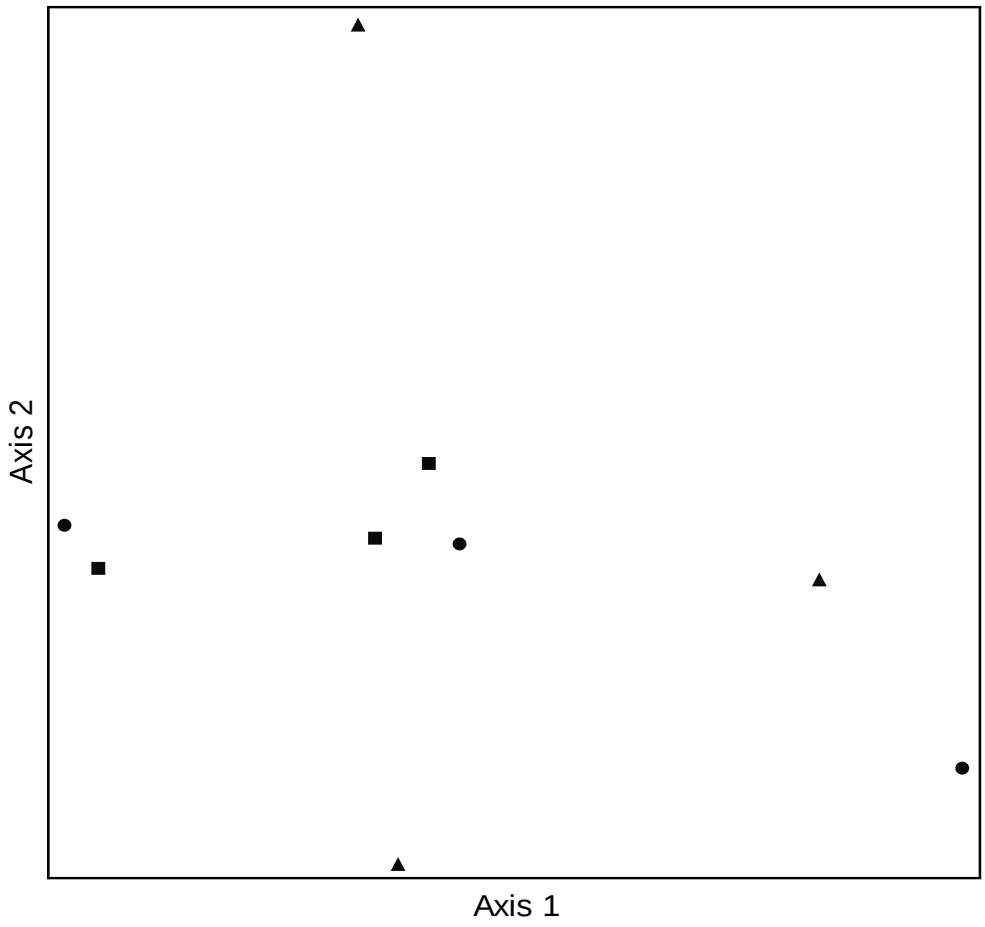

Figure 2.6 Nonmetric multidimensional scaling (NMS) ordination of the relative abundance of ectomycorrhizal (ECM) fungi found on short roots of spruce at three interior spruce sites that had been exposed to annual (squares), periodic (circles) or no (triangles) fertilization at Site 1.

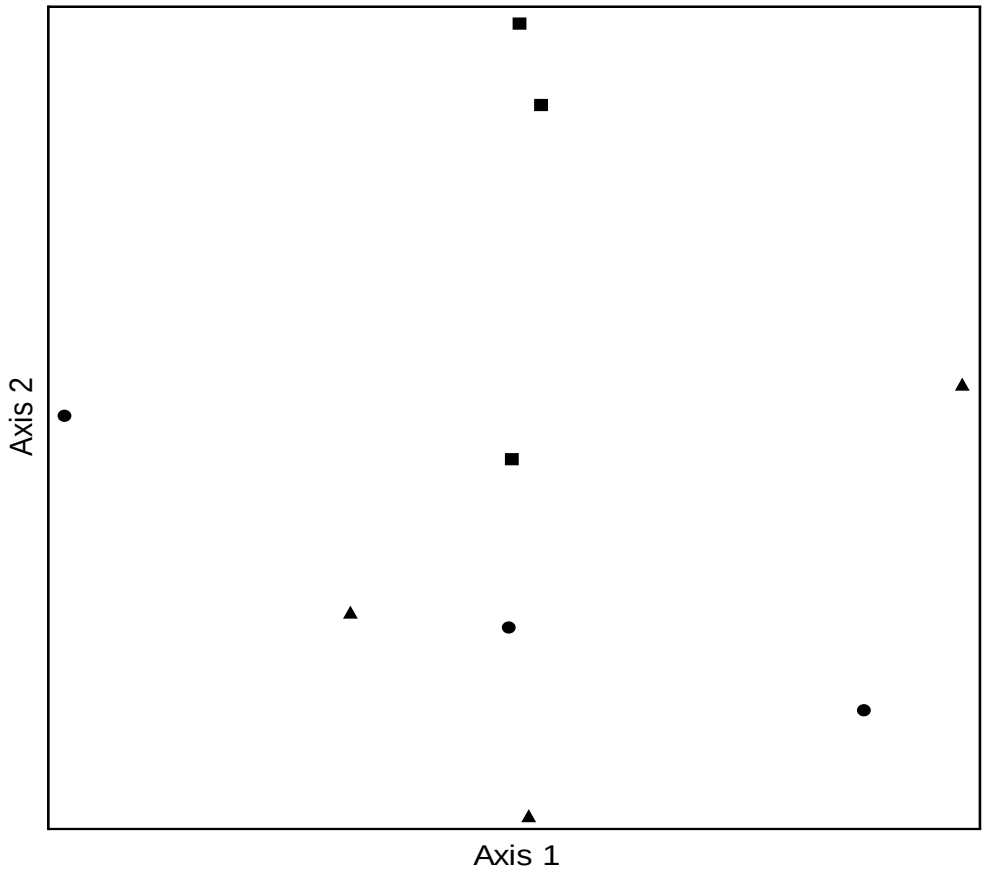

Figure 2.7 Nonmetric multidimensional scaling (NMS) ordination of the relative abundance of ectomycorrhizal (ECM) fungi found on short roots of spruce at three interior spruce sites that had been exposed to annual (squares), periodic (circles) or no (triangles) fertilization at Site 2.

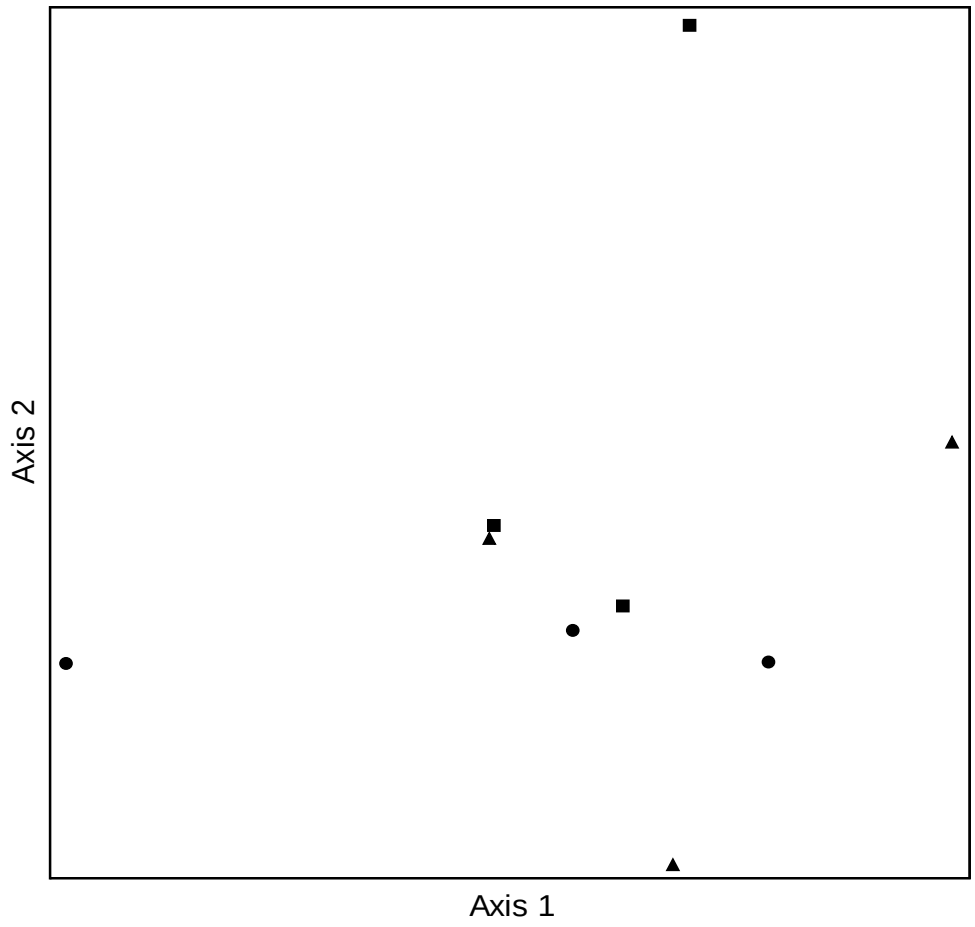

Figure 2.8 Nonmetric multidimensional scaling (NMS) ordination of the relative abundance of ectomycorrhizal (ECM) fungi found on short roots of spruce at three interior spruce sites that had been exposed to annual (squares), periodic (circles) or no (triangles) fertilization at Site 3.

