MICROBIAL COMMUNITIES AND ENZYME ACTIVITIES RELATED TO C AND N CYCLING IN FERTILIZED AND UNFERTILIZED FORESTS

by

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Abstract

The world's forest ecosystems store approximately 80% of above-ground and 40% of below-ground terrestrial C. There is scientific evidence that suggests N fertilization will lead to greater accumulation of humus or soil C. However, the underlying mechanism of long-term effects of fertilization on the soil microbial communities that drive decomposition and C sequestration processes is not clear. This study was conducted to address one possible mechanism for the reported enhanced C sequestration in fertilized forest soils, the suppression of decomposition.

The study was conducted at the fertilization demonstration plots of the Salal Cedar Hemlock Integrated Research Program. PH, moisture, N availability (NO_3^- and NH_4^+), microbial biomass C,N and P, phospholipid fatty acids (PLFA), and enzyme activities were measured in the forest floor and mineral soil of western red cedar stands and western hemlock stands ten years following fertilization with N or P, or both.

Results showed that forest floor had the largest effect on microbial and soil chemical variables, followed by forest type, and fertilization. N fertilization significantly increased overall bacterial PLFA abundance and reduced fungal PLFA abundance, while P fertilization significantly reduced AM fungal abundance in the organic layer of the hemlock stands. In addition, the stimulatory effect of N fertilization and inhibitory effect of P fertilization on phosphatase activity was still apparent 10 years after fertilization. Moreover, the effect of fertilization on microbial communities was more pronounced in the forest floor than at depth in the soil. Correlations between microbial community structure and function were weak.

After 10 years, fertilization had not inhibited enzyme activities related to lignin decomposition, but had a significant effect on microbial community composition. Future effort should be directed to long-term and *in situ* research to understand microbial processes at a fundamental level, as well as linking this research with external factors, such as C costs related to fertilization treatment and shorted rotation length, to understand how microbial processes contribute to the bigger picture of C cycle related with forestry.

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List of Abbreviations

ANPP	Annual Net Primary Production
AM fungi	Arbuscular Mycorrhizal Fungi
BEC	Biogeoclimatic Ecosystem Classification
C	Carbon
CWHvm	Coastal Western Hemlock Very Wet Maritime Subzone
DOPA	L-3, 4-dihydroxyphenylalanine
DEMO	Demonstration
GPP	Gross Primary Production
G+	Gram-positive Bacteria
G-	Gram-negative Bacteria
MANOVA	Multivariate Analysis of Variance
MRPP	Multi-response Permutation Procedures
MUB	4-methylumbelliferone
N	Nitrogen
NAGase	β-1, 4-N-acetylglucosaminidase
NPP	Net Primary Production
NH_4^+	Ammonium
NO_3^-	Nitrate
P	Phosphorus
PLFA	Phospholipid Fatty Acid
S	Sulfur
SCHIRP	Salal Cedar Hemlock Integrated Research Program

SOM	Soil Organic Matter
TBCF	Total Belowground Carbon Flux

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Dedication

To Gaia.

1 Introduction

The world's forest ecosystems store approximately 80% of above-ground and 40% of below-ground terrestrial carbon(C) in the form of both biomass and soil organic matter (SOM). Soils store twice as much carbon as vegetation in northern temperate forests and five times as much in boreal forests (Dixon et al. 1994; Hyvonen et al. 2007). There is scientific evidence that suggests that N fertilization of coniferous forests will suppress decomposition and increase C sequestration in humus (Frey et al. 2004; Allison et al. 2007b; Demoling et al. 2008). This has resulted in growing interest in using fertilization to increase the C sink potential of forest ecosystems and mitigate climate change [Johnson and Curtis 2001; Grayston(in press)]. However, the underlying mechanism of long-term effects of fertilization on the soil microbial communities that drive decomposition and C sequestration processes is not yet clear. This research aimed to solve a small piece of the puzzle by investigating the long term effect of fertilization on microbial community composition and enzyme activities, as well as identifying how forest type and soil layer affect microbial responses to fertilization in two coniferous forests on northern Vancouver Island, British Columbia.

2 Literature Review

2.1 Effect of Soil Layer and Forest Type on Belowground Communities

Both forest type and soil layer have substantial influences on the structure and function of belowground communities. There is no general consensus on which factor is more important, as they can both dominate depending on the biotic and abiotic conditions (Berg and Smalla 2009). Grayston and Prescott (2005) compared soil microbial communities under four tree species (*Thuja plicata, Tsuga heterophylla, Pseudotsuga menziesii, and Picea sitchensis*) and found that soil layer had the greatest effect on microbial communities, followed by site and tree species. In a similar study comparing microbial communities under Norway spruce (*Picea abies*), Douglas-fir (*Pseudotuga menziesii*), oak (*Quercus sessiflora*) and native forest [mixed stand dominated by oak and beech (*Fagus sylvatica*)], Lejon et al. (2005) attributed the differences in microbial community structure and biomass to soil physiochemical characteristics and organic matter composition. In addition, Leckie et al. (2004c) found distinct fungal communities in cedar-hemlock and hemlock-amabilis fir forests, and attributed this to the differences in rooting distributions and mycorrhizal associations among the plant species and layers.

With increasing soil depth, there may be decreased microbial biomass, decreased fungal-to-bacterial ratios, changes in microbial community composition, decreased hydrolytic enzyme activities and increased lignin degrading enzyme activities (Leckie et al. 2004b; Leckie et al. 2004c; Lejon et al. 2005; Brockett 2008). These changes could be attributed to decreased root density, decreased available carbon, as well as changes in microenvironment, trophic interactions, organic matter composition, and substrates that enzymes act upon (Berg et al. 1998; Fritze et al. 2000; Ekelund et al. 2001; Leckie et al. 2004c; Lejon et al. 2005; Daradick 2007). In addition, the decline in fungal-to-bacterial ratio with depth may also be related to decreases in plant tissues, as fatty acid 18:2ω6,9, a commonly used marker for fungi, is also found in plants (Leckie et al. 2004c).

Tree species have been shown to have significant effects on forest floor pH, nutrient concentrations and microbial community composition (Grayston and Prescott 2005; Prescott and Vesterdal 2005; Leckie et al. 2004b; Brockett 2008), although effects on total microbial biomass are less consistent (Myers et al. 2001; Priha et al. 2001; Leckie et al. 2004c; Grayston and Prescott 2005; Lejon et al. 2005). Direct effects of tree species on microbial communities include variation in litter quality and quantity (Augusto et al. 2002), nutrient uptake (Hobbie 1992; Lipson and Nasholm 2001) and root exudation (Grayston and Prescott 2005; Berg and Smalla 2009). Indirect effects include influences of tree species on herbivores, on the

development of understory vegetation and mycorrhizal associations, and on soil physical characteristics such as soil structure, texture and pedoclimate (Hobbie 1992; Leckie et al. 2004c; Malchair and Carnol 2009). Among these factors, root exudates are thought to be a key factor that contributes to plant influences on the microbial community, especially in the rhizosphere (Berg and Smalla 2009). Tree roots have been found to excrete 10% - 44% of the photosynthetically fixed carbon (Bais et al. 2006). These exudates are highly species-specific, and could serve as signals in plant-microorganism interactions that attract beneficial microbes (Shaw et al. 2006).

Cedar forest floors have been shown to have high bacterial biomass, low fungal biomass and high proportion of NO_3^-, while hemlock forest floors often have low pH and Ca and high proportion of NH_4^+ (Grayston and Prescott 2005; Prescott and Vesterdal 2005). The effects of cedar and hemlock on forest floor microbial communities were found to be more apparent in plots with higher growth rate, and may become more apparent over time (Leckie et al. 2004b). The differences in the proportions of NH_4^+ and NO_3^- and microbial community composition could be related to the high Ca content of red cedar litter, which may lead to forest floors with relatively high pH and base saturation that favor the growth of bacteria. Moreover, cedar has arbuscular mycorrhizal associations which do not contain the fatty acid 18:2ω6,9, and this may

also be attributed to the lower fungal biomass in cedar forest floors (Grayston and Prescott 2005; Prescott and Vesterdal 2005).

2.2 Effect of Fertilization on Aboveground Communities

Fertilization has been used extensively for the last few decades to increase tree growth and shorten rotation length (Brockley and Simpson 2004; Negrave et al. 2007). With a sound understanding of the factors that limit forest productivity on each specific site, fertilization can increase productivity of plantation forests, release the pressure from increasing timber demand and allow more native forests to be preserved (Fox 2000).

The specific effect of fertilization on tree productivity and understory species is determined by both external and internal factors. External factors include the different combinations of fertilizers, such as nitrogen (N) or N+ phosphorus (P), dose, frequency, duration, light limitation, the form of fertilizer, such as urea, NO_3^- or NH_4^+, and the initial nutrient content of the soil. Internal factors include differences among species and initial plant community structure.

2.2.1 Fertilizer Combinations

Distinct responses to different fertilizer combinations have been found among trees and understory species (Keith 1991). In a subalpine eucalypt forest, Keith (1991) found that N

addition resulted in increases in leaf fall, leaf area index and canopy leaf mass, while P fertilization had no significant impact on canopy leaf growth. He also found that stand basal area increased by 12% and 43% respectively, following N and P addition. Blevins et al. (2006) found that fifteen years after fertilization, the height of hemlock increased in plots that received N+P fertilization but declined in plots that received N fertilization only. The range of responses of different variables to P and N fertilization suggests these fertilizers might stimulate tree growth through different mechanisms.

Other studies on understory species have found lower sulfur (S) concentrations in salal leaves from plots receiving only N fertilization (Prescott et al. 1993), and increased growth of salal and fireweed in N+P treatments, but not with N alone (Bennett et al. 2004). The authors suggest that large N addition may induce nutrient imbalance, which could be alleviated by adding other nutrients such as P or S. Elevated concentrations of P or S could also increase immobilization of added N and, therefore, reduce the toxic effect of high inorganic N concentrations on roots and mycorrhizae (Turkington et al. 1998; Bennett et al. 2004).

2.2.2 Study Duration

There is evidence that initial plant community responses to fertilization do not always mirror long-term responses (Tilman 1988; Turkington et al. 1998). Turkington et al. (1998) found an initial increase in percent cover of Linnaea and Anemone following fertilization on

boreal forest vegetation, but then a decline of these two species after several seasons. In addition, Gilliam (2006) reviewed studies on the responses of herbaceous layers in forest ecosystems to excess nitrogen deposition and found short-term studies with low fertilizer additions reported no response to fertilization. However, in long-term studies he observed there was a significant decline in the diversity, density and biomass of herbs.

Similar studies have found that changes in herbaceous species composition were still occurring 10 years after fertilization (Inouye and Tilman 1995; Turkington et al.1998). In addition, Prescott et al. (1995) found reductions in ericaceous cover in a jack pine forest were still apparent 14 yrs after fertilization and 24 years after straw application. These long-lived effects might be attributed to the complete dominance of the herb layer of forests by perennial species, which store N in plant organs over a long time (Gilliam 2006). Therefore, long-term studies are critical to understand the effects of fertilization on systems with long-lived species and low dispersal rates (Turkington et al. 1998).

2.2.3 Light Limitation and Initial Soil Nutrient Concentration

The interaction between nutrient limitation and light limitation would partly explain the conflicting observations on the effects of fertilization on ground vegetation (Thomas et al. 1999). Thomas et al. (1999) reported an increase in overstory canopy cover, and decreases in understory light levels, understory vegetation cover and species diversity for 10-15 years

following fertilization of a Douglas-fir forest. Similarly, after 3 years of N fertilization in Adirondack forests, Hurd et al. (1998) found a significant decline in the cover of prominent herbaceous species *Oxalis acetosella* and *Huperzia lucidula*, which they attributed to the increased shading by the ferns. Contradictorily, Prescott et al. (1995) found a significant reduction in ericaceous ground cover in coastal western hemlock forests 14 years following N fertilization that could not be attributed to shading because of the high mortality of trees in the fertilized plots. Results from these studies suggest that initial stand structure, the understory community in question and the scale of shading, would likely affect the interaction between light limitation and nutrient limitation, therefore, alter the responses to nutrient additions (Thomas et al. 1999; Coomes and Grubb 2000; Gilliam 2006).

2.3 Effect of Fertilization on Belowground Communities

2.3.1 Mycorrhizae

Treseder (2004) perfomed a meta-analysis of mycorrhizal responses to additions of nitrogen and phosphorus and enhanced atmospheric CO_2 concentrations. They found that mycorrhizal abundance decreased 15% under N fertilization and 32% under P fertilization. However, fertilization effects varied significantly among studies. Such variance might be due to the duration of the studies, and the dominant mycorrhizal species present (Brandrud and

Timmermann 1998; Gilliam 2006). Brandrud and Timmermann (1998) presented a general model of mycorrhizal response to N enrichment in three phases: during stage 1, there was rapid loss of above-ground diversity and sporocarp production by mycorrhizal fungi; while below-ground mycorrhizae and fine-roots of host species were often unaffected. This stage could often be observed in short-term fertilization experiments in unpolluted forest. In stage 2, below-ground structures were affected variably, and N sensitive fungi disappeared. Lastly, stage 3 was characterized by a complete loss of sporocarp production and a sharp decline in the density of below-ground mycorrhizae and fine-roots of host species. Arnolds (1990) found that Phase 1 took about 7 years (1972-1979) to complete while Phase 2 took around 16 years to be firmly established (1972-1988). The author suggested that these three phrases needed to be adapted to take into account the different sensitivities of different mycorrhizal species to fertilization.

This model of mycorrhizal response to N amendment applies well to soils dominated by organic N cycling and with little N mineralization and no nitrification (Read and Perez-Moreno 2003; Gilliam 2006). Plants commonly supporting ericoid and ectomycorrhizal fungi with well-developed saprotrophic capabilities are especially well-adapted to this type of highly acidic, weathered and infertile soil, commonly found in many older forests (Read et al. 1996; Gilliam 2006). Studies have found measureable shifts in above-ground fungal (i.e. sporocarp)

communities, decreases in ectomycorrhizal diversity after adding N (Wallenda and Kottke 1998; Lilleskov et al.2002; Gilliam 2006) a significant decline in ericoid mycorrhizal infection along a N-deposition gradient (Yesmin et al.1996), and a pronounced decline in mycorrhizal abundance under high N application rates (Treseder 2004). More recently, Wright et al. (2009) found that N+P fertilization had a significant long-term effect on ectomycorrhizal species composition in a western hemlock forest, although there was no detectable difference in ectomycorrhizal fungal diversity. This may have been due to the large number of fungal species associated with the roots, which made it extremely difficult to reach sampling saturation.

For soils with high nitrification potential and limited or no saprotrophic capability, which are often dominated by plants supporting arbuscular mycorrhizal fungi (AM fungi) (Read and Perez-Moreno 2003; Gilliam 2006), fertilization might have little negative, or even positive impact on mycorrhizal fungi (Treseder et al. 2007). Treseder et al. (2007) found no significant decrease in abundance of ectomycorrhizal fungi and an increase in AM fungi root length after N fertilization in boreal forests. This suggests in some ecosystems, mycorrhizal growth maybe N-limited, therefore, adding N might increase mycorrhizal abundance (Treseder 2004). Alternatively, plants could be secondarily limited by other nutrients, such as phosphorus, and adding N could exaggerate such nutrient imbalance (Prescott et al. 1993; Bennett et al. 2004).

After N fertilization, plants may allocate more carbon to mycorrhizal fungi to improve uptake of other nutrients (Treseder et al. 2007).

2.3.2 Microbial Biomass, Activity and Community Structure

In general, fertilization tends to reduce microbial biomass and activity (Bowden et al. 2004; Pregitzer et al. 2007; Treseder 2008). A meta-analysis conducted by Treseder (2008) showed that for the microbial community as a whole (fungi + bacteria), N fertilization reduced biomass by an average of 15% in various ecosystems. Biomes, fertilizer type, ambient N deposition rates and methods of measuring biomass did not have a significant effect on microbial biomass response to N addition. However, studies that focused specifically on fungi or bacteria showed that fertilization did not significantly alter fungal or bacterial biomass. Positive effect on fungal biomass tended to be more frequent among studies with lower N load(< 250 kg N ha^{-1}) and shorter duration(<5 years), and a reduction in fungal biomass became more evident as N load and the duration of fertilization increased . The lack of response of bacterial biomass to fertilization was attributed to insufficient studies included in the meta-analysis. In this analysis, bacterial biomass was represented by only 11 studies, while total biomass and fungal biomass were represented by 29 and 16 studies, respectively. The potential mechanisms for N effects on microbial growth are summarized in Figure 1.1 (Treseder 2008).

Despite the general trend of microbial biomass decline with fertilization, there are disparate responses using different analytical methods and in different soil layers (Compton et al. 2004; Leckie et al. 2004a; Demoling et al. 2008). Compton et al. (2004) found that 10 years of chronic N addition strongly reduced microbial biomass in the O horizon. However, microbial biomass in the A horizon was relatively unaffected. Demoling et al. (2008) reported the effect of fertilization on microbial biomass in three coniferous forest soils using three different methods. Microbial biomass as measured using both fumigation-extraction and substrate-induced-respiration methods decreased by 40%, while biomass estimation using PLFA analysis only detected a decrease of 15%.

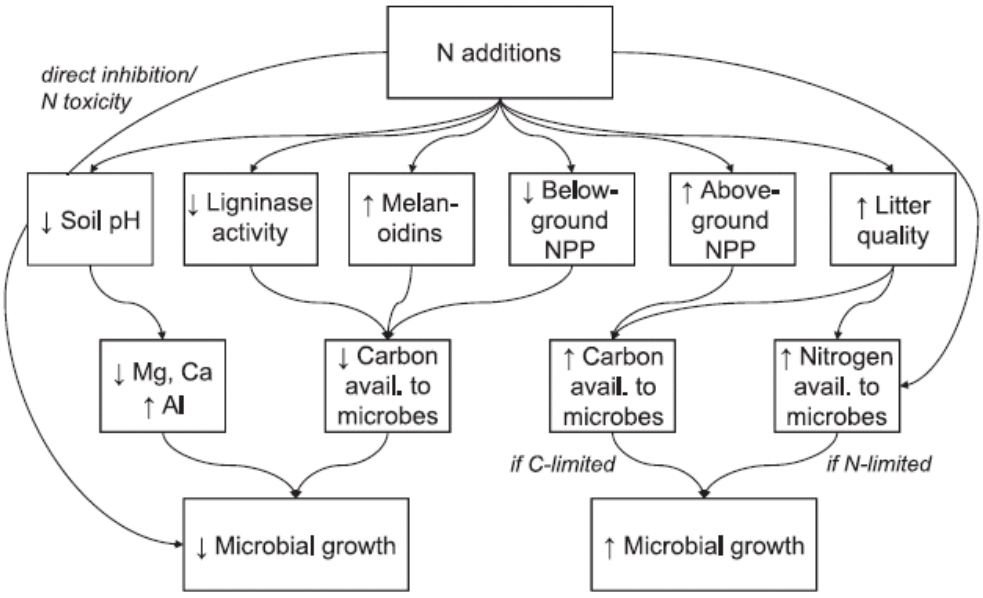

Figure 2.1 Potential mechanisms for N effects on microbial growth (from K.K. Treseder 2008)

2.3.3 Enzyme Activities

N fertilization has been shown to have both positive and negative effects on C-, N- and P-acquiring soil enzyme activities. Some studies found that N addition significantly stimulated β-glucosidase and cellobiosidase activities in hardwood forests (Carreiro et al. 2000; Saiya-Cork et al. 2002; Frey et al. 2004), suggesting N addition may increase the initial microbial breakdown of organic matter. Meanwhile, phenol oxidase activity was reduced significantly in the N-treated plots in these same forests (Carreiro et al. 2000; Sinsabaugh et al. 2002; Frey et al. 2004), illustrating the possibility that the suppression of lignin degrading enzyme activities might outweigh the stimulation of hydrolytic enzymes. Therefore, N addition might have a negative feedback on long-term decomposition. Other studies in forest and grassland ecosystems have shown neutral or positive effects of N on oxidative enzyme activities, and no correspondence between enzyme activity and decomposition across sites (Michel and Matzner 2003; Keeler et al. 2009).

2.4 Effect of Fertilization on Above and Below Ground Interactions

2.4.1 Carbon Allocation

Gross primary production (GPP) can be divided into five key components, which determine the annual carbon budget in forest ecosystems (Ryan et al. 2004; Litton et al. 2007). These five

key components are: foliage net primary productivity, wood net primary productivity, foliage autotrophic respiration, wood autotrophic respiration, and total belowground carbon flux (TBCF), which is the sum of root net primary production (NPP), root respiration, root exudates, and carbon consumed by mycorrhizae. Fertilization could shift C allocation patterns through altering any of these five components (Litton et al. 2007).

When assessing TBCF, it is essential to consider both biomass and turnover rate since changes in TBCF could result from increased root/mycorrhizae mortality and no change in production; no change in mortality and decreased production; or increased mortality in excess of increased production (Nadelhoffer 2000; Phillips and Fahey 2007). Keith et al. (1997) estimated turnover rates through biomass and respiration rates and found that after P fertilization of a mature eucalypt forest, there was an increase in coarse root construction, respiration and production, and a decrease in coarse root maintenance respiration, fine root production and fine root respiration. This resulted in an overall 19% decrease in belowground C allocation.

Aboveground components tend to increase after fertilization. Studies have found increased annual net primary production (ANPP), stem wood production and respiration, foliage ANPP, and foliage respiration (Keith 1991; Ryan et al. 1996; Keith et al. 1997; Maier et al.

