Chronic N and nutrient deposition impact on diversity and abundance of bacteria, fungi, and CH_4 cycling prokaryotes at a northern peat bog

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II. Abstract

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Anthropogenic N deposition may threaten peatland carbon storage capabilities. Increased deposition has been associated with plant community shifts and increased decomposition rates, potentially via the disruption of natural microbial communities. By examining peat soils from randomized, replicated treatment plots in a long-term simulated chronic nitrogen deposition experiment at the Mer Bleue Bog, my objective was to characterize how increased deposition impacts diversity and abundance of broad groups of microbial decomposers, and specific CH_4 cycling prokaryotes. Using fingerprinting approaches and qPCR of SSU rRNA and other functional genes, my data show that with increasing nutrient loading the bacterial and fungal community structure changed. Along the same gradient methanogen abundance decreased, however there were no corresponding changes in methanotroph community structure or abundance. My results provide new insight on the possible causes of higher CO_2 and CH_4 effluxes seen in situ following chronic nutrient loading.

Keywords

Peatland, Mer Bleue, nitrogen deposition, microbial community, bacteria, fungi, T-RFLP, qPCR, pyrosequencing, *pmoA, mmoX, mcrA,* methanotroph, methanogen, methane monooxygenase

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1 Introduction

Northern peatlands are important long term carbon (C) sinks as a result of a net primary production exceeding organic matter decomposition. In fact, peatlands covers only 3% of Earth surface, and have stored approximately one third of atmospheric C (approx. 450 Gt) in Earth's soils since the beginning of the Holocene; with long-term rate of C accumulation of up to 30 g C m^{-2} yr^{-1} (Gorham, 1991; Turunen et al., 2002). Climate and hydrological factors contribute to this net imbalance between peat production and decomposition. Peatlands are generally wet, creating an anoxic layer in the soil profile depleting election acceptors and promoting fermentation reactions and methanogenesis as the slow drivers of decomposition. Soils are typically cool at depth even in the growing season, which keeps metabolic rates low. Thus, peatlands play important roles in the global climate system through the exchange of both CO_2 and CH_4 The past and current function as C sinks is uncertain with current direct anthropogenic effects via drainage or mining, or indirect effects through broader climate (drought, warming, higher CO_2 concentrations) and other environmental changes including atmospheric pollution deposition (Limpens et al., 2011). Emissions of reactive nitrogen (N) (i.e. NO_x and resulting HNO_3, and NH_3^+) due to fossil fuel combustion and agricultural intensification have disrupted the input of available N to ecosystems in general: anthropogenic production has increased 9 fold in the last century (Galloway and Cowling, 2009). N and nutrient availability in peatlands may increase in other ways as well: while greenhouse gases rise, increases in temperature and altered precipitation can speed decomposition of stored organic matter and release N and other nutrients in available forms in the soil profile. This temperature effect has been shown to be comparable to an experimental application of almost 4 g N m^{-2} yr^{-1} for each 1°C increases (Limpens et al., 2011).

1.1 Roles of *Sphagnum* mosses in northern peatlands

Sphagnum mosses tolerate low nutrient conditions in bogs and poor fens and obtain their N and other nutrients from their own older tissues (Rydin and Clymo, 1989); this largely circumvents the need for N from decomposition of organic matter. *Sphagnum* mosses generally render soil inhospitable to microbes via recalcitrant litter and acidity generation and tight conservation of N. These factors also allow the *Sphagnum* mosses to outcompete many vascular plants, which rely more heavily on decomposition of soil organic material for nutrient supply (Malmer et al., 2003). However, through anthropogenic activities such as fossil fuel combustion and modern, high-intensity agriculture, reactive atmospheric N input has increased in some peatland regions. As more N is deposited onto peatlands, the N limitation is lifted. *Sphagnum* mosses lose ability to retain N and indeed many Sphagnum species cannot tolerate high N. Eventually vascular plants are able to become dominant. With longer stems and broader foliage, they can outcompete the shorter *Sphagnum* mosses for light (Berendse et al., 2001; Bubier et al., 2007). Increasing N deposition rates have been shown to lead to a progressive shift in vegetation whereas *Sphagnum* mosses were outcompeted by vascular plants, while gross photosynthesis and NPP remain unchanged (Larmola et al., 2013). The new plant community accumulates N in greater amounts, and releases N faster after tissue senescence, providing more nutrients to decomposers and the ecosystem at large (Bragazza et al., 2012; Larmola et al., 2013).

1.2 Nutrient loading in peatlands and atmospheric greenhouse gases

In peatlands, under a natural N gradient an increase in microbial CO_2 production and dissolved organic matter was observed (Bragazza et al., 2006). Additionally, in simulated N loading experiments, peat decomposition has been shown to increase (Basiliko et al., 2006) and

total NEE/C sequestration supported by the dominance of *Sphagnum* mosses, decrease despite that there was an overall increase, or little change in NPP by vascular plants (Bubier et al., 2007; Gunnarsson et al., 2008; Larmola et al., 2013). With the absence of the inhibitory capabilities of *Sphagnum* mosses and increases in nutrient supply from detritus and soil organic matter, the soil diversity and functioning of microflora will likely change. Observed increases in decomposition in the few short to medium-term peatland nutrient deposition loading experiments points to a more diverse microbial community that might help the vascular plants thrive even further, however these relationships have not yet been examined in detail. Root exudates and better litter quality are factors that can enhance soil fertility, providing more labile C and nutrients to promote microbial growth. Of course in peatlands, where the natural state is often one of nutrient limitation that promotes C sequestration, improved soil fertility is not inherently good, as it might be in intensively managed soils.

1.3 CO_2 and decomposers

Globally soil respiration by roots and soil microbes are the largest source of the flux of C from terrestrial ecosystems to the atmosphere. The IPCC estimates soil respiration releases of 107.7 Pg C yr^{-1} relative to photosynthetic inputs of 108.9 Pg C yr^{-1} (IPCC, 2013). Although this is indicative of some net sequestration in the terrestrial biosphere (especially in systems such as peatlands), any small increase in global soil respiration could increase microbial output of CO_2 that could equal and even surpass to C inputs (Cox et al., 2000). In the longest-running simulated N loading experiment at the Mer Bleue Bog, Ottawa, Canada, a net increase of 24–32% in ecosystem respiration (ER) and a loss of *Sphagnum* moss was observed that is likely driven by faster decomposition (Juutinen et al., 2010). This is somewhat paradoxical however, as it has also commonly been shown with increase N loading to forest soils with recalcitrant organic

matter, microbial biomass decreases as a results of N toxicity (Treseder, 2008) or due to formation of stable amino-quinone compounds (Ågren et al., 2001). Specifically in peatlands in the short-term however, increases in nutrients can promote increased microbial biomass (as observed in Mer Bleue bog in after 2 growing seasons; Basiliko et al., 2006). In the same field experiment in subsequent years, microbial activity remained elevated (Larmola et al., 2013) Increasing ER and decomposition rates could possibly mean more diverse and specialized microbes appearing to consume higher quality plant litters. Moreover residual peat lower in the soil profile could be exposed to decomposition as well thus potentially increasing CO_2 flux.

1.4 Methane and methanotrophs

Beyond cycling of CO_2, peatlands also typically emit the GHG methane (CH_4) that is produced in the anoxic soil layers by strictly anaerobic Euryarchaeota; however some to all CH_4 can be oxidized by aerobic CH_4 oxidation bacteria if there is an oxic zone in the peat profile. Per molecule, CH_4 absorbs more infrared radiation compared to CO_2, up to 21 times over a 100 year period. The IPCC reports that with a very high level of confidence, the atmospheric CH_4 has increases during the Industrial Era and is caused by anthropogenic activities, and it is accepted to be the 2nd most important GHG contributing to global warming (IPCC, 2013). Despite anthropogenic increases, wetlands are still the largest sources of CH_4 and drivers of interannual variability in atmospheric concentrations (177 to 284 Tg of CH_4 yr^{-1}). Unique attributes of wetland vegetation, water table depth, soil water content, and temperature affects CH_4 production and oxidation. Plants-mediated transport of CH_4 is responsible for 50-95% of methane fluxes in wetlands (Hanson and Hanson, 1996). CH_4 diffuses into the rhizomes and is transported through

the plants and to the atmosphere, bypassing the soil surface oxidation of methane by aerobic methanotrophic bacteria. Water table position affects decomposition processes and CH_4 fluxes by largely delineating the anoxic and oxic layers in peat. Moore and Knowles have observed experimentally that CH_4 emission rates decreased exponentially as the water table was lowered (Moore and Knowles, 1989). Soil water content is a major control on gas diffusion. Dryer soils have greater oxygen penetration and more rapid transport of CH_4 to soil methanotrophs, consequently, increasing CH_4 oxidation (Hanson and Hanson, 1996). Lastly, temperature was shown to link to higher CH_4 emissions; with rates higher as soil temperature rises (van Winden et al., 2012). However because there are a range of biotic (production, oxidation and plant transport) and abiotic factors that influence CH_4 emissions from peatlands, we do not have a complete understanding of CH_4 emission feedbacks to environmental change.

Aerobic methanotroph communities typically reside at the oxic-anoxic boundary layer (supplying O_2 from above and CH_4 from below), where they consume CH_4 chemoautotrophically with CH_4 (or occasionally other C1 compounds) as the sole source of C and electrons. This group of prokaryotes generally can be divided into two taxonomic groups. Based on their cell morphology, ultrastructure, phylogeny, and C-assimilation pathways: type I and type II (Hanson and Hanson, 1996). Type I methanotrophs belong to the gamma subdivision of the *Proteobacteria* and employ the ribulose monophosphate pathway for formaldehyde assimilation. As for Type II methanotroph, they form the beta subdivision of the *Proteobacteria* which uses the serine pathway for formaldehyde assimilation. Both groups might have favored conditions where they thrive respectively. Type I are typically found in conditions where CH_4 is limiting and with high N and copper concentration. Type II are the opposite, growing with low N and copper concentrations but relatively high CH_4 concentration (perhaps like in nutrient poor

peatlands) (Hanson and Hanson, 1996). All methanotroph contains key CH_4 monooxygenase (MMO) enzymes that catalyze the oxidation of CH_4 in the first step of CH_4 oxidation for redox and assimilation of CH_4-C (Hakemian and Rosenzweig, 2007).

1.5 Genes involved in methanotrophy

CH_4 monooxygenases can occur in two forms: a membrane bound particulate form (pMMO) and cytoplasmic soluble form (sMMO). It is known that copper plays a key role in the physiology and activity of aerobic methanotrophs as well as influencing which form of MMO is produced in some methanotroph species (Semrau et al., 2010; Vorobev et al., 2013). Copper is important in the production of pMMO and is found within its active site and is believe to enhance pMMO activities (Semrau et al., 1995). In environmental conditions where copper is absent, sMMO might be favored. This switch is called the "copper switch" (Semrau et al., 2013). Both MMO forms of enzyme have broad substrate specificities, especially sMMO, which can oxidize alkanes, alkenes, alicyclics, aromatics, ethers, heterocyclics and ammonia (Colby et al., 1977; Dalton, 1977). These processes are typically forms of co-metabolism, with the methanotroph gaining no energy or C. MMO catalyzes the first step in the oxidation of CH_4 by converting it to methanol. The pMMO contains copper and iron and is expressed via a three-gene operon, *pmoCAB*, which code for three integral membrane (Gilbert et al., 2000). *pmoA* is well studied and large dataset of sequences are available at GenBank from a number of environmental studies and isolates. It provides a useful molecular marker for methanotroph diversity studies. The pMMO is found in all methanotrophs except *Methylocella*, whereas sMMO is rarer, i.e. most methanotrophs rely solely on pMMO (Hanson and Hanson, 1996). The sMMO gene cluster is encoded by a six-gene operon: *mmoXYZ, mmoC, mmoB and mmoD* (gene on another operon) (Cardy et al., 1991). Recently, the sMMO cluster has been sequenced on *Methylococcus*

capsulatus (Bath), *Methylosinus trichosporium* OB3b, *Methylosinus sporium, Methylocystis sp., Methylocella silvestris* BL2 *and Methylomonas sp.* which provide a relatively small dataset. Both *pmoA* and *mmoX* have been shown to produce comparable phylogenies with the 16S phylogenies of the same organisms (Holmes et al., 1999; Kolb et al., 2003).

1.6 N deposition and methanotrophy

In the late 1980s it was observed that addition of NH_4NO_3 to temperate forest soils inhibits CH_4 uptake (Steudler et al., 1989). Subsequent studies showed this pattern in other soils (Bodelier and Laanbroek, 2004) and the reason is likely that ammonia and CH_4 monooxygenases are closely related phylogenetically and MMOs have been shown to bind NH_4 over CH_4 preferentially(Murrell et al., 1998). Additionally, the by- and end-products (hydroxylamine and nitrite) of nitrification have shown to be toxic to methanotrophs (Schnell and King, 1996). The N-effects-on- CH_4 -oxidation story in wetlands is not clear however: one decade after urea addition to rice paddies, CH_4 oxidation was shown to conclusively increase due to overcoming simple N limitation by the autotrophic methanotrophs that cannot access organic soil N (Bodelier et al., 2000). In peatlands, few studies have explored how mineral N and other nutrient additions influence methanotrophic bacteria and methanogenic archaea (Bodelier et al., 2000; Bodelier and Laanbroek, 2004).

In summary, with increasing N deposition from anthropogenic activities or indirectly through climate-change enhanced peat mineralization, a surge of unnatural amounts of available N and other nutrients may be fed into peatland systems. The tight controlled balance throughout the past millennia of peatlands being important CO_2 sinks and CH_4 sources may be stressed. Peatlands have stored an important amount of C, however inadvertent addition of bioavailable nutrients may allow opportunistic plants to change the landscape and in turn drive C fluxes.

Either via direct impacts from the N deposition or the indirect effects caused by plant community shifts, soil chemistry will likely change, with improved resources for microbial decomposers. Vanguard microbes will have sufficient energy to breakdown more robust complex molecules and mineralize nutrients for other microbial decomposers and higher trophic plants to thrive. As plants continue to shift, the new foliage provides more labile energy and C resources for the microbes as a result of litter fall and/or root exudates. This cycle will result in C loss from the system, both as CO_2 and CH_4. A key concern is that under higher nutrient availability, peatlands will no longer act as a C sink and might produce and emit more CH_4. The poorly understood mechanisms of N inhibition of CH_4 oxidation may also contribute to anticipated changes in GHG cycling.

2 MSc thesis objectives

My objective is to characterize microbial communities following simulated chronic N loading (with and without other nutrients) in the Mer Bleue Bog in two steps: (1) Investigate broad changes in microbial decomposer phylogenetic diversity and community structure associated with the previously reported shifts in vegetation and litter chemistry over the past 15 years, and characterize abundances of bacteria and fungi and linkages to peat mineralization and (2) target specific CH_4 oxidizing bacterial and methanogen communities and activities in the same nutrient loading experiment.

I predict that mineral N added at the Mer Bleue bog will be assimilated quickly by the vegetation. As a result, nutrients provided from plant litters will be more labile, enhancing microbial decomposition and shifting the community structure in favour of more abundant and diverse bacteria over fungi. In vitro patterns illustrated in my work will link to colleagues'

ongoing work that is illustrating increasing CO_2 effluxes in situ and that C storing capabilities are weakening. Also, CH_4 monooxygenases will selectively oxidize the ammonia added (and/or naturally mineralized); leading to reduced biomass, diversity and activity of methanotrophs. Both predictions hypothetically would support scenarios of increased atmospheric CO_2 and CH_4 arising from microbial feedbacks in peatlands and further driving climate change. However, ecosystems have many complex feedback mechanisms that I still do not understand. Looking at the microbial response to N and other nutrient addition to a bog is a first step in understanding the complex feedbacks between increased N pollution and the terrestrial biosphere.

2.1 Thesis structure

This thesis consists of two subsequent journal style chapters. Chapter one describes my work on Mer Bleue Bog's bacterial and fungal communities subjected to chronic N loading, while chapter 2 evaluates the methanotroph and methanogen communities and their potential impact on overall methane dynamics.

3 Chapter 1

3.1 Introduction

Peatlands accounts for just 3% of the Earth's terrestrial surface but have stored more than one third of atmospheric C over the Holocene epoch (Gorham, 1991). Plant growth exceeds decomposition and thus they are long-term net sinks of atmospheric CO_2. Soil decomposition is slowed or halted by many environmental factors: low pH (Williams et al., 2000), cold (Rydin and Jeglum, 2013), waterlogged anoxia, chemically complex substrates, potential nutrient limitation (Moore and Basiliko, 2006) and high phenolic concentration from *Sphagnum* mosses toxic to microbes (Yavitt, 2000; Jassey et al., 2011). Constrained nutrient availability is an important factor among the list. With increasing human activities, fossil fuel combustion and intensive agricultural practices have dramatically increased reactive atmospheric N concentrations. Through dry or wet deposition, peatlands are fertilized, thus providing often limiting nutrients to the vegetation community and ultimately increasing plant tissue decomposition (Turunen et al., 2004; Larmola et al., 2013). In bog and poor fen sites that predominate peatlands across Canada, taller and denser vascular plants become more dominant and shade *Sphagnum* mosses under N and combined nutrient loading. As *Sphagnum* mosses are lost, new plant tissues including belowground roots that are typically more nutrient rich would become the source of litters to soils and antibiotic *Sphagnum* leachate concentrations would diminish (Preston et al., 2012) possibly changing microbial communities. Plant community shifts under increased N have been relatively well documented in both North American and Northern European contexts; however subsequent effects of the vegetation shift on microbial communities and ultimately C losses in nutrient poor peatlands are not clear.