Two major gradients captured most of the variation in the ECM fungal communities, the first two dimensions containing 34.1% and 11.9% (cumulative = 46.0%) at Site 1, 37.7% and 13.4% (cumulative = 51.1%) at Site 2 and 36.3% and 12.5% (cumulative = 48.8%) at Site 3. Higher dimensionality improved the models very little. NMS ordination did not result in clear separation of plots based on fertilization rate, although the annual plots were grouped somewhat (Fig 2.6, 2.7, 2.8). MRPP analyses of both relative abundance and frequency detected no difference in community composition among treatments at any site ($P \geq 0.5$). Overall the ECM fungal communities did not appear to be structured by either fertilization treatment or any of the

aspects of soil solution chemistry including; Aluminum (Al), Boron (B), Copper (Cu), Magnesium (Mg), Manganese (Mn) and Nitrate (NO_3), measured by Harrison (2011) (Fig 2.9, 2.10, 2.11). A relationship between the ECM communities in the annual treatments and the nutrient vectors at each site can be observed at each site (Fig 2.9, 2.10, 2.11); however, this relationship is very weak.

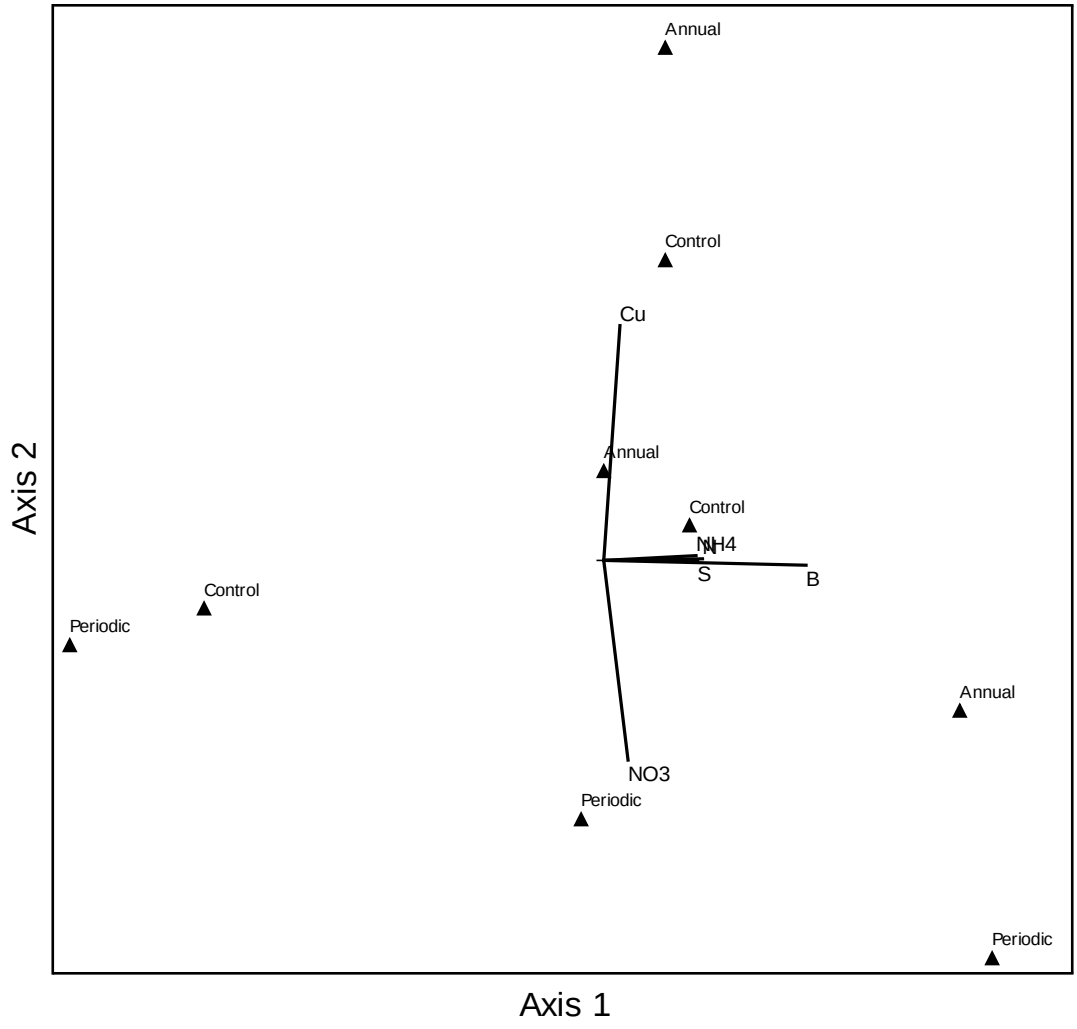

Figure 2.9 Nonmetric multidimensional scaling (NMS) ordination bi-plot of soil chemistry of forest floors from Site 1. The points represent the ECM community from each plot at each Site. The length and direction of the line vectors represent the degree to which the specific soil properties correspond with each axis.