2004; Ryan et al. 2004; Stape et al. 2004). One exception was that Keith et al. (1997) found a decrease in ANPP foliage after P fertilization (Keith et al. 1997).

Litton et al. (2007) reviewed existing literature on annual carbon budgets for forest ecosystems and found that with increasing resources, partitioning to aboveground production increased and to TBCF decreased. However, very few studies in this review provided estimates for all five key components (as discussed above) when answering allocation questions. The estimate for the TBCF component was particularly weak, as none of these studies provided information on root exudates or carbon used by mycorrhizae, which could be a key portion of TBCF. Moreover, when estimating TBCF using data from the reviewed studies, four different methods with distinct assumptions were used by the author (Litton et al. 2007).

The lack of standard procedures for assessing TBCF is largely due to the difficulty in tracking belowground carbon flow, i.e. how to measure carbon used for producing root NPP, root exudates and mycorrhizae, how to separate root respiration from microbial respiration, and how to estimate turnover of roots and microbes (Litton et al. 2007). Such difficulty may be addressed by refining stable isotope techniques for *in situ* investigations of above-belowground carbon flow (Boschker et al. 1998; Radajewski et al. 2000; Treonis et al. 2004).

2.4.2 Decomposition Process

The key factors that control the decomposition process include moisture, temperature, organic matter quality, N availability, soil texture, and availability of exogenous labile C for microorganisms (Prescott 2005). Among these factors, fertilization has direct impact on N availability, and indirect impact on organic matter quality (e.g. increasing N concentration in green leaves (Pregitzer et al. 2007), soil texture (e.g. affecting microbes involved in forming and stabilizing soil aggregates) (Oades 1993; Degens 1997; Guggenberger et al. 1999) and available labile C (e.g. reducing carbon allocation to roots and therefore reducing root exudates, or increasing dissolved organic carbon from litters) (Prescott 2005; Litton et al. 2007; Phillips and Fahey 2007).

Knorr et al. (2005) conducted a meta-analysis to examine the effects of N enrichment on litter decomposition. The analysis showed that N additions inhibited litter decomposition when fertilization rates were 2 to 20 times greater than the anthropogenic N deposition level, when litter quality was low (>20% lignin), and when ambient N deposition was around 5 to 10 kg N ha^{-1} yr^{-1}. In addition, decomposition was stimulated when litter quality was high (<10% lignin), and when ambient N deposition was low (< 5 kg N ha^{-1} yr^{-1}). Moreover, litter decomposition responses to N additions were not affected by climate, fertilizer type, or the mesh size of the litterbags used in the studies. The meta-analysis also revealed that N additions stimulated mass

loss by up to 7% when litters had been decomposing for less than 24 months, but inhibited decay by 18% when litters had been decomposing for greater than 24 months (Figure 2.2), suggesting long-term studies tend to reveal different response patterns compared to short-term studies (Knorr et al. 2005), as early decay rates may not accurately represent the entire decay process (Prescott 2005).

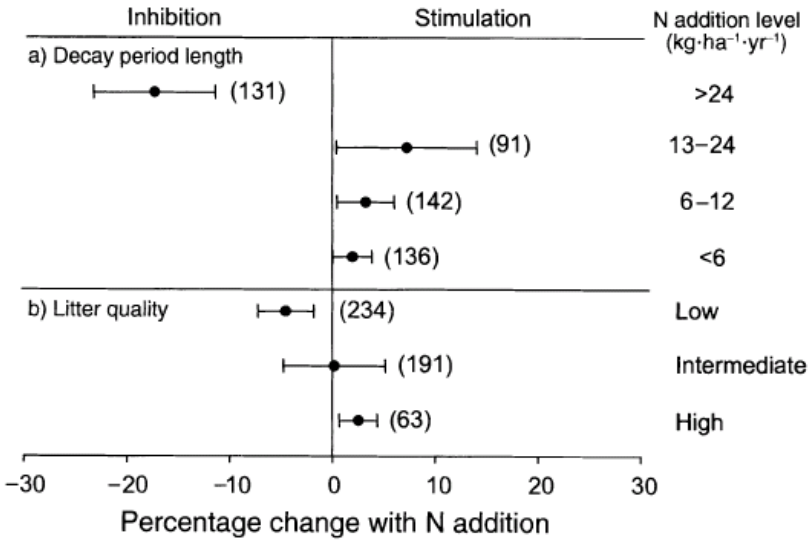

Figure 2.2 Response of litter mass remaining to N additions when the data were grouped by (a) decay period length (in months), and (b) initial litter quality (low, intermediate, high) (from M. Knorr et al. 2005).

Large-scale and long-term ecosystem implications of the above direct and indirect impacts may be dominated by feedback loops (Compton et al. 2004; Hyvonen et al. 2007). In the short term, N fertilization may increase N concentration in litter (Pregitzer et al. 2007), stimulate

cellulose-degrading enzymes activities and the decomposition of high quality litters (Carreiro et al. 2000; Frey et al. 2004), accelerate formation of recalcitrant compounds (Ågren et al. 2001), suppress fungal communities, reduce lignin degrading enzyme production and inhibit lignin degradation (Frey et al. 2004; Allison et al. 2007b; Demoling et al. 2008). Collectively, changes in these small-scale processes may lead to greater accumulation of humus at later stages of decomposition (Prescott et al. 2004). As over-accumulation of humus limits nutrient availability to plants (Prescott et al. 2000b), plants may allocate more C belowground, which would provide more C for mycorrhizae and more labile carbon through root exudates, thus re-activate fungal communities in systems that are more C-limited than N-limited.

2.5 Introduction to the Study

This study investigated the response of soil microbial community composition and enzyme activities, related to cycling of C, N and P, to a range of fertilization regimes in plots of western hemlock and western red cedar. Through this analysis the aim was to try to assess the long-term effect of fertilization on the soil microbial communities that drive decomposition and sequestration processes and one possible mechanism of enhanced soil C storage after fertilization, a change in the enzyme activities involved in decomposition processes. A study by Yolova (2007) demonstrated that fertilization of the western hemlock stands at SCHIRP with 200Kg N ha^{-1} would increase soil C sequestration by 12-16% at rotation through increased

humus accumulation in these stands. In my study, key site factors (pH, moisture), N availability (NO_3^-, NH_4^+), microbial biomass C, N and P were measured. Phospholipid fatty acid (PLFA) analysis was used to assess microbial community composition, and enzyme profiling was used to characterize the functional diversity of these communities. The objectives of this study are:

1. To investigate one possible mechanism for the reported enhanced sequestration of C in fertilized forest soils, by investigating whether fertilization inhibits enzyme activities related to lignin decomposition in fertilized and unfertilized plots of two coniferous forests.
2. To examine links among soil layers, forest types, fertilization and the soil microbial community structure and function related to activities of their enzymes involved in C,N and P cycling, and to identify how forest type and soil layer affect microbial responses to fertilization.

2.6 Specific Hypotheses

Hypothesis 1: Organic and mineral layers will have distinct microbial biomass and enzyme activities, as they provide different substrates and microenvironment to microbes.

Hypothesis 1a: Microbial biomass and hydrolytic enzyme activities will decrease with depth, while lignin degrading enzyme activities will increase with depth.

Hypothesis 2: Cedar and hemlock stands will have distinct microbial activities and composition due to the effect of tree species.

Hypothesis 2a: Cedar stands will have a higher bacterial and AM fungal abundance than hemlock stands.

Hypothesis 3: N fertilization will increase bacterial and decrease fungal abundance.

Hypothesis 4: N fertilization will stimulate cellobiosidase, glucosidase and phosphatase activities, but suppress NAGase, phenol oxidase and peroxidase enzyme activities.

Hypothesis 5: The effect of N fertilization on these enzyme activities will be less pronounced in plots that also receive P and/or micronutrients, in particular, P fertilization will suppress phosphatase activity.

Hypothesis 6: Microbial communities will respond to fertilization differently in the different forest stands and soil layers if hypothesis 1 and 2 are not rejected.

Hypothesis 6a: Microbial communities in hemlock stands will be more sensitive to P fertilization since P is an important limiting factor for hemlock productivity at these sites.

Hypothesis 7: There will be strong correlations between components of measured biological variables (microbial community structure and function) and environmental variables (pH and moisture).

3 Materials and Methods

3.1 Research Sites

The study sites were located within the SCHIRP research forests near Port McNeill B.C (50°60′N, 127°35′W) and operated by Western Forest Products Inc. The ecosystem is in the very wet maritime subzone (CWHvm) of the Coastal Western Hemlock biogeoclimatic zone (Green and Klinka 1994). Mean daily temperature ranges from 3.0 °C in January to 14.1 °C in August. The average annual precipitation is 1700 mm, which falls predominately as rain from October to March. Topography is gentle with elevations less than 300 m above sea level (Prescott et al. 1993). Forest floors are about 1 m in depth and predominantly hemimors or lignomors (Green et al. 1993). Mineral soils are well- to poorly-drained humo ferric podzols with overlying unconsolidated morainal and fluvial outwash material (Prescott et al. 1993). Concentration (%) of C is around 48% in LF and H horizons, 14% in Bhf1 horizon, and 7% in Bhf2 horizon. Concentration of N is around 1% in LF and H horizons, 0.5% in Bhf1 horizon, and 0.2% in Bhf2 horizon (Cade-Menun et al. 2000, Prescott et al. 1993). A detailed soil profile description is given in Table 3.1.

The samples were taken in the Salal Cedar Hemlock Integrated Research Program (SCHIRP) fertilization demonstration (DEMO) plots, which were established in 1987 in seven-year-old western red cedar (*Thuja plicata*) and western hemlock (*Tsuga heterophylla*) plantations. All

sites had been clearcut and slash burned prior to planting. Each plot was 25 m by 25 m (Appendix 1). The first broadcast fertilization application was applied in 1987 and the second one in 1997. Three types of fertilizer were used: N as urea, P as triple superphosphate, and micronutrient mix (kg ha^{-1}) as 99 P, 102 K, 129 Ca, 51 Mg, 50 S, 9 Fe, 3.5 Mn, 1.5 Cu, 1.5 B and 1.0 Mo (Blevins and Prescott 2002).

Table 3.1 Soil profile description of the cedar-hemlock ecosystem at SCHIRP (adapted from Germain, 1985).

Horizon	**Depth (cm)**	**Description**
LF	27 - 26	mixture of coniferous and moss litter; loose consistency; many fine roots and mycorrizal hyphae; abrupt wavy boundary to
H	22 - 0	black, dark reddish brown; highly decomposed organic matter; granular, slightly greasy in lower horizon; abundant roots of all sizes; abrupt wavy boundary to
Ae	0 - 3	grey to brown; sandy loam; medium subangular blocky; friable; few fine-roots; clear, broken boundary to
Bhf	3 - 18	reddish brown, strong brown; sandy loam; weak medium subangular blocky; friable when moist; non-sticky and slightly plastic, wet; few to abundant fine and medium roots; abrupt wavy boundary to
Bf	18 - 40	strong brown, yellowish brown, gravelly sandy loam; weak medium subangular block; friable when moist; non-sticky and non-plastic, wet; few fine roots; some medium to large roots; clear wavy boundary to
Bfgj	40 - 60	yellowish brown, brownish yellow; gravelly sandy loam, weak, medium and coarse subangular blocky, firm when moist; non-sticky and non-plastic, wet; very few roots; abrupt wavy boundary to
BCc	60+	olive grey, strongly cemented to indurated gravelly sandy loam; no roots

3.2 Sampling Design

An unbalanced factorial design with three rates of nitrogen fertilization (0 kg ha^{-1}, 200 kg ha^{-1}, 300 kg ha^{-1}), two rates of phosphorus (0 kg ha^{-1}, 100 kg ha^{-1}), and an additional phosphorus with micronutrients were selected for a combination of nine treatments. Each treatment had three replicate plots within each forest type. There were a total of 108 composited samples. Soil samples were taken in August 2007. Five random sampling locations were selected within each replicate plots. At each sampling location, we used a two-inch diameter corer and pushed it into the H horizon to 50 cm depth, removed the soil from the corer to a Ziploc bag for organic soil samples, and repeated core sampling until we hit the mineral soil. We sampled the top 50 cm of mineral soil in a similar fashion and put it into another Ziploc bag for mineral soil sample. Same procedures were repeated for the other 4 locations. After that, we composited all organic sub-samples from 5 locations into one organic soil sample, and all mineral soil sub-samples into one mineral soil sample. 300 grams of the composited and homogenized sample was then brought back for laboratory analyses. Samples were placed in plastic bags and stored in a cooler with ice packs immediately after sampling and during transport. All samples were transported to the field fridge within the same day of collection and stored at 4 °C until sample preparation.

3.3 Laboratory Sample Analysis

On return to the UBC laboratory (within a week of collection), each composite sample was sieved to <2 mm to remove stones and roots. Sieved samples were then frozen at -20 °C for later laboratory analyses. Soil pH was measured using a pH meter in a matrix of 0.01 mol L^{-1} $CaCl_2$ (Hendershot et al. 1993). Soil gravimetric moisture was measured by weighing the samples before and after oven-drying at 105 °C for 48 hours.

3.3.1 Microbial Biomass Carbon, Nitrogen and Phosphorus Analyses

Microbial biomass C, N and P were measured via chloroform-fumigation-extraction and alkaline persulfate oxidation techniques adapted from Basiliko et al. (2007) and Bengtson et al. (2007). For each sample, approximately 5 g of sieved soil was fumigated and incubated in a sealed dessicator in the dark for 24 hours. After fumigation, fumigated samples and control samples (the other 5 g of sieved soil without fumigation) were extracted with 50 mL of K_2SO_4 (0.5 mol L^{-1}) and added to Nalgene bottles. The bottles were put on the shaker for an hour. After that, extracts were filtered using Whatman 42 filter paper.

10 mL of the extracts from the control samples were used for NO_3^- and NH_4^+ analyses. 1 mL of the extracts from both the control and fumigated samples were diluted 10 times and used for total organic C and total N analyses. For total phosphorus analysis, 15 mL potassium

persulfate solution and 5 mL distilled water were added to 5 mL of the extracts from both control and fumigated samples. All the extracts for total P were autoclaved before analysis.

The NO_3^- and NH_4^+ analyses were done by the Lachat QuickChem® FIA+ (Lachat 8000, Lachat Instruments, USA) using a modified version of QuickChem Method # 10-115-01-1-A. total organic C and total N analyses were done on a Lachat combustion, and total P was done on a Lachat Flow Injection. Microbial biomass C, N and P were calculated from the difference between the amount of total C, N and P extracted from fumigated and unfumigated samples and was expressed as $\mu g\ g^{-1}$ soil.

3.3.2 Enzyme Assays

The activities of hydrolytic enzymes involved in breakdown of cellulose, chitin and organic P were assessed using fluorimetric enzyme assays. The activities of two lignin-degrading enzymes, phenol oxidase and peroxidase, were assessed using colorimetric assays. Both fluorimetric and colorimetric assays quantify enzyme activities based on reactions colour change. Fluorimetric enzyme assays used 4-methylumbelliferone (MUB), a fluorescent compound, while colorimetric assays used L-3,4 dihydroxyphenylalanine (L-DOPA) as reaction substrates to quantify such change. The reaction substrates and description of the functions of the enzymes assayed in this study are summarized in Table 3.2 (Saiya-Cork et al. 2002; Sinsabaugh et al. 2002; Grayston et al. 2004; Keeler et al. 2009).

Frozen soil samples were ground in a mortar for 20 seconds. The pestle and mortar were rinsed with 70% ethanol and then with distilled H_2O after each sample. 0.5 g of the ground soil samples were then transferred into 125mL screw cap Nalgene bottles. 50 mL of 0.05 mol L^{-1} sodium acetate buffer (pH 5.0) and approximately 30 glass beads were added to each soil sample bottle. The bottles were shaken for an hour on a high speed shaker. After that, additional 50 mL of buffer was added to each sample. The soil suspensions were kept at 4 °C for up to 24 hours until ready to use.

Table 3.2 Enzyme description and assay substrates

Enzyme	Assay Substrate	Function Description
Cellobiosidase	4-MUB-β-D-cellobioside	depolymerizes cellulose into cellobiose
Glucosidase	4-MUB-β-D-glucoside	hydrolyzes cellobiose to form glucose
NAGase	4-MUB-N-acetyl-β-D glucosaminide	breaks down chitin for acquisition of organic N
Phosphatase	4-MUB-phosphate	hydrolyzes bound organic phosphorus
Phenol oxidase	L-3,4 dihydroxyphenylalanine(L-DOPA)	degrades polyphenolic compounds such as lignin
Peroxidase	L-3,4 dihydroxyphenylalanine(L-DOPA)	degrades polyphenolic compounds such as lignin

For the fluorimetric enzyme assays, a 10 µm concentration of 4-MUB standard substrate and one hundred milliliters of 4-MUB synthetic substrates (200 µm) were prepared in sterile

water and kept at 4°C for up to a week, except for 4-MUB-phosphate which was prepared fresh each time. 96-well black microplates were prepared with 16 replicates (16 wells) for each soil sample. The following was added to individual wells on the same microplate for each sample: 16 replicates of soil sample (200 µL of soil suspension and 50 µL of MUB substrate), 16 replicates of a standard positive control (200 µL of sodium acetate buffer and 50 µL of MUB standard), 8 replicates of a substrate negative control (200 µL of sodium acetate buffer and 50 µL of MUB substrate), 8 replicates of a quench standard (200 µL of soil suspension and 50 µL of MUB standard), 8 replicates of a soil background (200 µL of soil suspension and 50 µL of buffer), and 8 replicates of a buffer negative control (250 µL of buffer).

Plates were incubated at 20 °C in the dark for 2 hours for phosphatase, 3 hours for glucosidase and NAGase, and 7 hours for cellobiosidase activity assays. A 20 µl of 0.5 mol L^{-1} NaOH was added to each well at the end of the incubation to stop the reaction. A CytoFluor™ fluorimeter (CytoFluor™ II, Applied Biosystems, USA) was used to read the microplates. The CytoFluor™ was set to provide excitation wavelength at 360/40 nm, emission wavelength at 460/40 nm and a mixing period of 5 seconds. Potential enzyme activities were calculated as the amount of substrate (nmol) converted per hour per gram of soil sample (nmol h^{-1} g^{-1}). The actual rate was counted as 0 if the calculated activity rate was negative.

For the colorimetric enzyme assay, 0.493 g of DOPA was added into 100 mL of 50 mM acetate buffer (pH 5.0) to make up 100 mL of 25 mM L-DOPA solution. The solution was kept at 4 ºC in the dark (for up to 24 hours) until needed. A separate set of 96-well clear microplates (Costar microplate 3370, Corning Life Sciences, USA) were used for phenol oxidase and peroxidase assays. The following was added to individual wells on the same microplate for each phenol oxidase sample: 16 replicates of soil sample (200 µL of soil suspension and 50 µL of L-DOPA), 8 replicates of soil background (200 µL of soil suspension and 50 µL of buffer), 8 replicates of a substrate negative control (200 µL of buffer and 50 µL of L-DOPA) and 8 replicates of buffer negative control (250 µL of buffer). For peroxidase assay, 10 µL of 0.3% H_2O_2 was added to the wells after 50 µL of DOPA was added.

Plates were incubated at 20 ºC in the dark for 5 hours for peroxidase activity assays, and 18 hours for phenol oxidase activity assays. After incubation, the plates were read in a SpectraMax 340 microplate spectrophotometer (Molecular Devices, USA). Results were recorded using the Softmax Pro software. Wavelength was set to 460 nm with the "automix option" on. Potential activity was calculated as the amount of substrate (nmol) converted per hour per g of sample (nmol h^{-1} g^{-1}). Peroxidase values include phenol oxidase activity. Phenol oxidase activity was subtracted from initial peroxidase values to get peroxidase activity alone. The actual rate was counted as 0 if the calculated activity rate was negative.

3.3.3 Phospholipid Fatty Acid (PLFA) Analysis

Phospholipid fatty acid analysis (PLFA) was used to assess microbial community structure. This method provides insight to the relative proportions of specific microbial groups, such as fungi, actinomycetes, bacteria, Gram-positive bacteria, Gram-negative bacteria, within the community (Allison et al. 2007a). Following the procedure described by Bligh and Dyer (1959) and Frostegard et al. (1991), lipids were extracted from soil samples (~ 1.5 g for mineral soil, 1.0 g for organic soil) in a single-phase mixture of citrate buffer (0.15 mol L^{-1} anhydrous citric acid, pH 4.0), chloroform and methanol(0.8:1:2 by volume). The organic phase, which contains lipids, was retained using a SPE silica column (Accubond II Silica Catridges, Agilent Technologies, UBC). Subsequent additions of chloroform, acetone, and methanol were then added to the column to fractionate the lipid into neutral, glycol-, and polar (phosphor-) lipids. The methanol fraction, which contains the polar lipid fractions, was mixed with the internal standard, methyl nonadecanoate (Sigma N5377, Sigma-Aldrich, Canada). The mixture was subject to a mild alkaline methanolysis to yield fatty acid methyl esters (FAMEs). The FAMEs were separated and analyzed by a gas chromatograph (Agilent 6890N, Agilent Inc., USA) with a mass selective detector (Agilent 5973N, Agilent Inc., USA). Fatty acids were quantified by comparing peak areas from the samples with the peak areas of a bacterial acid methyl ester (BAMS) standard mix (1:20 by volume) (Supelco UK, UK) on the resulting chromatogram.