Initial reports indicated that inorganic N addition to soil organic matter across a large range of ecosystems slows decomposition, particularly with nutrient poor plant litters (Fog, 1988). Agren et al. (2001) explains these observation through decreased decomposer growth rate, altered microbial community with increased decomposer efficiency and more rapid formation of recalcitrant compounds. However in more recent studies focussing on peat soils, groups have shown the opposite (Basiliko et al., 2006; Bragazza et al., 2006; Larmola et al., 2013). Bragazza et al. showed that higher atmospheric N deposition in European peatlands has increased CO_2 emissions and dissolved organic C release. They theorized that increasing N availability favored microbial decomposition by removing N limiting constrains on microbial metabolism and a positive feedback on microbial enzymatic activity through better litter quality. Both Larmola et al. (2013) and Basiliko et al. (2006) showed that in a longer and shorter-term fertilization experiment respectively, the increased N impacts microbial communities in a bog via increased mineralization of C and increased microbial biomass, both supporting overall increased decomposition in a nutrient deprived bog ecosystem where decomposition rates are typically very slow. Additionally, the *Sphagnum* moss loss causes other environmental changes in nutrient poor peatlands. Bogs with high nutrient loading become wetter as *Sphagnum* is lost though relative depression of the peat. Higher litter quality has appeared as a results of shrub and other vascular plant functional type appearance (Wang, Murphy, et al., 2014). Chloroform fumigation-extraction and phospholipid fatty acid analyses have provided coarse-scale microbial biomass and microbial community data to help shed light on N loading impact on microbial community (Basiliko et al., 2006). However, there is little detailed information on how fungal and bacterial communities are affected by nutrient loading in peatlands.

Soil microbial communities are highly diverse and complex and fingerprinting methods provide a good overview to assess the community structure of dominant members. Terminal restriction length fragment polymorphism (T-RFLP) is a fingerprinting method based restriction fragments (digested with nucleases) of target gene of interest, and 16S and 18S small subunit (SSU) ribosomal RNA (rRNA) genes or group-specific functional genes are commonly assayed. T-RFLP is a highly reproducible technique for monitoring spatial and temporal changes and has proven to be effective in differentiating between different microbial diversity in different environments (Tiedje et al., 1999; Lukow et al., 2000). Other techniques to understand complex microbial interactions involve dividing them into ecological meaningful groups. Characterizing microorganisms by major decomposer groups- namely fungi and bacteria- both in terms of fingerprinting, but also quantification of gene copies using qPCR can help simplify a complex microbial community and evaluate its dynamics. Bacteria and fungi are known to respond differently to nutrient availability in peatlands (Myers et al., 2012), and thus this differentiation is relevant to the current study.

The Mer Bleue Bog, a large ombrotrophic site located east of Ottawa, ON, has been the focus of more than 100 of studies dealing with plant community, hydrology, C and CH_4 exchange, and decomposition. This includes the longest chronic nutrient amendment experiment to assess N and other nutrient impacts on C exchanges. Here, my objective is to characterize the microbial community and its response to chronic nutrient loading at the Mer Bleue Bog from two sampling year (2013 and 2014). I characterized the microbial community structure using T-RFLP and the relative proportions of fungi to bacteria ratio using a quantitative polymerase chain reaction. This built on the work of Larmola et al. (2013) who at Mer Bleue demonstrated that higher ecosystem-level respiration was counteracting increased NPP under enhanced N and

combined nutrient loading. Our data should reflect changes in vegetation shift shown at Larmola et al.'s paper. Moreover, given the length of the nutrient loading and substantial plant-community, hydrological, and soil chemical changes seen, treatment effects on microbial communities should not change much from one sampling year to the next.

3.2 Methods

3.2.1 Experimental site and sampling

The Mer Bleue bog (45°24' N latitude, 75°31' W longitude), located 10 km east of Ottawa, Canada, is a raised ombrotrophic peat bog complex with hummock-hollow patterned topography. It is mainly Sphagnum dominated with a shrub overstory. The water table depth averages 30 cm beneath the surface of the hummocks. Background N deposition is relatively high in Canada with an approximate annual deposition of 1.5 N g^{-2} yr^{-1}. Regional total N deposition rate from a wet atmospheric N deposition was estimated to be 0.6 to 0.8 g N m^2 yr^{-1} (Basiliko et al., 2006). Average water table measured for 2013 was positioned at -35.3 cm and -32 cm for 2014.

3.2.2 Field Fertilization

Field fertilization experiments were established in 2000-2001 (Bubier et al., 2007). Fertilization plots (9 m^2 in area) were set up in a 400 m^2 section of the bog with little variation in micro-topography and vegetation. Solutions of N were added every three weeks as ammonium nitrate (NH_4NO_3) at 1.6**,** 3.2 and 6.4 g N m^{-2} yr^{-1}, approximately 5, 10 and 20 times the ambient growing season atmospheric deposition. Phosphorus and potassium (PK) were added as KH_2PO_4, equivalent to 6.3 g K and 5.0 g P m^{-2} yr^{-1}. Control plots received P and K solution or distilled

H_2O. Triplicate plots for sampling were identified as Control, PK, 5N, 5N+PK, 10N, 10N+PK, 20N and 20N+PK.

3.2.3 Sampling

For each plot, three cores were taken using a Russian corer that was washed with distilled water between plots. Soil 5-15 cm below the live vegetation was sampled and stored in a sterile plastic sampling bag. Each bag was homogenized by mixing the sample mechanically by hand. Sample bags were placed in a cooler with ice until they were transported to Laurentian University where they were stored at 4°C. Samples used for 2013 and 2014 are listed in Table 3-1.

3.2.4 Soil Chemistry

Samples were sent to analytical laboratories (Soil and Plant Analytical Lab at the Great Lakes Forestry Centre in Sault Ste.Marie, Ontario) for analysis of total C, N and elements (P, K, Ca, Mg, Fe, Cu, Mn, Zn, Al, Na, and S). Total C and N were determined using a solid phase combustion analyzer while other elements were measured using the EPA 3051A acid digestion protocol and then determined using a Varian ICP-OES spectrometer.

3.2.5 DNA extraction

DNA was extracted from peat samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA). All DNA samples used in subsequent experiments were pooled from three extractions. Homogenization was performed using a 5 minute cycle on a 16 tube MiniBeadbeater TM (Biospec Products Inc., Bartlesville, OK). Samples were stored at -20°C for analysis at a later date.

3.2.6 Terminal restriction fragment length polymorphism (T-RFLP) analyses

PCR reactions were performed using a MWG AG Biotech Primus 96+ Thermocycler. Previously determined optimized primer combinations and cycles from Preston et al. (2012) were utilized for the amplification of bacterial 16S and fungal 18S ribosomal RNA genes. Bacterial and fungal T-RFLP was performed as described by Preston et al., (2012). Fragment size analysis was completed at the Agriculture and Food Laboratory in Guelph University of Guelph Agriculture and Food Laboratory (Guelph, ON). T-RFLP data were preprocessed in R using a custom function created using the algorithm described by Ishii, Kadota and Senoo (2009) with slight modifications to accommodate the data format supplied by Guelph. The cutoff distance was set to 2 bp and the final output was expressed as proportion total peak height per T-RF by sample (Kaplan and Kitts, 2003).

3.2.7 Quantitative polymerase chain reaction (q-PCR) amplification

The bacterial and fungal SSU rRNA genes were q-PCR-amplified using oligonucleotide primers that broadly targets members of the domain *Bacteria* and the kingdom Fungi following methods described in (Fierer et al., 2005). qPCR assays were conducted in polypropylene 96–well plates on an Agilent Technologies Stratagene MX3005P qPCR system. Each 20 µl reaction contained the following: 10 µl of Thermo Scientific DyNAmo HS SYBR green qPCR 2X Master Mix, 0.5 µl of each primer sets 9 µl of dH_2O. PCR conditions for bacterial SSU rRNA were 15 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 30 sec at 50°C for *Bacteria* and 53°C for *Fungi*, and 72°C for 1 min. All reactions including sets of standards were performed in triplicate. After each qPCR run, melting curve analysis was performed to verify the presence of the desired amplicon and not from primer-dimers or other artifacts.

3.2.8 Statistical analyses

3.2.8.1 T-RFLP-based community analysis

Microbial communities between samples were compared using DCA with the vegan package provided by R statistical software (R Core Team, 2013; Oksanen et al., 2014). OTU peaks were analyzed in the context of relative abundance as well as just presence or absence. An adonis test using an eigenvalue method was performed to determine whether there were significant differences between groupings (Oksanen et al., 2014). An agglomerative hierarchical cluster analysis based on Ward's method of dissimilarity calculation was used to provide another visual indication on relatedness among the sites/treatments. Environmental vector fitting was applied to DCA to visually represent effect of N amendment on peat chemistry in relation to shifts in microbial community structure. The environmental vector fitting was performed using the vegan package in R.

3.2.8.2 qPCR analysis

Standard curves were produced using triplicate 10-fold dilutions of DNA from pure culture *E.coli* for *Bacteria* and *S. cerevisiae* for fungi. At least three nonzero standard concentrations per assay were used with concentration ranging from 10^{-2} to 10 ng of DNA per reaction. Target copy numbers were calculated from the standard curves using threshold cycle value (CT). For all qPCR assays, there was a linear relationship between the log of the DNA copy number and the calculated threshold cycle value (CT) across the specified concentration range ($R^2 > 0.95$). Amplification efficiencies, calculated using Pfaffl's (2001) formula $E = 10^{[-1/slope]}$, varied from 1.9 to 2.0 across both *Bacteria* and *Fungi* qPCR assays. Estimated gene fragment copies were used to calculate the F/B ratio. Linear regression analysis were performed on those ratio compared to N deposition level with and without P and K.

3.3 Results

3.3.1 Microbial community structure

In terms of weather, 2013 was a relatively average year, while 2014 was wetter and colder. Water table data were significantly different between both sampling years (Figure 3-1; $P = 0.01$). Moreover, significant differences were also observed between the summer months (June to August) from both years ($P < 0.001$). Chemical analysis of peat showed a decrease in C/N ratio as N was added (Figure 3-12). Total C did not change among treatments however. Total N showed a significant increase between control and N plots with 20 N+PK being significantly different to 20N without the addition of PK (Table 3-3; Figure 3-12). Other elemental compositions are shown on **Error! Reference source not found.**and certain trends were observed. Significant decreases of Ca, Mn and Ba and a decreasing trend of Mg and Zn were observed as increasingly more N was added (Table 3-3, **Error! Reference source not found.**; **Error! Reference source not found.**). A significant increase in K was observed with increasing N (**Error! Reference source not found.**).

Adonis tests of the T-RFLP data of microbial community structure in both sampling years indicated significant differences between the N fertilization plots and controls (Table 3-2). N with a fixed amount of added of P and K also showed a significant difference as well in both sampling years (Table 3-2) compared to N plots without P and K. To visualize these difference, a detrended correspondence analysis (DCA) of the T-RFLP data was used and revealed grouping of Control and low N fertilization plots (5N, 10N), separated from high N fertilization plots (20N, 20N+ PK) in the 2013 samples (Figure 3-2). Additionally, hierarchical clustering using Ward's method also presented a similar pattern as the DCA (Figure 3-2). When observing the

microbial groups individually, bacterial communities in 2013 and 2014 were significantly different between control and low N plots compared to high N plots using both Adonis tests and as visualized via multivariate techniques (Table 3-2; Figure 3-3; Figure 3-6). In 2013, N+PK treatments had significantly different bacterial communities as visualized on the cluster analysis in Figure 3-3 and Figure 3-6 and confirmed through the Adonis tests (Table 3-2). However in 2014, bacterial communities were not significantly different between N plots and N + PK plots. As for fungal communities, 2013 samples showed no significant differences between control and low N plots versus high N plots as depicted in the DCA and cluster analysis (Table 3-2; Figure 3-4). However, fungal communities were significant different when comparing N only plots and N + PK plots. In 2014, fungal communities were significant different between control and low N treatments relative to high N treatments (Table 3-2; Figure 3-7). Addition of PK did not affect the fungal community composition.

3.3.2 Quantitative analyses of gene copy numbers and F/B ratios

For all qPCR assays, amplification efficiencies varied from 1.7 to 2.0. A linear relationship between the log of the DNA standards and the calculated threshold cycle value was observed across the diluted concentration ($R^2 > 0.95$). These values were consistent with those reported in the literature (Fierer et al., 2005).

Figure 3-8 and Figure 3-10 show F/B estimated from relative abundance of Bacterial 16S and Fungal 18S in both sampling years (2013, 2014). Using a linear regression to display the effect of the N fertilization, 2013 samples showed no significant effect from fertilization, however, P and K addition significantly decrease the F/B ratio in all N plots (Figure 3-8). In the

following year, the F/B ratio decreased with increasing N addition (Figure 3-10). The addition of P and K decreased the F/B ratios on average but differences between N fertilization alone and with the addition of P and K were not significant.

In 2013, fungal 18S rRNA gene copy numbers remained unchanged as N fertilization increased (**Figure 3-9**). Addition of P and K also had no significant effect. Bacterial 16S rRNA copy number increased as N fertilization increased with the addition of P and K. Without P and K, bacterial gene copy numbers remained unchanged. In 2014, fungal 18S rRNA copy numbers decrease with and without the addition of P and K (Figure 3-11). Bacterial copy numbers increased with and without the addition of P and K.

In general, the addition of P and K enhanced the effect of N deposition whether it increased or decreased the overall group-specific gene copy numbers.

3.4 Discussion

Although my T-RFLP data do not provide specific phylogenetic information on microbial groups, they do indicate overall broad changes to microbial community structure. My data suggest that there are significant microbial community changes as N deposition increases, but not at low N addition rates (Figure 3-2; Figure 3-5); however I note that my study site already receives relatively high N deposition, which might mean less polluted sites would not experience the same effects over the same time frame. These data build on patterns in two previous studies at Mer Bleue (Basiliko et al., 2006; Larmola et al., 2013). Both studies showed higher level of microbial respiration rate in the 20N+PK plots relative to control. In 2013, changes in microbial communities could be attributed to altered community structure (with addition of P and K) as Adonis tests indicate significant changes in bacterial community structure (Table 3-2, Figure

3-3) and also shown in our qPCR data (Figure 3-9). In 2014, changes in microbial communities, were attributed to both bacteria and fungi community structure (Table 3-2, Figure 3-6Figure 3-7Figure 3-11). Based on qPCR data, the possible increase in decomposition could be the results of an increase in bacterial numbers and bacterial community diversity (Figure 3-3,Figure **3-9**). Peat C/N ratios appeared to be decreasing, which fits conceptually with additional N inputs, but perhaps was not statistically not significant ($P < 0.11$), primarily due to the variability between plots. As the C/N ratio drops from 52.21 to 33.93 (Average C/N ratio of Control and 20N+PK plots respectively), the soil condition becomes more favorable for microbial decomposition (Manzoni et al., 2008); increase N uptake by microorganisms could have caused the significant decrease in C/N ratio observed in my data (Figure 3-12). As N deposition increases, the nutrient poor bog is slowly becoming relatively nutrient rich, supporting bacteria that have higher biomass N requirements and that are superior competitors in terms of C mineralization over fungi in nutrient rich peatlands (Myers et al., 2012).

Atmospheric N deposition onto low nutrient soil such as in peatlands has been shown to decrease ectomycorrhizal fungal species richness (Lilleskov et al., 2011).My data show similar patterns where high N+PK plots have decreased fungal diversity compared to controls in 2014 (Figure Figure **3-11**). Perhaps N added as nitrate and ammonium allowed plants to uptake the nutrient without the help of fungal partner to access organic N sources that predominate in unimpacted bogs and poor fens. Mycorrhizal fungi are abundant in soils with low N availability and are known to form symbiotic relationships with *Ericaceae*, which are abundant in the higher N plots (with and without P and K) (Selosse et al., 2007; Larmola et al., 2013). Additionally, dark septate endophytic fungi might be important in control and low N plots as they contribute to nutrient and C cycling within the plant hosts; they produce the extracellular enzyme machinery

required to breakdown complex plant polymers, including phenolic compounds (Caldwell et al., 2000). As vegetation cover changes and nutrient become available, the endophytic fungi might not be needed. The decreasing fungal numbers could be a result in loss of mycorrhizal fungi and/or dark septate endophytic fungi (Figure 3-11). Additional data on the identity of the fungal taxa would be needed to confirm that claim, and T-RFLP defined taxa are known to be quite coarse in phylogenetic resolution.