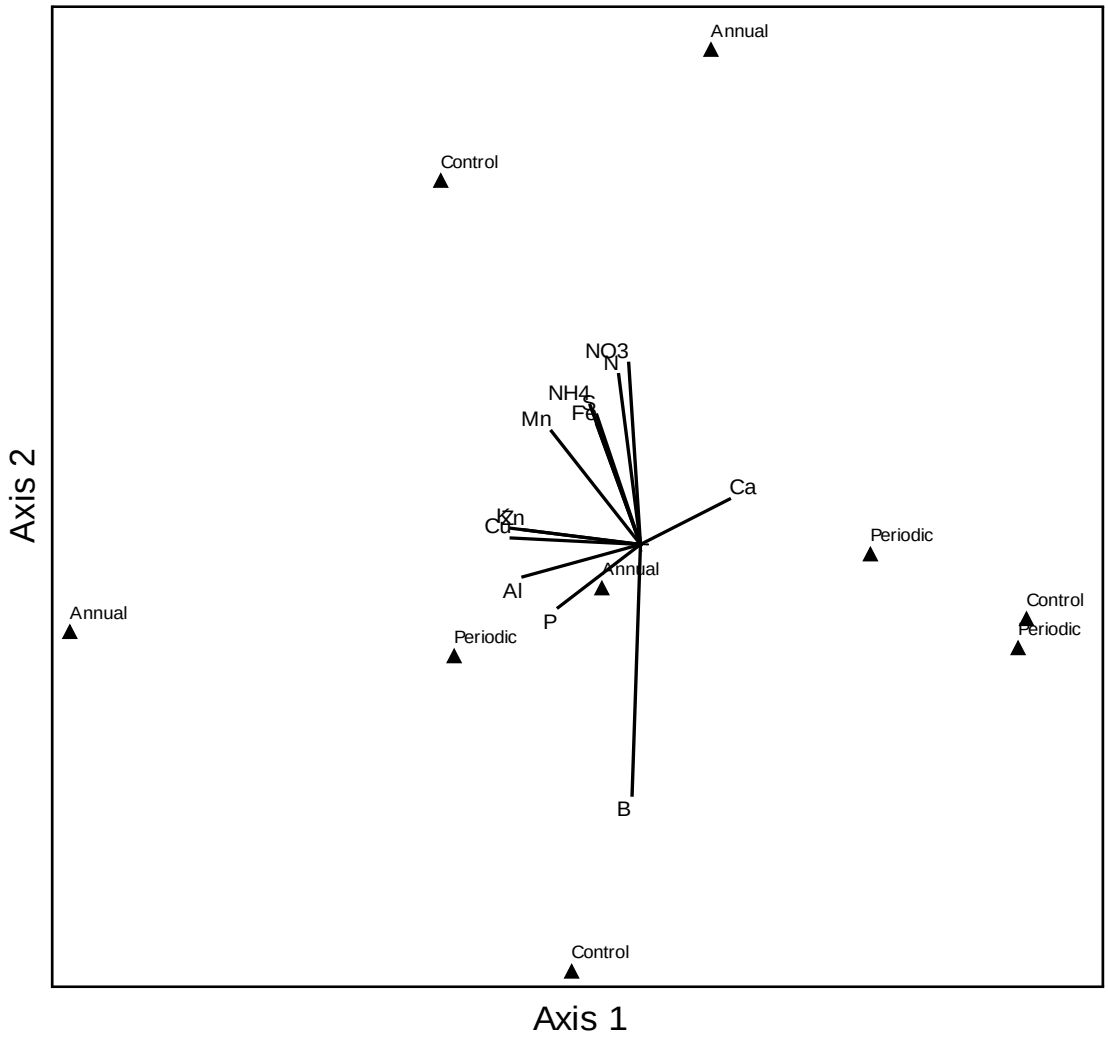

Figure 2.10 Nonmetric multidimensional scaling (NMS) ordination bi-plot of soil chemistry of forest floors from Site 2. The points represent the ECM community from each plot at each Site. The length and direction of the line vectors represent the degree to which the specific soil properties correspond with each axis.

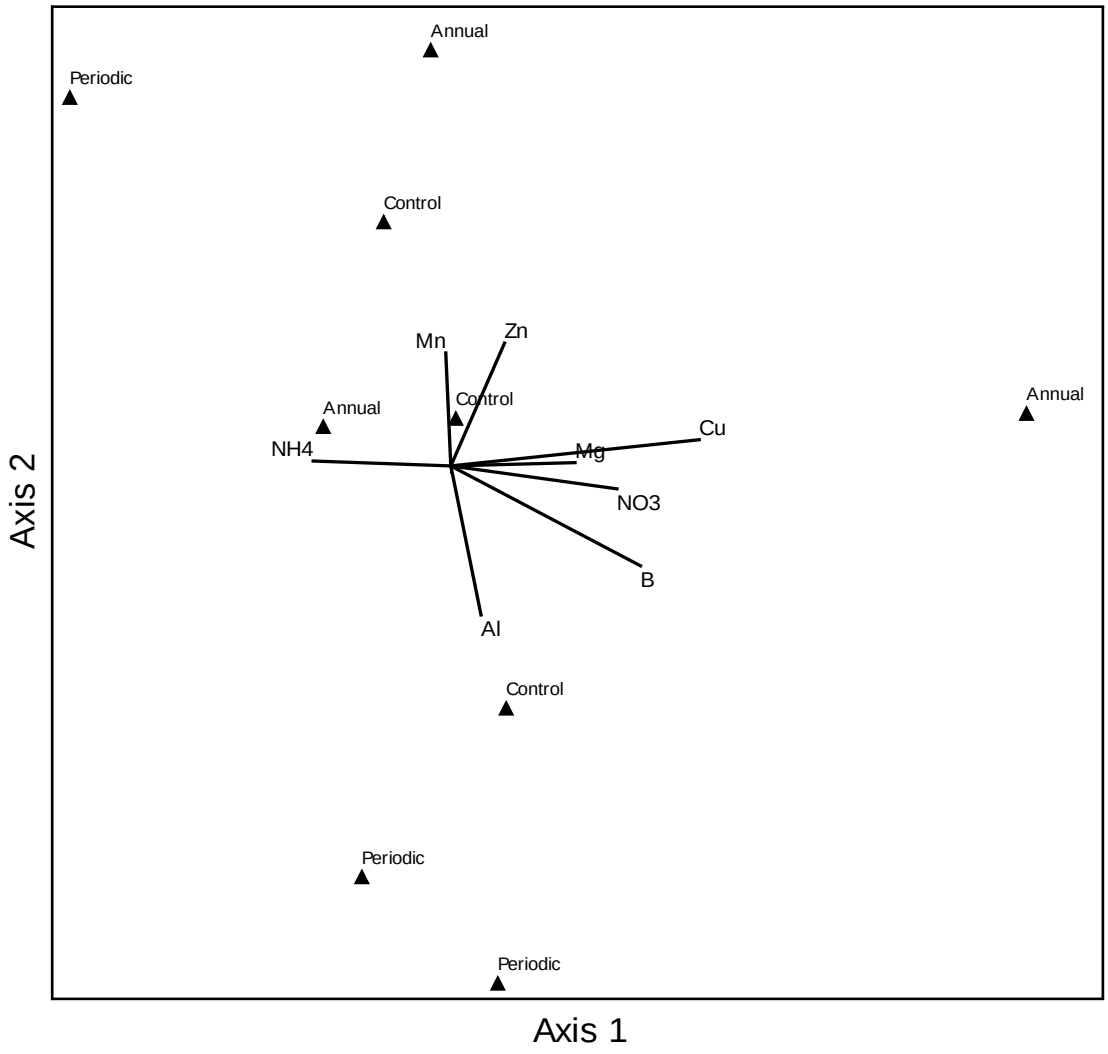

Figure 2.11 Nonmetric multidimensional scaling (NMS) ordination bi-plot of soil chemistry of forest floors from Site 3. The points represent the ECM community from each plot at each Site. The length and direction of the line vectors represent the degree to which the specific soil properties correspond with each axis.

Discussion

Overall, we found that annual fertilization had marginal effects, and fertilization every six years had no detectable effects on fine root length and ECM fungal community structure at three interior spruce forests. No fertilization effect was detected in the evenness and diversity of the ECM fungi colonizing roots; however, root length density and the relative abundance of *Tylospora* mycorrhizas differed among treatments at one site. This was opposite to our predicted

increase in fine root length and a decrease in relative abundance and frequency of the dominant genera *Cortinarius* sp. and *Piloderma* sp. in response to fertilization.

Fine root length

In only the mineral soil, fine root length decreased in response to the annual fertilization treatment at Site 3. No other responses in fine root length were observed. In forests, fine-root length production, standing fine root biomass and mortality responses to fertilization are variable and responses can depend on root diameter, forest type, tree species, depth of soil, branching order, mean annual precipitation and temperature (Majdi and Nylund 1996, Majdi *et al.* 2001, Majdi 2001, Majdi and Ohrivic 2004, Majdi and Andersson 2005, Bakker *et al.* 2008, Berch *et al*. 2006, 2009). For example, in a 34 year-old *Picea abies* forest in Northern Sweden, Majdi and Andersson (2005) observed an increase in standing root biomass, fine-root production and N turnover of spruce roots in both forest floor and mineral soil with addition of liquid N, P and micronutrient elements over 10 years. However in another long-term spruce fertilization study conducted by Majdi and Kangas (1997) 5-6 years of fertilization with N, S and micronutrients led to lower fine-root production and an increase in fine-root mortality at soil levels between 0-20 cm.

In 2002, Berch *et al*. (2006) sampled roots from Sheridan Creek, one of the pine sites of the Maximum Productivity Study; in 2004, they sampled from Crow Creek (listed as Site 1 in my study) (Berch *et al.* 2009). Effects of long-term fertilization had different effects on fine root length at the two sites. At the pine site a significant decline in fine root length was observed in response to increasing fertilization (Berch *et al.* 2006) while at the spruce site annual fertilization resulted in a marginal increase ($P = 0.09$) in fine root length (Berch *et al.* 2009). The difference

between pine and spruce may, in part, be explained by the decreased mycorrhizal colonization rate observed in the pine site and the unaffected colonization rate observed in the spruce site in response to increased fertilization. As mycorrhizal roots live longer than non-mycorrhizal roots (King *et al.* 2001, Yan and Chen 2010), the decline in mycorrhizal colonization in pine roots may have resulted in increased root turnover and lower biomass. No changes in colonization of spruce roots were observed in my study, based on approximately 30% of my samples (data not shown). Given the small sample size, these results must be treated with caution; however, they would be consistent with the results of Berch *et al.* (2006) and could explain the low sensitivity of spruce root length to high fertilization frequencies. Although Berch *et al.* (2009) detected a marginal increase in fine-root length and we found none, this may be attributable to differences in methodology. We assessed fine root length in forest floor and mineral soil separately while Berch *et al.* (2009) used combined samples. The results for Site 1 in Figure 2.6a are not incompatible with the minor effects observed by Berch *et al.* (2009).