Based on the corresponding peak area, the abundance of each PLFA was expressed as the concentration of fatty acids per gram of freeze-dried soil (nmol g^{-1}). Fatty acids were designated as the ratio of the total number of C atoms to the number of double bonds present in the molecule. The prefixes “a”, “i” and “cy” indicates anteiso-, isobranching, and cyclopropane fatty acid, respectively. “10Me” refers to a methyl group on the tenth C atom from the methyl end of the molecule. Suffix “ω” indicates the position of the double bonds from the methyl end of the molecule. “c” and “t” refer to Cis and trans configurations (Frostegård et al. 1993; Zelles and Bai 1993).

The PLFAs i15:0, a15:0, i16:0, i17:0, a17:0 were used as biomarkers for Gram-positive bacteria. The PLFAs i16:1ω7c, 16:1ω9c, 16:1ω7c, i17:1ω8c, cy17:0, 18:1ω7c, 18:1ω5c, cy19:0 were used for Gram-negative bacteria (Baath et al. 1992; Frostegård et al. 1993; Zogg et al. 1997; Zelles 1999; Fritze et al. 2000). The PLFAs 10Me19:0, 10Me16:0, 10Me17:0 and 10Me18:0 were used for actinomycetes (Baath et al. 1992; Coleman et al. 1993; Allison et al. 2007b). For total bacteria, PLFAs i15:0, a15:0, 15:00, i16:1ω7c, i16:0, 16:1ω9c, 16:1ω7c, i17:1ω8c, i17:0, a17:0, cy17:0, 17:00, 18:1ω7c,18:1ω5c,18:00 and cy19:0 were chosen. PLFAs 18:2ω6,9 and 16:1ω5c were used for total fungi and AM fungi, respectively (Baath et al. 1992; Coleman et al. 1993; Frostegård et al. 1993; Zogg et al. 1997; Zelles 1999; Fritze et al. 2000; Allison et al. 2007b). The relative abundance of each PLFA (nmol % g^{-1}), calculated as the

absolute abundance of each PLFA divided by total PLFAs, was used in this study for further comparison of microbial community composition.

3.4 Statistical Analysis

3.4.1 Data Screening and Transformation Attempts

Data were tested for normality and equal variance using Kolmogorov–Smirnov and Bartlett's tests in SAS 9.1 (SAS Institute Inc, USA). The microbial community datasets (biomass, enzyme activities, PLFAs) and most of the site variables (NH_4^+, soil moisture) did not meet the assumptions of normal distribution and equal variances. Various transformations were tried to normalize the data, but none were successful. Therefore, all data were left untransformed and were analyzed using nonparametric techniques.

3.4.2 Multi-response Permutation Procedures (MRPP) and Correlation Analysis

Multi-response permutation procedures (MRPP) (PC-ORD 5.0, MjM Software, USA) is a nonparametric procedure for testing the hypothesis of no difference between two or more groups. It is similar to multivariate analysis of variance (MANOVA), but more advantageous for this particular study for three reasons. First of all, it does not require meeting the assumptions of multivariate normality and equal variances. Secondly, it can be used on unbalanced design, such as the unbalanced P fertilization treatment in this study (i.e. 18 plots with P fertilization vs.

9 plots without P fertilization). Thirdly, it can be used to perform both non- parametric univariate and multivariate analyses (McCune et al. 2002).

In this study, MRPP with pair-wise comparisons was used to test for significant differences in all measured variables among (i) organic and mineral layers, (ii) cedar and hemlock forests, (iii) three levels of N fertilization, (iv) two levels of P fertilization, (v) two levels of micronutrient fertilization, and (vi) nine treatments (NxPxmicronutrients). Both Sørensen (Bray-Curtis) and Euclidean distance measures were used and compared in all MRPP analyses. After 812 MRPP runs, Sørensen (Bray-Curtis) showed higher consistency in distinguishing ecological distinct groups, possibly because it retains sensitivity in more heterogeneous data sets and gives less weight to outliers (McCune et al. 2002). Therefore, all final MRPP results presented in this study were using Sørensen (Bray-Curtis) distance measured.

MRPP provides not only the traditional p-value, but also the chance-corrected within-group agreement (A) (e.g. group size $n_1 = 54$ when comparing two soil layers or two forest types, and group size $n_2 = 36$ when comparing three levels of N fertilization), which describes within-group homogeneity compared to the random expectation. When all items within groups are identical, A=1. When heterogeneity within groups equals random expectation, A=0. In community ecology, an A>0.3 is considered fairly high, while commonly A is below 0.1. In this study, MRPP results with a p-value < 0.05 will be considered statistical significant

(McCune et al. 2002). The significant differences will be interpreted with caution, if a small p comes with a small A (< 0.1). Spearman's rank test (JMP 8, SAS Institute Inc, USA) was used for non-parametric correlation analysis. Correlations were considered strong when correlation coefficient $|\rho| > 0.3$.

4 Results

4.1 Effect of Soil layer, Forest Type and Fertilization Treatments on Soil Microbial Community Structure and Activity and Soil Chemical Parameters

Soil layer was the most significant factor affecting soil microbial community activity and soil chemistry in these plots (Table 4.1 - 4.2). Soil moisture, NH_4^+ and NO_3^- availability, microbial biomass P, and enzyme activities such as NAGase, phosphatase, β –glucosidase, and cellubiosidase were significantly higher in the organic soil layer, while pH, microbial biomass N and peroxidase enzyme activity were significantly higher in the mineral soil layer (Table 4.3).

Forest type was the second most important factor affecting microbial community structure and function. Total enzyme activities and total PLFAs were both significantly different between western red cedar stands and western hemlock stands (Table 4.1-4.2). Phenol oxidase enzyme activity and abundance of Gram-positive bacteria, Gram-negative bacteria, actinomycetes, total bacteria and AM fungi were significantly higher in western red cedar stands than in western hemlock stands, while phosphatase activity was significantly higher in the western hemlock stands (Table 4.4).

Table 4.1 MRPP statistics for effect of soil layer, forest type and fertilization on measured soil variables.

Measured variable		Soil layer	Forest type	N fertilization	P fertilization	Micro fertilization	NxPxMicro
pH	p value	**0.0000**	0.2659	0.9231	1.0000	1.0000	0.7618
	A value	0.0688	0.0015	-0.0087	-0.0054	-0.0061	-0.0134
Moisture%	p value	**0.0000**	0.3998	0.4359	0.1401	0.8488	0.7184
	A value	0.3851	-0.0013	-0.0015	0.0069	-0.0058	-0.0147
Microbial C	p value	0.3598	0.6591	0.4246	0.7653	0.6732	0.3653
	A value	0.0000	-0.0041	-0.0006	-0.0051	-0.0043	0.0047
Microbial N	p value	**0.0000**	0.5917	0.6271	0.1754	0.6647	0.7120
	A value	0.2032	-0.0040	-0.0059	0.0054	-0.0048	-0.0149
Microbial P	p value	**0.0000**	0.2010	0.4718	0.3910	0.7140	0.8092
	A value	0.1734	0.0036	-0.0010	-0.0001	-0.0037	-0.0144
NO_3^-	p value	**0.0000**	0.0638	0.1484	0.2197	1.0000	0.5494
	A value	0.1089	0.0101	0.0080	0.0030	-0.0060	-0.0039
NH_4^+	p value	**0.0000**	**0.0244**	0.9766	0.4159	0.8151	0.8966
	A value	0.2833	0.0183	-0.0110	-0.0011	-0.0051	-0.0226
NAGase	p value	**0.0000**	0.1575	1.0000	0.0727	0.5142	0.8409
	A value	0.2322	0.0055	-0.0142	0.0109	-0.0024	-0.0192
Phosphatase	p value	**0.0000**	**0.0179**	**0.0074**	0.1075	0.3091	**0.0422**
	A value	0.0787	0.0190	0.0314	0.0077	0.0011	0.0353
β -glucosidase	p value	**0.0000**	0.0853	0.6560	1.0000	0.8975	0.6415
	A value	0.0774	0.0087	-0.0046	-0.0066	-0.0055	-0.0078
Cellubiosidase	p value	**0.0010**	0.0830	0.3796	0.6554	0.7576	0.0884
	A value	0.0352	0.0091	0.0008	-0.0035	-0.0045	0.0253
Peroxidase	p value	**0.0203**	0.1147	0.1185	0.6666	0.7232	0.4475
	A value	0.0264	0.0095	0.0142	-0.0051	-0.0056	-0.0001
Phenol oxidase	p value	0.3003	**0.0249**	0.7447	0.7542	0.4686	0.9089
	A value	0.0008	0.0245	-0.0088	-0.0058	-0.0027	-0.0304
All enzymes	p value	**0.0000**	**0.0250**	0.2314	0.2201	0.5693	0.3918
	A value	0.0684	0.0101	0.0035	0.0024	-0.0015	0.0022

Note: *p values ≤ 0.05 are in bold. Group size n = 54 for soil layer and forest type, n = 36 for nitrogen (N) fertilization, n_1= 72, n_2=36 (unbalanced design) for phosphorus fertilization (P) and micronutrient (Micro) fertilization, and n = 12 for individual treatment effect (NxPxMicro).*

Table 4.2 MRPP statistics for effect of forest type and fertilization on PLFAs.

Measured variables		Forest type	N fertilization	P fertilization	Micro fertilization	NxPxMicro
G+%	p value	**0.0000**	0.1713	0.1254	0.3374	0.1312
	A value	0.0928	0.0109	0.0097	0.0006	0.0313
G-%	p value	**0.0491**	**0.0401**	0.5522	1.0000	0.2103
	A value	0.0156	0.0237	-0.0033	-0.0072	0.0177
Bacteria%	p value	**0.0262**	**0.0433**	0.5782	1.0000	0.0727
	A value	0.0189	0.0209	-0.0033	-0.0065	0.0332
Fungi%	p value	0.5195	**0.0520**	0.7032	0.1973	**0.0160**
	A value	-0.0031	0.0264	-0.0064	0.0065	0.0749
AM fungi%	p value	**0.0000**	0.9779	0.2143	0.7753	0.8213
	A value	0.2154	-0.0203	0.0062	-0.0084	-0.0314
Actinomycetes%	p value	0.1923	0.5088	0.6175	0.4709	0.6030
	A value	0.0074	-0.0045	-0.0064	-0.0033	-0.0132
All Fatty Acids	p value	**0.0005**	**0.0342**	0.5401	0.8862	0.0700
	A value	0.0356	0.0173	-0.0021	-0.0048	0.0258

Note: *p values ≤ 0.05 are in bold. Group size n = 27 for forest type, n = 18 for nitrogen (N) fertilization, n_1= 36, n_2=18 (unbalanced design) for phosphorus fertilization (P) and micronutrient (Micro) fertilization, and n = 6 for individual treatment effect (NxPxMicro). "%" refers to relative abundance of certain microbial group. For example, G+% refers to relative abundance of Gram-positive bacteria. Relative abundance will be referred to as "abundance" for simplicity throughout this thesis.*

Table 4.3 Significant effect of soil layer on the measured environmental and microbial variables.

Measured variable	Organic layer	Mineral layer
pH	**3.26(0.06)b**	**3.55(0.05)a**
Moisture%	**244.66(9.66)a**	**87.63(5.88)b**
NH_4^+(μg g^{-1} soil)	**119.26(8.16)a**	**25.92(2.82)b**
NO_3^-(μg g^{-1} soil)	**1.56(0.25)a**	**0.69(0.05)b**
Microbial C(μg g^{-1} soil)	1929.27(282.21)	1724.33(244.68)
Microbial N(μg g^{-1} soil)	**22.28(6.21)b**	**30.93(5.89)a**
Microbial P(μg g^{-1} soil)	**191.55(18.05)a**	**31.27(9.31)b**
NAGase(nmol $h^{-1}g^{-1}$ soil)	**1590.26(106.61)a**	**578.41(67.21)b**
Phosphatase(nmol $h^{-1}g^{-1}$ soil)	**1871.21(190.82)a**	**731.95(83.90)b**
β -glucosidase(nmol $h^{-1}g^{-1}$ soil)	**267.56(26.69)a**	**139.56(26.80)b**
Cellubiosidase(nmol $h^{-1}g^{-1}$ soil)	**47.81(5.25)a**	**35.30(7.77)b**
Peroxidase(nmol $h^{-1}g^{-1}$ soil)	**1047.99(337.75)b**	**3277.01(638.14)a**
Phenol oxidase(nmol $h^{-1}g^{-1}$ soil)	1020.62(509.38)	590.50(182.30)

Note: *Values are means (group size n=54) with one standard error given in parentheses. Means that were significantly different ($p \leq 0.05$) are in bold. Comparison is between two soil layers.*

Table 4.4 Significant effect of forest type on the measured environmental and microbial variables.

Measured variable	Western red cedar	Western hemlock
pH	3.37(0.05)	3.45(0.06)
Moisture%	160.59(13.74)	171.70(14.00)
NH_4^+ (μg g^{-1} soil)	**74.39(7.28)a**	**70.79(10.19)b**
NO_3^- (μg g^{-1} soil)	1.21(0.25)	1.03(0.10)
Microbial C(μg g^{-1} soil)	1750.6(176.8)	1903.0(330.6)
Microbial N(μg g^{-1} soil)	19.12(4.02)	34.10(7.49)
Microbial P(μg g^{-1} soil)	96.59(15.97)	126.23(19.82)
Cellobiosidase(nmol $h^{-1}g^{-1}$ soil)	37.87(5.20)	45.24(7.87)
NAGase(nmol $h^{-1}g^{-1}$ soil)	999.41(119.47)	1169.26(104.61)
Glucosidase(nmol $h^{-1}g^{-1}$ soil)	187.97(23.58)	219.15(187.52)
Phosphatase(nmol $h^{-1}g^{-1}$ soil)	**1006.67(132.66)b**	**1596.50(187.52)a**
Peroxidase(nmol $h^{-1}g^{-1}$ soil)	1640.81(466.39)	2684.19(581.78)
Phenol oxidase(nmol $h^{-1}g^{-1}$ soil)	**1433.72(474.05)a**	**177.40(235.53)b**
G+%	**0.158(0.003)a**	**0.139(0.002)b**
G-%	**0.391(0.005)a**	**0.372(0.007)b**
Actinomycetes%	0.043(0.002)	0.048(0.004)
Bacteria%	**0.614(0.008)a**	**0.593(0.006)b**
AM Fungi%	**0.040(0.003)a**	**0.026(0.001)b**
Fungi%	0.055(0.003)	0.057(0.003)

Note: *Means that were significantly different ($p \leq 0.05$) are in bold (Comparison is between two forest types). Group size n=54 for all variables except for PLFA variables; n=27 for PLFA variables. "%" refers to relative abundance of certain microbial group. For example, G+% refers to relative abundance of Gram-positive bacteria. Relative abundance will be referred to as "abundance" for simplicity throughout this thesis.*

N fertilization had a significant effect on phosphatase activity, abundance of Gram-negative bacteria, total bacteria and fungi, and total PLFAs (Table 4.1-4.2). The abundance of fungi was significantly higher in control plots than in N200 plots, but not in the N300 plots. Since N200 and N300 had very similar abundance of fungi, the difference in statistical significance is likely due to the larger standard error and smaller A value from of the N300 treatment (Table 4.7). In contrast, phosphatase activity and the abundance of Gram-negative bacteria and total bacteria were significantly higher in plots that received N fertilization than in the unfertilized control plots (Table 4.5-4.6). On the other hand, fertilization with P and micronutrients did not have any significant effect on microbial community structure or activity. When looking at fertilization with the three fertilizers combined, only phosphatase activity was significantly different among nine treatments (Appendix 2).

Table 4.5 Pair-wise MRPP comparison of phosphatase activity, PLFA patterns (all fatty acids), abundance of Gram-positive bacteria (G-%), total bacteria (Bacteria%) and total fungi (Fungi%) with three levels of N fertilization(N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} 300=300 kg ha^{-1}). Group size n=36 for phosphatase activity, n = 18 for abundance of Gram-negative bacteria, total bacteria and total fungi.

Levels Compared	Variables	T-test	A	P value
N0 vs. N200	Phosphatase	-0.83	0.01	**0.16**
	G-%	-1.79	0.04	**0.06**
	Bacteria%	-2.34	0.05	**0.04**
	Fungi%	-2.80	0.06	**0.02**
	All fatty acids	-2.43	0.03	**0.03**
N0 vs. N300	Phosphatase	-5.77	0.05	**0.00**
	G-%	-2.61	0.03	**0.03**
	Bacteria%	-2.24	0.02	**0.04**
	Fungi%	-1.24	0.02	**0.11**
	All fatty acids	-2.54	0.02	**0.03**
N200 vs. N300	Phosphatase	-1.06	0.01	**0.13**
	G-%	-0.07	0.00	**0.36**
	Bacteria%	0.29	0.00	**0.48**
	Fungi%	-0.01	0.00	**0.41**
	All fatty acids	0.28	0.00	**0.51**

Table 4.6 Significant effect of N fertilization on phosphatase activity and abundance of Gram-negative bacteria (G-%), total bacteria (bacteria%) and total fungi (fungi%) with three levels of N fertilization. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} 300=300 kg ha^{-1})

	Phosphatase(nmol h^{-1} g^{-1} soil)	**G-%**	**Bacteria%**	**Fungi%**
N300	1722.51(245.21)a	0.389(0.010)a	0.616(0.010)a	0.052(0.004)ab
N200	1237.98(198.66)ab	0.386(0.007)ab	0.608(0.006)a	0.053(0.003)b
N0	944.26(155.24)b	0.371(0.087)b	0.588(0.139)b	0.063(0.015)a

Note: *Values are means with one standard error given in parentheses. Means with the same letter were not significant different (p>0.0167= 0.05/3) (comparison is between three N levels). Group size n=36 for phosphatase activity, n = 18 for abundance of Gram-negative bacteria, total bacteria and total fungi.*

4.2 Effect of Fertilization on Environmental and Microbial Variables in Individual Forest Types and Soil Layers

4.2.1 PH and Moisture%

Soil pH in both soil layers from both forest stands was not significantly different 10 years after fertilization (Figs 4.1, 4.2). Although soil moisture appeared to be significantly reduced by N fertilization in the organic layer of the western hemlock stand (Fig. 4.3), this was likely an

artifact of sampling time. Most of the samples from the unfertilized western hemlock plots were taken on a day when there was a short heavy precipitation event. Soil moisture was not affected in either soil layer by fertilization in the western red cedar stands (Fig. 4.4).

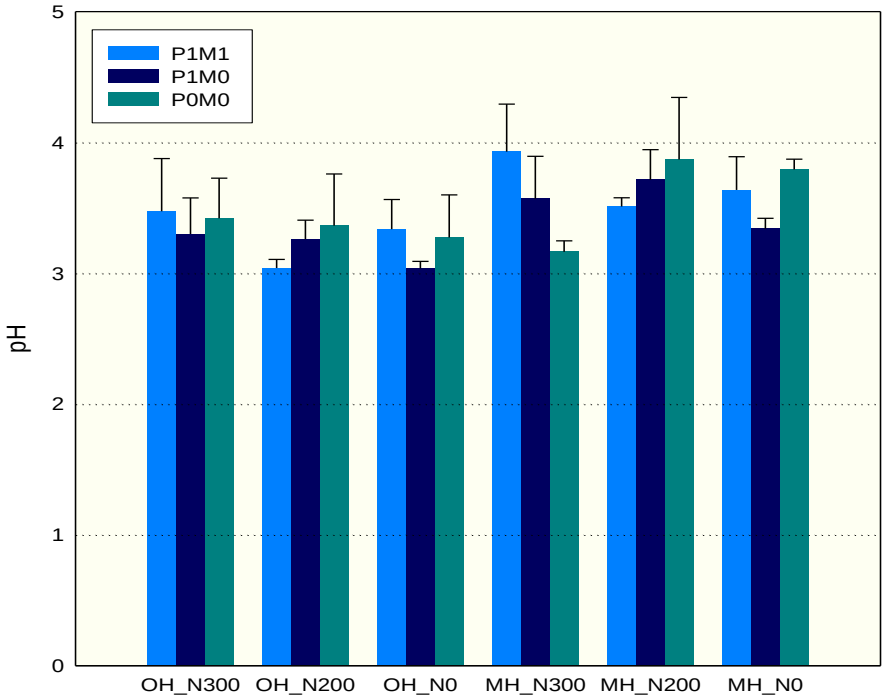

Figure 4.1 Mean pH of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1} , P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors

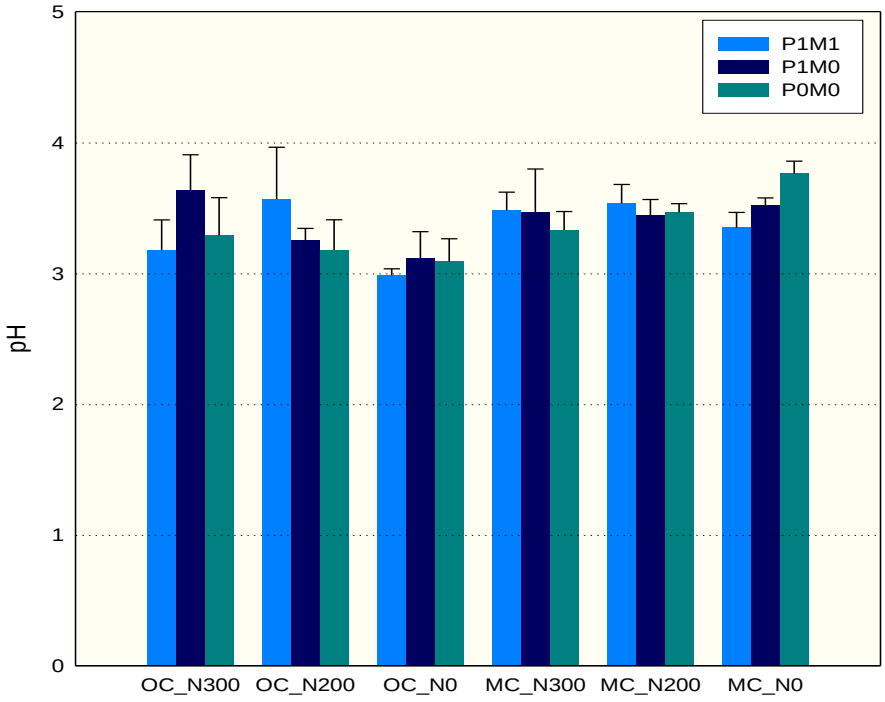

Figure 4.2 Mean pH of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1} , P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

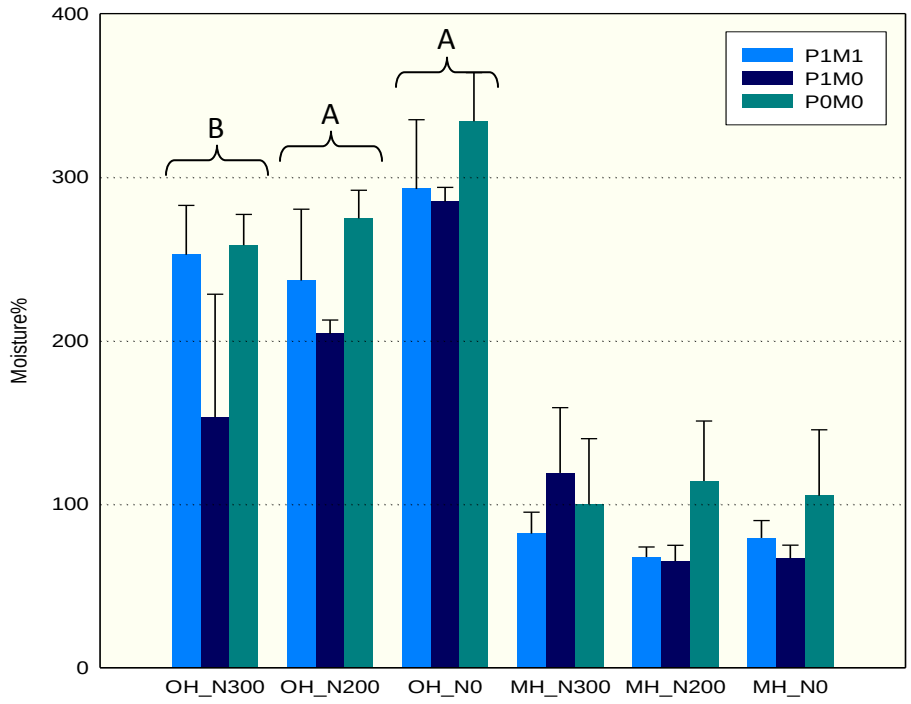

Figure 4.3 Mean moisture content of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} , 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different P<0.05.