My data suggest P is limiting as P was potentially assimilated in foliage and remained at same levels in soil compared to controls, whereas K accumulated and remained in the soils (Table 3-3). It has been suggested that Mer Bleue, being in a relatively elevated atmospheric N deposition region, could be P limited (Larmola et al., 2013). As a naturally N-limited ecosystem and past studies suggested P to be the second most limiting nutrient to primary producers in our peatlands, could the increase addition of N alleviated the ecosystem from N limitation and become P limited instead? (Basiliko et al., 2006). My data has shown the synergistic role of P and K on N deposition effects. This supports Larmola et al's finding; microbial respiration was significantly altered in high N plots with the addition of P and K (Table 3-2). The addition of P and K has enhanced effects of N deposition as shown via T-RFLP and qPCR data. These data were also in agreement with previous studies where P and K seems to enhance ER, microbial biomass (Basiliko et al., 2006), shrub biomass production and peat decomposition (Larmola et al., 2013). Wang et al. (2014) have shown resorption of foliar nutrient in shrubs being more responsive to addition of N and PK than N or PK alone. Without knowledge on the species composition, particularly for example mycorrhizal fungi, it would be difficult to ascertain if the microbial changes are due to P additions. A follow-up study that includes ultra-deep high

throughput amplicon sequencing data is underway, where I will be able to better identify microbial groups altered by nutrient additions with higher phylogenetic resolution.

3.4.1 Plant community shift influence microbial communities

The microbial community changes (Figure 3-2, Figure Figure **3-4**) generally corresponded with the vegetation shifts observed in previous studies at Mer Bleue (Bubier et al., 2007; Juutinen et al., 2010; Larmola et al., 2013). Loss of *Sphagnum* moss could be an important factor allowing diversification of microbial communities. *Sphagnum* moss renders conditions inhospitable to other bryophytes and most mesophilic microbes by sequestering N leaving the soil nutrient poor, acidifying through ion exchange, through the production of highly anti-microbial secondary compounds, and because its litter chemical composition is very resistant to decay, similar to woody tissue (Moore and Basiliko, 2006; Rydin and Jeglum, 2013). With higher N loading, the available N concentration is high and allows vascular plants to thrive. The *Sphagnum* moss cover gradually diminishes as vanguard vascular plants compete for light through larger foliage. It was also observed in the higher 20N plots that microbial communities are significantly different. The 20N and 20N+PK plots have little to no *Sphagnum* moss left (Larmola et al., 2013).

As mentioned earlier, *Sphagnum* moss has numerous properties that allow then to thrive in poor nutrient conditions as well as create non palatable litter for the soil microbiota (Limpens and Berendse, 2003). Loss of *Sphagnum* moss and increases in vascular plants shift the litter type and quality and removes inhibitory properties that might keep microbial activities slow (Larmola et al., 2013). Shrubs and graminoids dominate the higher N and N+PK plots and litters are more labile permitting rapid microbial mineralization and associated release of nutrients lower in the peat profile (Limpens and Berendse, 2003; Bragazza et al., 2009). Additional nutrients provided

by the litter shifts the microbial community as it adapted to new resources (Bragazza et al., 2007). Data show changes in microbial communities in high N+PK plots (Figure 3-2, Figure Figure **3-5**) and in concordance with data from Larmola et al. (2013), there is evidence to suggest that the microbial community changed in accordance with the presence of higher litter quality. Microbial diversification potentially provides a broader suite of metabolic capabilities, consistent with findings from substrate utilization assays used by Larmola et al. (2013). However, this speculation requires more in-depth genetic analyses of the soil microbial community. Regardless, changing microbial communities from a *Sphagnum* dominated bog with low decomposition to a shrub and graminoid dominated system with greater microbial diversity can possibly shift the bog's C storing capabilities via altered decomposer communities, consistent with very high ER values reported in the same fertilization plots prior (Bubier et al., 2007; Larmola et al., 2013).

3.4.2 Interannual variability

Mer Bleue was exposed to significant changes in weather conditions and as a result, water tables were significantly different between both sampling years. As shown in our Figure 3-1, changes in water table during the summer months were significant higher in 2014 compare to 2013. During the 6 year span at Mer Bleue, Roulet et al (2007) have shown significant difference in NEE, DOC and CH_4 output affecting C balance every year. They blame variation on climatic conditions causing large interannual and interseasonal C balance ranging from 50 to 105 g m^{-2} yr^{-1}. I would expect my data to be skewed as a result of the difference in water table observed.

In 2013, there was a significant impact from N fertilization: as N fertilization increased, microbial communities shifted (Figure 3-2, Figure Figure **3-5**). The same pattern was not observed in 2014, despite that the fertilization experiment had run 1 year longer. Increasing water table might have attenuated N effects on fungal communities. Previous studies show that

drought and lowered water tables cause increased ecosystem respiration and reduced primary production, creating a net effect of C export (Waddington and Roulet, 1996; Carroll and Crill, 1997; Bubier et al., 2003). A similar pattern was observed in our study sites where ecosystem respiration was higher with lower water table (Bubier et al., 2007). As water table rises and recreates zone of anoxia, oxidation potential is reduced, negatively impacting microbial communities, thus potentially decreasing decomposition. Another way water table might affect microbial community is through the changes in vegetation. Conversely, high water table restrict electron acceptors and nutrients in the catotelm and increase fermentations and methanogenesis. Peatland hydrology has tremendous implications in the biogeochemistry of these systems, specifically at Mer Bleue (Roulet et al., 2007), it is perhaps not surprising that interannual patterns were not consistent, given how wet 2014 was. Data suggests that microbial community dynamic are sensitive to annual environmental fluctuations, in particular water tables, as well as the longer-term effects of nutrient deposition.

3.5 Conclusions and future research

Changes in microbial community composition fit with my predictions, since high levels of N were added and vegetation shifts had occurred prior to this study. T-RFLP data provided a broad picture of certain microbial groups (bacteria and fungi) and although it is a generally suitable first step to obtain a rapid fingerprint of microbial communities, ongoing work using next-generation sequencing will cover gaps and provide better information to explore direct and indirect effects from N or P and K additions to the microbial communities. In fact, Illumina sequence data was obtained, I am in the process of analyzing those data.

Microbial community are adaptable to their environment and my study site was a fair example. Moore et al (2011) have observed significant difference in CH_4 fluxes between each year and water table might be the main drivers of those changes. My data suggest water table has affected my 2014 data as it was an abnormally wet year. A continuous monitoring could potentially help decipher true vs. environmental microbial community differences and understand the impact of N and nutrient at Mer Bleue.

In its current state, *Sphagnum* moss at Mer Bleue appears to be actively inhibiting microbial diversity by altering the environmental parameters of the soils and therefore, allowing this ecosystem to sequester C. Changes in litter quality from shifting vegetation and (directly or indirectly) increased nutrient availability are the likely factors shaping an altered microbial community. Increased C quality and nutrients shifts the microbial community and provides altered resources and habitats for new microbes to establish. In light of prior work on microbial functional abilities by Larmola et al. (2013), these new microorganisms appear to bring other functional tools that further decompose the peat soil; decomposition provide more nutrients for plants and a cycle is formed; weakening the C source of peatlands.

3.6 Figures

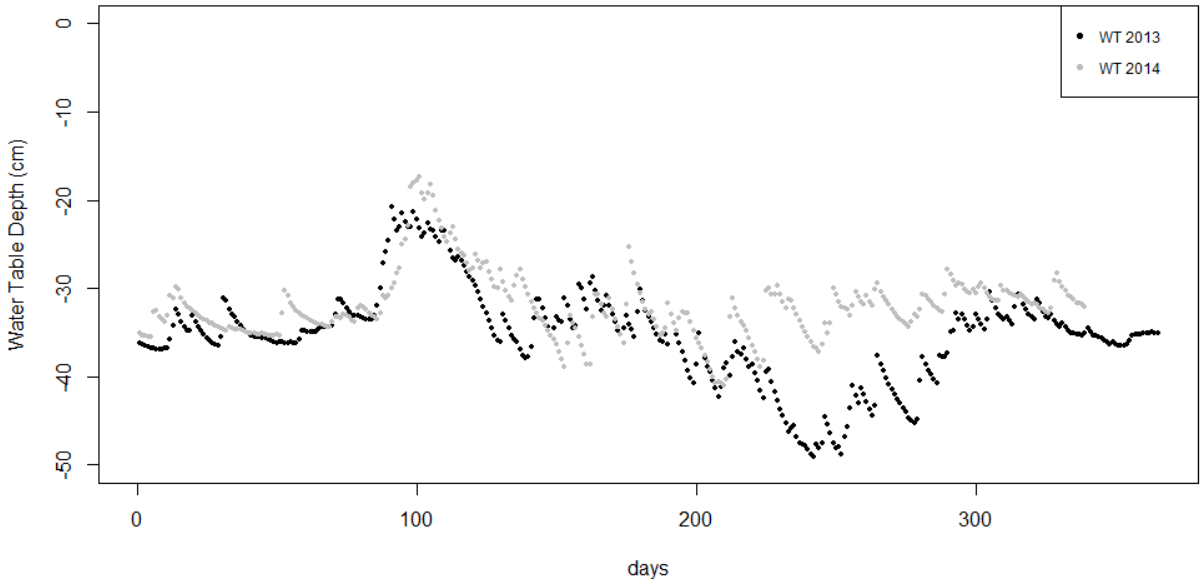


Figure 3-1 - Water table data for 2013 and 2014. Measurements were taken every 30 mins. Daily average water tables are shown above. Significant difference using Tukey's HSD were observed for average annual water table.

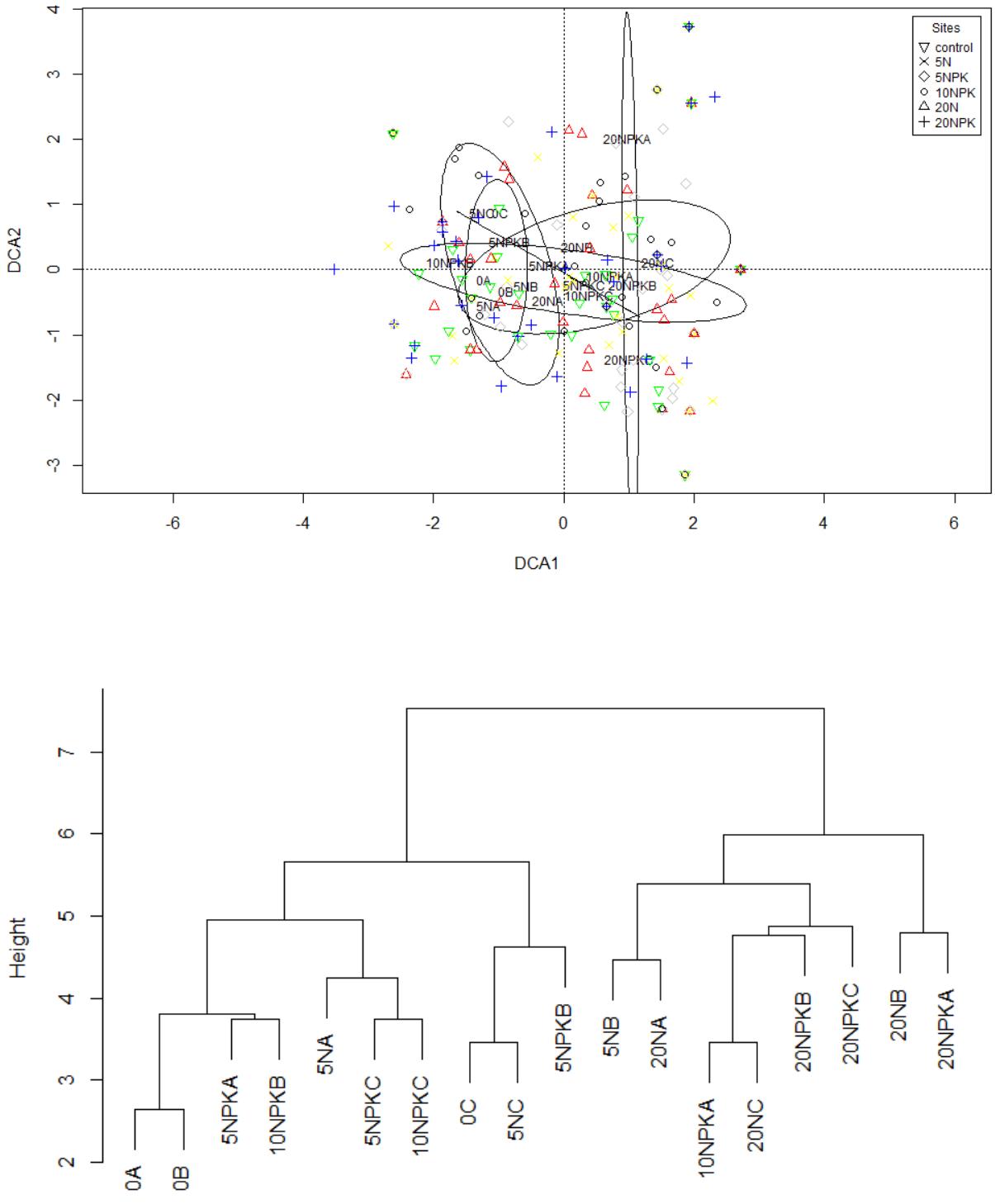


Figure 3-2 – (top) DCA ordination of Bacterial 16S and Fungal 18S T-RFLP communities from 2013 soil samples. (bottom) Hierarchal clustering using Ward's methods of Bacterial 16S and Fungal 18S T-RFLP communities from 2013 N fertilization samples; 0 = control.

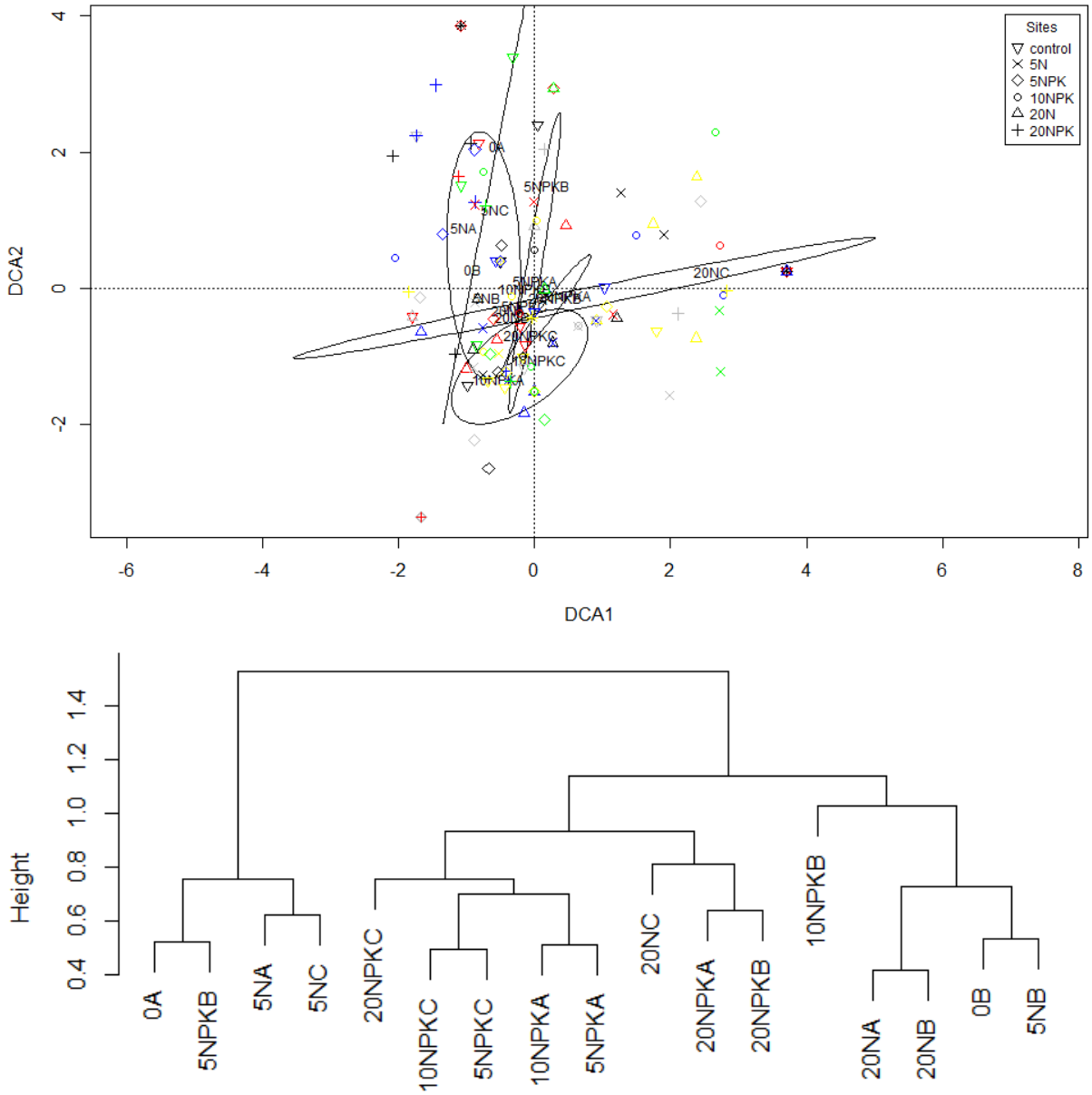


Figure 3-3 – (Top) DCA ordination of Bacterial 16S T-RFLP communities from 2013 soil samples. (bottom) Hierarchal clustering using Ward's methods of Bacterial 16S T-RFLP communities from 2013 N fertilization samples; 0 = control.