The reduction in fine root length in mineral soil with annual fertilization at Site 3 may be attributable to the decrease in pH observed with increasing fertilization at that site. Harrison (2011) found that control soils, between 0-10 cm, at Site 3 had lower pH than soils at other sites and appeared to have much lower buffering capacity as well. Low pH can result in an increase in Al^{+3} in the soil solution. As well as inhibiting root elongation (Yuan and Chen 2010), high soluble aluminium can, in turn, bind with P, making it insoluble and thereby reducing amount of P taken up by roots. Phosphorus is especially important for root elongation, so reduction in uptake can lead to a reduction in fine root production (Yuan and Chen 2010 and references therein).

A very different, and speculative, explanation for the reduction in root length with annual fertilization at Site 3 is that trees allocated less carbon belowground at this site. Percent increase in leaf area index (m^2 leaf m^{-2} ground surface) was higher at Site 3 compared to S1 in response to the annual fertilization treatment (Brockley and Simpson 2004). In nutrient-rich soils, plants often shift their allocation from belowground to aboveground and that could have been happening here. This explanation would be contradictory to the previous one, which ascribes the reduction in root growth to soil toxicity, whereas this explanation assumes luxury uptake of nutrients by roots.

Diversity, richness and evenness of spruce ectomycorrhizas

Across all three sites diversity and evenness were not obviously affected by fertilization, whereas species richness tended to decline at Site 3 in response to the annual fertilization treatment. In oak (Avis *et al.* 2003, Baxter *et al.* 1999), spruce (Fransson *et al.* 2000, Peter *et al.* 2001, Berch *et al.* 2009) and hemlock (Wright *et al.* 2009) forests no change in ECM fungal species richness, evenness or diversity were observed on root systems in response to N additions. However, ECM fungal diversity (Frey *et al.* 2004) and richness (Lilleskov *et al.* 2002, Berch *et al.* 2006) was reduced in response to continuous long-term N fertilization in other pine and spruce dominated forests. For example, following 10 years of annual fertilization 40% fewer ECM fungal types were observed in plots subjected to high N additions compared to control plots at the Sheridan Creek pine site of the Maximum Productivity Study (Berch *et al.* 2006). The lack of response in diversity and evenness observed in my study are consistent with other fertilization studies occurring in spruce forests (Peter *et al.* 2001, Berch *et al.* 2009). Obviously, this indicates that any shift in species composition involves species replacement or compensatory changes in

relative abundance of individual species, rather than whole scale changes in evenness/dominance patterns.

Colonization of roots by ECM fungi decreases in nutrient-rich environments (Jones *et al.* 1990, Berch *et al.* 2006). With fewer ectomycorrhizas being formed, the number of ECM fungal species on the roots would be expected to decrease. A reduction in colonization was not observed in my study (data not shown). The variable responses of ECM fungal species diversity and richness cited above and those observed among the sites in this study indicate that responses to fertilization may be dependent on the ecosystem to which nutrients are being added. Soil analysis at Site 1 and Site 3 (Harrison 2011; soils at Site 2 were not extracted by Harrison) reveal large increases in N pools, soil acidity and a significant decline in pH at Site 3, but not Site 1, with annual fertilization. This may explain the marginal decline in OTU richness observed at Site 3 with annual fertilization.

Effects of fertilization on overall ectomycorrhizal community composition

A shift in the species composition of ECM fungal communities is a very common response to fertilization (Karen and Nylund 1997, Fransson *et al.* 2000, Peter *et al.* 2001, Frey *et al.* 2004, Baxter *et al.* 2006, Berch *et al.* 2006, Lilleskov *et al.* 2008, Wright *et al.* 2009). In particular, a relationship between the quality and availability of N and ECM community structure has been firmly established (Lilleskov and Parrent 2007, Treseder *et al.* 2008, Kranabetter *et al.* 2009). As reviewed by Lilleskov *et al.* (2011), ECM fungal genera that are capable of growing on protein as a sole source of N, and those that are classified as hydrophobic medium-distance fringe exploration types (Agerer 2001) typically decline in response to fertilization or N deposition. Along a natural productivity gradient in BC, Kranabetter *et al.* (2009) also found that the

composition of ECM fungal communities changed with soil N status. Lilleskov *et al.* (2011) and references therein defined those fungi that declined with anthropogenic N depositions as 'nitrophobic' and those that increased with anthropogenic N depositions as 'nitrophilic'. Although repeated fertilization increased available N in the soil solution at our sites (Harrison 2011), MRPP detected no significant shift in the overall ECM fungal community in fertilized plots. Likewise, Berch *et al.* (2009) had found no detectable changes to the ECM community at Site 1 after 10 years of fertilization.

Although no change in the overall ECM community was detected by MRPP, annual fertilization resulted in a significant reduction in both frequency and abundance of *Tylospora* mycorrhizas at Site 3. *Tylospora* is classified as a short-distance exploration type, with hydrophilic extramatrical hyphae and no rhizomorphs (Hobbie and Agerer 2010) and displays variability in its protein use (Lilleskov *et al.* 2011). As less investment in mycelium is required, it is expected that *Tylospora* would be of lower carbon cost to its host, relative to ECM fungi forming more complex mycelium and rhizomorphs (Lilleskov *et al.* 2011). In situations where belowground C allocation is reduced in response to increased N availability (Ericsson 1995) it is proposed that *Tylospora* would be favoured (Lilleskov *et al.* 2011). This contrasts with results of other studies on forest fertilization. For example, in response to atmospheric N deposition over 27 years, *Tylospora fibrillosa* was not observed in the low N sites, but was a dominant taxon in the high N sites in a white spruce (*Picea glauca*) forest (Lilleskov *et al.* 2002). Similarly, in response to 4 years of 100 kg ha^{-1} per year of ammonium sulphate in a 30-year-old Norway spruce (*Picea abies* (L.) Karst) stand, the relative frequency of *Tylospora fibrillosa* increased (Karen and Nylund 1997). In response to 150 kg ha^{-1} yr^{-1} of ammonium nitrate additions in a 35-year-old subalpine spruce forest, Peter *et al.* (2001) observed no change in relative abundance of

Tylospora asterophora and an increase in relative frequency for only the first two years. In reviewing these studies, Lilleskov *et al.* (2011) concluded that, as a genus, *Tylospora* was tolerant to N additions. The studies cited above were either shorter fertilization trials, approximately 4 years with large fertilization applications, between 100-150 kg ha^{-1} yr^{-1} (Karen and Nylund 1997, Peter *et al.* 2001), or long-term fertilization trials (27 years), with smaller fertilization applications (approximately 20 kg ha^{-1} yr^{-1} at the high N sites and approximately 1 kg ha^{-1} yr^{-1} at the low N sites; Lilleskov *et al.* 2002). The Maximum Productivity Study used 100 kg ha^{-1} yr^{-1} of urea-N in the annual fertilization treatment over a period of 14 years. The differences in the duration of fertilization and amount of fertilizer used may have resulted in the different results seen in this study, or alternatively it may be due to the difference in genera studied. In a meta-analysis by Treseder (2008), declines in abundances of microbes and fungi were more evident in studies of longer durations and with higher total amounts of N added. When considering my study compared to atmospheric N deposition studies (Lilleskov *et al.* 2002), the differing responses observed may also be due to the pulsed nature of the fertilizer inputs compared to the chronic low-level inputs involved in atmospheric N deposition.

Importantly, the relative abundance of *Tylospora* mycorrhizas declined only at Site 3. This is the same site where a significant reduction in fine root length was detected and lends further support to Harrison (2011)'s conclusion that this site, with its low buffering capacity, has responded more extremely to fertilization than the other sites. The further acidification at Site 3 resulting from annual fertilization may have been responsible for the decline in *Tylospora* mycorrhizas. Following 15-18 applications of sulphuric acid rain (pH = 2.7 - 2.8) over 6 years in an 80-year-old *Picea abies* forest, the frequency of *Tylospora* spp. decreased significantly (Quian *et al.*

1998). Consequently the lower initial pH at Site 3 compared to Site 1 and Site 2, followed by a further decrease in pH may explain the sensitivity of *Tylospora* at Site 3.

Other dominant ECM fungal genera observed in this study were *Amphinema* spp., *Cortinarius* spp., *Cenococcum* spp., *Piloderma* spp. and *Lactarius* spp. These fungal genera are similar to those found in other studies of northern temperate forests (Karen and Nylund 1997, Fransson *et al.* 2000, Peter *et al.* 2001, Avis *et al.* 2003, Frey *et al.* 2004, Lilleskov *et al.* 2008, Wright et al. 2009). Contrary to expectations, I did not detect a reduction in *Cortinarius* or *Piloderma* mycorrhizas, both of which are considered sensitive to N by Lilleskov et al. (2011). *Amphinema byssoides* has been labelled as highly nitrophillic by Kranabetter *et al.* (2009) based on its distribution across natural productivity gradients in BC. As such, we would expect it to become more abundant in fertilized plots, but this was not observed. *Cenococcum geophilum* is considered to be weakly sensitive (Lilleskov *et al.* 2011) and appears to show preference for a particular range of N levels (Kranabetter *et al*. 2009). Based on foliar N concentrations, higher percent colonization by *Cenococcum geophilum* was observed between 11 and 13 N g kg^{-1} (Kranabetter *et al*. 2009). As foliar N concentrations of the annual fertilization treatment were found within in this range, it is surprising that an increase in *Cenococcum geophilum* with increasing fertilization was not observed.