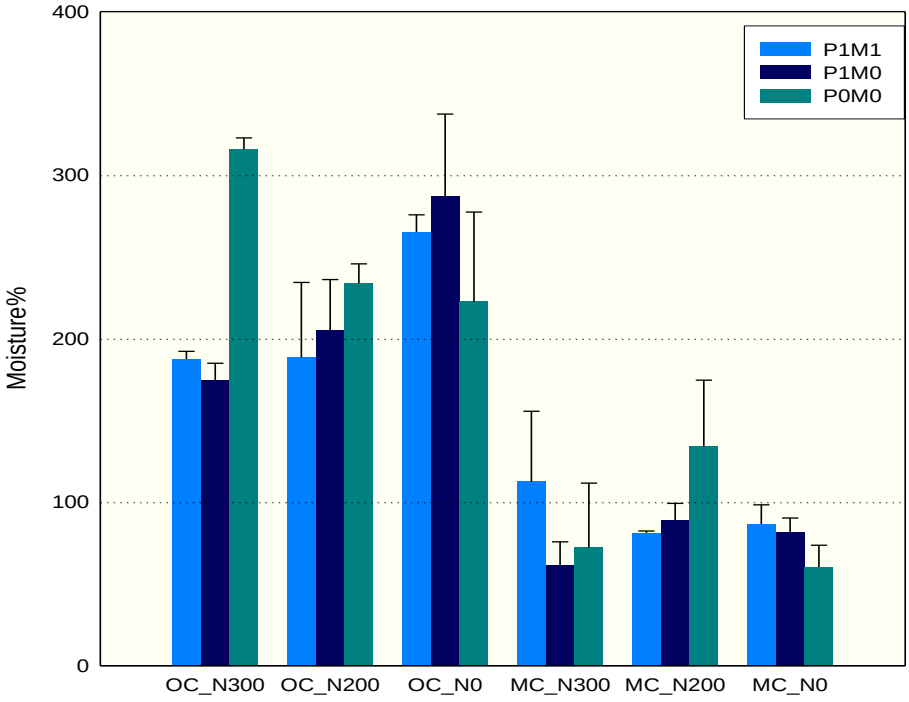

Figure 4.4 Mean moisture content of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} , 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

4.2.2 NO_3^- and NH_4^+ Availability

Fertilization had a significant effect on NH_4^+ and NO_3^- availability in the organic layers of both forest stands, but did not affect these two variables significantly in the mineral soil layers (Figs 4.5 - 4.8). In the organic layer of the western hemlock stands, NH_4^+ availability was higher ($A = 0.07$, $p = 0.02$) in the P0 plots (149±19 µg g^{-1} soil) than in the P100 plots (111±26 µg g^{-1} soil), while NO_3^- availability was significantly affected by N fertilization ($A = 0.09$, $p = 0.02$). The

highest availability was found in the N200 plots, and the lowest was found in N300 plots (Table 4.7, Fig 4.7), owing to a few of the N200 samples with unusually high level of NO_3^- availability. In the organic layer of the western red cedar stands, NO_3^- availability increased as N fertilization level increased (Table 4.7, Fig 4.8).

Table 4.7 NO_3^- availability ($\mu g\ g^{-1}$ soil) in the organic layer of western red cedar and western hemlock stands following fertilization with three levels of N (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} 300=300 kg ha^{-1}).

	N0	**N200**	**N300**
Cedar Stands Organic Layer	0.85(0.08)b	1.11(0.28)ab	3.11(1.05)a
Hemlock Stands Organic Layer	1.42(0.17)ab	2.01(0.32)a	1.10(0.17)b

Note: *Values are means (n= 9) with one standard error given in parentheses. Means with the same letter were not significant different (p<0.0167, 0.05/3) (comparison is between three N levels).*

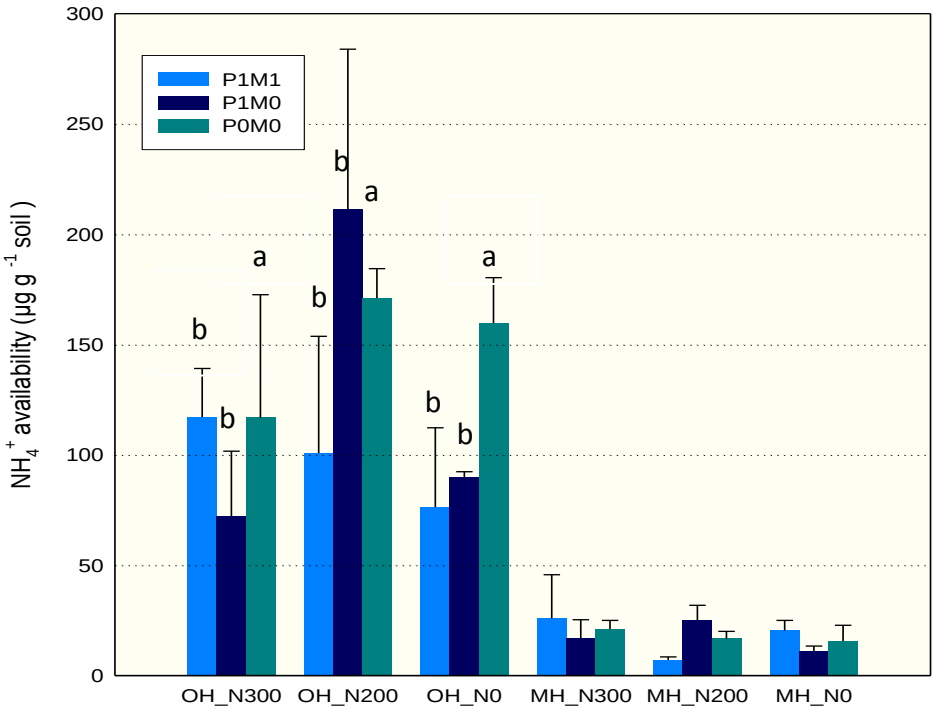

Figure 4.5 Mean NH_4^+ availability (µg g^{-1} soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

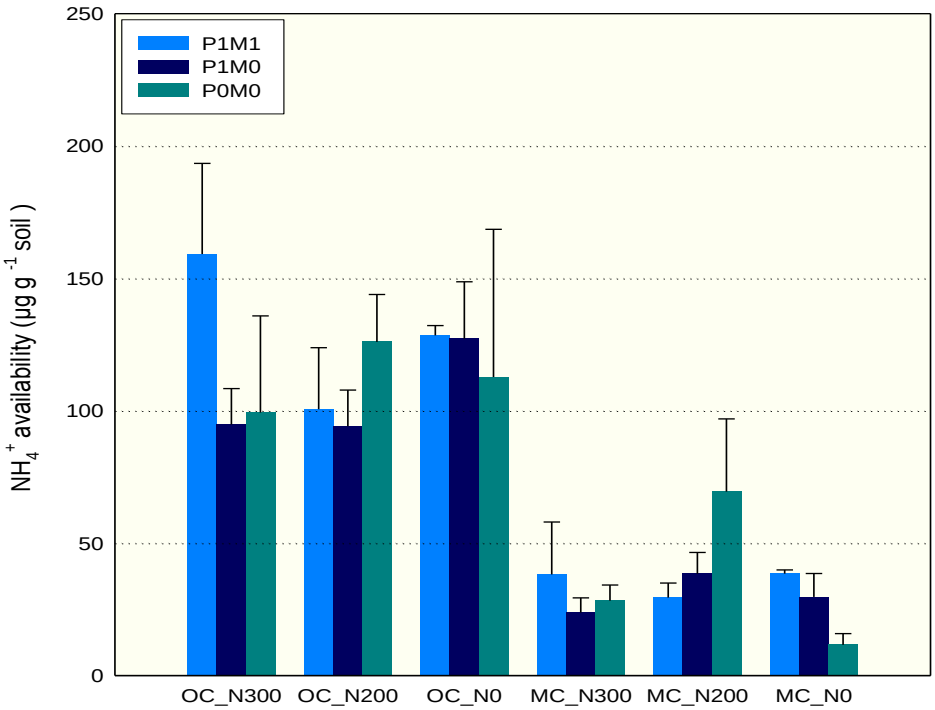

Figure 4.6 Mean NH_4^+availability (µg g^{-1} soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

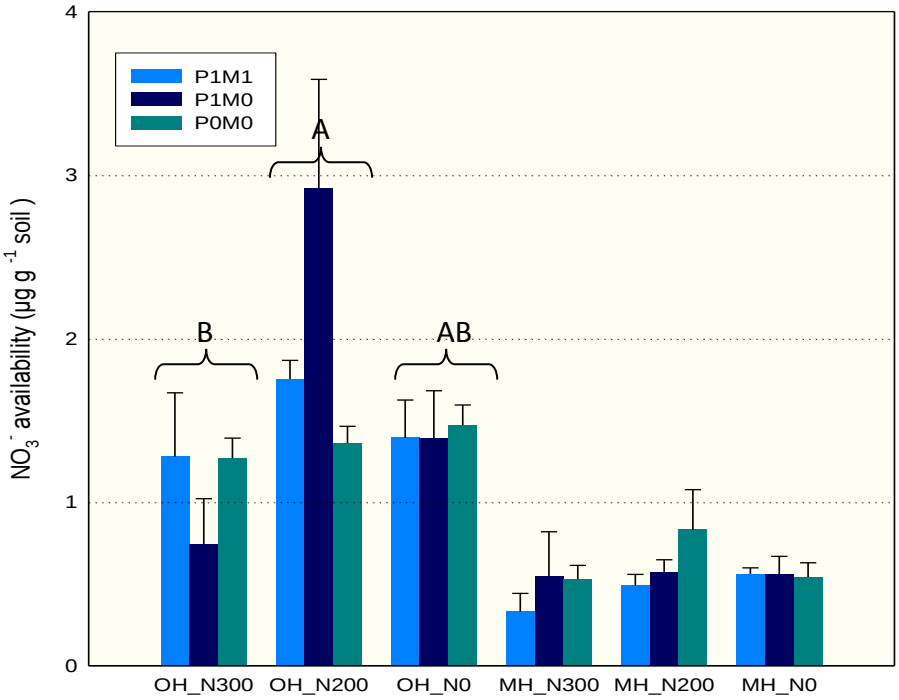

Figure 4.7 Mean NO_3^- availability (µg g^{-1} soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

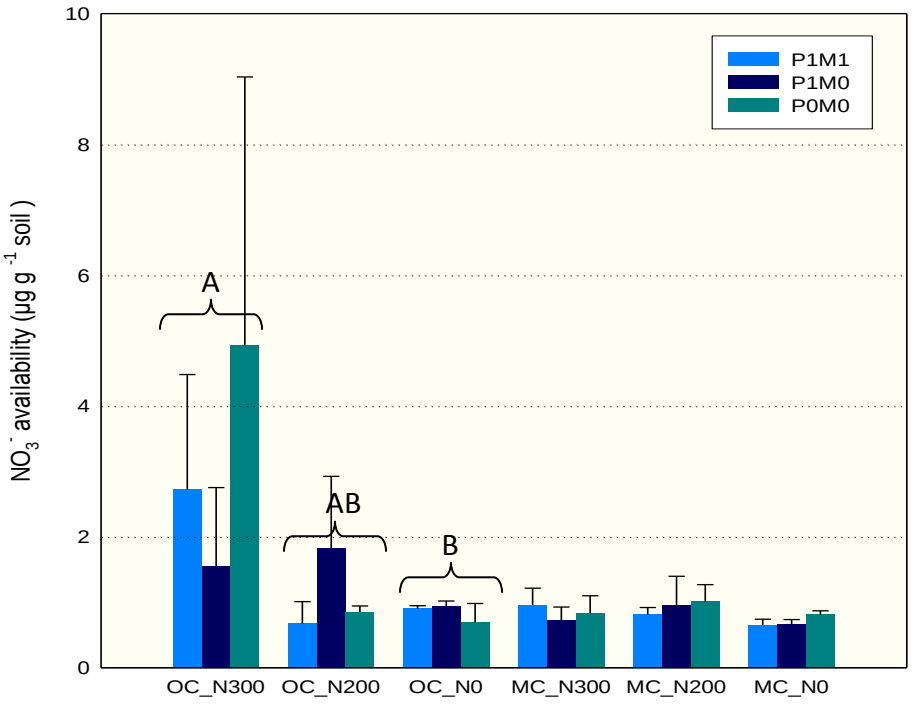

Figure 4.8 Mean NO_3^- availability (µg g^{-1} soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

4.2.3 Microbial Biomass C, N and P

Microbial biomass carbon was not affected by fertilization in either soil layer of western hemlock or western red cedar (Figs 4.9 and 4.10). Microbial biomass N on the other hand, was significantly affected by P fertilization in the organic layer of the western red cedar stand (p = 0.03, A = 0.11) (Fig 4.12). Microbial biomass N was significantly higher in the organic layer of

cedar stands receiving 100 kg ha^{-1} P (32.43± 10.47 µg g^{-1} soil) than in those receiving 0P (0.18 ± 0.08 µg g^{-1} soil).

Microbial biomass P was significantly affected by micronutrient fertilization in the organic layer of the western hemlock stands (p = 0.01, A=0.07) (Fig 4.13), and by N fertilization in the organic layer of the western red cedar stands (p= 0.03, A=0.08) (Fig 4.14). In the organic layer of the western hemlock stands, microbial biomass P was significantly higher in plots that received micronutrients (341 ± 138 µg g^{-1} soil) than in the plots that did not receive micronutrients (168 ± 110 µg g^{-1} soil). In the organic layer of the western red cedar stands, microbial biomass P was higher in plots receiving no N fertilizer than in the N fertilized plots. A similar higher microbial biomass P in the organic layer of the western hemlock stands in non-N fertilized plots was also observed, though it was not significant (Table 4.8). However, the large variation among replicates had limited my ability to make sound interpretation of these patterns.

Table 4.8 Microbial biomass P (µg g^{-1} soil) in the organic layer of western red cedar and western hemlock stands following fertilization with three levels of N (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1})

	N0	**N200**	**N300**
Cedar Stands Organic Layer	167(26)a	178(49)ab	121(42)b
Hemlock Stands Organic Layer	241(64)	213(39)	221(41)

Note: *Values are means (n= 9) with one standard error given in parentheses. Means with the same letter were not significant different (p<0.0167, 0.05/3) (comparison is between three N levels).*

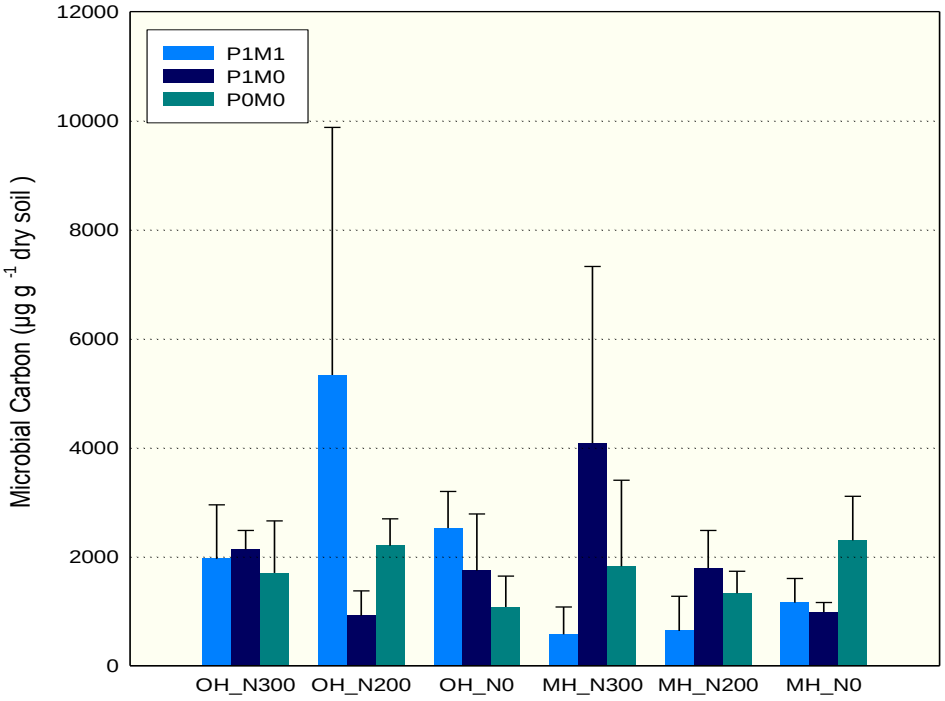

Figure 4.9 Mean microbial biomass carbon concentration (µg g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

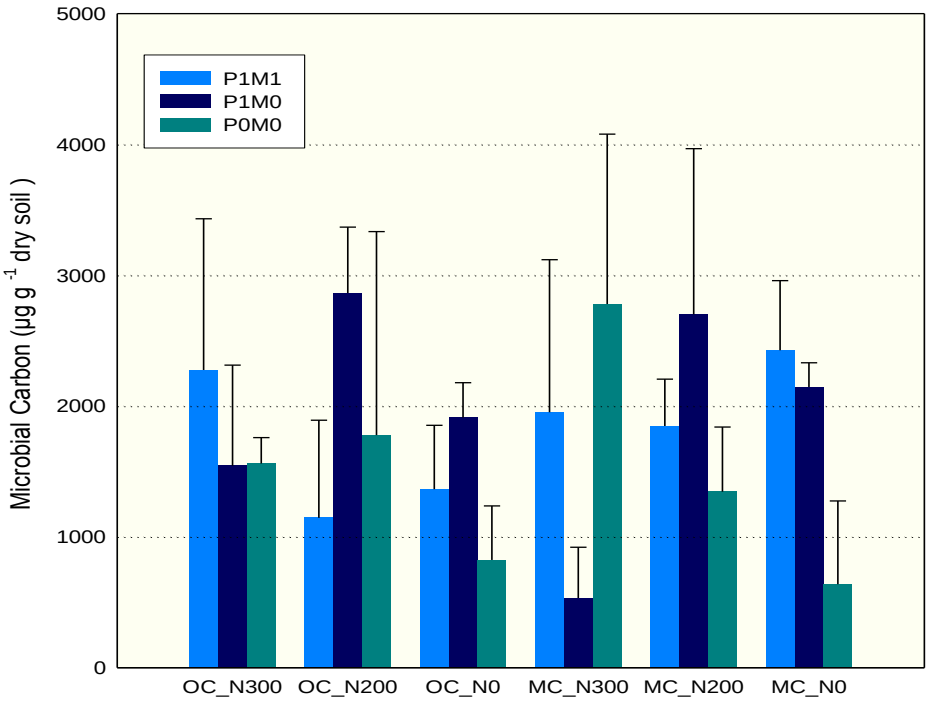

Figure 4.10 Mean microbial biomass carbon concentration (µg g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