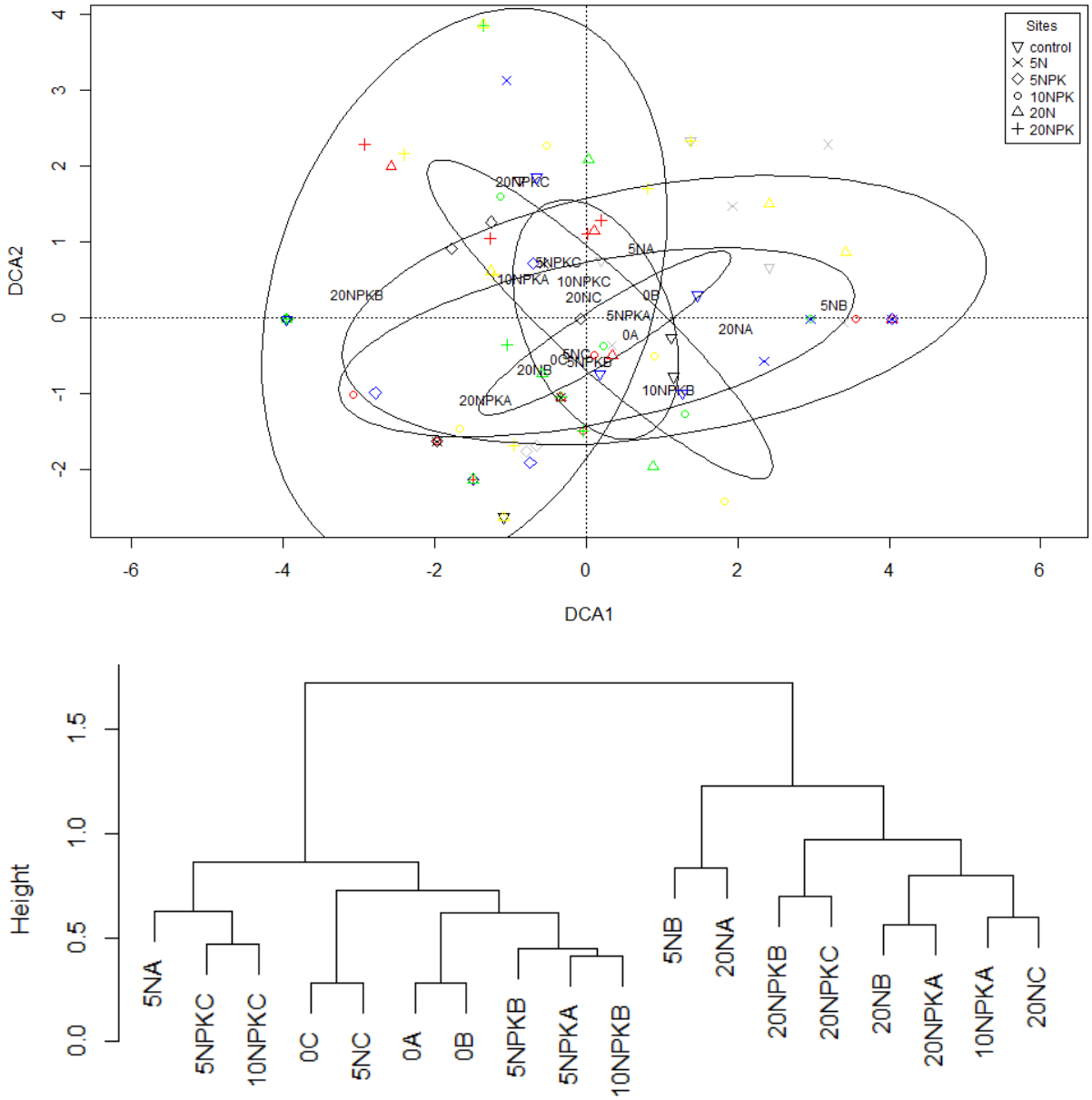


Figure 3-4 – (Top) DCA ordination of Fungal 18S T-RFLP communities from 2013 soil samples. . (bottom) Hierarchal clustering using Ward's methods of Fungal 18S T-RFLP communities from 2013 N fertilization samples; 0 = control.

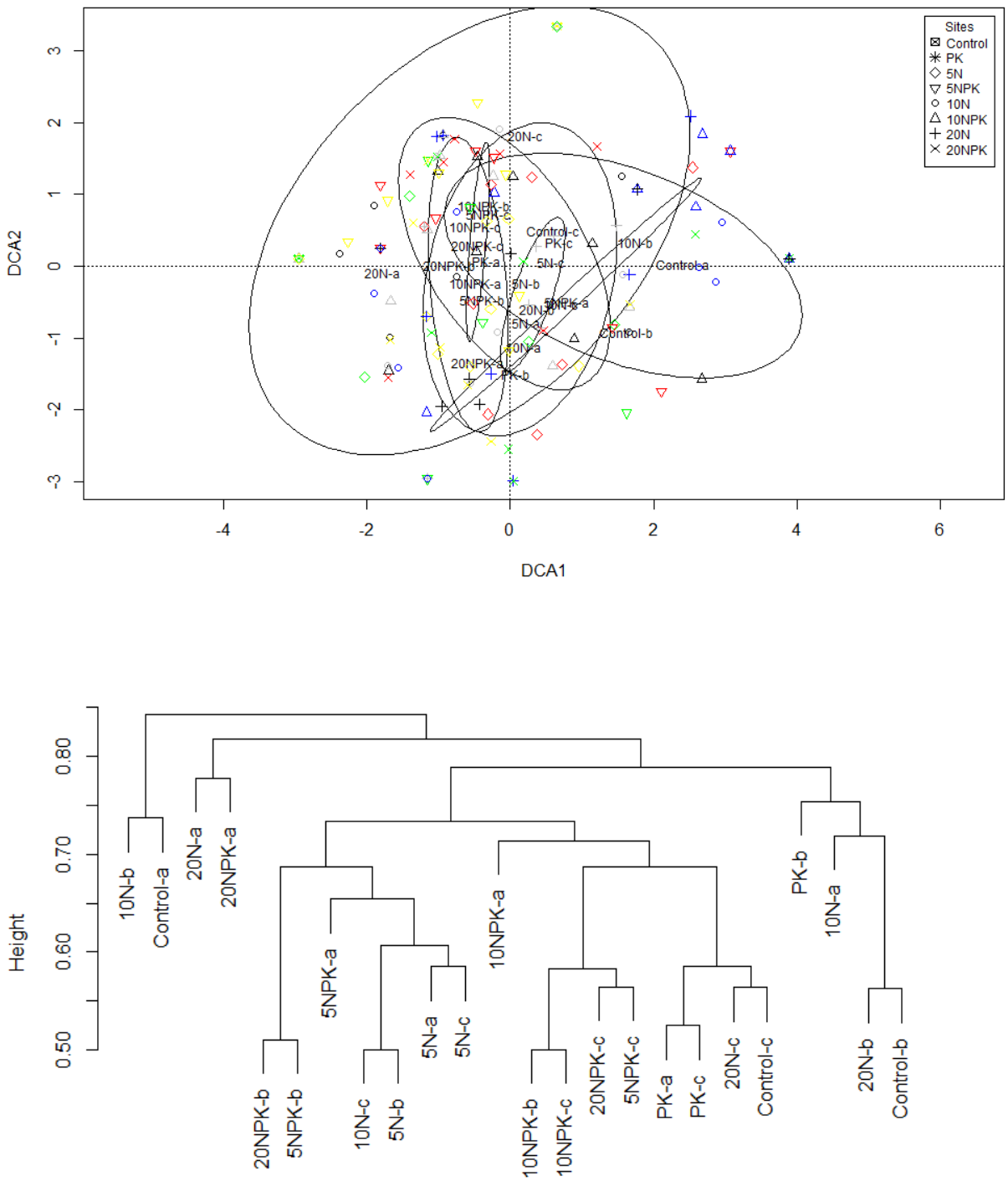


Figure 3-5 – (Top) DCA ordination of Bacterial 16S and Fungal 18S T-RFLP communities from 2014 soil samples. (Bottom) Hierarchal clustering using Ward's methods of Bacterial 16S and Fungal 18S T-RFLP communities from 2014 N fertilization samples

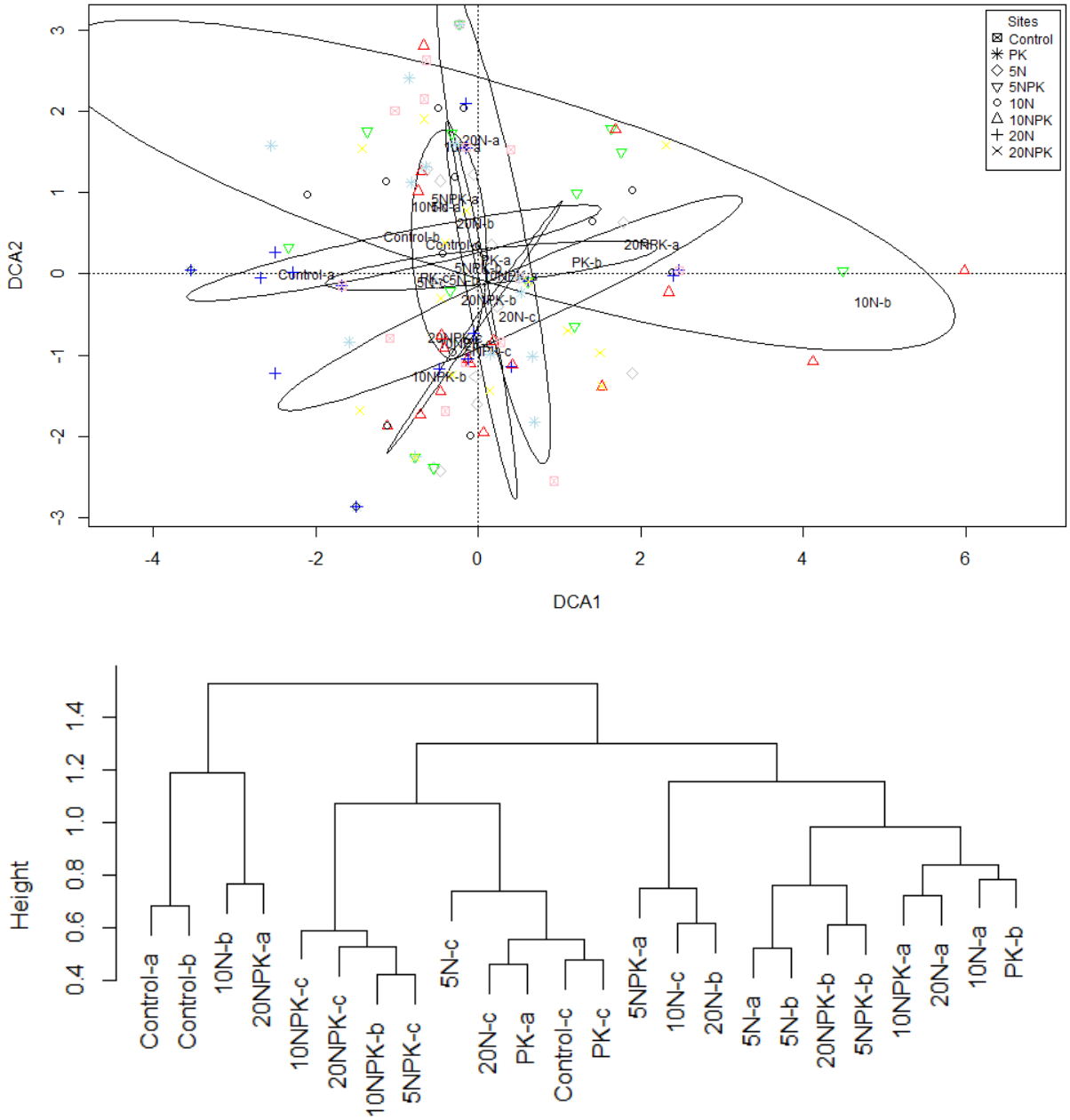


Figure 3-6 - (Top) DCA ordination of Bacterial 16S T-RFLP communities from 2014 soil samples. (Bottom) Hierarchal clustering using Ward's methods of Bacterial 16S T-RFLP communities from 2014 N fertilization samples

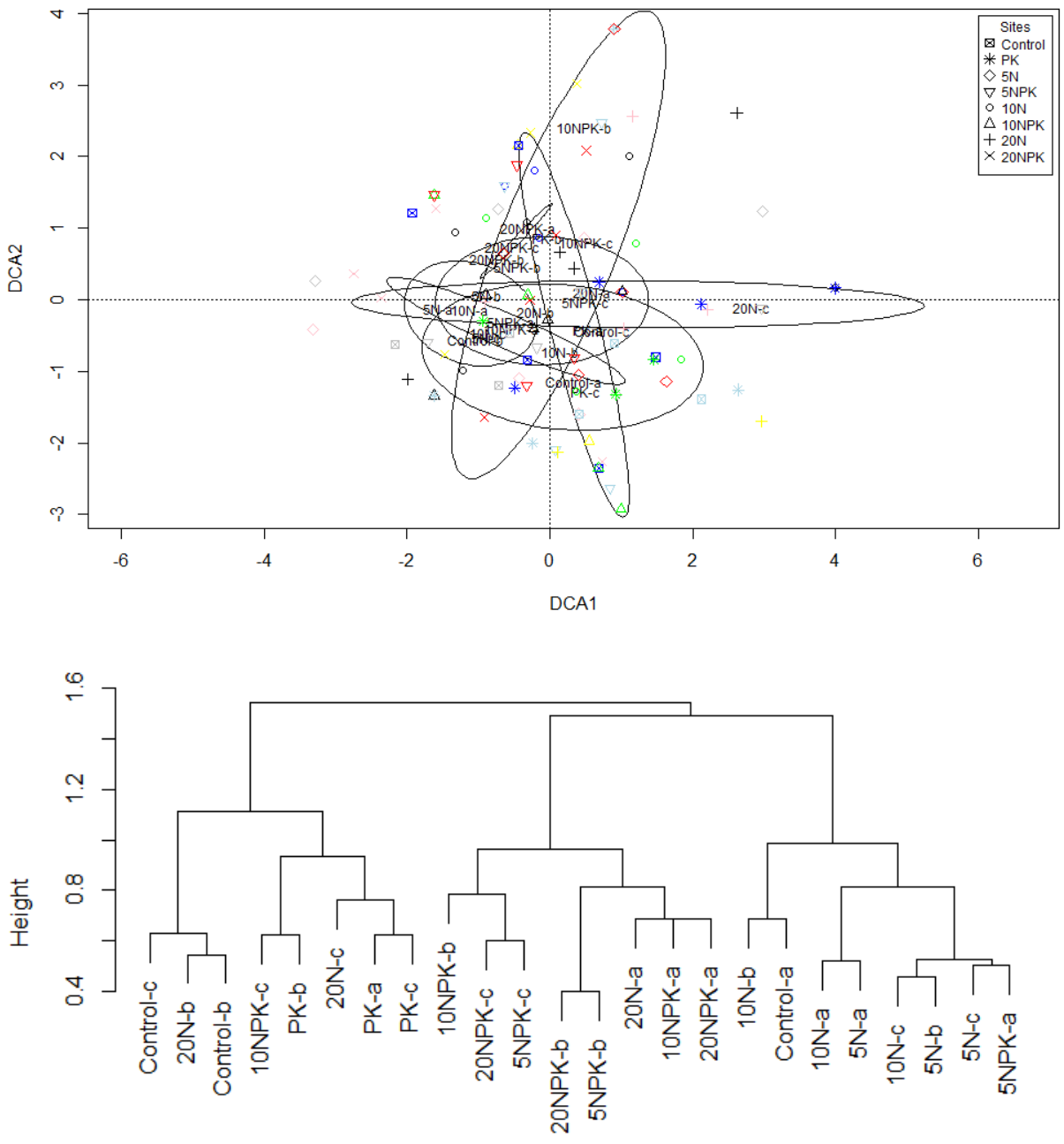


Figure 3-7 - (Top) DCA ordination of Fungal 18S T-RFLP communities from 2014 soil samples. (Bottom) Hierarchal clustering using Ward's methods of Fungal 18S T-RFLP communities from 2014 N fertilization samples