Interestingly in the study conducted by Berch *et al.* (2009), *Wilcoxina* sp. was also considered a dominant player and yet was not observed at all in this study. Stands were approximately 20 years of age in the study conducted by Berch *et al.* (2006 & 2009) and approximately 25 years of age in my study. Abundances and frequencies of many ECM fungal genera/species can be influenced by the successional stage of the forest (Twieg *et al.* 2009), and so the differences in stand age may be responsible for the disappearance of *Wilcoxina* mycorrhizas. Karen and Nylund

(1997) surveyed a 30-year-old Norway spruce stand and, similar to my study, no *Wilcoxina* spp. was observed, even though the composition of the rest of the community was similar. *Wilcoxina* was observed in a 12-year-old Norway spruce stand (Korkama *et al.* 2006) and in newly planted spruce seedlings (Hagerman *et al.* 1999) though data from the latter study was based on the E-strain mycorrhizas, which matched closely to the morphology of *Wilcoxina*. These observations add to the evidence that *Wilcoxina* is an early-stage ECM fungal genus.

The apparent insensitivity of several genera of ECM fungi in our study to frequent addition of fertilizer may be an artefact of analyzing the relative abundance of the fungus at the genus level. I took this approach because (i) most species were too rare or spatially heterogeneous to assess at the species level and (ii) Lilleskov et al.'s (2011) review of N addition studies found some very consistent patterns in response at the genus level. Nevertheless, there is no question that ECM fungal species within the same genus can vary structurally (e.g., differentiation of the mycelia and rhizomorphs, and their exploration types) and functionally. For example, although most *Lactarius* spp. belongs to the contact exploration type, some species belong to the medium-distance, smooth types (Agerer 2001). Kranabetter *et al*. (2009) found that some *Cortinarius* spp. increased in abundance along a natural productivity gradient (i.e., increased N), while other decreased. In my samples, we detected seven OTUs of *Cortinarius*, seven of *Piloderma* and two of *Amphinema*. Therefore it is quite possible that these OTUs responded differently to nutrient enrichment and the net effect was no detectable change at the genus level. However, when we evaluated the communities as a whole, we used OTUs, not genera, so any major shift in species composition would have been detected. The fact that no overall change in community was detected suggests that we did not miss any major change by comparing relative abundance at the genus level.

The findings reported in this study and those by Berch *et al.* (2009) are suggestive of an ECM fungal community that is stable under conditions of nutrient enrichment. A community that does not change in composition or diversity with disturbance and/or stress is more stable compared to one that changes (Lawrence 2008). Both resilience, defined as the ability of an ecosystem to return to its pre-disturbed state, and resistance, defined as the ability of the ecosystem to resist change following disturbance, are components of stability (Barbour *et al.* 1998).

Community stability in the form of both resilience (Allison *et al.* 2008, Girvan *et al.* 2005) and resistance (Girvan *et al.* 2005, Allison *et al.* 2010) has been observed among bacterial, fungal, algal and forest communities and typically shows a positive relationship with species diversity (Frank and McNaughton 1991). Soil microbial communities are highly diverse and that may be why they tend to be relatively stable. For example, following experimental warming in a boreal ecosystem in Alaska, no changes in soil fungal communities were observed (Allison *et al.* 2010). Similarly strong genetic and functional resistance were observed in bacterial communities in response to contamination of soil by copper (Girvan *et al.* 2005). With increasing diversity, resistance in both bacterial (Girvan *et al.* 2005) and plant (Frank and McNaughton 1991) communities increased in response to perturbations of benzene amendment and drought, respectively. Our study of spruce sites detected lower ECM diversity than a study of similar treatments at pine sites in interior BC (Jones *et al*., submitted). Contrary to expectations of the resistance-diversity relationship, the more diverse ECM communities in pine stands appear to be less stable when exposed to the same fertilizer regimes than the ECM communities in spruce stands.

Mathematical modeling, based on the assumption that populations commonly reach an equilibrium state, suggests increased productivity results in an increase in community stability

(Stone *et al*. 1996). As productivity, defined as tree height and stand volume, was affected more by fertilization in the spruce compared to the pine (Brockley and Simpson 2004) sites, this may explain the different responses between studies. As nonequilibrium conditions appear to be dominant in many ecosystems (Stone *et al.* 1996) this productivity-stability hypothesis needs to be considered with caution.

As mentioned above, resistance is the ability of a community to remain substantially unchanged in response to perturbation and/or stress. In this study, fertilization was our perturbation and minimal changes in ECM fungal community were observed in response. As such, it would appear that this community is resistant to fertilization. It is possible, however, that the community had initially changed in response to fertilization and then returned to its original composition as a result of resilience.

CHAPTER 3: CONCLUSION

General discussion

A total of four objectives were addressed in this thesis. Objective one was to evaluate total spruce fine root length response to fertilization, in both the forest floor and mineral soil. My results indicate a marginal fertilization effect on fine root length in mineral soil at Site 3 and no effect at Site 1 or Site 2. Across all three sites, fertilization effect on fine root length in the forest floor was not observed. Objective two was to determine whether ECM fungal OTU diversity and richness responded to fertilization. My results indicate that diversity was not affected by fertilization at any of the three sites and richness was only marginally affected by annual fertilization at Site 3. Objective three was to evaluate the effects of fertilization on the relative abundance and frequency of some of the dominant ECM fungal genera. My results indicate that the relative abundance and frequency of *Tylospora* spp. declined in response to the annual fertilization treatment at Site 3; however, no other effects were observed for the remaining dominant genera across all three sites. Objective four was to assess whether changes in soil chemistry, as a result of fertilization, influenced the ECM fungal community structure. My results indicated only a very weak relationship between the ECM fungal communities in the annual treatment and nutrient vectors.

Management implications

Although both single and continuous fertilization applications yield increases in tree yield, long-term sustained growth in temperate and boreal forests is only attained with regular fertilization (Tamm *et al*. 1999 and Bergh *et al*. 2006). For economic reasons, forest managers are more likely to apply fertilizer every few years, rather than annually, to achieve these long-term

increases in timber yield. My study suggests that this type of fertilizer regime is not likely to elicit any ECM fungal belowground response. Thus based on these results at these sites, forest management practices employing periodic fertilization would generate aboveground productivity with no belowground disturbance with respect to ECM fungi; however, my study also indicated that fertilizer responses will be site specific. Consequently, the effects of fertilization on the ECM fungal communities and their function should be carefully evaluated by site.

As this study assessed the implications of large-scale fertilization on the soil system, it is important that we understand, not only which ECM fungal taxa were present, but also what they were doing. In the present experiment, no statistically significant reduction in species richness (i.e., the number of ectomycorrhizal OTUs) was observed in response to either annual or periodic fertilization across all sites. Therefore, based on the view that functional trait diversity is, to some extent, correlated with phylogenetic diversity (Taylor et al. 2000; Lilleskov et al. 2002b), the ECM communities in my fertilized plots may not have lost functionality. As part of the Maximum Productivity Study, enzymatic activities of dominant ECM fungi were studied in the pine stands (Jones *et al.* submitted). In response to annual fertilization at one pine site, both *Piloderma* spp. and *Cenococcum* spp., whose abundances were not affected by fertilization, showed an increase in their associated activities of cellobiohydrolase, ß-glucosidase, xylosidase, leucine amino peptidase and laccase per unit of mycorrhizal surface area. Total soil N was positively related to the enzymatic activities of *Cenococcum* mycorrhizae, while pH had a negative influence on enzyme activities associated with *Piloderma*. Jones *et al*. (submitted) concluded that the shifts in ECM fungal communities in response to fertilization did not influence the ability of the ecosystem to degrade soil organic matter and, hence, did not result in a loss of functionality.