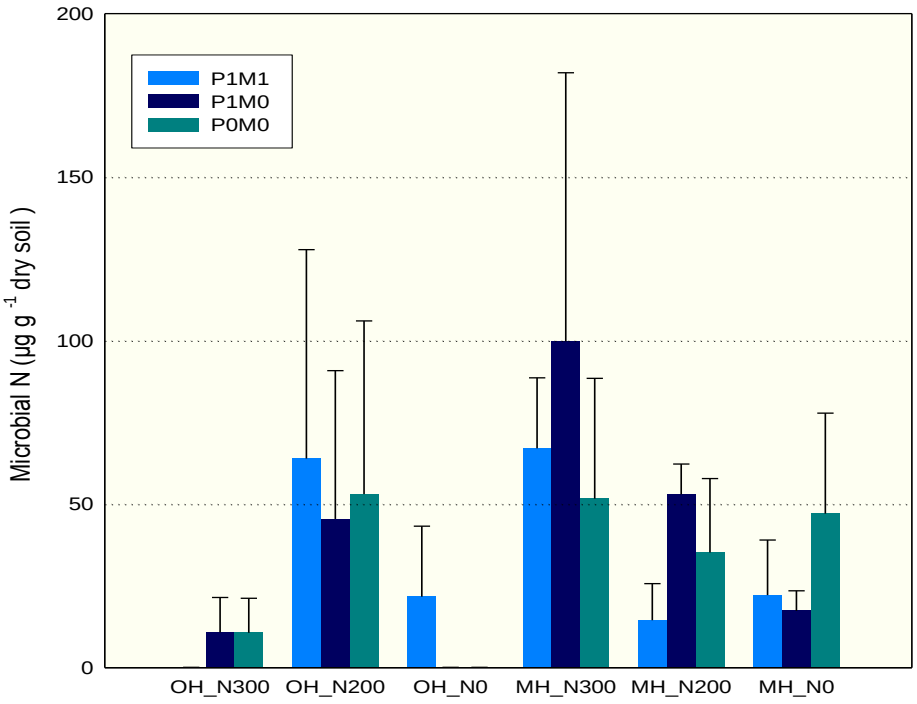

Figure 4.11 Mean microbial biomass N concentration (µg g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

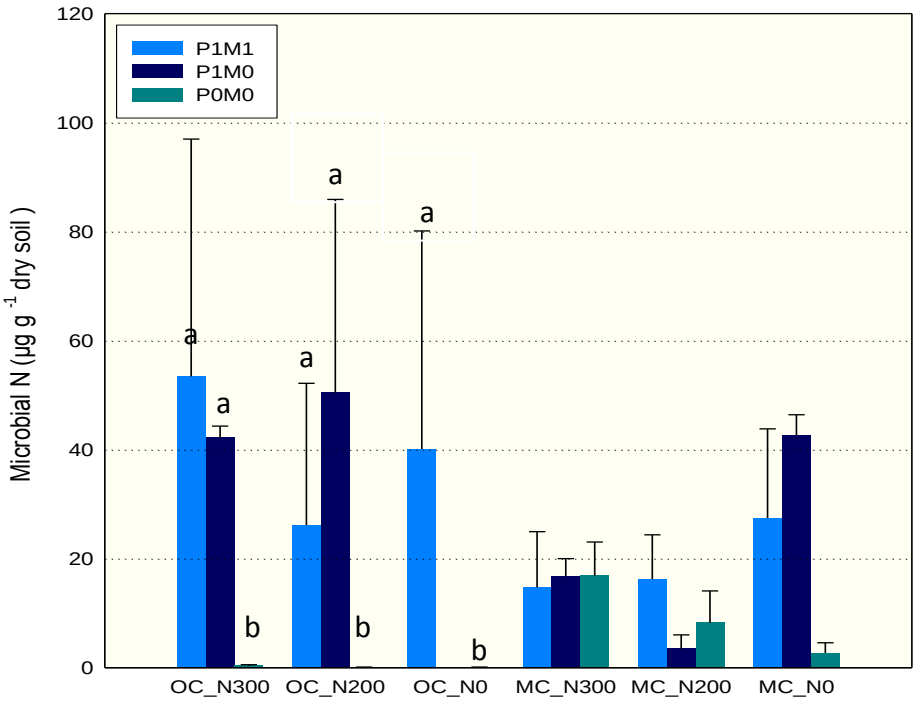

Figure 4.12 Mean microbial biomass N concentration (µg g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} , 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

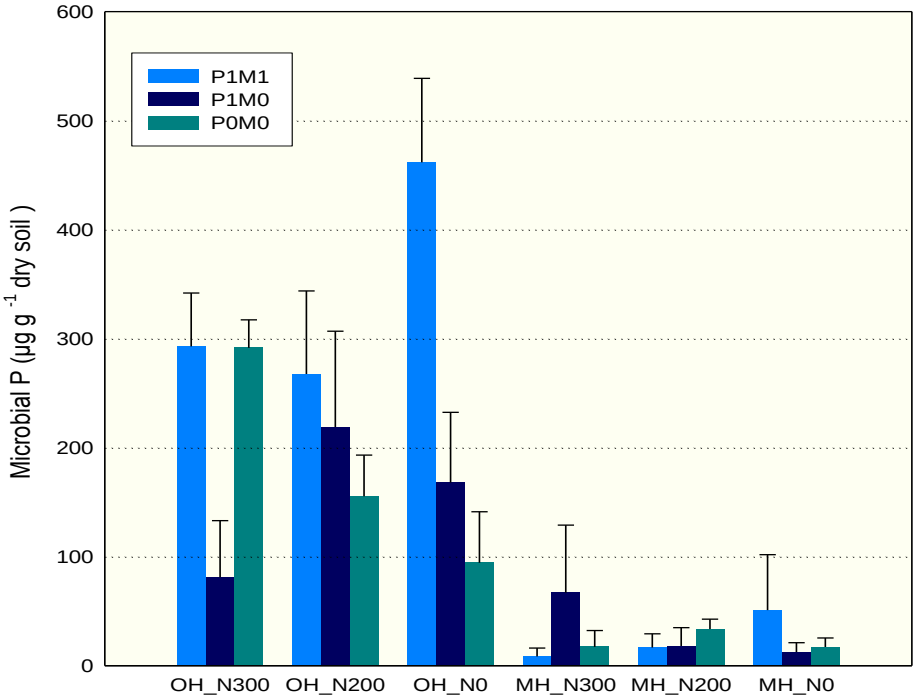

Figure 4.13 Mean microbial biomass P concentration (µg g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

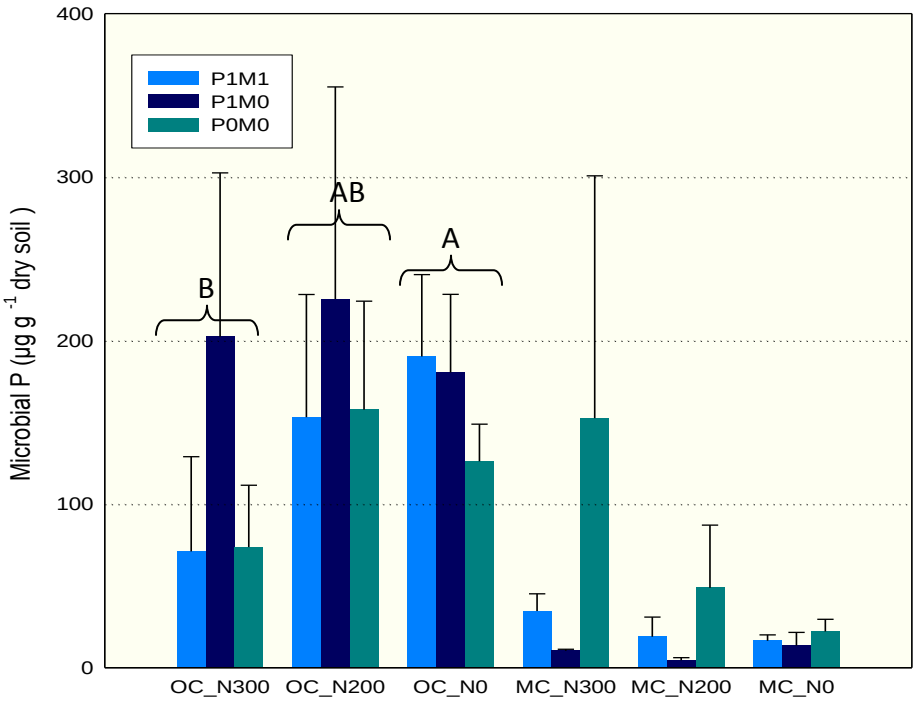

Figure 4.14 Mean microbial biomass P concentration (µg g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

4.2.4 Enzyme Activities

Total enzyme activities were significantly affected by N fertilization (A = 0.06, p = 0.02) and P fertilization (A = 0.04, p = 0.03) in the in the organic layer of the western hemlock stands. Pairwise MRPP revealed that enzyme activities in the plots receiving 300 kg N ha^{-1} were significantly different from the plots receiving no N fertilizer (A= 0.10, p= 0.004). When looking

at individual enzyme activity, fertilization did not affect the activities of cellobiosidase, NAGase, glucosidase and phenol oxidase in either soil layer of cedar or hemlock (Figs 4.15-4.22). Fertilization had a significant effect on phosphatase activities in the organic layers of both the western hemlock and western red cedar stands (Fig 4.23, 4.24), and peroxidase activities in the in the organic layer of the western hemlock stands (Fig 4.25).

Phosphatase activity was significantly affected by N fertilization in the organic layer of the western red cedar stands ($p= 0.08$, $A = 0.04$) and increased as the concentration of N fertilizer increased (Table 4.9, Fig 4.24). In the organic layer of the western hemlock stands, phosphatase activity was significantly lower ($p = 0.02$, $A = 0.07$) in plots that received P fertilizer than in plots that received no P fertilizer (Table 4.10, Fig 4.23).

N fertilization significantly affected peroxidase activity in the organic layer of the western hemlock stands ($p =0.02$, $A = 0.15$). Peroxidase activity was significantly higher in plots that received 300 kg N ha^{-1} than in plots that received no N fertilizer ($p= 0.01$, $A= 0.21$). Both peroxidase and phenol oxidase seemed to be more active in mineral soil layers than in organic layers (Figure 4.21, 4.22, 4.25, 4.26). However there was large variation among replicates, making it difficult to make sound comparisons between treatments.

Table 4.9 Phosphatase activity (nmol $h^{-1}g^{-1}$ soil) in the organic and mineral soil layers of western red cedar and western hemlock stands following fertilization with three levels of N (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, O- organic layer, M- mineral layer, C- western red cedar stands, H- western hemlock stands)

N Level	OC	MC	OH	MH
0	1031(354)b	458(162)	1446(282)	842(222)
200	1583(342)ab	384(85)	1968(549)	1017(295)
300	1985(370)a	599(178)	3214(604)	1092(156)

***Note**: Values are means (n= 9) with one standard error given in parentheses. Means with the same letter were not significant different (p<0.0167, 0.05/3) (comparison is between three N levels).*

Table 4.10 Phosphatase activity (nmol $h^{-1}g^{-1}$ soil) in the organic and mineral soil layers of western red cedar and western hemlock stands following fertilization with P and micronutrients. (P-phosphorus, 0=0 kg ha^{-1}; 100-100 kg ha^{-1}, Micro – micronutrients, 0-did not receive micronutrients, 1-received micronutrients, O- organic layer, M- mineral layer, C- western red cedar stands, H- western hemlock stands)

P	**OC**	**OH**	**MC**	**MH**
0	1731(439)	3332(573)a	572(167)	1354(268)
100	1434(236)	1648(302)b	435.8(95)	7988(125)
Micro	**OC**	**OH**	**MC**	**MH**
0	1655(310)	2334(405)	521(118)	1124(190)
1	1289(334)	1960(427)	400(135)	701(177)

***Note**: Values are means with one standard error given in parentheses (P0 & Micro1 group size $n_1 = 9$, P100 and Micro 0 group size $n_2 = 18$, unbalanced design). Means with the same letter were not significant different ($p<0.0167$, 0.05/3) One comparison is between two P levels. The other one is between two micronutrient levels.*

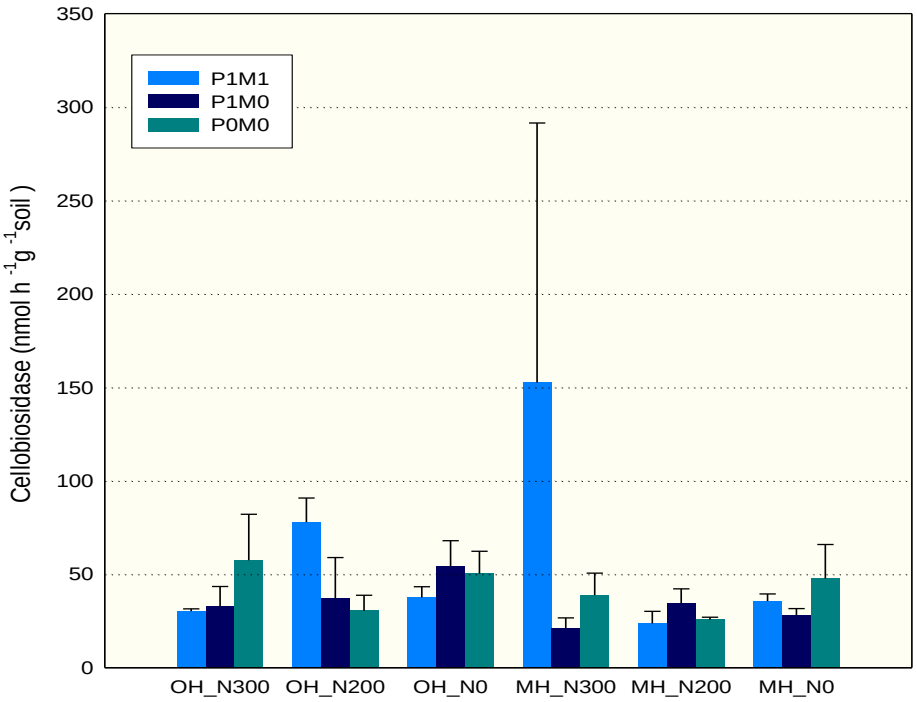

Figure 4.15 Mean cellobiosidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

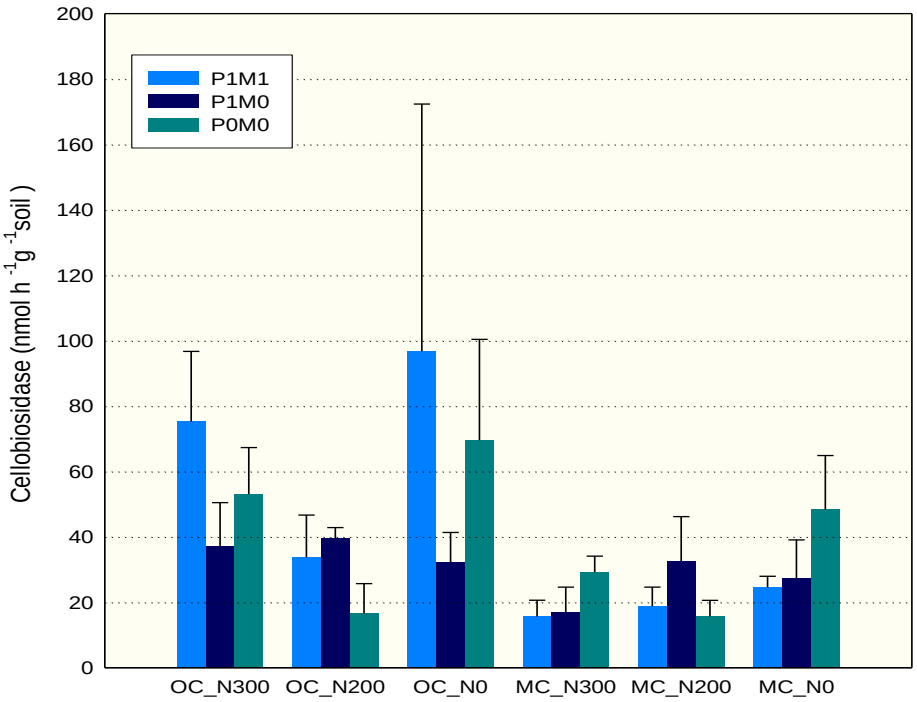

Figure 4.16 Mean cellobiosidase activity (nmol h $^{-1}$g $^{-1}$ soil) of western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