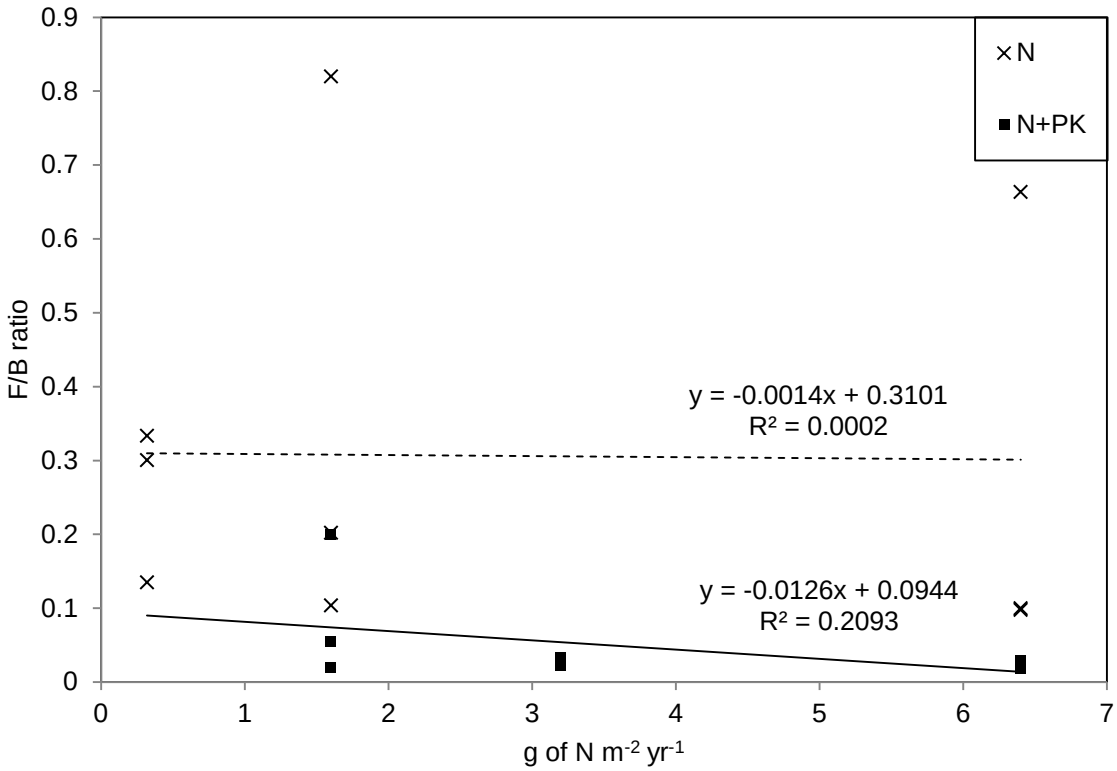


Figure 3-8 - Linear regression plot of 2013 samples comparing Fungal-Bacterial ratios to g of N deposited using qPCR data of Bacteria 16S and Fungi 18S gene copy number (Regression line: N only = dotted ; N+PK = solid).

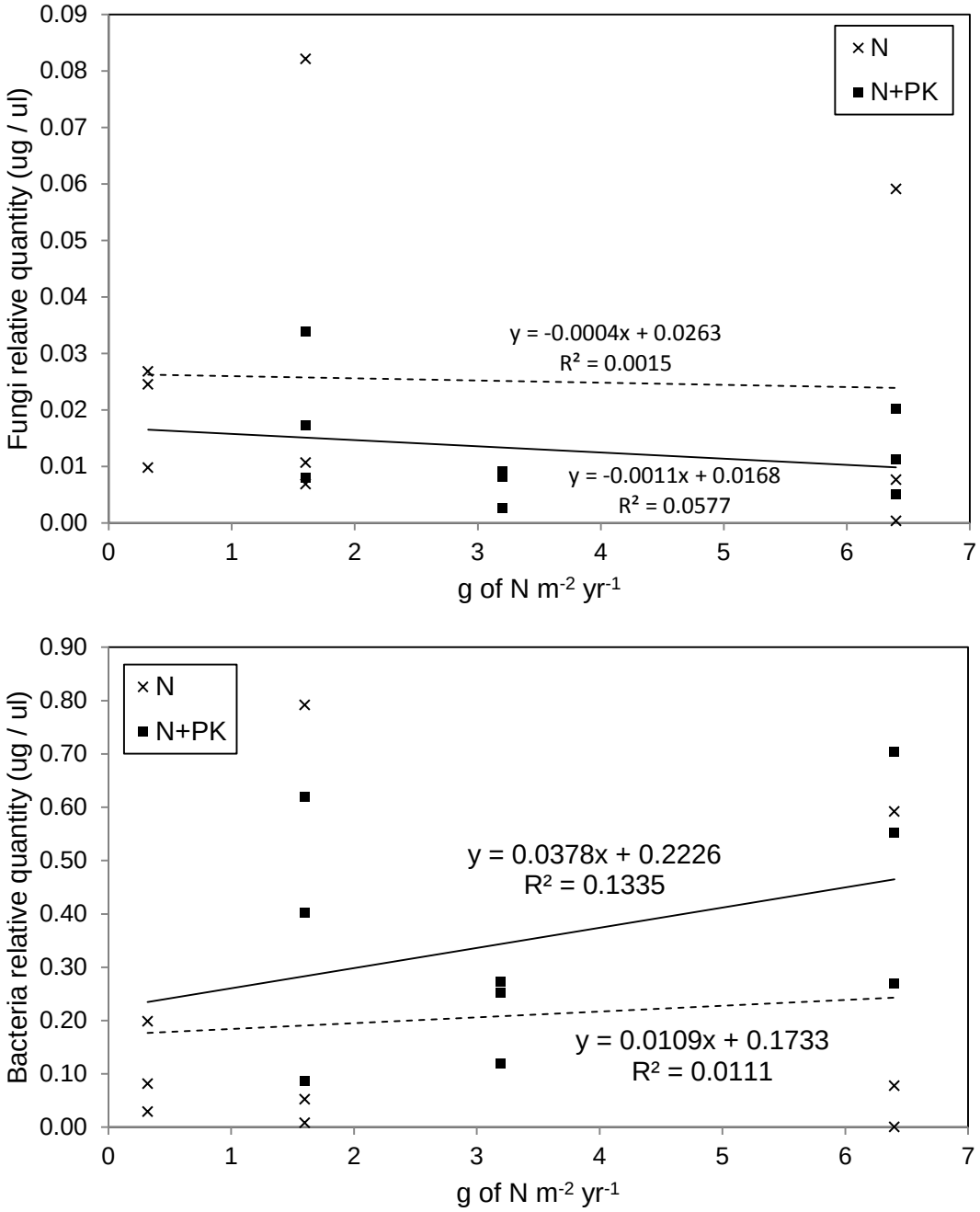


Figure 3-9 - (top) Regression plot of Fungi 18S rRNA copy number vs N added with and without PK of 2013 sampling plots. (bottom) Regression plot of Bacteria 16S rRNA copy number vs N added with and without PK of 2013 sampling plots. (Regression line: N only = dotted; N+PK = solid).

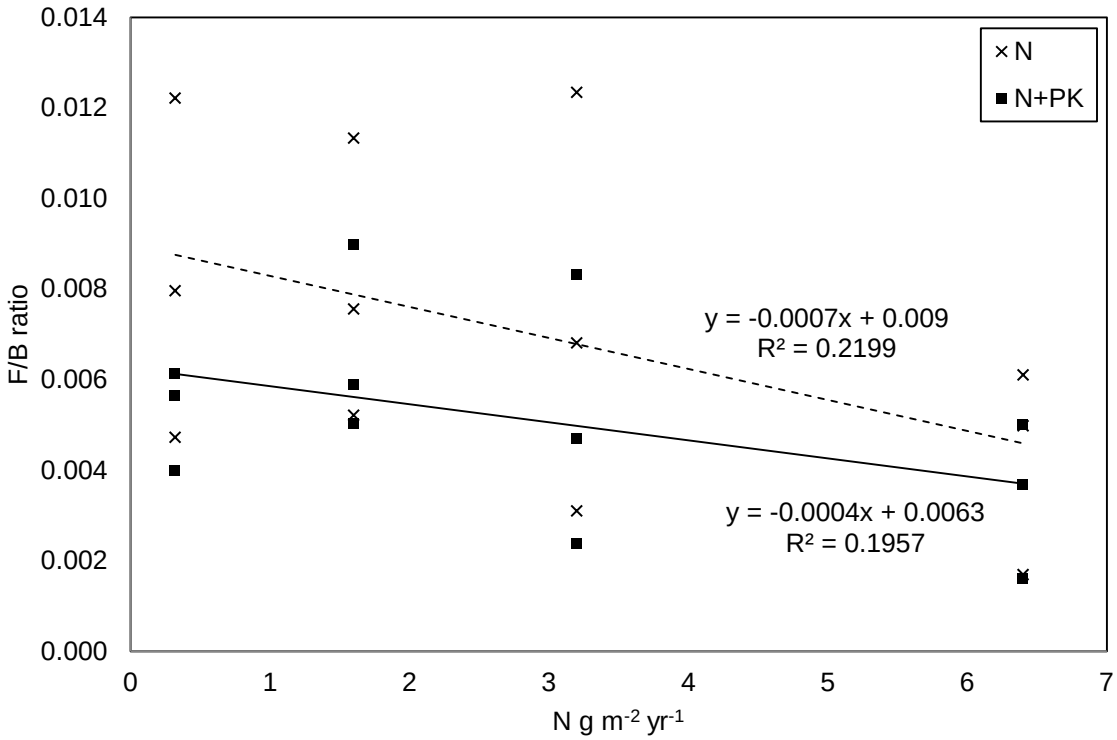
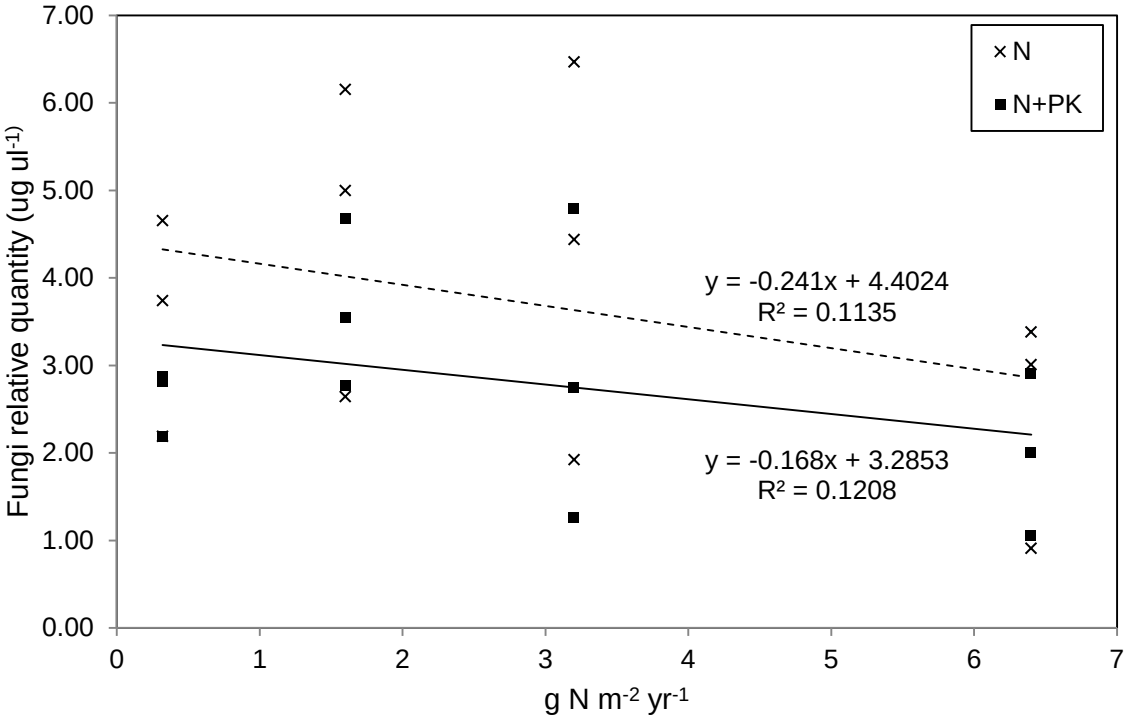


Figure 3-10 - Linear regression plot of 2014 samples comparing Fungal-Bacterial ratios to g of N deposited using qPCR data of Bacteria 16S and Fungi 18S gene copy number (Regression line: N only = dotted ; N+PK = solid).



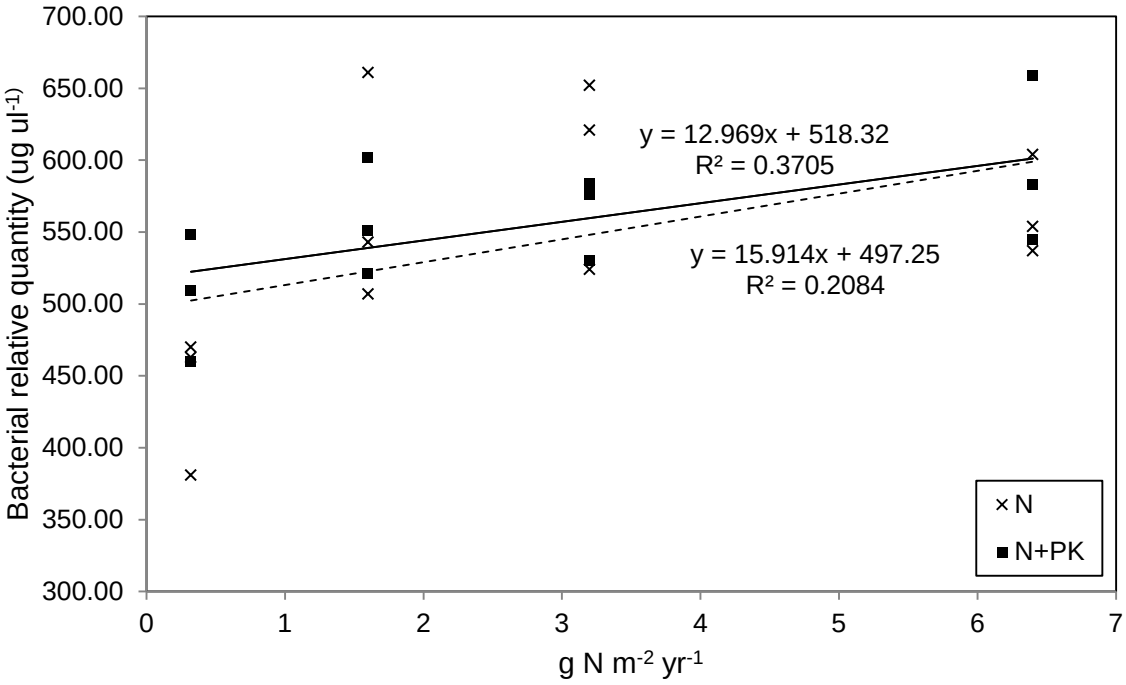


Figure 3-11 (top) Regression plot of Fungi 18S rRNA copy number vs N added with and without PK of 2014 sampling plots. (bottom) Regression plot of Bacteria 16S rRNA copy number vs N added with and without PK of 2014 sampling plots. (Regression line: N only = dotted; N+PK = solid).

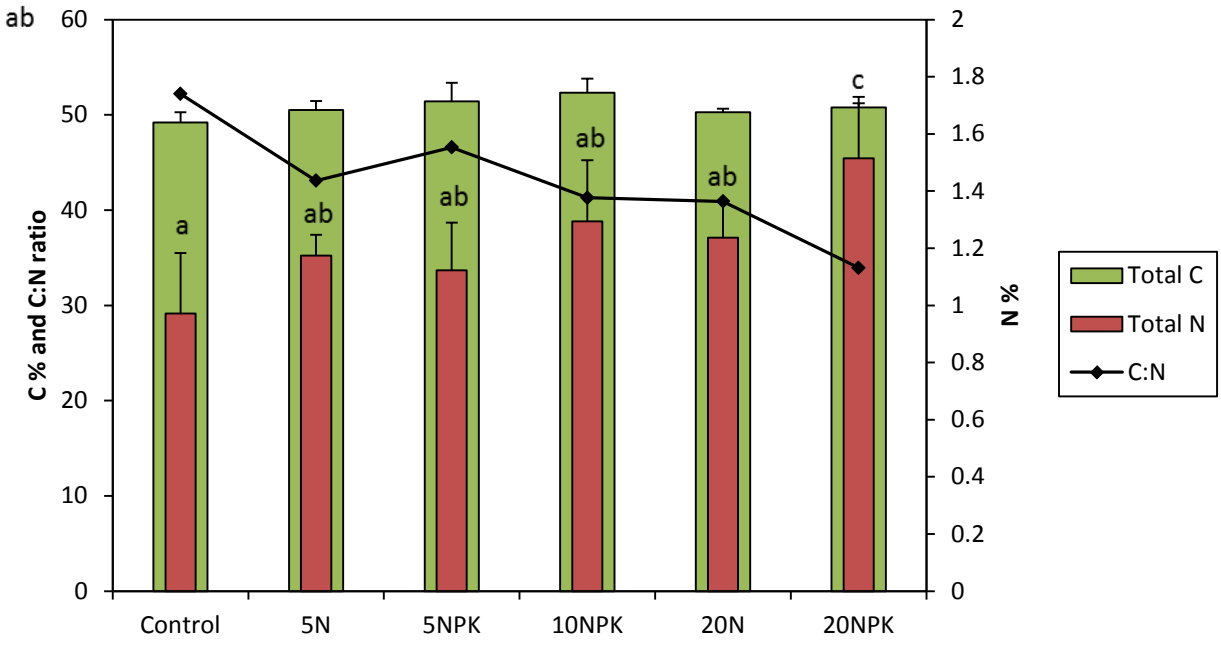


Figure 3-12 - Barplot representing total C and N (%) of total soil. Standard errors are represented by error bars. Lineplot represents C/N ratio.