With the exception of *Tylospora* spp. in the annual treatment at Site 3, no effects on the relative abundances and frequencies of the dominant ECM fungal genera were observed in my study. Although ECM fungi may change their associated enzymatic activities in response to fertilization, it seems unlikely that such minor changes in community composition would affect nutrient cycling. Although not presented, I conducted enzyme assays on some of the dominant genera found in this study. These results remain to be analyzed. Nevertheless, my study, like most other studies of ECM fungi, detected a large number of rare species. New techniques, such as high-throughput sequencing and more sophisticated "community bioinformatics", will expand our knowledge of the abundance and distribution of these rare species; however, we currently know little about the contribution of rare species to the functioning of ECM fungal communities (Peay *et al.* 2008). Consequently, we cannot conclude with any certainty whether losing some of these species would be a threat to the function of our forests. As a result, further study of the effect of long-term forest fertilization on ECM fungal physiology is warranted.

Assumptions and limitations

Fungal communities are highly diverse and poorly described. The difficulty lies in the ability to accurately describe members of these communities due to their highly cryptic nature and microscopic size. In the past, ECM fungal ecology relied solely on assessing fruiting structures in the field (Peay *et al.* 2008). However as fruiting bodies do not accurately represent the belowground fungal assemblages, the use of molecular tools, more specifically, amplification of fungal DNA using fungal-specific primers, has become a widely accepted technique for alleviating these difficulties (Gardes and Bruns 1996). The use of unique morphological traits of ECM root tips has also been used to identify ECM fungi in ECM fungal community studies (Karen and Nylund 1997, Baxter 1999). There is considerable variation in the detail with which

researchers examine ectomycorrhizas: from more simplistic observations of colour, shape and texture to more detailed anatomical features of the mantle, extramatrial hyphae, Hartig net and rhizomorphs (Agerer 1987-2001, Goodman *et al.* 1996). When morphological analyses are performed at the more detailed level, researchers can be more confident in their sorting and categorizing of ECM fungal tips. It should also be noted that typically within the same sample, morphotypes almost always turn out to be formed by the same fungus even under more basic morphological sorting procedures (Horton 2001).

To alleviate some of these potential issues, I used a combination of the more detailed morphological approach and direct sequencing. Although all ECM fungal tips were morphologically identified, as is typical in studies of ECM communities, only representative tips were sequenced. In this process, a predefined region of the genome, in this case the ITS region of the fungal DNA, is amplified, cleaned and sequenced, and its similarity compared against a database. There are three main assumptions made during this process (Nilsson *et al.* 2006): (i) that there is a satisfactory number of sample sequences within the database, (ii) that those sequences have been identified and annotated correctly, and (iii) that the process involved in naming has been consistent, commonly adopted and easily understood. Unfortunately when it comes to fungi these assumptions are rarely met (Nilsson *et al.* 2006). Of the estimated 1.5 million fungal species, less than 1% have had their ITS region sequenced, and as many as 20% of the sequences in the NCBI database are incorrectly labelled (Nilsson *et al*. 2006 and references therein). As new sequences being added to the database are being named based on sequences in the database, the cycle of misidentification is perpetuated. I used a combination of morphological and molecular methods to reduce these uncertainties.

Newly developed molecular tools have, without question, augmented our understanding of ECM fungal ecology; however, there remain many unanswered questions, specifically when assessing the functionality of these systems. Although understanding which ECM fungi form the ECM community is important, understanding the functional roles they play within that community is even more important. Unfortunately the functionality of these ECM fungal species is a relatively recent area of study. One of the approaches that have been used to address functionality is to quantify the activities of wall-bound enzymes in the ectomycorrhizosphere (Koide and Zabir 2000, Courty *et al.* 2007). Breakdown of organic molecules in soil by hydrolytic and oxidative enzymes secreted by ECM fungi and their associated bacterial flora is thought to be a key contribution of ECM fungi to nutrient cycling (Read and Perez-Moreno 2002). Accordingly, measurements of the activities of such enzymes should be one measure of function of the ECM fungal community. Such studies are beginning to show that ECM fungal species, and often genera, have predictable enzyme profiles. In my study, the enzymatic activity of some of the dominant ectomycorrhizas was assessed in fertilized and unfertilized plots; however, those data remain to be analyzed. As such this study was not able to assess fully the effects of fertilization on the functionality of the system.

Accurately estimating species richness in ECM fungal communities is challenging. Based on an extrapolation of a 6 to 1 ratio of fungal to plant species, Hawksworth (1991) estimated there to be 1.5 million fungal species globally and, when allowing for cryptic species, the number tripled to 5.1 million. In the tropics, Fröhlich and Hyde (1999) estimated a ratio of 33 fungal species to 1 plant species. Thus the Kingdom Fungi is one of the most species rich of the major eukaryotic lineages (Peay *et al.* 2008). Almost all field studies of ECM fungi, including this one, do not sample intensively enough for asymptotes to be reached on species accumulation curves.

Typically sampling is limited by financial and time constraints, but even highly intensive, large-scale sampling (O'Brien *et al.* 2005, Fierer *et al.* 2007) fail to detect sufficient ECM fungi for accurate estimates of total species richness. Peay *et al.* (2008 and references therein) suggest that the inability to reach the estimated fungal species asymptote is a potential explanation for the difficulties in making comparisons between treatments and/or sites in studies. Consequently results pertaining to treatment effects on ECM fungal communities in this study must be interpreted cautiously.

In conjunction with the high species richness found in ECM fungal communities, the high spatial and temporal variability observed (Lilleskov *et al.* 2003) makes it challenging to detect species frequently enough to be able to draw substantial conclusions about effects on their abundance. Based on species-specific patterns and community similarity, this patchiness occurs at relatively small scales. Consequently the patchiness of ECM fungi has huge implications for effective sampling strategies. Lilleskov *et al.* (2004) found that samples collected less than 2.6 m apart in seven mature forests contained more similar ECM fungal communities than those taken further apart. This means that by sampling at or above 2.6 m spacing, one would be able to account for the background dissimilarity and as such will yield the maximum information regarding community characterization. Though samples were collected randomly they appeared on average to be above 2.6 m apart. As such I can be more confident in my abilities to account for background dissimilarity. Lilleskov *et al.* (2004) also noted the large differences in distribution patterns of individual taxa among soil cores. For example some species display distinct clumps, such as *Rhizopogon* sp., while others, such as *Cenococcum geophilum,* have a more even distribution of root tips. There appear to be a variety of processes contributing to these species-specific spatial patterns including differences in growth rate of genets and patterns of genet

establishment (Lilleskov *et al.* 2004). For example studies looking at genet size in sporocarp-based studies found that genets can range in size from tens of centimetres (*Rhizopogon*) to tens of meters (*Suillus).* Genets of *Rhizopogon vesiculosus* had a span of 0-20.9 m (mean = 13.9 $\pm$ 5.4 m) in interior Douglas-fir forest (Beiler *et al.* 2011) Sites can vary in their autocorrelation distances (Izzo *et al.* 2005). Determining the autocorrelation distance of ECM fungal communities in the interior spruce stands prior to assessing their response to treatments would have allowed us to design a more effective sampling strategy. However, due to budget and time constraints this was not a feasible approach for my study and, consequently, as many samples as could be processed in a 6 month-period were randomly collected per plot.

Interpreting the results of nitrogen fertilization experiments can be difficult. The observed responses in this study and those of others may not solely be explained by fertilization but may also be explained by other interacting factors such as changes in abiotic and biotic factors including soil chemistry (Harrison 2011) and mesofaunal communities (Berch *et al.* 2006, 2009). For example changes in soil chemistry may result in a shift of C allocation from belowground to aboveground components (Albaugh *et al.* 1998) and affect the aboveground plant community composition and biomass (Tamm *et al.* 1999), which may result in differences in litter quality. Changes in mesofaunal communities in response to fertilization, as observed by Berch *et al.* (2006, 2009), may result in an increased amount of mycorrhizal herbivory thereby decreasing percent colonization. Consequently, it is difficult to attribute any changes observed in ECM fungal community composition to a direct effect of fertilization and this makes it is extremely challenging to predict the impacts of alterations in ECM fungal communities on long-term ecosystem vigour.

As an important part of the experimental design, conducting a power analysis and an estimation of sample size is key to determine whether your sample size may be too high or too low. The experiment will lack the accuracy to produce reliable results if the sample size is too low. Additionally, much time and resources will be unnecessarily used with very little gain if the sample size is too large. As the sampling for this thesis occurred before I began the project a power analysis was not conducted. Additionally, to account for the multiple comparisons a P value of less than 0.003 should have been used when concluding a difference significant, thus reducing my chance of making a Type I error; i.e., a P value of 0.05 may increase my chances of rejecting a true hypothesis. However by reducing the P value I am also increasing my chances of making a Type II error; i.e., not rejecting a false hypothesis. As my results appear to be occurring in response to the same treatment at the same site I have more assurance that the results found were as a result of what was actually occurring in response to the treatment.