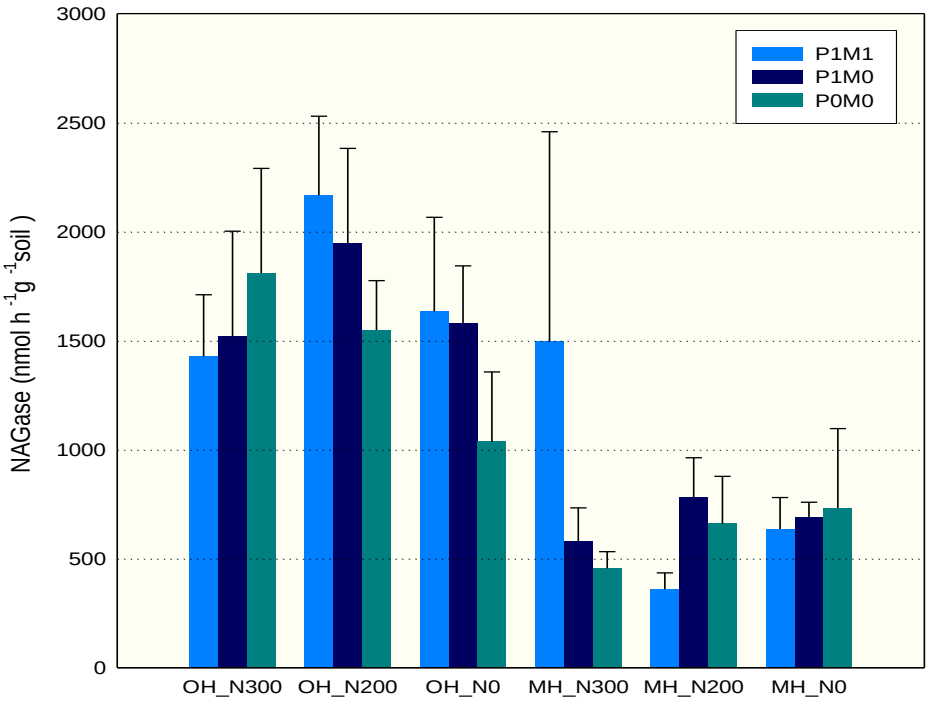

Figure 4.17 Mean NAGase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

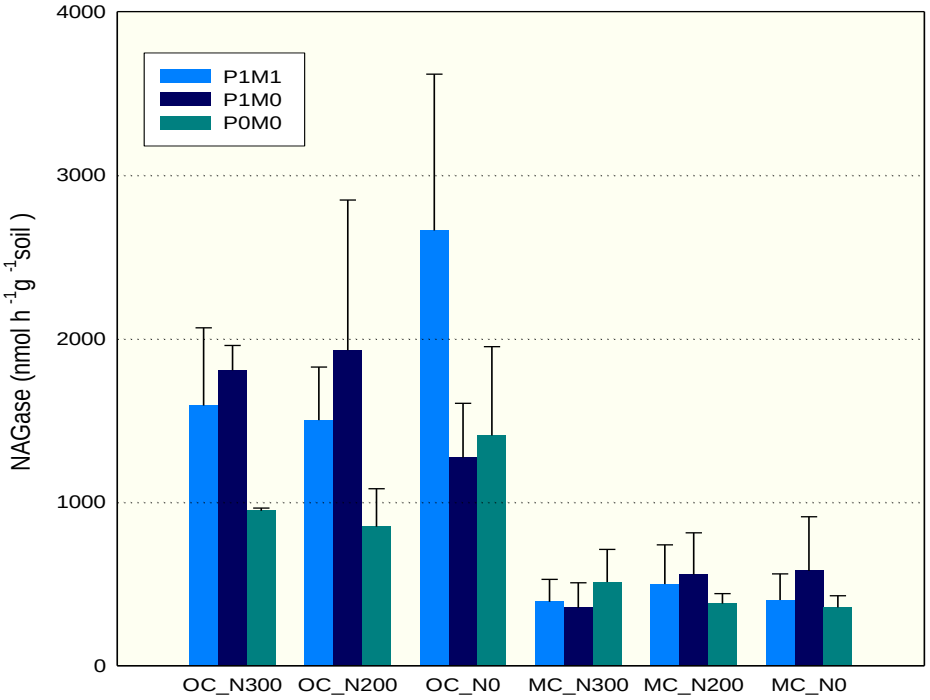

Figure 4.18 Mean NAGase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

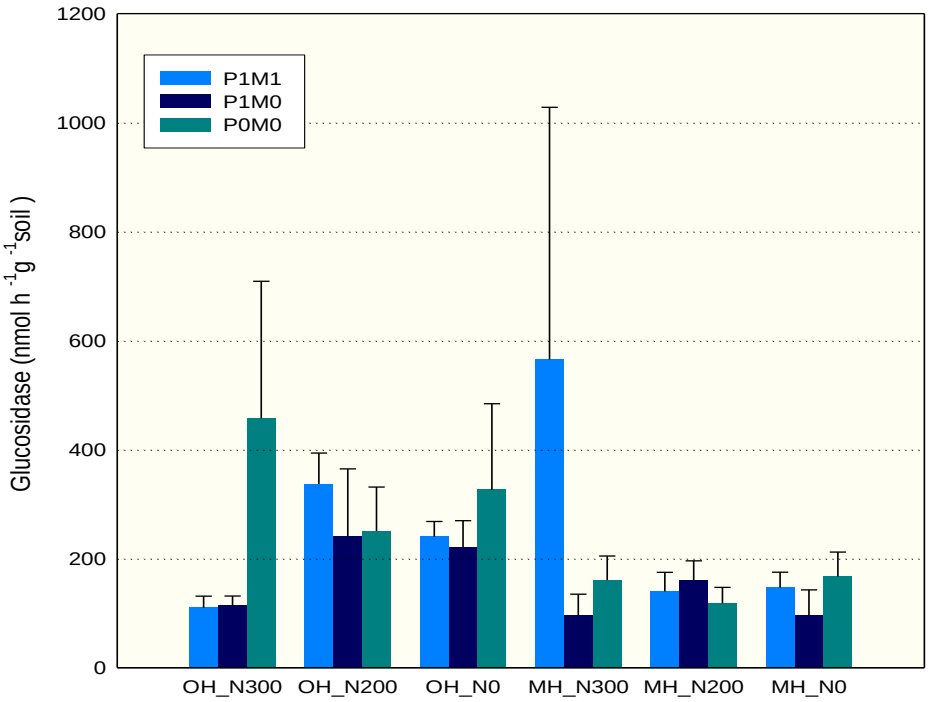

Figure 4.19 Mean glucosidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

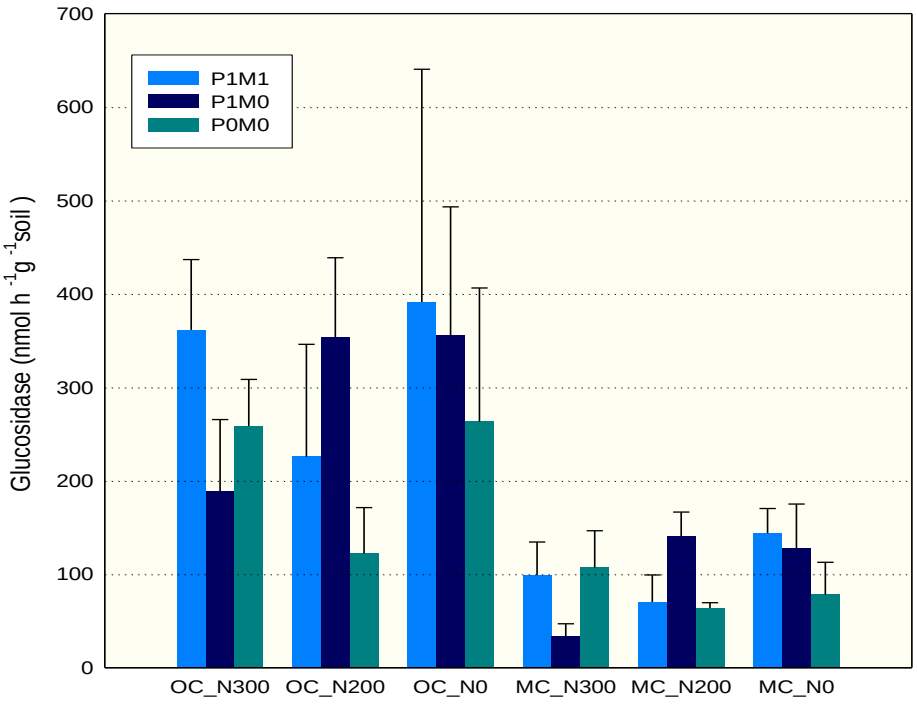

Figure 4.20 Mean glucosidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

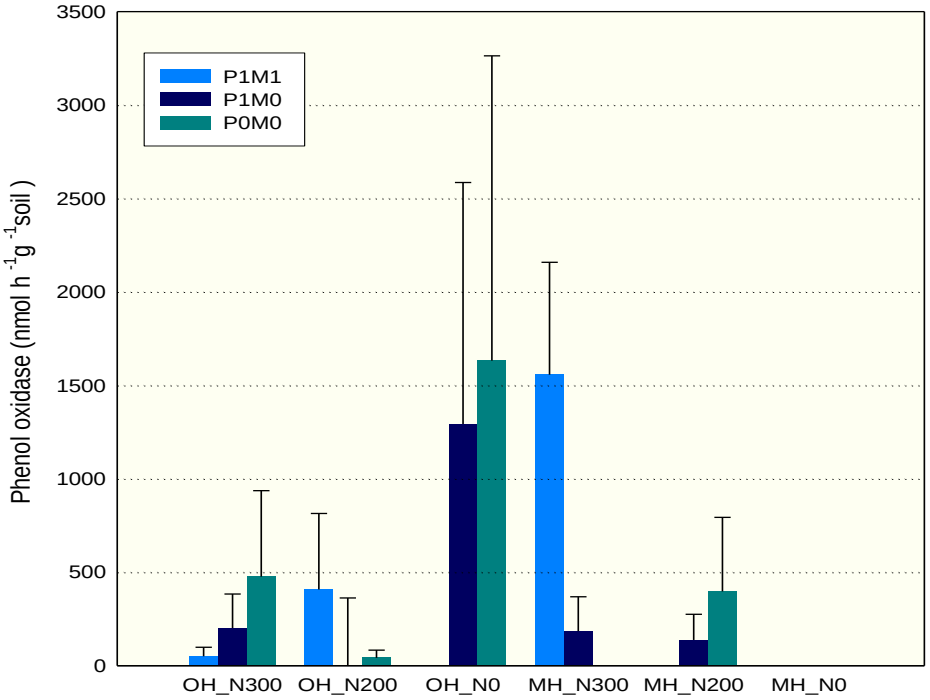

Figure 4.21 Mean phenol oxidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

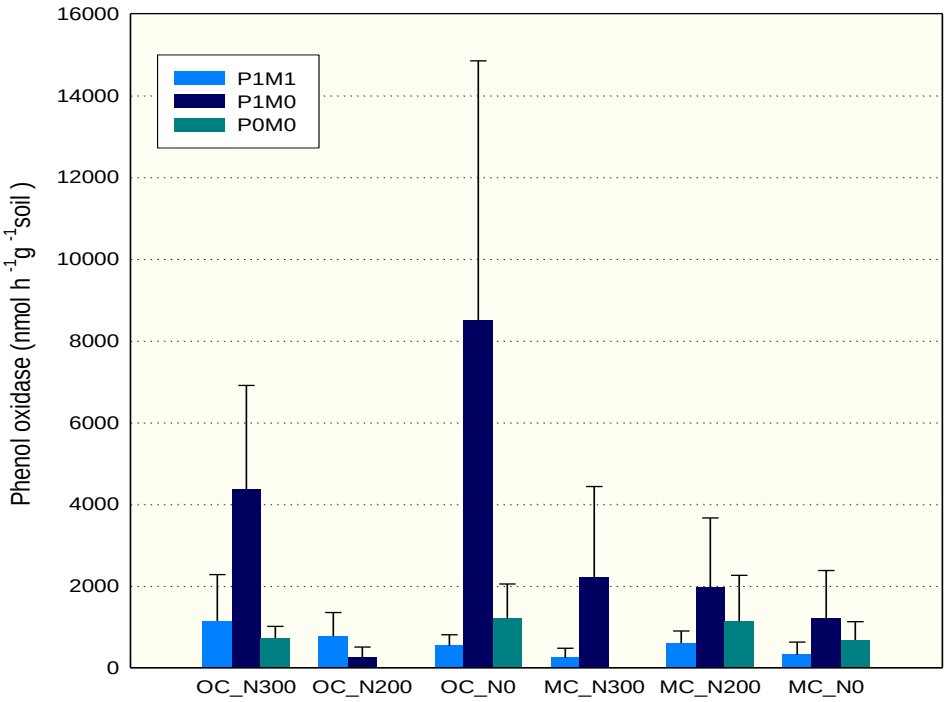

Figure 4.22 Mean phenol oxidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

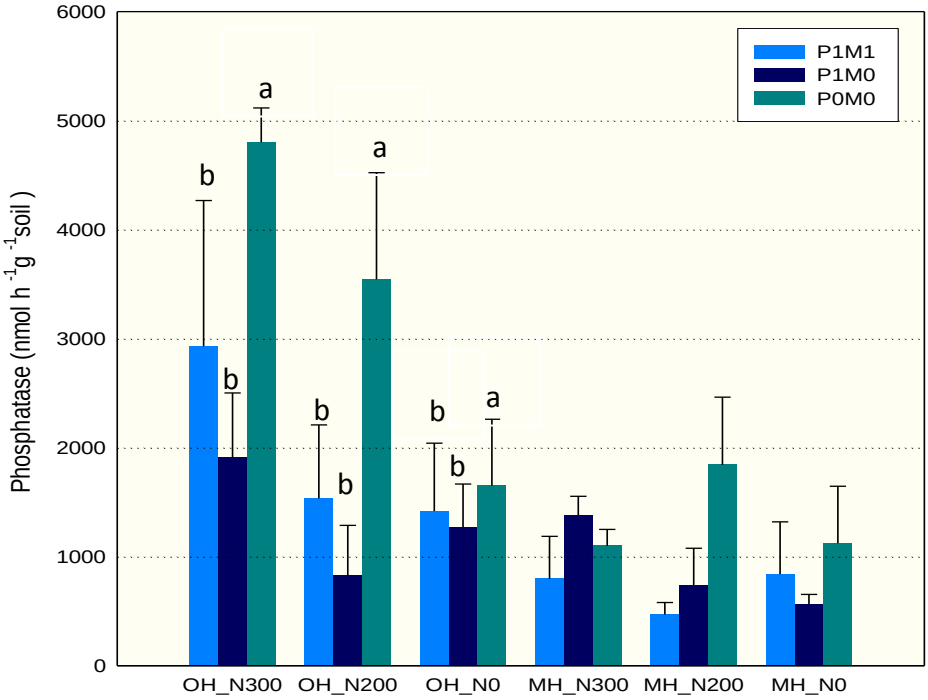

Figure 4.23 Mean phosphatase activity (nmol h $^{-1}$g $^{-1}$ soil) of western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} , 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different.

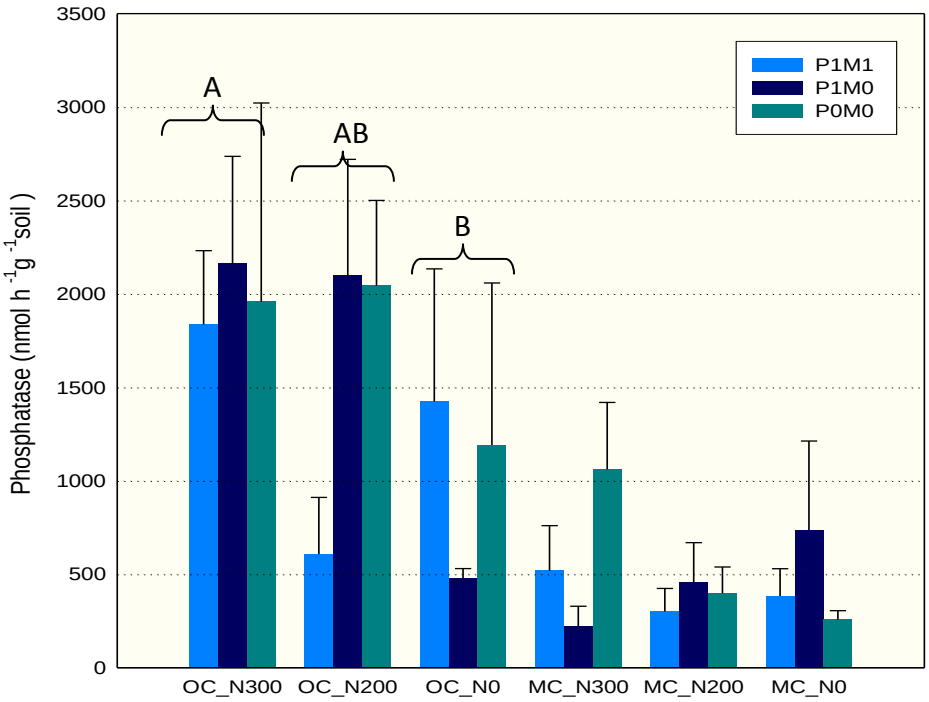

Figure 4.24 Mean phosphatase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

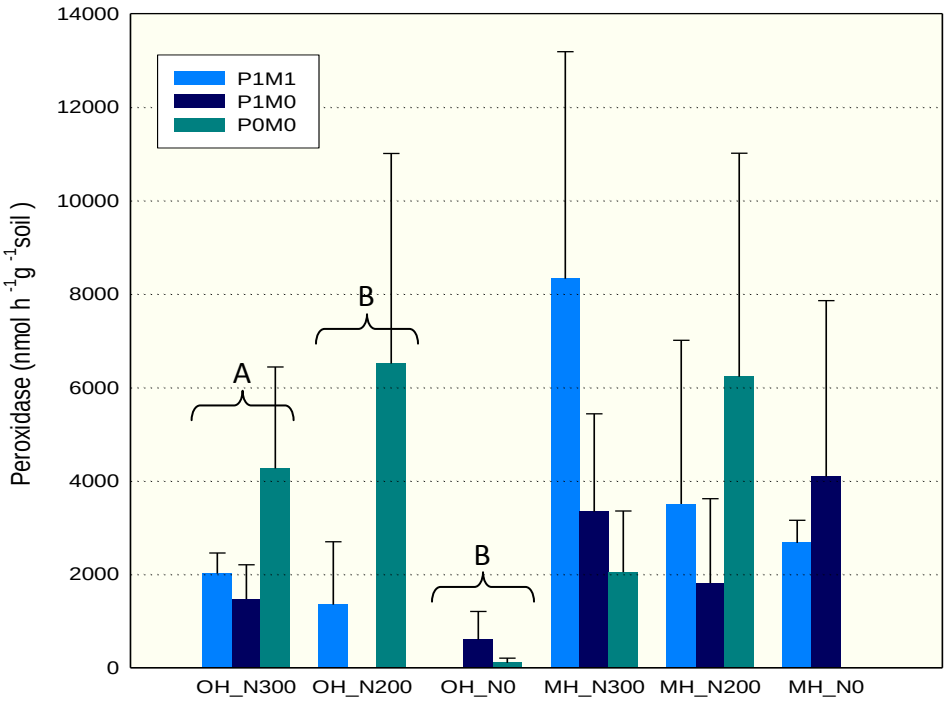

Figure 4.25 Mean peroxidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western hemlock forest organic layer (OH) and mineral soil layer (MH) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

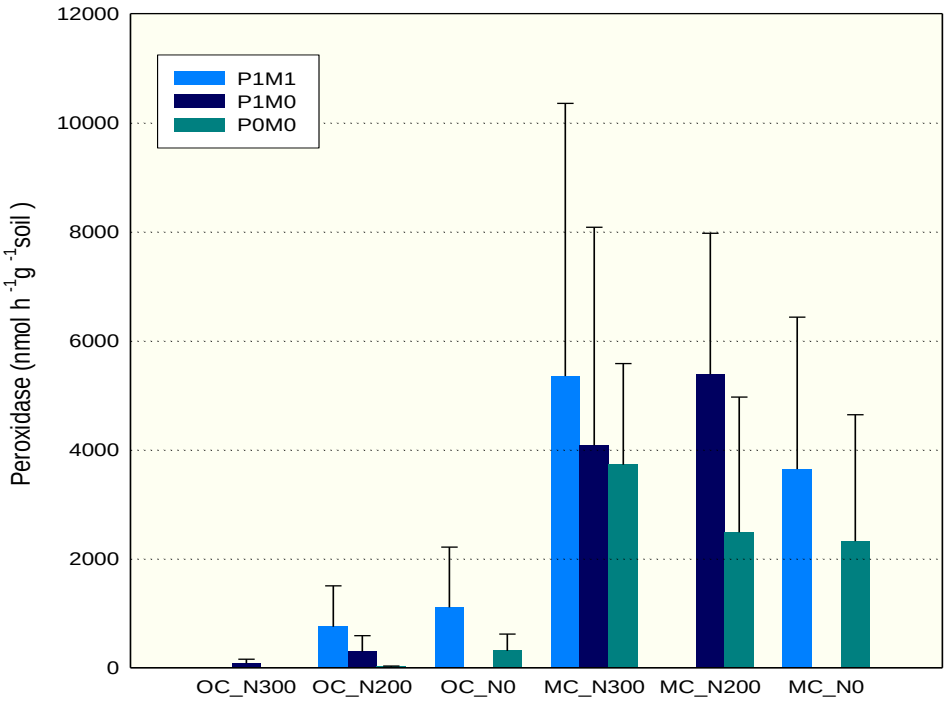

Figure 4.26 Mean peroxidase activity (nmol h $^{-1}$g $^{-1}$ soil) of the western red cedar forest organic layer (OC) and mineral soil layer (MC) in nine fertilization treatments (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} ,300=300 kg ha-1, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

4.2.5 Microbial Community Composition

N fertilization had a significant impact on the abundance of different groups in the organic layer of the cedar stands, but not in the organic layer of the hemlock stands (p=0.0326, A= 0.0246). N fertilization reduced the abundance of fungal PLFA (Fig 4.27, Table 4.11) and increased the total bacterial PLFA (Fig 4.28, Table 4.12) in the organic layer of the cedar stands.

Similar responses were also found in the organic layer of the hemlock stand, though they were not significant (Table 4.11 - 4.12). P fertilization on the other hand, significantly reduced the abundance of AM fungal PLFA in the organic layer of the hemlock stands ($p = 0.047$, $A= 0.06$) (Fig 4.29, Table 13). The abundances of Gram-positive bacteria, Gram-negative bacteria and actinomycetes were not significantly affected by fertilization in individual soil layers (Figs 4.30-4.32).

Table 4.11 Abundance of total fungi (fungi%) and AM fungi (AM fungi%) in the organic layer of western red cedar and western hemlock stands following fertilization with three levels of N. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, O- organic layer, C- western red cedar stands, H- western hemlock stands)

N level	OC Fungi%	OC AM Fungi%	OH Fungi%	OH AM Fungi%
0	0.0634(0.0062)a	0.0410(0.0057)	0.0635(0.0042)	0.0265(0.0012)
200	0.0523(0.0017)b	0.0394(0.0046)	0.0535(0.0053)	0.0260(0.0016)
300	0.0473(0.0075)ab	0.0401(0.0036)	0.0554(0.0041)	0.0259(0.0017)

Note: *Values are means (n=9) with one standard error given in parentheses. Means with the same letter were not significant different (p>0.0167= 0.05/3) (comparison is between three N levels).*

Table 4.12 Abundance of total bacteria (bacteria%) in the organic layer of western red cedar and western hemlock stands following fertilization with three levels of N. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1} , 300=300 kg ha^{-1}, O- organic layer, C- western red cedar stands, H-western hemlock stands)

N level	OC Bacteria%	OH Bacteria%
0	0.5928(0.0154)b	0.5836(0.0089)
200	0.6127(0.0079)ab	0.6028(0.0102)
300	0.6420(0.0120)a	0.5932(0.0102)

Note: *Values are means (n=9) with one standard error given in parentheses. Means with the same letter were not significant different (p>0.0167= 0.05/3) (comparison is between three N levels).*

Table 4.13 Abundance of AM fungi (AM Fungi%) and total fungi (Fungi%) in the organic layer of western red cedar and western hemlock stands following fertilization with P. (P-phosphorus, 0=0 kg ha^{-1}, 100-100 kg ha^{-1}, O- organic layer, C- western red cedar stands, H- western hemlock stands)

P level	OC Fungi%	OC AM Fungi%	OH Fungi%	OH AM Fungi%
0	0.0564(0.0045)	0.0412(0.0062)	0.0539(0.0051)	0.0283(0.0012)a
100	0.0542(0.0047)	0.0397(0.0027)	0.0592(0.0031)	0.0248(0.0010)b

***Note**: Values are means (P0 group size n_1 = 9, P100 group size n_2 =18, unbalanced design) with one standard error given in parentheses. Means without letter were not significant different (p>0.05) (the comparison is between two P levels).*