3.7 Tables

Table 3-1 - List of plots sampled in both sampling years

Sample year	***Plots (N only)***	***Plots (N+PK)***
2013	dH_2O (control)	**N/A**
	5N	5N+PK
	N/A	10N+PK
	20N	20N+PK
2014	dH_2O (control)	dH_2O+PK
	5N	5N+PK
	10N	10N+PK
	20N	20N+PK

Table 3-2 - Adonis testing results of microbial (combination of bacterial and fungal), bacterial and fungal TRFLP based community data. Significant different ($p < 0.05$) are indicated with an asterix.

Sample Year	***Species***	***Site effect***		***PK addition effect***	
		R^2	*p*	R^2	*p*
2013	*Microbial*	0.34	0.013*	0.061	0.017*
	Bacterial	0.35	0.012*	0.061	0.012*
	Fungal	0.32	0.166	0.061	0.018*
2014	*Microbial*	0.35	0.007*	0.061	0.017*
	Bacterial	0.34	0.024*	0.056	0.062
	Fungal	0.36	0.003*	0.056	0.051

Table 3-3- Peat soil chemistry as percentage of element by dry weight. Values are mean of results percentage from sample. Standard errors are indicated in parentheses. Letters indicates significant difference among treatments (ANOVA)

	Total N (%)	**Total C (%)**	**P (%)**	**K (%)**
Control	0.97 (0.21)[a]	49.21 (1.06)[a]	0.06 (0.01)[a]	0.12 (0.01)[ab]
5N	1.18 (0.07)[ab]	50.50 (0.96)[a]	0.06 (0.01)[a]	0.12 (0.01) [ab]
5NPK	1.12 (0.17)[ab]	51.40 (1.95)[a]	0.08 (0.04)[a]	0.16 (0.06) [ab]
10NPK	1.30 (0.21)[ab]	52.32 (1.50)[a]	0.09 (0.04)[a]	0.14 (0.08) [ab]
20N	1.24 (0.13)[ab]	50.27 (0.36)[a]	0.05 (0.01)[a]	0.08 (0.01)[a]
20NPK	1.51 (0.21)[b]	50.77 (0.45)[a]	0.10 (0.02)[a]	0.20 (0.02)[b]

4 Chapter 2

4.1 Introduction

Peatlands account for 3% of the total land surface but have stored more than one third of the World's soil C (Gorham, 1991). Peatlands are generally acidic, waterlogged and cold, which prevents rapid decomposition and renders the soil nutrient limited. Peat soils are depleted in oxygen through some or all of the soil profile, resulting in ideal conditions for anaerobic CH_4 production. Considering the land coverage of peatlands, they are important players in the production of atmospheric CH_4, releasing 10% of total emission annually. Anaerobic CH_4 producers are coupled with the presence of methanotroph (CH_4 oxidizers). Most methanotroph are found in the upper aerobic zones, but enigmatic anaerobic CH_4 oxidizers might also be important in peatlands (Gupta et al. 2013). They are important in controlling the amount of CH_4 that is release, it is believed that 90% of CH_4 produced anaerobically by the methanogens are consumed, thus mitigating net CH_4 emissions substantially (Shannon and White, 1996).

Methanotrophs are ubiquitous gram-negative *Proteobacteria* but are generally found in wet or moist area. They are unique in their ability to uses CH_4 as their soil sources of C and energy (Hanson and Hanson, 1996). Naturally, they are an important player in mitigating CH_4 flux in both water/aquatic systems and soils/terrestrial system. Methanotrophs oxidize CH_4 through a series of biochemical steps; where the first step is the conversion of CH_4 to methanol carried by CH_4 monooxygenases (MMO). There are two different types of MMO: particulate (pMMO) and soluble (sMMO; Murrell et al., 2000). The pMMO operon contains three consecutive open reading frames (*pmoC*, *pmoA* and *pmoB*) in both type I and type II methanotrophs (that differ in use of serine versus RuMP assimilation pathways; Stolyar et al., 1999; Gilbert et al., 2000). Most methanotrophs encode the pMMO operon, whereas a restricted few solely encode the sMMO operon (contains numerous gene including *mmoX*), such as the

genus of acidophilic, uniquely facultative methanotrophs *Methylocella* found in peatlands (Dedysh et al., 2005). It is suggested that this genus of methanotroph can play a predominant role in CH_4 cycling in acidic *sphagnum* bogs (Dedysh et al., 2001). *Methylocella* have the ability to utilize other C sources such as acetate, pyruvate, succinate, malate, and ethanol (Dedysh et al., 2005). This relatively recent discovery after methanotrophs had been thought to solely utilize CH_4 for many decades shows that there are likely still many gaps in what is known about methanotroph ecology and implications for CH_4 dynamics wetlands.

Bogs are largely anoxic and are home to microorganisms capable of producing CH_4 through anaerobic respiration. These methanogens are key organisms in C cycling in any ecosystem. In fact, methanogens thrive in nutrient stressed environment as they are capable utilizing other electron acceptors allowing them to decompose organic material in anoxic habitats such as wetlands where electron acceptors are limited (Thauer, 1998a). This process involved numerous enzyme and cofactors including Methyl-Coenzyme M Reductase (MCR) coded by the *mcrA* gene (Thauer, 1998b). The MCR enzyme catalyzes the reduction of methyl-coenzyme M with coenzyme B (HS–CoB) to CH_4. This gene is unique to methanogens and is considered as a good alternative to 16S rRNA in phylogenetic analysis of methanogen and other culture independent techniques (Thauer, 1998b; Luthon et al., 2002). It was also reported that this gene could be a good candidate for qPCR analysis (Steinberg and Regan, 2009) and could be used to understand N deposition impact on methanogen population dynamics.

Atmospheric N deposition has increased substantially through fossil fuel combustion and intensive agricultural practices. The excess reactive N has the ability to change the plant communities of bogs as N is often limiting as a plant nutrient. At the Mer Bleue Bog, the effect

of N deposition on plant composition is well documented (Bubier et al., 2007). Plant functional type and environmental conditions including soil temperature, precipitation, water table position, and N deposition all can impact CH_4 fluxes in boreal peatlands (Moore and Knowles, 1989; Larmola et al., 2010; Moore et al., 2011). In turn, fluctuations of greenhouse gases concentration in the atmosphere, like CH_4, will lead to significant climatic and related environmental changes (IPCC, 2013). Reports indicate a rise in temperature of 1°C is equivalent to an increase in 4 N g ha^{-1} through increased rates of mineralization of stored N (Limpens et al., 2011); regardless of if excess N come from deposition or mineralization, the resulting effects could impacts the capacity of naturally nutrient poor (bog and poor fen) peatlands to store C. Past studies have demonstrated increased CH_4 emissions with additional N; however, much remains to be understood about the impacts of excess N on CH_4 cycling microbiota in peatlands.

Methanotrophs are capable of utilizing NH_4 ions as a substrate and oxidizing into NO_2 and NO_3. In a hypothetical system, where atmospheric N is increased, NH_4 deposited could compete with CH_4 for the active site leading to CH_4 oxidation inhibition (Dunfield and Knowles, 1995; Bodelier and Laanbroek, 2004; Nyerges et al., 2010) potentially leading to an increase in atmospheric CH_4. However, the exact mechanism is still unknown (Bodelier, 2011). Other reports indicate the inorganic N addition decrease atmospheric CH_4 emission through inhibition of methanogens. Competition from nitrate-reducing bacteria for organic C availability and by-products toxic to methanogen could decrease methanogen population (Klüber and Conrad, 1998; Fumoto et al., 2007). Liu and Greaver (2009) have compared 109 studies on wetlands with comparable environmental (climate) and physical factors (vegetation and soil types) have found that CH_4 emission increased by 95% over all ecosystem type including undrained wetlands but

CH_4 was not significant in drained wetlands. At Mer Bleue, it was reported that inorganic N deposition increase atmospheric CH_4 emission which is consistent with the studies above.

New technological advances in molecular biological tools and computational tools can aide immensely in exploring microbial communities. In fact, considerable progress on development of methods in microbial community analyses and activities have provided new insights on methanotrophs in peatlands with high-throughput sequencing technologies (Deng et al., 2013) and quantitative real-time PCR (qPCR) (Dedysh et al., 2005). Genes involved in the oxidation of CH_4 are well-conserved and can make good phylogenetic markers (McDonald et al., 2008). A commonly used functional marker gene is *pmoA*, which encodes the β-subunit of pMMO (belonging to the class of copper-containing membrane-bound monooxygenase) enzymes). A second commonly used functional marker is the *mmoX* gene, which encodes the α subunit of the sMMO enzyme. sMMO is a cytoplasmic enzyme that has a broader range of substrates. Both *pmoA* and *mmoX* have been shown to be congruent with 16S rRNA markers; thus both genes are good candidates as robust phylogenetic markers. Since *pmoA* genes have been sequenced for a larger number of methanotrophs in different environment setting, and because all known genera of methanotrophs except *Methylocella* contain *pmoA* (Degelmann et al., 2010), it is a good phylogenetic marker tool in sequence-based studies of methanotroph ecology.

The objective of this chapter is to characterize the dynamics and feedbacks of methanotroph communities and CH_4 cycling activities to N fertilization at the Mer Bleue Bog near Ottawa, Canada. I used physiological incubations to observe CH_4 oxidation and production potentials from soils collected across the experimental plots. To understand inorganic N addition on methanotrophy, I characterized methanotroph community structure using high throughput

amplicon sequencing of the *pmoA* gene and qPCR of *pmoA* and *mmoX* genes. Additionally, to complement CH_4 production potential, I also characterized *mcrA* gene copy number using qPCR to observe the N addition effect on methanogen.

Methanotroph communities will diversify by the increase N deposition. N addition has increase overall microbial diversity and therefore releases nutrient that was unavailable in lower N plots. Nutrient increases might increase overall methanotroph diversity. However, CH_4 consumption will be inhibited by the inorganic N addition as reports explained above have indicated. As for CH_4 production potential, inorganic N would decrease methanogen population through toxicity from nitrate-reducing bacteria by product. But, latest results at Mer Bleue concerning CH_4 fluxes showed an increase in CH_4 release due to vascular plants aerenchyma and higher water table.

4.2 Methods

4.2.1 Experimental site and sampling

The Mer Bleue bog (45°24' N latitude, 75°31' W longitude), located 10 km east of Ottawa, Canada, is a large raised ombrotrophic peat bog complex with hummock-hollow patterned topography. It is sphagnum moss dominated with an evergreen shrub overstory. Background N deposition is among the highest in Canada with an approximate annual deposition of 1.5 N g^{-2} yr^{-1}. Regional total N deposition rate from a wet atmospheric N deposition was estimated to be 0.6 to 0.8 g N m^2 yr^{-1} (Keith and Dillon, 1989; Sisterson et al., 1994). The water table depth typically averages 30 cm beneath the surface of the hummocks and in the 2014 growing season when I collected samples it was -32.0 cm.

CH_4 oxidizing bacteria are assumed to be found in soil depths above the oxic- anoxic interface or in close proximity to oxygen conducting plants issue within the anoxic layer. This interface allows both the presence of CH_4 and O_2 (Henckel et al., 2000). For that reason, samples at 30-40 cm depths were used to study the impact of N and/or P and K on methanotrophs. On the other hand, methanogenesis is an strictly anaerobic heterotrophic process utilizing a select range of small organic molecules or H_2 and CO_2(Freitag and Prosser, 2009). Therefore sampling at the depth below the oxic-anoxic layer (50-60 cm depth) should target active methanogenic communities, as they are in a zone of anoxia, but still relatively "fresh" peat and C and redox substrates, versus in more recalcitrant organic matter at lower depths.

4.2.2 Field Fertilization

Field fertilization experiments were established in 2000-2001 (Bubier et al., 2007). Fertilization plots (9 m^2 in area each; 3 replicates per treatment) were set up in a 400 m^2 section of the bog with little variation in micro-topography and vegetation. Solutions of N were added every three weeks as ammonium nitrate (NH_4NO_3) at 1.6**,** 3.2 and 6.4 g N m^{-2} yr^{-1}, approximately 5, 10 and 20 times the ambient growing season atmospheric deposition. Phosphorus and K were added as potassium dihydrogen phosphate (KH_2PO_4), equivalent to 6.3 g K and 5.0 g P m^{-2} yr^{-1}. Control plots received P and K solution or distilled H_2O. Plots were identified as Control, PK, 5N, 5N+PK, 10N, 10N+PK, 20N and 20N+PK. Treatment plots were relatively large, close to each other and located in a homogenous area of vegetation and topography. Also, large concentration of nutrients was added to those plots. As a result, I assumed that any consistent observed difference across replicates were due to fertilization treatments.

For each plot, three cores were taken in 2014 using a Russian corer that was thoroughly washed between samples. Soil depths of 30-40 and 50-60 cm below the vegetation layer representing the horizons above and below the water table were places in sterile plastic bags. Each bag was homogenized by mixing the sample mechanically by hand. Sample bags were placed in a cooler with ice until they were transported to Laurentian University where they were stored at 4°C.

4.2.3 DNA extraction

DNA was extracted from peat samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA). All DNA samples used in subsequent experiments were pooled from three extractions. Peat disruption and homogenization was performed using a 5 minute cycle on a 16 tube MiniBeadbeater TM (Biospec Products Inc., Bartlesville, OK). DNA samples were stored at -20°C for analysis at a later date.

4.2.4 Pyrotag sequencing

Pyrotag sequencing of pmoA gene fragments on the Roche 454 platform (at Molecular Research LLP) was completed on samples from the 30 to 40 cm depth horizon per Dowd et al. (2008). Forward primer A189 and reverse primer mb661 (Table 4-1) were used and amplified an approximately 470-bp internal section of *pmoA* as described by Costello et al. (1999). All replicated plots were treated as individual samples.

4.2.5 CH_4 oxidation

Mason© glass jars of 250 ml volume were used as microcosms for measuring soil CH_4 oxidation potential. Twenty grams of moist peat from each sample was put in a jar sealed with a modified metal lid with an incorporated butyl rubber septa. For each sample, ultra-high pure CH_4

was added 1% (v/v) CH_4 (10 000 ppm CH_4) and incubated in the dark at room temperature for a two days. 10 ml of headspace air was sampled at 0, 6, 12, 18, 24 hours post-CH_4 addition. To maintain pressure within the jar, 10 ml of room air was injected into the jar. Jars were shaken prior to sampling. Syringes were injected into a SRI 8100C gas chromatograph (Torrence, CA, USA) with a flame ionization detector. After the incubation period, jars were weight prior- and post-drying where they were placed in an oven at 70°C overnight. Dry peat mass and headspace volume was also measured. Chromatograph peak data were converted to µg CH_4 g^{-1} dry peat and µg CO_2 g^{-1} dry peat based on known standards. CH_4 oxidation potentials were calculated by linear regression of µg CH_4 g^{-1} dry peat as a function of time. CO_2 production potential was calculated by linear regression of µg CO_2 g^{-1} dry peat as a function of time. Cumulative CH_4 oxidation potentials and CO_2 production potential were corrected for loss of mass of gasses during multiple samplings.

4.2.6 CH_4 production potential

Falcon© tubes of 50 ml volume were used as a mesocosm for measuring soil CH_4 production potential. For each sample 10 g of moist peat was placed in the tubes and sealed with a modified plastic cap with an incorporated butyl rubber septa under anoxic conditions in a glove box. For each sample, tubes were incubated in a controlled incubator at 25°C for 80 days. 5 ml of headspace air was sampled at 0, 40 and 80 days post-incubation. To maintain negative pressure within the jar, 5 ml of N_2 was injected into the jar. Jars were shaken prior to sampling. Syringes were injected into a SRI 8100C gas chromatograph (Torrence, CA, USA) with a flame ionization detector. After the incubation period, jars were weight prior- and post-drying where they were placed in an oven at 70°C overnight. Dry peat mass and headspace volume was also measured. Chromatograph peak data was converted to µg CH_4 g^{-1} dry peat based on known standards. CH_4

production potential was calculated by linear regression of µg CH_4 g^{-1} dry peat as a function of time. Cumulative CH_4 production potentials were corrected for multiple sampling.