Suggestions for further research

According to Lilleskov *et al.* (2011), most species within an ECM fungal genus display some functional redundancy; that is, they are similar in function. Consequently assessing the effects of fertilization at the genus level is a reasonable approach. However, the review conducted by Lilleskov *et al.* (2011 and references therein) indicated that there were species within some genera that varied in their response to N enrichment. Accordingly increased sampling intensity so that all taxa could be identified and their relative abundance/frequencies calculated would most likely result in the ability to assess fertilization effects at the species level.

Regardless of a lack of ECM community change it is possible that the functional role of dominant ECM fungi may be altered in response to fertilization and as such, altering the

functional role of that particular ecosystem. As such it is important to understand if these species were changing in function and, if so, in what way. Some enzyme data was collected for this system; however, the same taxa were not always located at each plot. Therefore, this data set will not allow us to determine the effect of fertilization on individual ECM fungal taxa. Having a more extensive set of physiological data, including all the dominant taxa, would be beneficial to assess any changes in functional roles of these species and/or genera. Therefore, if I could continue this work, I would sample more intensely to ensure that all dominant ECM fungal taxa were represented and replicated for yielding more statistical significance.

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APPENDICES

Appendix A: Experimental plot layout

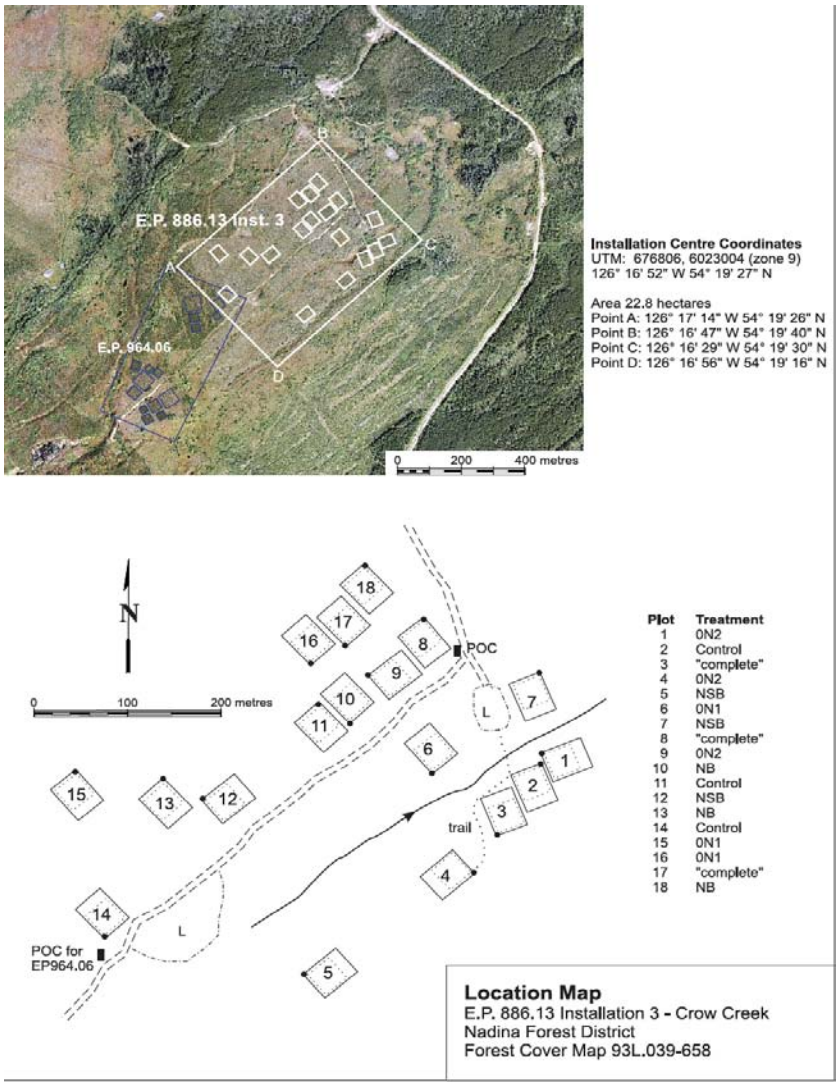

Figure A.1 Experimental plot layout for sampling at Site 1 (Crow Creek). Treatment legend (on map = this study): control = control, complete = periodic, ON1 = annual.

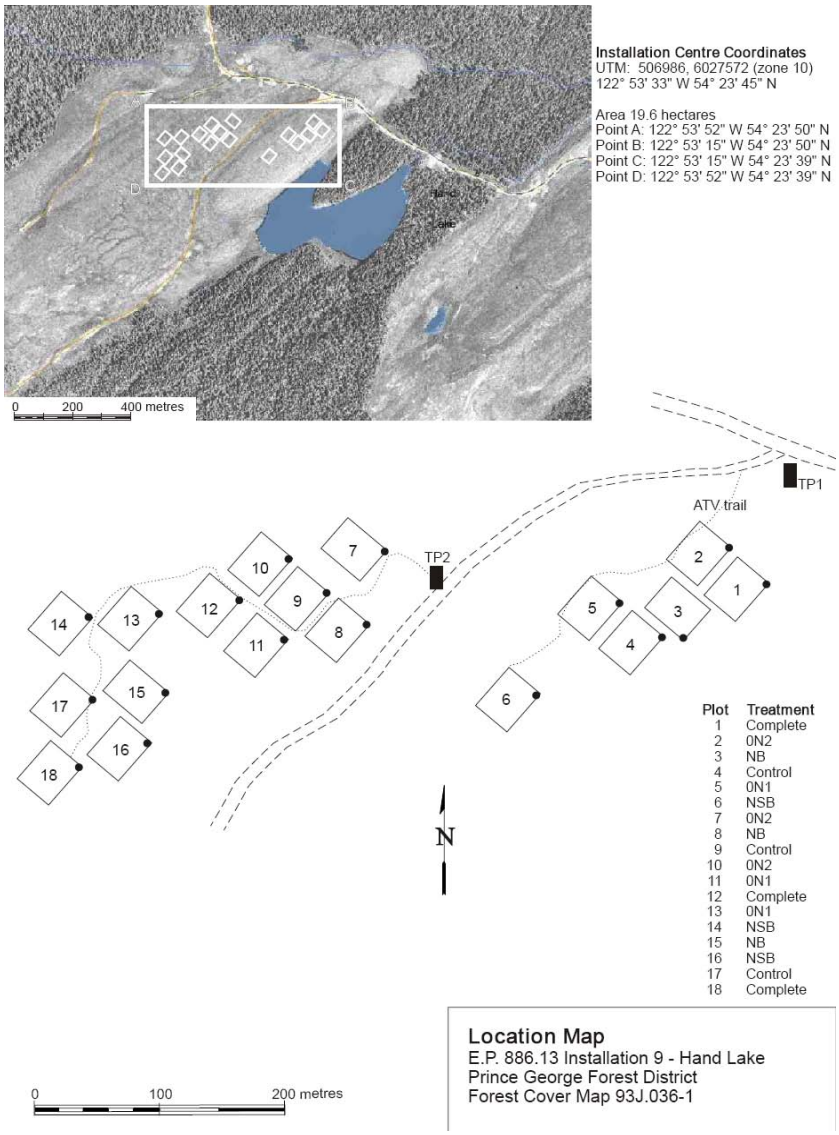

Figure A.2 Experimental plot layout for sampling at Site 2 (Hand Lake). Treatment legend (on map = this study): control = control, complete = periodic, ON1 = annual.