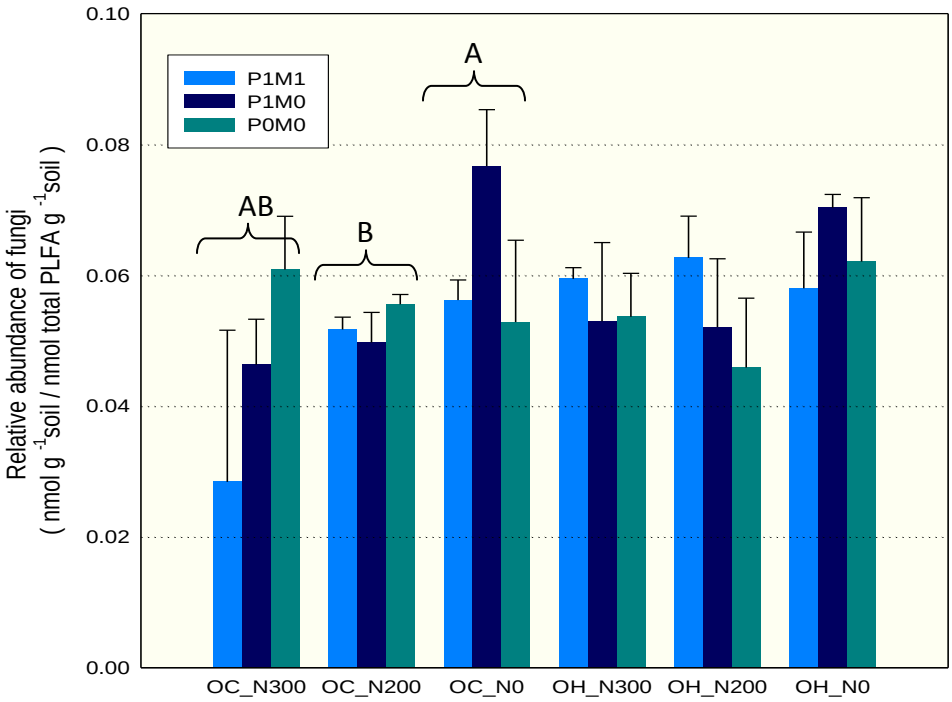

Figure 4.27 Mean abundance of total fungi (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

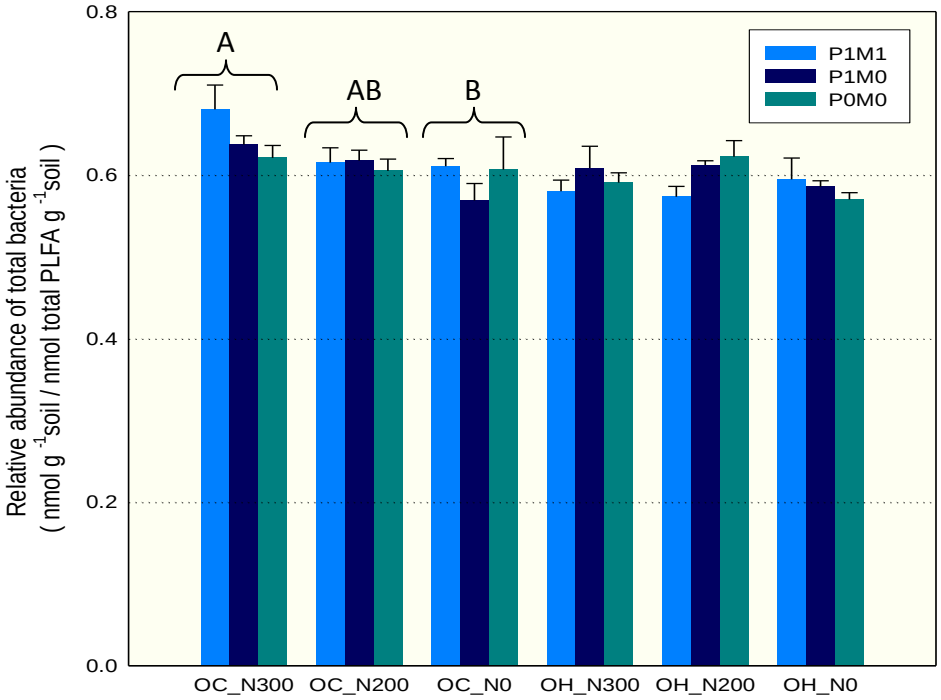

Figure 4.28 Mean abundance of total bacteria (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different (p<0.0167=0.05/3).

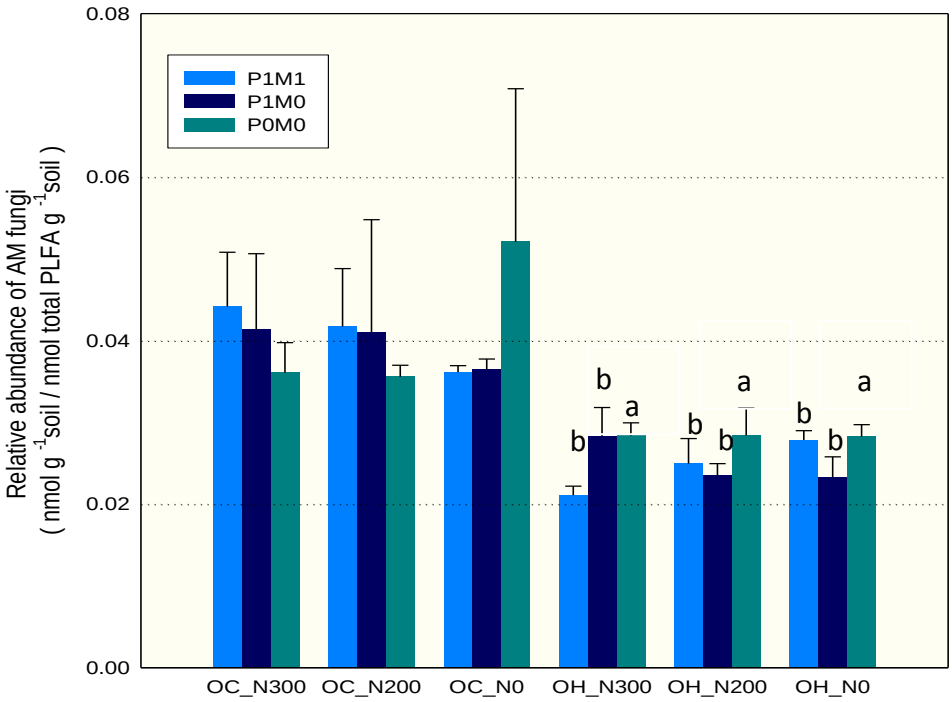

Figure 4.29 Mean abundance of AM fungi (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors with different letters are significantly different.

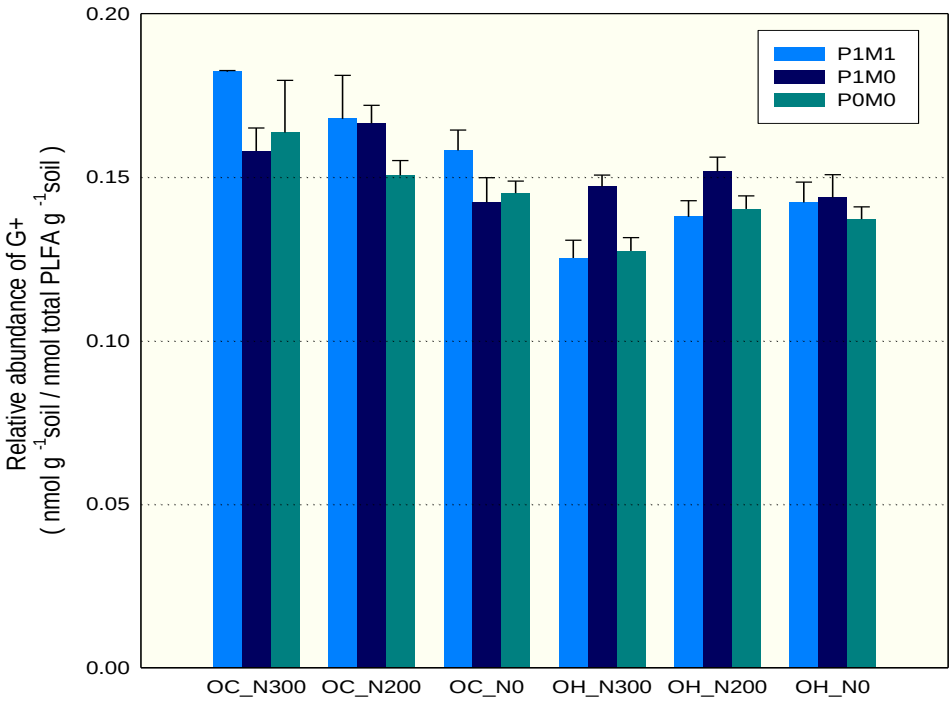

Figure 4.30 Mean abundance of Gram-positive bacteria (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

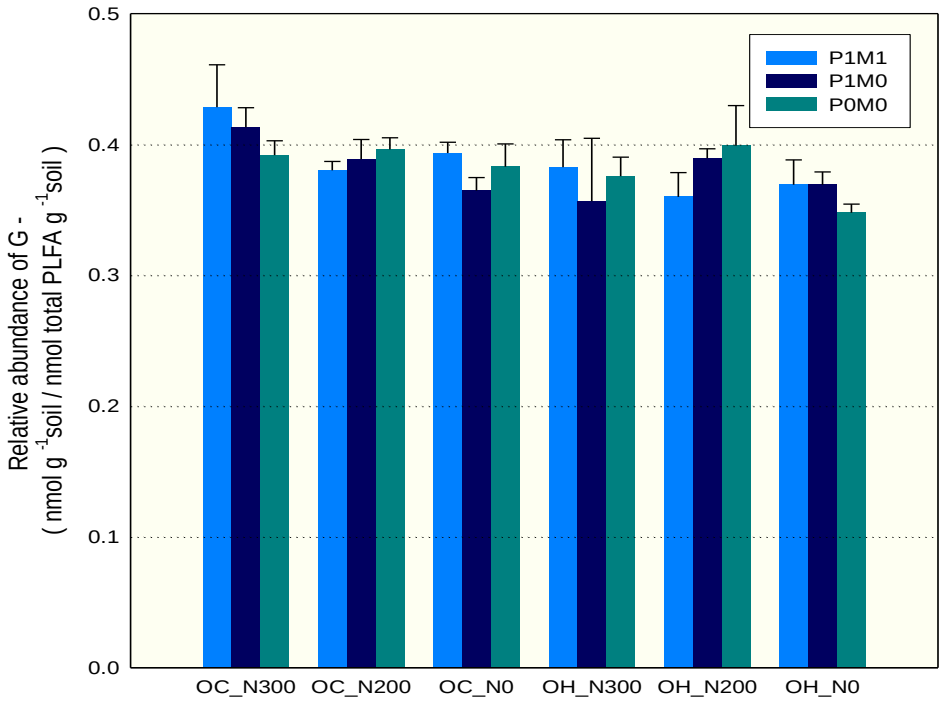

Figure 4.31 Mean abundance of Gram-negative bacteria (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

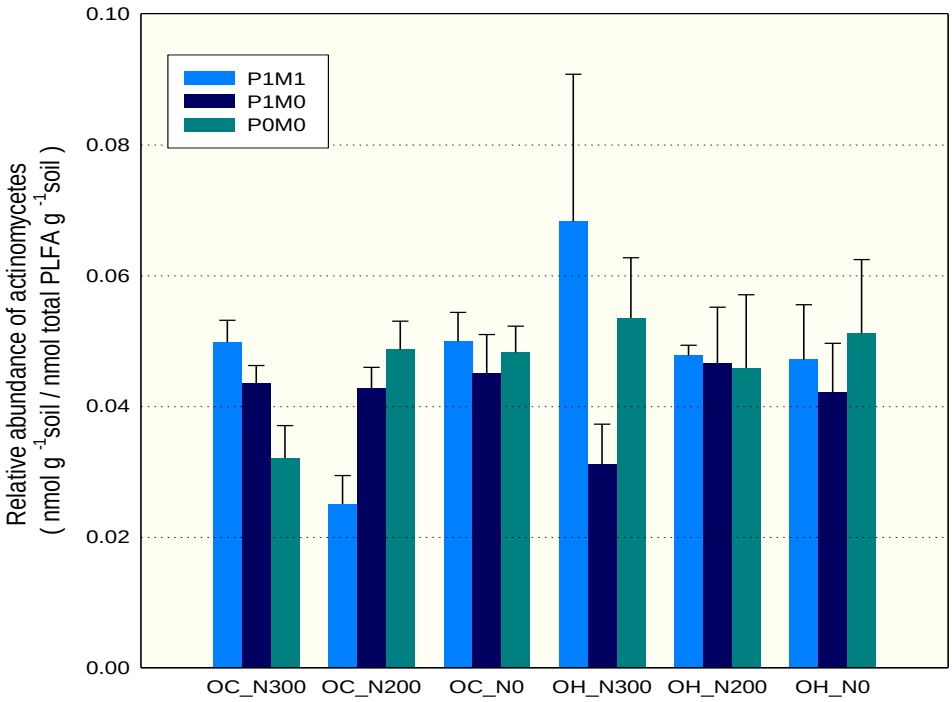

Figure 4.32 Mean abundance of actinomycetes (nmol g $^{-1}$ soil / nmol total PLFA g $^{-1}$ soil) of the western cedar organic layer (OC) and western hemlock organic layer (OH) in nine fertilization treatments. (N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}). Values are means of 3 samples. Error bars indicate standard errors.

4.2.6 Correlations between Environmental Variables, Microbial Biomass, Enzyme Activities and PLFAs

There were strong correlations between soil moisture and many of the microbial variables measured in the organic layers of the two forest stands. Microbial N, NAGase activity,

abundance of Gram-negative, Gram-positive and total bacteria were negatively correlated with moisture, while the abundance of total fungi was positively correlated with moisture (Table 4.14 - 4.15). In addition, the abundance of Gram-negative, Gram-positive and total bacteria, and phenol oxidase activity were found to be negatively correlated with NO_3^- concentration, while microbial biomass P was positively correlated with NO_3^- concentration. Moreover, pH was positively correlated with phosphatase activity and microbial biomass N, while NH_4^+ was positively correlated with only the abundance of actinomycetes (Table 4.14 - 4.15).

There were strong correlations amongst many of the microbial community structure variables measured in the organic layers of the two forest stands. Microbial biomass N, abundance of total bacteria, Gram-negative bacteria and Gram-positive bacteria were all positively correlated with each other and negatively correlated with abundance of total fungi. AM fungi on the other hand, only showed strong negative correlation with the abundance of actinomycetes (Table 4.16). Correlations between microbial community function and structure variables were weak, with only one detected negative correlation between peroxidase activity and the abundance of Gram-positive bacteria (Table 4.17). However, significant correlations were found amongst functional variables in the organic layer. β-glucosidase, cellobiosidase, NAGase and phenol oxidase activity were all positively correlated. Peroxidase activity was positively correlated with phosphatase activity (Table 4.18).

Table 4.14 Spearman's rank correlations between measured biological variables and environmental variables in organic layer. Numbers in bold indicate strong correlations (p>0.25).

Measure variable	NH_4^+	NO_3^-	pH	Moisture%
Microbial C	0.0786	-0.2143	0.1379	-0.013
Microbial N	-0.2535	-0.0784	**0.3442**	**-0.4074**
Microbial P	-0.2263	**0.2988**	-0.0644	0.0896
Cellobiosidase	-0.1166	0.1089	-0.1712	-0.1532
NAGase	-0.1738	-0.2151	0.0685	**-0.459**
Glucosidase	-0.0871	0.0632	-0.0558	-0.0876
Phosphatase	0.0309	-0.2329	**0.2731**	-0.1226
Peroxidase	-0.0683	-0.0669	0.0838	-0.0839
Phenol oxidase	-0.0916	**-0.2854**	-0.0857	-0.2267

Table 4.15 Spearman's rank correlations between measured environmental variables and PLFA signatures in organic layer. Numbers in bold indicate strong correlations (p>0.25).

	NH_4^+	NO_3^-	pH	Moisture%
G-%	-0.0902	**-0.4495**	0.132	**-0.4665**
G+%	-0.162	**-0.2882**	-0.0881	**-0.4337**
Actinomycetes%	**0.2826**	0.1756	-0.1666	0.0196
Total bacteria%	-0.1538	**-0.2967**	0.0205	**-0.5513**
AM fungi%	-0.0069	-0.1793	-0.0241	0.0414
Total fungi%	-0.0027	0.2455	-0.2354	**0.4597**

Table 4.16 Spearman's rank correlations between microbial C (MB-C), N (MB-C), P (MB-P) and PLFA signatures in the organic layer. Numbers in bold indicate strong correlations (p>0.25).

	Actinomycete	G-	G+	Total bacteria	Total fungi	AM fungi	MB-C	MB-N	MB-P
Actinomycetes	1.00	-0.11	-0.10	-0.14	-0.16	**-0.28**	0.08	-0.18	0.09
G-		1.00	**0.35**	**0.82**	**-0.62**	0.15	0.11	**0.31**	-0.19
G+			1.00	**0.61**	**-0.32**	0.29	0.10	**0.29**	-0.25
Total bacteria				1.00	**-0.73**	0.23	0.12	**0.38**	-0.23
Total fungi					1.00	-0.12	-0.13	**-0.45**	0.20
AM fungi						1	-0.10	0.13	-0.15
MB_ C							1	**0.27**	0.09
MB_ N								1.00	-0.04

Table 4.17 Spearman's rank correlations between enzymes and PLFA signatures in the organic layer. Numbers in bold indicate strong correlations (p>0.25).

	Cellobiosidase	**Glucosidase**	**NAGase**	**Peroxidase**	**Phenol oxidase**	**Phosphatase**
Actinomycetes	0.04	0.07	0.06	0.10	-0.13	-0.03
AM Fungi	-0.07	-0.04	-0.25	-0.23	0.13	-0.22
Total fungi	0.02	0.03	-0.24	-0.24	-0.04	-0.14
G-	0.09	0.05	0.22	0.18	0.23	0.23
G+	0.06	0.14	0.12	**-0.33**	0.14	-0.12
Total bacteria	0.12	0.08	0.26	0.03	0.18	0.13

Table 4.18 Spearman's rank correlations between individual enzyme activities. Numbers in bold indicate strong correlations (p>0.25).

	Glucosidase	NAGase	Cellobiosidase	Phosphatase	Peroxidase	Phenol oxidase
Glucosidase	1.00	**0.44**	**0.78**	0.17	-0.07	**0.36**
NAGase		1.00	**0.33**	**0.28**	0.15	**0.33**
Cellobiosidase			1.00	0.07	-0.03	**0.42**
Phosphatase				1.00	**0.40**	-0.03
Peroxidase						-0.03

5 Discussion

5.1 Effect of Soil Layer on Measured Variables

Hypothesis 1: Organic and mineral layers will have distinct microbial biomass and enzyme activities, as they provide different substrates and microenvironment to microbes.

Hypothesis 1a: Microbial biomass and hydrolytic enzyme activities will decrease with depth, while lignin degrading enzyme activities will increase with depth.

Hypothesis 1 was partially supported since most of the measured variables were significantly different between mineral and organic layers (Table 4.1). The decreases in moisture, NO_3^- and NH_4^+ availability, microbial biomass P, as well as enzyme activities (except for peroxidase) in deeper soil layers are consistent with findings from the other studies (Table 4.2)(Leckie et al. 2004b; Bengtson et al. 2007). Previous studies have associated these decreases in microbial biomass and enzyme activities with changes in pH, moisture, temperature, microenvironment or the quantity and quality of carbon and nutrient sources (Kelliher et al. 2004; Leckie et al. 2004b; Bengtson et al. 2005). In this study, hydrolytic enzyme activities had neutral or even negative correlations with moisture in the organic soil layer (Table 4.14). Since the mean moisture content in the organic layer was 245% (dry weight basis), close to the value Prescott (in press) suggested as a tentative threshold for decomposition effects [30% and 80% (wet weight basis)], this suggests that in our study sites during the rain fall events,

high moisture content may limit microbial activities in the organic soil layer, and carbon and other nutrient sources would become more important once the primary constraints are relieved (Kelliher et al. 2004; Bengtson et al. 2005; Pietikainen et al. 2005).

Our hypothesis 1a was partially supported, as peroxidase activity was significantly higher in mineral soil layers (Table 4.1, 4.3), however the variation among replicates was large, making it difficult to make sound interpretation of these patterns. Brockett (2008) also found peroxidase activity increased with depth (Brockett et al. 2008). This could be attributed to increased recalcitrant materials in deeper soil layer, which provide more substrates for peroxidase. In addition, we did not find significantly higher microbial C in organic soil layers than in mineral layers, but found significantly higher microbial N in mineral soil layers (Table 4.1, 4.3). These were contradictory to other studies, as was the expectation that overall biomass would decrease with depth (Leckie et al.2004b; Bengtson et al.2007). However, Leckie et al. (2004b) had found that fungal to bacterial ratios decreased with depth across a range of ecosystems. Since bacteria are generally richer in protein and have a lower C/N ratio than fungi, decreased fungal to bacterial ratios may contribute to the higher microbial biomass N in the mineral layer.

5.2 Effect of Forest Type on Measured Variables

Hypothesis 2: Cedar and hemlock stands will have distinct microbial activities and composition due to the effect of tree species.

Hypothesis 2a: Cedar stands will have a higher bacterial and AM fungal abundance than hemlock stands.

Our hypothesis 2 was supported since total enzyme activities and total PLFAs were significantly different in cedar and hemlock forests (Table 4.1). The difference in microbial community composition (Table 4.4), i.e. higher total bacteria, higher AM fungal abundance and narrower fungi abundance in cedar forest floors, was consistent with our hypothesis 2a, and with findings from other studies (Turner and Franz 1985; Grayston and Prescott 2005). In the previous studies, the difference in microbial composition was associated mainly with moisture and pH. Higher moisture content in cedar forest floors may favor water stress-tolerant bacteria, and high Ca content in cedar litter may contribute to the higher pH in cedar forest floors, which would favor the growth bacteria over fungi (Frostegård and Baath 1996; Killham 2001; Grayston and Prescott 2005; Prescott and Vesterdal 2005). However, moisture and pH did not explain the difference in composition in this study, as both moisture and pH in cedar stands were slightly lower than hemlock stands (Table 4.4), and moisture was negatively correlated with Gram-negative bacteria, Gram-positive bacteria, and total bacteria in organic layers (Table 4.15).