4.2.7 Quantitative polymerase chain reaction

The *pmoA* and *mmoX* functional genes markers were q-PCR-amplified using oligonucleotide primers and following methods described in Costello and Lidstrom (1999) and Rahman et al. (2011) respectively. Primer sequences are described in Table 4-1. qPCR assays were conducted in polypropylene 96–well plates on an Agilent Technologies Stratagene MX3005P qPCR system. Each 20 µl reaction contained the following: 10 µl of Thermo Scientific DyNAmo HS SYBR green qPCR 2X Master Mix, 0.5 µl of each primer sets 9 µl of dH_2O. All PCR have a heating step of 15 mins at 95°C. PCR conditions for *pmoA* were 45 cycles of 95°C for 30 sec, a two-step annealing at 56°C for 30 sec and 60°C for 30 sec, and extension at 77°C for 30 sec. Data were collected at the end of extension phase. As for *mmoX*, 45 cycles of melting at 95°C for 15 sec and an annealing at 68°C with no extension. Data was collected at the end of annealing phase. Finally, PCR conditions for *mcrA* were 45 cycles of melting at 95°C for 15 sec and an annealing at 68°C with no extension. Data was collected at the end of annealing phase. All reactions, including sets of standards, were performed in triplicate. After each qPCR run, melting curve analysis was performed to verify the presence of the desired amplicon and to confirm products were not from primer-dimers or other artifacts. Three dilutions of *Methylococcus capsulatus* (Foster and David) ATCC ® 19069 ™ and *Methylocella silvestris* (Dunfield) DSMZ 15510 DNA were used as a standard for *pmoA* and *mmoX* respectively.

To evaluate the gene copy abundance with the 50 – 60 cm anoxic zone of our soil samples, the *mcrA* functional gene marker were q-PCR-amplified using oligonucleotide primers characterized that targets methanogens community following methods described in Steinberg and

Regan (2008). Primers are described in (Table 4-1). PCR conditions for *mcrA* were 15 min at 95°C, followed by 45 cycles of melting at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec. Data was collected at the end of annealing phase. *Methanoregula Boonei* 6A8 (Brauer et al., 2010) was used as a standard for *mcrA*.

4.2.8 Statistical analyses

Molecular Amplicon sequence datasets obtained were then analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Data were quality filtered using QIIME default parameters (quality score = 25, min length=200, max length = 1000). Additional quality filtering and OTU clustering was performed with the Usearch 5.2.236 program, which utilizes the UCHIME algorithm to identify chimera sequences for removal against *pmoA* methanotroph dataset from Dumont et al. (Edgar, 2010; Edgar et al., 2011; Dumont et al., 2014). De novo OTU picking with uclust was used to form the representative OTU dataset (Edgar, 2010). Bacterial taxonomy was assigned using the RDP classification algorithm against *pmoA* dataset of Dumont et al. (2014). Muscle was used to produce a denovo alignment of all OTU sequences and produce a phylogenetic tree (Edgar, 2004). 48 Final OTUs represented potential species based on unique *pmoA* sequences that were discriminated at a level of 97% similarity.

Pyrotag phylogenetic and methanotroph community analyses data were analyzed in QIIME pipeline and R packages phyloseq and (McMurdie and Holmes, 2013; R Core Team, 2013; Oksanen et al., 2014). Microbial communities between samples were compared using a non-metric multidimensional scaling analysis (NMDS) with the vegan package. OTU were analyzed in the context of relative abundance. Using QIIME, area chart plot were generated to observed relative species abundance across our samples. An agglomerative hierarchical cluster

analysis based on Ward's method of dissimilarity calculation was used to provide another visual indication on relatedness among the treatments.

Gas analyses results were analysed using regression analysis using standard R packages. Regression analysis was performed to determine the effect of N on activity measurements.

Standard curves were produced using triplicate 10-fold dilutions of DNA from pure culture *M. capsulatus* for *pmoA* and *M. silvestris* for *mmoX*. At least three nonzero standard concentrations per assay were used with concentration ranging from 10^{-2} to 10 ng of DNA per reaction. Target copy numbers were calculated from the standard curves using threshold cycle value (CT). For all qPCR assays, there was a linear relationship between the log of the DNA concentration and the calculated threshold cycle value (CT) across the specified concentration range ($R^2 > 0.95$). Linear regression analysis were performed on those ratio compared to N deposition level with and without P and K.

4.3 Results

4.3.1 Methanotroph communities

A total of 83 161 *pmoA* reads across the 27 samples/experimental plots were generated forming the sequence dataset. Usearch OTU picking parameters was able to identify 1 206 distinct OTUs. More than 50% were unmatched sequence to the BLAST database and, upon further investigation; they were deemed as computational artifact and were discarded from further analyses.

There were not significant *pmoA*-based community differences in methanotroph communities among the fertilization treatments (Figure 4-3, Figure 4-4). NMDS showed 95%

confidence interval ellipses that are overlapping by treatments and controls, meaning N addition or P and K addition had no significant impact on *pmoA*-containing methanotroph community. Similarly cluster analysis of plots provides also demonstrated no observed patterns by treatment; thus no effects can be attributed to additions of N and/or P and K (Figure 4-3). Moreover, phylogenetic identification of across all plots illustrated that most fall within a few select genera (Figure 4-2); with *Methylocystis sp* and *Methylomonas sp* representing the majority of the *pmoA*-containing methanotrophs and with *Methylocystis* dominating across all plots. A stacked area chart reveals the coarse-scale phylogenetic assemblages of methanotroph communities among all treatment plots; there were no clear effects of N and/or PK loading on methanotroph communities (Figure 4-1).

4.3.2 CH_4 dynamics

At 30 – 40 cm depth, I observed no significant effects of N and/or PK additions on methanotrophy (Figure 4-5); oxidation rates were low and variability among treatments was high Figure 4-5). In some instances, I observed slight CH_4 production (control, 10N, 10N+PK, 20N+PK). At 50 – 60 cm depth, anaerobic CH_4 production also showed no significant changes with N addition (Figure 4-6). Gas production rates among all plots were small and highly variable. Addition of P and K also had no significant effect on CH_4 production rate. However, we observed a decreasing trend in both N only addition and N with addition of P and K plots.

4.3.3 Functional gene abundance

My data indicated no significant changes in *mmoX* or *pmoA* copy number from the N and/or P and K fertilization (Figure 4-7, Figure 6-8). However CH_4 production marker gene *mcrA* copy number significantly decreased with increasing N addition (P =0.021, Figure 4-10).

Addition of P and K (alone or with N) had no effect on *mcrA* copy number. The ratio of *mmoX* gene to *pmoA* gene, we found that there is no significant different as a measure of increase N addition. Interestingly, the addition of P and K change the sign of the slope as depicted in Figure 4-9.

4.4 Discussion

4.4.1 Methanotroph communities

Our purpose was to investigate the effect of N addition on peatlands CH_4-related microbial communities; observing effects on methanotroph and methanogens. Both are key organisms in the generation of this potent greenhouse gas. The addition of N as ammonia and nitrate could have dire consequence in releasing more CH_4. We have shown that with the addition of nutrient, the methanotroph communities did not change significantly or increase in *pmoA* gene copy numbers (Figure 4-4, Figure 4-5, Figure 4-7, Figure 4-8). Our results do not reflect a decreasing pattern as other studies has shown, but the drastic physical changes or the wetter year might have skewed our data.

I observed a difference in methanotroph communities following N addition and/or P and K addition (Figure 4-3, Figure 4-4). I would have expected a changed in methanotrophic community due to the changes in vegetation communities seen in the same experimental plots as described by Larmola et al. (2013) and earlier by Bubier et al (2007) and Basiliko et al. (2006). Methanotrophs are effectively lithoautotrophs naturally found in nutrient poor environments where CH_4 is abundant, like in wetlands. Moreover, *Sphagnum* moss are known to have methanotroph symbionts in their tissues (Basiliko et al., 2004). With the loss of *Sphagnum*

mosses observed in the high N and NPK plots, I would have expected to observe a change methanotroph community structure and MOB gene abundance.

Members of the genera *Methylomonas* and *Methylocystis* dominated the methanotroph communities regardless of treatments (Figure 4-1, Figure 4-2). This is similar to a pattern seen in a Dutch peat bog (Kip et al., 2011). *Methylocystis sp*. are commonly found in ecosystems where the soil is acid (pH < 5) and CH_4 is present (Dedysh et al., 2007; Belova et al., 2013). *Methylomonas* have also been found in other bogs (Chen et al., 2008; Kip et al., 2011). *Methylocystis* and *Methylomonas* have both MMO genes (*mmoX* and *pmoA*), and their predominance could explain why *mmoX* (Figure 4-7) and *pmoA* (Figure 4-8) qPCR data remained unchanged across treatments.

Many factors could explain *Methylocystis* dominance in our plots. As mentioned before, possibly the most important factor is the acidity of the peat soils. Other studies has reported *Methylocystis* to be particularly abundant in acidic peat soils (Chen et al., 2008). Mer Bleue bog pore water was acidic (pH = 4.3). Another factor, would be the versatility of *Methylocystis* in multicarbon compounds metabolism capabilities. Belova et al. (2013) demonstrated *Methylocystis sps.* survivability when CH_4 availability is limited. It was able to grow on acetate alone, disproving earlier data on it being an obligate methanotroph. This strategy could be a reason for it being dominating in our soils, if CH_4 concentrations are low. This would make sense considering aerenchyma present driving CH_4 from below water table straight to the atmosphere, and the low numbers of *mcrA* gene copies and low rates of CH_4 production observed.

4.4.2 CH_4 dynamics

We have observed decrease in methanogen abundance (Figure 4-10) but in change abundance in both soluble and particulate MMO. As methanotroph thrive on CH_4 release by the methanogen, both population dynamics would be synchronized. We have not observed in our experiment. However, our samples were taken in an abnormally wet year. Roulet et al (2012) have observed Mer Bleue's NEE for the past decade and claim that water table could be a significant factor in NEE at Mer Bleue. The same impact factor could explain our abnormal results.

Surprisingly, despite changes in vegetation cover and broad-scale microbial communities reported earlier in the Mer Bleue fertilization experiments (Basiliko et al., 2006; Larmola et al., 2013), my data show no changes in CH_4 oxidation potential as N deposition increased (Figure 4-5, Figure 4-7, Figure 4-8). However this finding is perhaps consistent with the lack of change in MOB community structure. Application of inorganic N has been documented as detrimental to atmospheric CH_4 uptake by soils (Bodelier and Laanbroek, 2004). In our fertilization experiments, inorganic N is deposited , potentially providing enough ammonia or ammonium substrate to compete with CH_4 for MMO active sites (Nyerges and Stein, 2009). As well, Osmotic effects due to salt additions and inhibition by nitrate intermediate molecule, nitrite, which is toxic to methanotrophs, were also potentially expected, but not observed. Another idea would be the inorganic N was quickly consumed by overlaying organisms (vascular plants and microbes) and have not reach the 30-40 cm depth. In fact, methanotrophic communities are able to adapt to small changes in N concentrations through successional changes of species of methanotrophs. Nyerges et al (2010) have shown how one species of methanotroph is inhibited by inorganic N addition, but another species thrives in higher N conditions. However, since we have not observed any significant changes in methanotrophic communities among our

experimental plots (Figure 6-2), maybe there is an upper threshold of inorganic N for methanotrophic communities to efficiently consume CH_4 and mitigate CH_4 release in bogs.

Addition of N decreased methanogen population as shown in our qPCR data of *mcrA* functional marker gene (Figure 4-10). These data are in accord with past data that have shown a significant decrease in or even absence of, CH_4 production with increasing N (Armés, 2009). Armés (2009) stated that nutrient and N added might not have reached the anoxic zones which could explain our observation on decreasing *mcrA.* Although the addition of N on methanogens in peatlands is not well known, changes in plant communities could be a cause. Anoxia is required for the production of CH_4 and can cause oxygen stress in methanogens (Kiener and Leisinger, 1983). Sedges, such as *E.vaginatum,* and other vascular plants that increased greatly with fertilization could facilitate the transfer of oxygen from aboveground to the rhizosphere through aerenchyma in their roots (Murphy and Moore, 2010). Obtaining data O_2 levels at different depths in situ could help explain this potential explanation for having seen reduced *mcrA* gene copy numbers. With the decrease in *mcrA* gene copy number, it is not surprising to observe a decreasing pattern in CH_4 production potential in mesocosm (Figure 4-6). These data shows the complexity of the microbial community and changes that occurs as a result of N deposition.

In situ CH_4 emissions and underlying microbial production and consumption processes are closely linked to water table levels. As water tables rise, one usually observes an increase in CH_4 production and emissions (Moore et al., 2011). In 2014, water table measured at Mer Bleue was unusually high (Figure 3-1). While, I should have observed an increase in CH_4 production potential, my data indicate the opposite. In fact, a water table rise in higher N plots is due to a depression of the peat layer that is at large decomposing more quickly than control plots (Larmola et al., 2013), bringing the vegetation layer closer to the water table; the roots and

rhizosphere are also closer. If one considers the argument that the new, highly abundant vascular plants are providing O_2 via root systems to the peat profile, then I should have observed a decrease in *mcrA* abundance/methanogen populations, as conditions are no longer favourable. It would seem that N deposition is indirectly affecting the methanogen population via the vegetation shift. Following the same logic, as the methanogen population decreases, CH_4 concentration at the oxic-anoxic zone would be lower. However interestingly this did not lead to changes in methanotroph abundance as described by my *mmoX* and *pmoA* qPCR data or to CH_4 oxidation rates.

4.5 Conclusion

Contrary to my initial hypotheses, my data indicated no changes in methanotrophy, concurrent with an unchanged methanotroph community after prolonged increased N deposition and /or P and K; consequently the net effect of N fertilization on *in situ* CH_4 dynamics might be dictated by the nutrient effects on CH_4 production. My data show CH_4 production potential in controlled experiment has not changed significantly but that *mcrA* gene copy number was significantly reduced with N addition. Past experiments also showed a decrease in CH_4 production in vitro, however, field CH_4 fluxes data at Mer Bleue indicate increasing CH_4 efflux as N deposition increased (Armés, 2009). CH_4 dynamics seemed to be the result of a much more complex system where additional environmental data are needed to fully understand inconsistencies between in vitro approaches here and in situ gas fluxes. Moreover, more comprehensive understanding on exact function of inorganic N on methanotrophy and methanogenesis could provide additional clues as to the possible dynamics in a growing population and increasing fossil fuels usage. While using next-generation sequencing does

provide information on microbial population dynamics, being able to successfully culture the various group of methanotrophs and methanogens could help us understand the functions of me

4.6 Figures

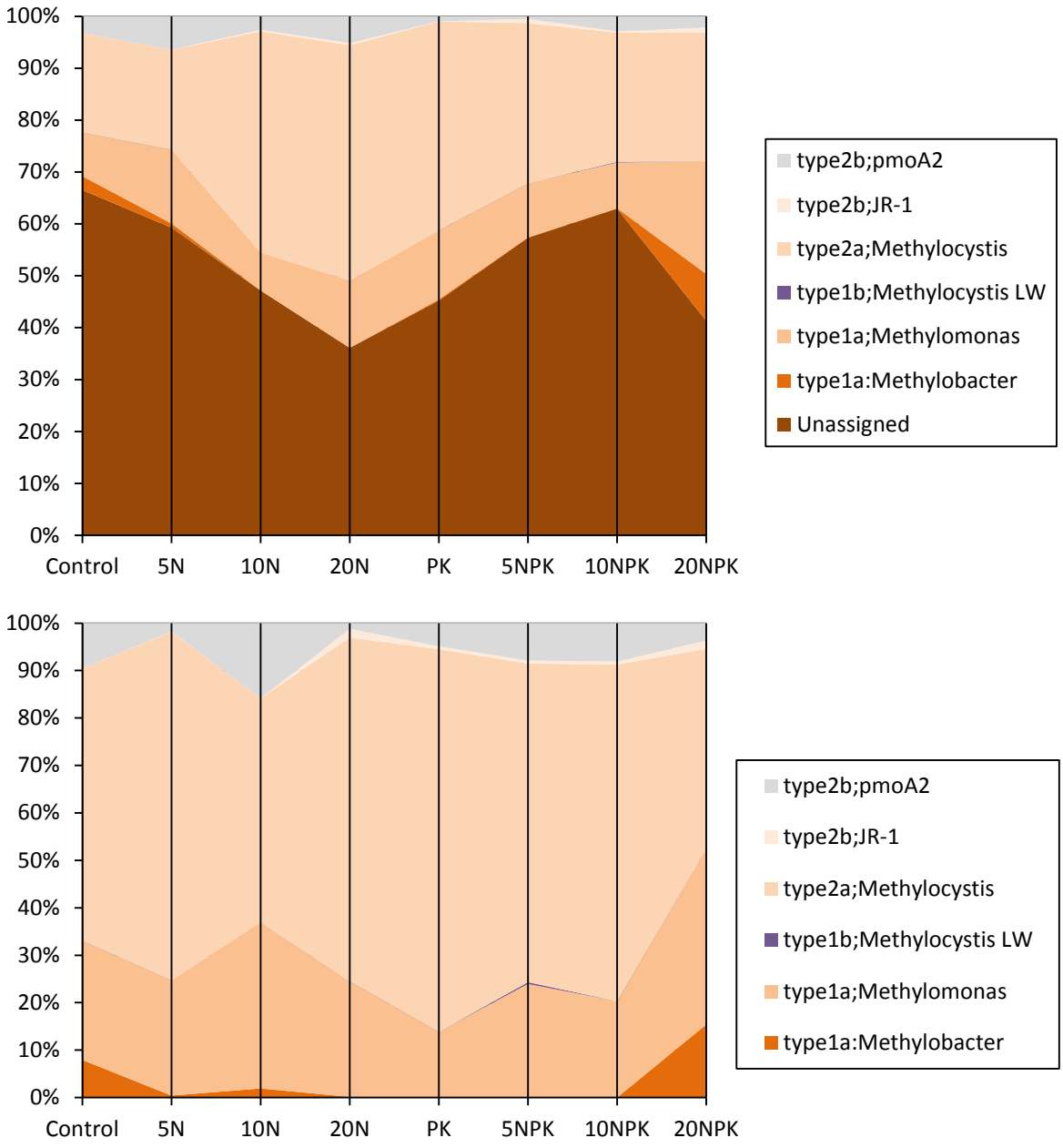


Figure 4-1 - Stacked area chart of *pmoA*-based phylogenetic groups detected in each treatment. (Top) original OTUs including the unassigned/ non-pmoA sequences. (Bottom) abundance without the unassigned sequences.