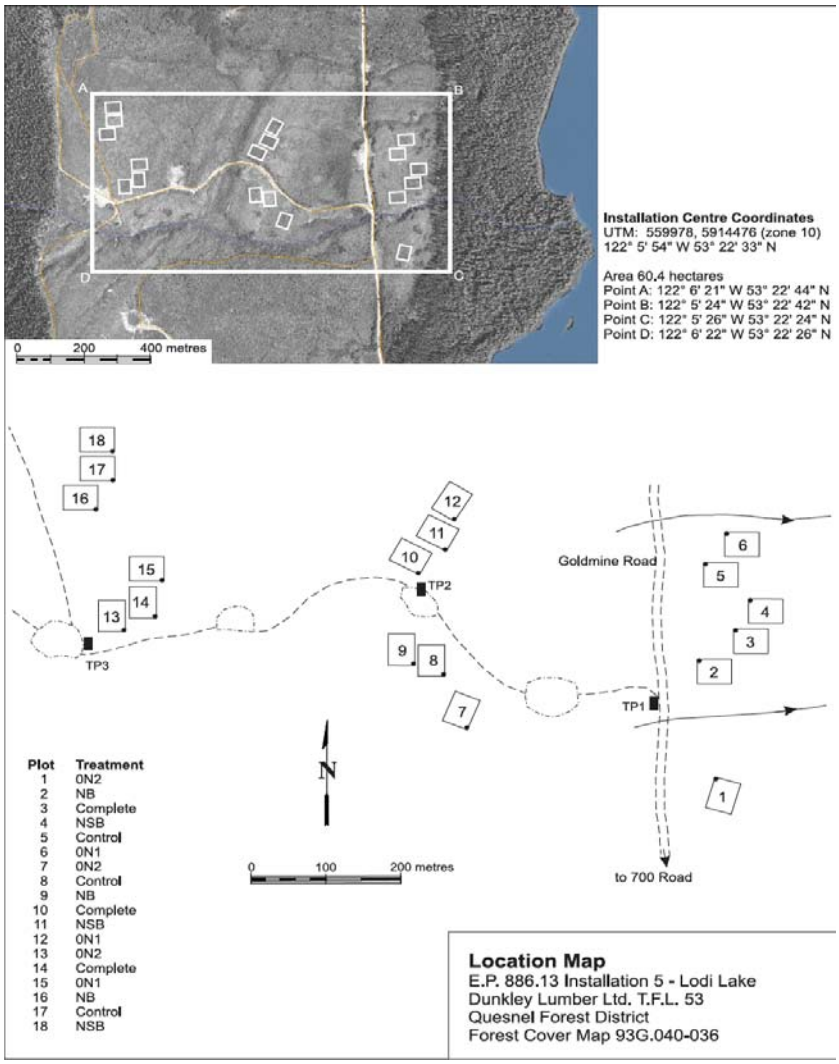

Figure A.3 Experimental plot layout for sampling at Site 3 (Lodi Lake). Treatment legend (on map = this study): control = control, complete = periodic, ON1 = annual.

Appendix B: Accession numbers

Table B.1 Accession numbers for the best BLAST matches for fungal sequences (OTUs and their relative abundances by treatment and site. Associated total aligned base pair numbers and percent similarities provided. C = control, P = periodic, A = annual treatment.

OTU name	Best NCBI or UNITE BLAST match	Accession number of BLAST match	Total base pairs aligned	% Similarity to best matched sequence	Mean relative abundance of OTU (% of root tips per treatment)								
					Site 1			Site 2			Site 3		
					C	P	A	C	P	A	C	P	A
Amphinema byssoides	*Amphinema byssoides*	EF493272.1	469	97%	6.4	21.5	14.0	4.3	3.7	7.2	11.3	4.66	3.89
Amphinema sp.2	*Amphinema* sp.	AY838271.1	533	95%	0.1	3.2	2.6	5.7	1.9	0	0	0	0
Cenococcum geophilum	*Cenococcum geophilum*	EU427331.1	490	99%	6.6	24.7	16.6	18.3	13.1	22.4	28.18	21.27	14.68
Cortinarius caninus	*Cortinarius caninus*	AY669646.1	641	99%	1.2	0	5.0	10.5	13.1	4.4	3.1	0.56	1.58
Cortinarius sertipes	*Cortinarius sertipes*	FJ039540.1	561	99%	0	1.4	3.6	2.5	3.1	3.2	0.1	0.14	0
Cortinarius croceus	*Cortinarius croceus*	GQ159909.1	586	100%	6.3	4.0	0	0.7	1.0	1.0	0.17	0	0
Cortinarius sp.4	*Cortinarius venetus*	GQ159862.1	530	99%	1.1	0.6	0.4	2.9	2.4	1.6	2.6	0	1.79
Cortinarius sp.5	*Cortinarius* sp.	FJ717584.1	556	99%	0	0	0	0	0	0	2.1	0	0
Cortinarius sp.6	*Cortinarius flexipes*	FJ717557.1	467	95%	0	0	0	0	0	0	0	0.69	0
Cortinarius sp.7	*Cortinarius fulvescens*	GQ159914.1	490	98%	3.2	0	0	0	0.5	0	0	0	0
Hebeloma sp. 1	*Hebeloma mesophaeum*	FJ845404.1	563	99%	0	0	0	0.2	0.2	0.1	0	0	0
Hebeloma sp.2	*Hebeloma velutipes*	AY818351.1	631	95%	0	0	0	0	0	1.5	0	0	0

Hystorangium sp.1	*Hystorangium separabile*	EU563921.1	592	99%	7.8	0.5	0	1.9	0.1	0.5	4.17	3.3	0
Hygrophorus sp.1	*Hygrophorus olivaceoalbus*	FJ845410.1	550	100%	0	1.2	0.4	0.5	0.45	0	0	3.3	0
Hygrophorus sp.2	*Hygrophorus pustulatus*	FJ8445412.1	471	91%	0	0.6	0	0.37	0.5	0	0	0	3.9
Inocybe sp.1	*Inocybe napipes*	HG604254.1	462	99%	0.5	0	1.1	1.0	2.27	1.1	0	0	0
Inocybe sp.2	*Inocybe johannae*	EU326177.1	465	96%	0	0	0	0	0	0.32	0	0.42	0
Lactarius deliciosus	*Lactarius deliciosus*	EF685056.1	618	99%	8.1	8.0	4.1	15.5	21.9	10.1	9.4	13.4	13.6
Lactarius sp.2	Lactarius sp.	EF685095.1	499	99%	2.2	1.3	23.0	0.88	0	3.8	1.8	1.8	3.3
Leccinum sp.1	*Leccinum manzanitae*	JF899565.1	308	100%	0	0	0	1.57	0	0	0	0	0
Piloderma olivaceum	*Piloderma olivaceum*	DQ469291.1	568	97%	11.2	12.8	15.6	10.6	3.7	5.6	13.39	17.2	9.4
Piloderma lanatum	*Piloderma lanatum*	DQ469288.1	540	98%	4.0	4.2	1.3	3.24	3.33	0.21	4.4	7.0	5.55
Piloderma sp.3	*Piloderma* sp.	AF476984.1	572	100%	4.0	4.7	1.3	0	0	1.7	0	0.83	0
Piloderma sp.4	*Piloderma* sp.	FN669235.1	540	90%	0	5.4	0.5	0	0	1.46	0	3.1	0
Piloderma sp.5	*Piloderma fallax*	DQ179125	547	94%	2.4	0	0	1.2	0	0	0	0	0
Piloderma sp.6	*Piloderma olivaceum*	DQ469291.1	563	97%	0	0	0	0	0.6	0	0	0	0
Piloderma sp.7	*Piloderma lanatum*	DQ469288.1	542	89%	0	0	0	0	0	0	0.05	0	0
Russula sp.1	*Russula versicolor*	FJ84531.1	503	99%	1.1	2.1	0	0	0	0	0	0	0
Russula sp.2	Russula decolorans	FJ845432.1	582	99%	0	0	0	0	0	0	0.17	2.08	0
Sebacina sp.1	*Sebacina* sp.	GQ907120.1	578	92%	1.0	0	0	0.46	0	0	0	0	0
Tomentella sp.1	*Tomentella* sp.	HQ215807.1	561	99%	0	0	0.2	0	0.7	1.35	0	0.83	1.5

Tomentella sp.2	*Tomentella* sp.	AF272944.1	554	97%	0	3.1	0	0.09	0.12	1.4	0	0	0
Trichophaea sp.1	*Trichophaea gregaria*	UDB000989	438	92%	0.7	0	0	0	0	0	0	0	0
Tuber sp.1	*Tuber stuposa*	UDB000245	561	99%	0	0	0	0	0.9	1.0	0	0	0
Tuber sp.2	*Tuber pacificum*	EU837241.1	454	92%	0	2.5	0	0	0	0	0	0	0
Tylospora asterophora	*Tylospora asterophora*	UDB000841	571	97%	5.5	5.0	7.0	6.3	11.0	6.2	10.1	15.3	0
Tylospora fibrillosa	*Tylospora fibrillosa*	AF052562.1	537	97%	0.4	3.5	7.0	0	1.4	7.2	0	4.4	6.7
Tylospora sp.3	*Tylospora asterophora*	HM190017.1	563	97%	0.4	3.5	0.4	0	0.5	4.4	0	0.14	0
Tylospora sp.4	*Tylospora* sp.	UDB002468	499	89%	0	0	9.7	0	0	0	0	0	0

Appendix C: Sequencing success rate

Table C.1 Extraction and sequencing success based on number of first or second ECM fungal tip used.

	Number of Tips	
	First tip	Second tip
DNA extraction attempted	359	89
DNA extraction successful	200	29
Sequences > 97% & 450 bp	103	5
Sequences < 97% &/or 450 bp	97	24
Total number of tips used	449	