This suggested that the influence of trees species on forest floor chemistry could be highly site-specific, and that direct impacts from the trees (e.g. litter, root exudates), rather than indirect impacts (e.g. changes in soil pH), may be responsible for the specificity and resiliency of microbial communities associated with cedar stands.

Distinct enzyme activity patterns in the two forest types may be related to the difference in microbial community composition, although it is not clear which microbial groups had caused changes in enzyme activity. For example, higher phenol oxidase activity and lower fungal abundance in cedar stands (Table 4.4) were contradictory with our expectation that lower fungal abundance may lead to lower phenol oxidase activity, and suggested that other factors, such as broader C:N ratio and high lignin content of cedar foliar litter(Prescott 2005, Prescott and Vesterdal 2005), might have contributed to the lower phenol oxidase activity.

5.3 Effect of Fertilization on Measured Variables

Hypothesis 3: N fertilization will increase bacterial and decrease fungal abundance.

N fertilization showed a significant effect on microbial community composition, including increases in abundance of Gram-negative bacteria and total bacteria, and decrease in abundance of fungi, which supported our hypothesis (Table 4.2, 4.5, 4.6, 4.11, 4.12, Fig 4.27, 4.28). Decreases in fungal biomass after N addition have been found in many forest soils

(Treseder 2008). Decreased microbial growth after N addition was often attributed to N toxicity, decreased soil pH, decreased ligninase activity, increased melanoidins, or decreased belowground net primary production (NPP) (Treseder 2008). In this study, since N availability in these sites was relatively low, and N did not have a significant effect on pH and lignin-degrading enzyme activities, the decrease in abundance of fungi is more likely related to the increased melanoidins, decreased belowground NPP, or the increased resource competition from the bacterial community.

The increase in abundance of bacteria on the other hand, may be attributed to increased labile carbon from aboveground NPP, or increased N availability from both N fertilization and increased litter quality (Treseder 2008). Contradictory to a recent meta-analysis that found an average 15% decline in microbial biomass after N additions, there was no decrease in total microbial biomass in our study, which is likely because the increase in bacterial community had offset the decrease in fungal community.

Hypothesis 4: N fertilization will stimulate cellobiosidase, glucosidase and phosphatase activities, but suppress NAGase, phenol oxidase and peroxidase enzyme activities.

Hypothesis 5: The effect of N fertilization on these enzyme activities will be less pronounced in plots that also receive P and/or micronutrients, in particular, P fertilization will suppress phosphatase activity.

Contradictory to our hypothesis 4, fertilization did not have a significant effect on microbial biomass or most of the enzyme activities, except for phosphatase (Table 4.1, 4.5, 4.6, 4.9, Fig 4.23, 24). The increase in phosphatase activity after N fertilization is consistent with other studies (Olander and Vitousek 2000; Wang et al. 2008; Keeler et al. 2009). Previous researchers suggested that the addition of N in N-limited systems may have stimulated microbial growth and increased the demand for P, which led to stimulation of P-acquiring enzymes. In addition, the trend of decreased phosphatase activity after P fertilization (Table 4.10, Fig 4.23), supported our hypothesis 5 and could be attributed to the inhibition of phosphatase synthesis by increased availability of inorganic P (Olander and Vitousek 2000; Wang et al. 2008; Keeler et al. 2009). Alternatively, decreased phosphatase activity following P fertilization in this study may be related to the reduction in AM fungi after P fertilization.

There was little evidence of suppression of NAGase activity following N fertilization in our study. Enowashu et al. (2009) conducted a N reduction study in a 75-year-old Norway spruce

plantation through establishing “clean rain” plots, where through-fall was collected and deionised to pre-industrial level. They found mixed responses of N-cycling enzymes to reduced N treatment in a Norway spruce forest, in particular, a trend of lower NAGase activity or no significant reaction under N reduction treatment. They attributed this mixed responses to different turnover times of peptides and different microbial acquisition of organic N compounds.

Contradictory to several previous studies that showed significant negative effects of N on lignin-degrading enzymes (Carreiro et al. 2000; Frey et al. 2004), peroxidase activity increased significantly with N fertilization in the organic layer of the western hemlock stands (Fig 4.25). However, there were large variations in peroxidase and phenol oxidase activities among replicates (Figure 4.21, 4.22, 4.25, 4.26). Other studies have also reported neutral or positive effects of N fertilizer/deposition on enzymes involved in lignin degradation (Michel and Matzner. 2003; Enowashu et al. 2009), as well as low or undetectable level of phenol oxidase activity. The authors suggested that more temporally intensive sampling is required to elucidate the reasons (Sinsabaugh et al. 2005; Finzi et al. 2006; Sinsabaugh et al.2008; Keeler et al. 2009). These effects maybe attributed to microbes that regulate the production of lignin-degrading enzymes, but do not respond negatively to N addition. It could also due to the effects of other site-specific environmental variables, which may outweigh the effect of N

availability (Knorr et al. 2005; Prescott 2005), or because inhibition of lignin-degrading enzymes following fertilization is relatively short-term and the effects may have disappeared 10 years after the initial treatment.

Hypothesis 6: Microbial communities will respond to fertilization differently in the different forest stands and soil layers if hypothesis 1 and 2 are not rejected.

Hypothesis 6a: Microbial communities in hemlock stands will be more sensitive to P fertilization since P is an important limiting factor for hemlock productivity on these sites.

Consistent with our hypothesis 6, the effect of fertilization on microbial communities was more pronounced in the forest floor than at depth in soil. The different responses in two forests on the other hand, varied mostly in the degree of response (whether it was significant or insignificant), not in the pattern of response (increased or decreased) (Table 4.9 - 4.13). In addition, consistent with hypothesis 6a, the inhibition of P fertilization on phosphatase activity was more profound in hemlock stands than in cedar stands (Table 4.10, Fig 4.23-24). Belvins et al. (2006) found hemlock stands had greater responsiveness to P fertilization. This suggests that P is an important limiting factor for hemlock on these sites.

5.4 Correlations between Measured Variables

Hypothesis 7: There will be strong correlations between measured biological variables and environmental variables.

Hypothesis 7 is supported as physiochemical soil properties appeared to correlate closely with microbial community structure (i.e. abundance of fungi, bacteria, actinomycetes)(Table 4.15). The importance of physiochemical soil properties such as moisture, pH and nutrient concentrations in shaping microbial community structure has been found in various studies (Högberg et al. 2007; Lauber et al. 2008). Lauber et al. (2008) found that bacterial community composition was closely correlated with soil texture and pH, while fungal community composition was most sensitive to soil nutrient availability. Hogberg et al. (2007) found that soil pH and C-to-N ratio, along with the response of trees to these two factors had a strong influence on soil microbial community structure and activity.

Both moisture and NO_3^- had a negative correlation with the abundance of Gram-positive, Gram-negative and total bacteria (Table 4.15). This finding was contradictory to the expectation that higher N availability would favor bacteria over fungi, and that bacteria, in particular Gram-positive bacteria, would have strong water stress tolerance (Grayston and Prescott. 2005; Högberg et al. 2007). Dimitriu and Grayston (2009) quantified bacterial compositional diversity patterns in ten boreal forest sites and found that the *α-Proteobacteria* that dominated most

sites decreased with increasing moisture content. Therefore, the negative correlations between moisture and the bacterial abundance maybe attributed to temporary high water stress from heavy rainfall during the sampling period.

The strong positive relationships between glucosidase, cellobiosidase and NAGase enzyme activities and strong negative relationship between Gram-negative bacteria and peroxidase activity were consistent with previous studies (Table 4.17-18), but the overall correlation patterns between PLFA signatures, enzyme activities and total biomass have been inconsistent (Brockett 2008; Dewi 2009). There are several possible explanations for such inconsistency. First of all, some enzymes are produced by a very specific group of microorganisms, such as white rot basidiomycete fungi and their production of lignin degrading enzymes (Keeler et al. 2009), whereas other enzymes such as phosphatase could also be secreted by plants and soil fauna (Tabatabai and Dick 2002; Grayston and Prescott 2005), therefore these enzymes may not correlate well with total biomass. Likewise, many of the fatty acids are also unspecific. For example, the PLFA 18:2ω6,9, a commonly used marker for fungi, was also commonly found in soil fauna and eukaryotes(Zelles 1997; Ruess et al. 2002; Grayston and Prescott 2005). Secondly, similar to physiochemical soil properties, the distribution of microorganisms and enzymes could vary both spatially and temporally in forest ecosystems (Bengtson et al. 2007; Lauber et al. 2008). It is possible that certain group of microorganisms may have high turnover rate over

certain time period, or are metabolically inactive, while the enzymes they produced can persist in soil long after the death of the producing cell (Šnajdr et al. 2008). Thirdly, disturbances such as heavy rainfall, harvesting, or even soil sampling may alter the spatial distribution of microbial hotspots, and therefore obscure the real correlation patterns.

5.5 Study Limitations

5.5.1 Experimental Design and Sampling

Two of the weaknesses of this study were its complex unbalanced design with five control factors and non-parametric data. Together they made it difficult to analyze with MRPP and related techniques. As recommended by McCune et al. (2002), the data was analyzed piecewise with MRPP and was sliced in various ways to answer different questions regarding soil layer, forest types and fertilization effects. However, this approach sacrificed the ability to analyze interaction terms, and very often resulted in relative small A values (< 0.1). In addition, there were a few rainfall events occurred that during the sampling period, which might have affected our measured microbial variables.

5.5.2 Limitations in Enzyme Assays and PLFA Analysis

One of major challenges in enzyme assays and PLFA analysis is to ascribe microbial processes/components to specific microorganisms, since there are huge uncertainties associated with large numbers of unspecific fatty acids, as well as enzymes and fatty acids that

could be found in microbes, soil fauna and plants (Zelles 1997; Ruess et al. 2002; Tabatabai and Dick 2002; Marschner 2007). In addition, potential enzyme activities were measured using artificial substrates at saturating concentration and under optimal pH, temperature and moisture conditions, which may not represent the *in situ* activities (Caldwell 2005). Moreover, only six enzyme activities were measured in this study, which might represent only a small portion of total activities that produced by a large number of enzymes in the soil (Caldwell 2005). Lastly, PLFA soil samples in this study were stored for about a year before lab analysis. Such prolonged storage time might have reduced PLFA concentrations and altered PLFA profiles (Wu et al. 2009).

5.6 Recommendations for Future Studies

Experimental design and lab assays:

- Avoid unbalanced multi- factorial design by assigning equal number of replicates to each tested factor.
- Use more natural substrates for measuring enzyme activities, such as phytates or nucleic acids for phosphastase, would to better understand *in situ* activity patterns (Caldwell 2005).
- Avoid prolonged storage of PLFA samples.

Long term and collaborative research:

- Combine enzyme imprinted system (Dong et al. 2007) and labeling technique (Boschker et

al. 1998; Treonis et al. 2004) to identify spatial and temporal variances of enzyme activities in soil, trace above-below ground resource movement, and link microbial function and structure *in situ.*

- Link effect of fertilization on decomposition and carbon sequestration processes with other factors that would also affect green house gas emissions related with forestry, such as the emission of N_2O and oxidation of CH_4, shorter rotation length that leads to reduced total forest ecosystem carbon (Seely et al. 2002), energy costs for producing fertilization, implementing silviculture treatments and processing wood products.
- Instead of asking questions such as "should residues be removed for biomass burning to offset fossil fuel combustion if they have no long-term positive effect on mineral soil C?" or "how do C and economic costs of fertilization compare with the costs of leaving residues on site?" (Johnson and Curtis 2001), researchers should resist the dangerous allure of global warming technofixes and seek to understand intertwined underlying mechanisms. Only when we better understand the complex relationships between ecosystem components will we be able to assess the effects of management practices on the ability of forest ecosystems to mitigate climate change.

6 Conclusions

Soil layer had the largest effect on microbial and soil chemical variables, followed by forest type, and fertilization. Moisture, NH_4^+ and NO_3^- availability, microbial biomass P and most of the enzyme activities (except for peroxidase and phenol oxidase) decreased significantly with depth. Cedar and hemlock stands had significantly different enzyme activities and microbial composition. More specifically, bacteria and AM fungal PLFAs were more abundant in cedar stands than in hemlock stands, while phosphatase activity was significantly higher in hemlock stands.

Fertilization had a significant long-term effect on microbial community composition. N fertilization significantly increased the abundance of bacterial PLFA and reduced the abundance of fungal PLFA, while P fertilization significantly reduced the abundance of AM fungal PLFA in the organic layer of the hemlock stands. On the other hand, the responses of enzyme activities to fertilization observed in other studies were not apparent in this study and so would appear to be short-term in nature, except for phosphatase activity. The stimulatory effect of N fertilization and inhibitory effect of P fertilization on phosphatase activity were apparent 10 years after fertilization.

Microbial communities responded to fertilization differently in different forests and soil layers, although the communities varied mostly in the degree of response (whether it was

significant or insignificant), not in the pattern of response (increased or decreased). The effect of fertilization on microbial communities was more pronounced in the forest floor than at depth in the soil. Microbial communities in hemlock stands were more sensitive to P fertilization than those in cedar stands.

Strong correlations were found between microbial variables and environmental variables, especially between moisture and microbial variables. On the contrary, correlations between microbial community structure and function were weak.

This study provided a comparison of microbial communities and their enzyme activities related to the cycling of C and N in fertilized and unfertilized plots of two coniferous forests. Enzyme activities measured under optimal pH and moisture conditions provided good comparisons among samples, but may not reflect actual activities in the field. In addition, this study provided information about the variability of microbial community in two forest stands and soil layers, and identified how this variability would affect long-term microbial response to fertilization. Results from this study stress not only the importance of conducting long-term and *in situ* research to understand microbial processes at a fundamental level, but also the importance of examining the implications of this research in a bigger context by linking them with other factors such as net carbon lost related to producing and applying fertilizer, shorter

rotation time that would reduce total forest ecosystem carbon, and greenhouse gas emissions related to fertilization and increased forest harvesting.

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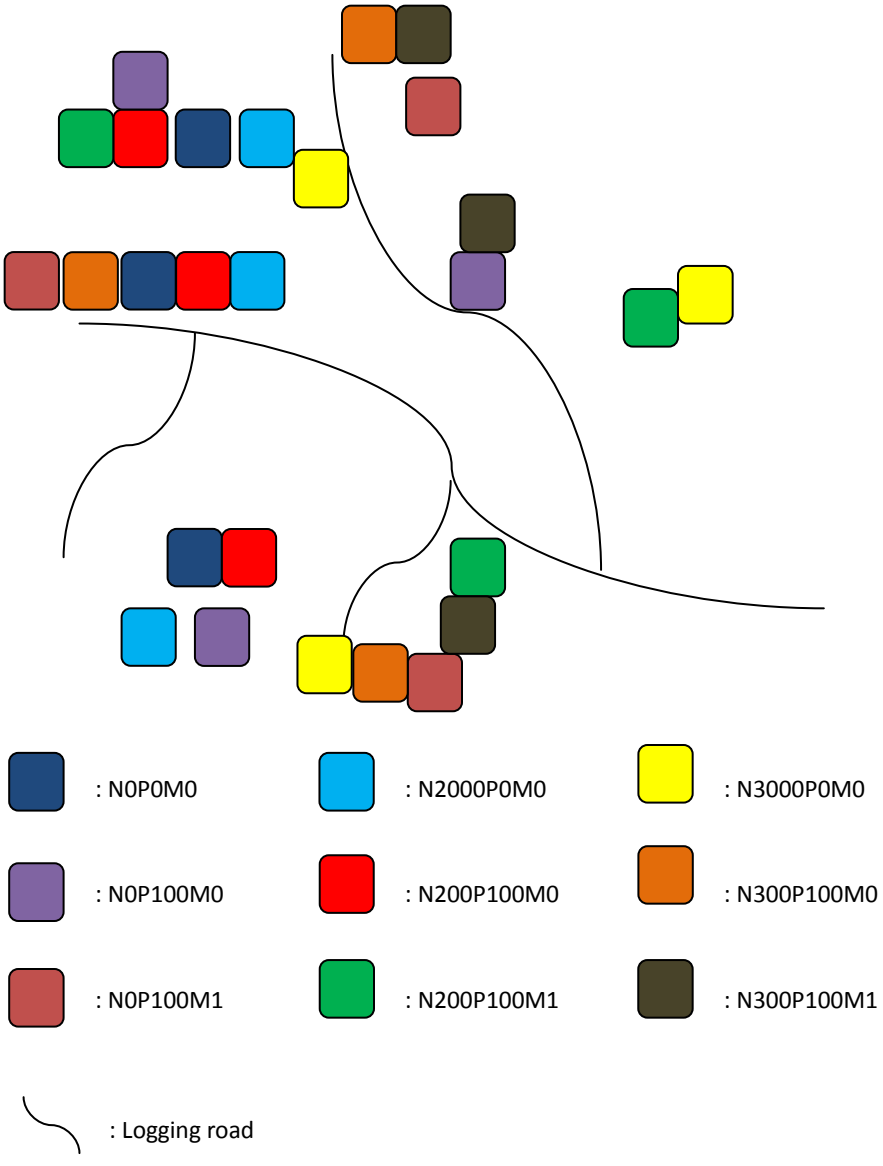
Appendices

Appendix 1. Map of Sample Plots of Random Block Design Treatments.



Note: Plots were 25m in diameter. N-nitrogen, 0=0 kg ha^{-1}, 200=200 kg ha^{-1}, 300=300 kg ha^{-1}, P-phosphorus, M-micronutrients, 0=0 kg ha^{-1}, 1=100 kg ha^{-1}

Appendix 2. MRPP Statistic for Effects of Nine Fertilization Treatments on Phosphatase Activity

********** MRPP finished **********

Phosphatase_Treatment_Sorensen
Groups were defined by values of: Treatment

Input data has: 108 Samples by 1 soil var

Weighting option: C(I) = n(I)/sum(n(I))

Distance measure: Sorensen (Bray-Curtis)

Chance-corrected within-group agreement, A = 0.03529000

A = 1 - (observed delta/expected delta)

Amax = 1 when all items are identical within groups (delta=0)

A = 0 when heterogeneity within groups equals expectation by chance

A < 0 with more heterogeneity within groups than expected by chance

Probability of a smaller or equal delta, p = 0.04216844

PAIRWISE COMPARISONS

Note: p values not corrected for multiple comparisons.

Group	Codes	Compared	T	A	p
1	vs.	2	0.01050601	-0.00037225	0.36413698
1	vs.	3	0.48357494	-0.01661752	0.59546196
1	vs.	4	-1.16102021	0.04123012	0.11160046
1	vs.	5	0.03558024	-0.00116123	0.38630996
1	vs.	6	0.57499877	-0.01829378	0.65550782

Group Codes Compared			T	A	p
1	vs.	7	-2.45213205	0.07801525	0.03120152
1	vs.	8	-1.40599153	0.04581673	0.08859901
1	vs.	9	-0.62293760	0.01902805	0.19620640
2	vs.	3	0.66460594	-0.02085929	0.71262707
2	vs.	4	-3.10609355	0.10293664	0.01727407
2	vs.	5	-0.75833151	0.02269792	0.18096536
2	vs.	6	0.30435288	-0.00894670	0.52517169
2	vs.	7	-3.38057165	0.10792757	0.01141267
2	vs.	8	-2.73313009	0.08551359	0.02514585
2	vs.	9	-1.75946251	0.04862581	0.06322077
3	vs.	4	-1.16499032	0.03643936	0.11473950
3	vs.	5	0.73865475	-0.02288993	0.75452600
3	vs.	6	0.59895396	-0.01726581	0.67628776
3	vs.	7	-1.36995094	0.04130262	0.09427389
3	vs.	8	-1.20938840	0.03378276	0.11086388
3	vs.	9	0.30456012	-0.00853453	0.51629257
4	vs.	5	-0.52583146	0.01582041	0.22876991
4	vs.	6	-2.39664469	0.07126730	0.03284106
4	vs.	7	0.36767366	-0.01126650	0.53501117
4	vs.	8	0.83711341	-0.02249659	0.81382057
4	vs.	9	0.61954131	-0.01697648	0.68544051
5	vs.	6	0.33194936	-0.01004014	0.52850457

Group Codes Compared			T	A	p
5	vs.	7	-1.00836837	0.02905657	0.14310997
5	vs.	8	-0.36737204	0.01026074	0.26860851
5	vs.	9	0.80985329	-0.02390798	0.79717665
6	vs.	7	-3.23522157	0.08969318	0.01143703
6	vs.	8	-2.27334291	0.06368211	0.03709056
6	vs.	9	-0.63526141	0.01808242	0.20866398
7	vs.	8	-0.04804682	0.00127705	0.37089122
7	vs.	9	0.41580305	-0.01195713	0.56153459
8	vs.	9	0.53088416	-0.01303836	0.64098822

Group 1: N - 0 Kg ha^{-1}, P - 0 Kg ha^{-1}, Micro – without micronutrient

Group 2: N - 0 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro - without micronutrient

Group 3: N - 0 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro – with micronutrient

Group 4: N - 100 Kg ha^{-1}, P - 0 Kg ha^{-1}, Micro - without micronutrient

Group 5: N - 100 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro – without micronutrient

Group 6: N - 100 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro - with micronutrient

Group 7: N - 200 Kg ha^{-1}, P - 0 Kg ha^{-1}, Micro – without micronutrient

Group 8: N - 200 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro - without micronutrient

Group 9: N - 200 Kg ha^{-1}, P - 100 Kg ha^{-1}, Micro – with micronutrient