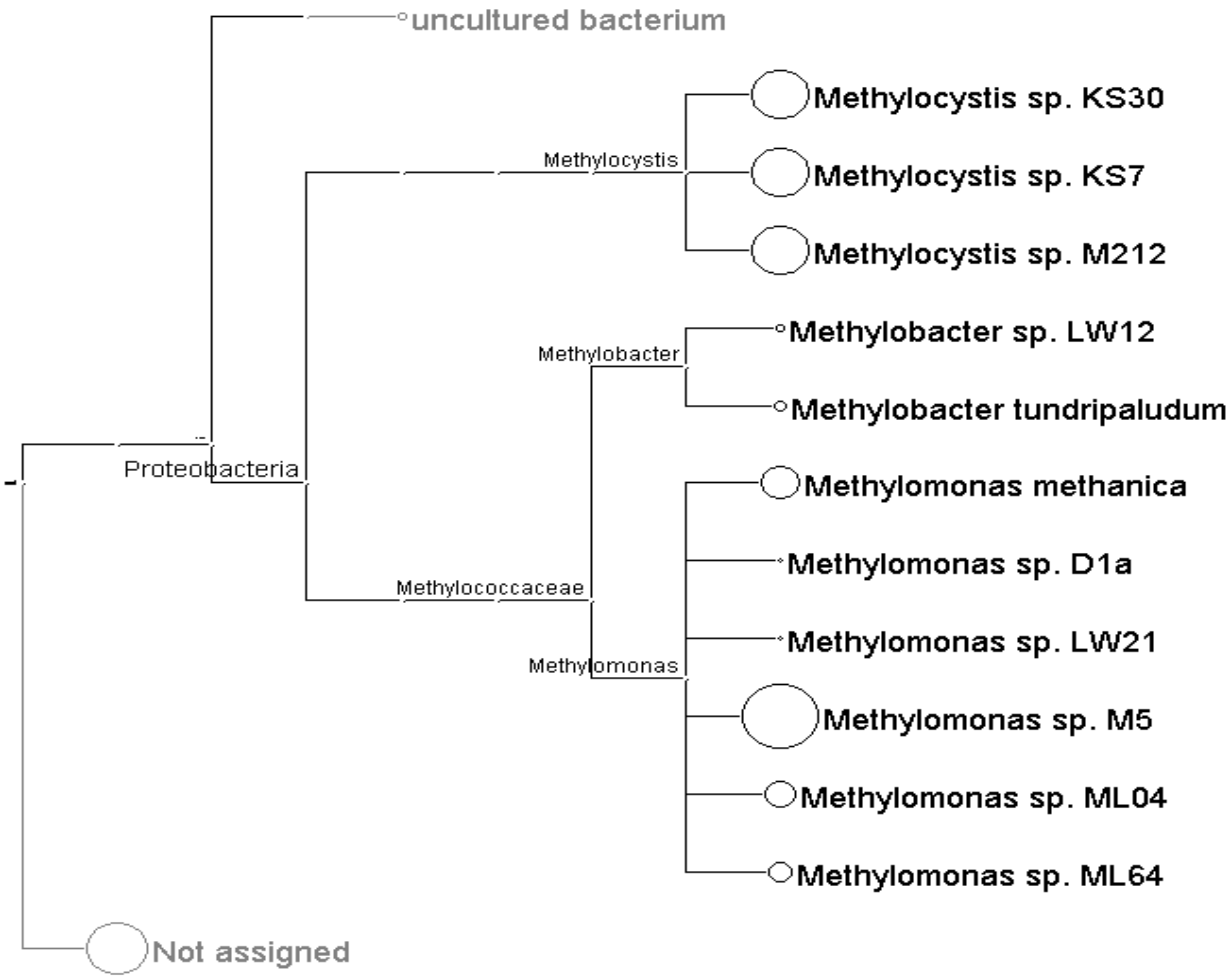


Figure 4-2 - Phylogenetic tree grouping *pmoA* sequences (across all treatments) obtained in this study. Circle sizes are proportional to number of OTUs.

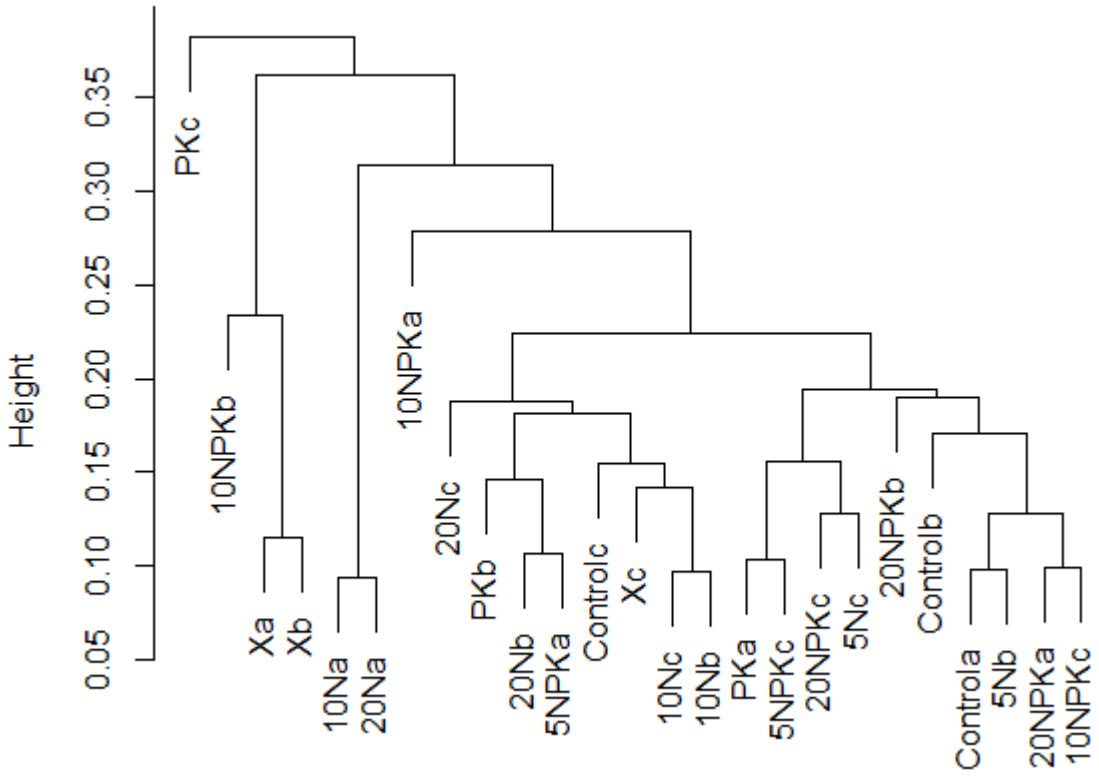


Figure 4-3 Hierarchal clustering using Ward's methods of *pmoA* based methanotroph communities across all plots. No significant clustering was observed by treatment.

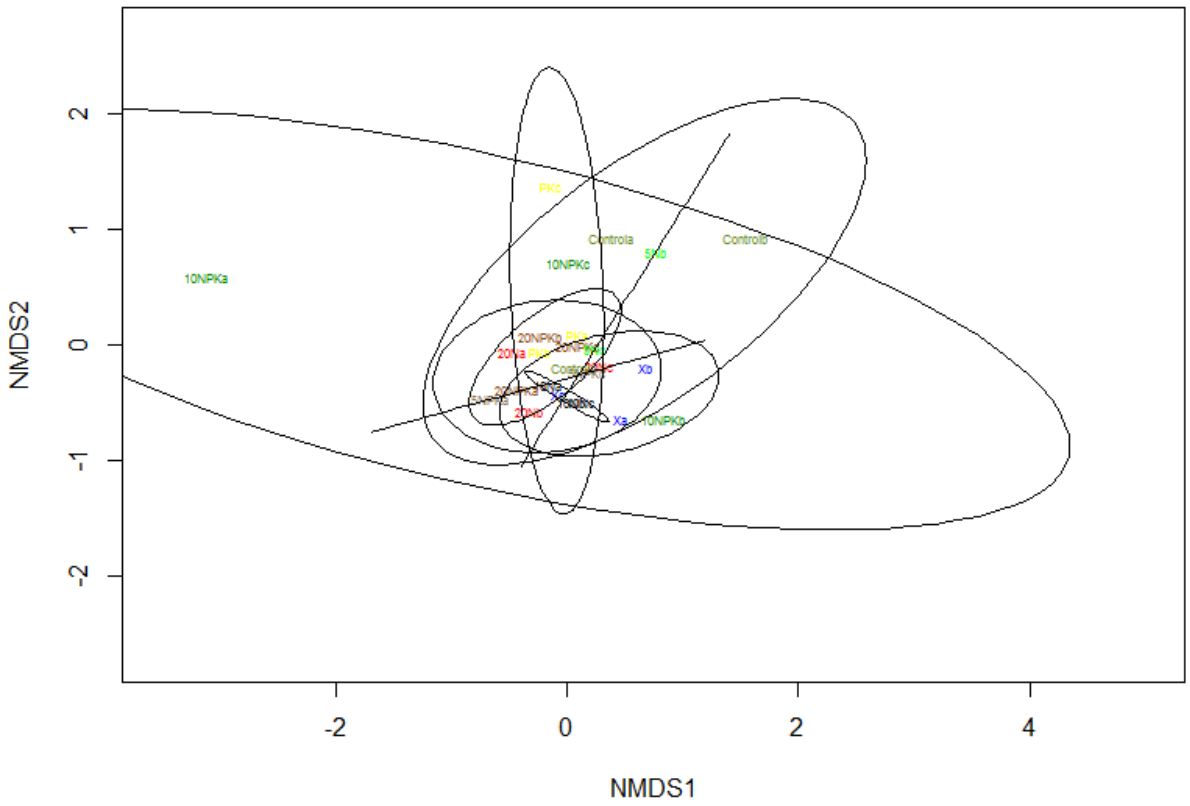


Figure 4-4 NMDS analysis of *pmoA* based methanotroph communities. Ellipses of each treatment show 95% confidence intervals.

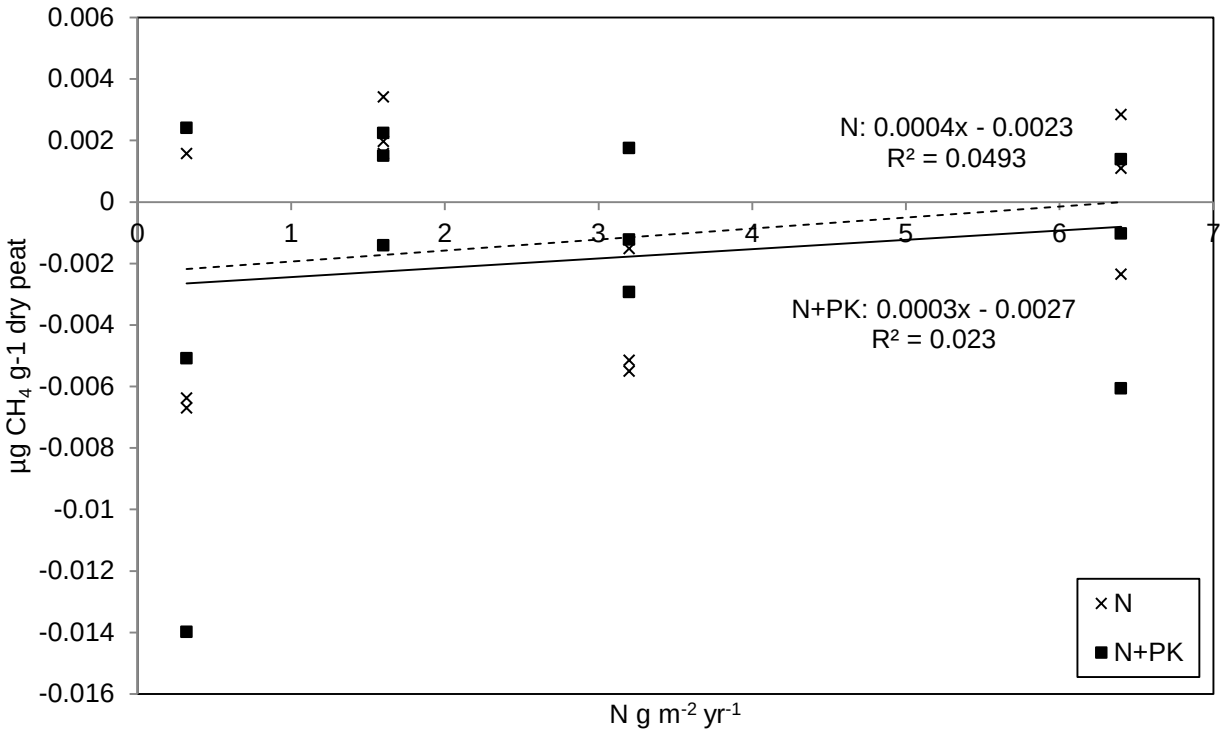


Figure 4-5- Rates of CH_4 oxidation potential across increasing N addition treatments at 30-40 cm depth. Dotted line: No addition of PK; solid line: With addition of PK.

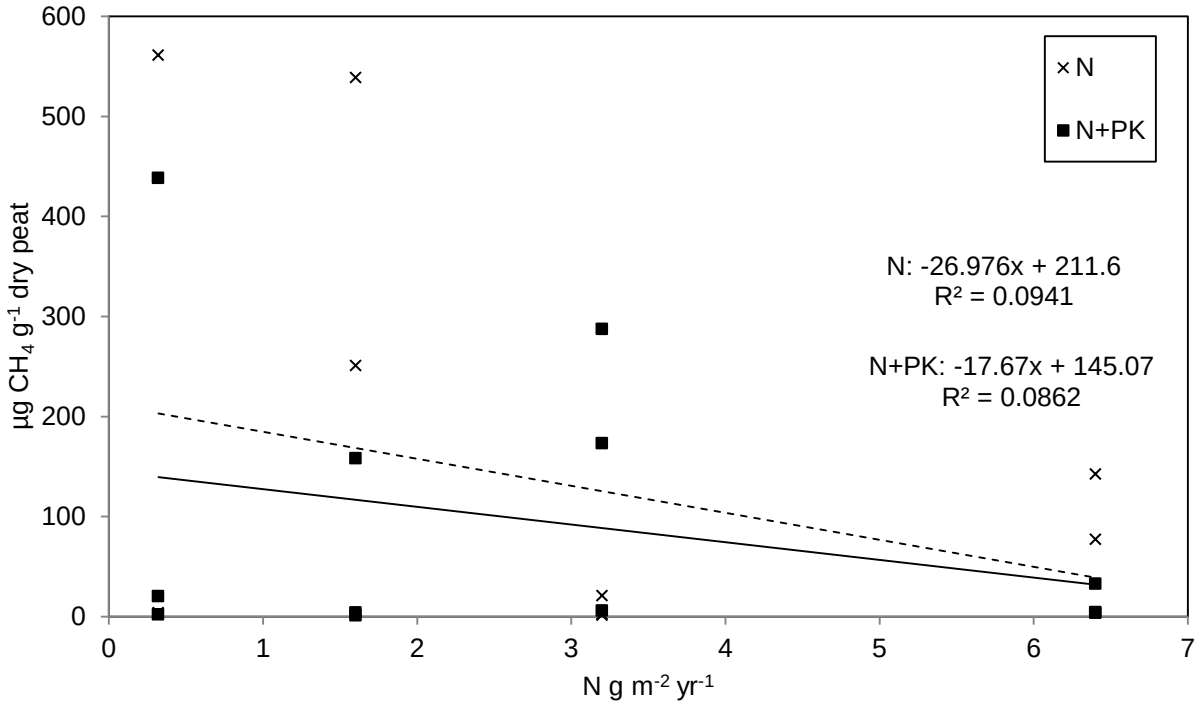


Figure 4-6 - Rates of CH_4 production potential across increasing N addition treatments at 50-60 cm depth. Dotted line: No addition of PK; solid line: With addition of PK.

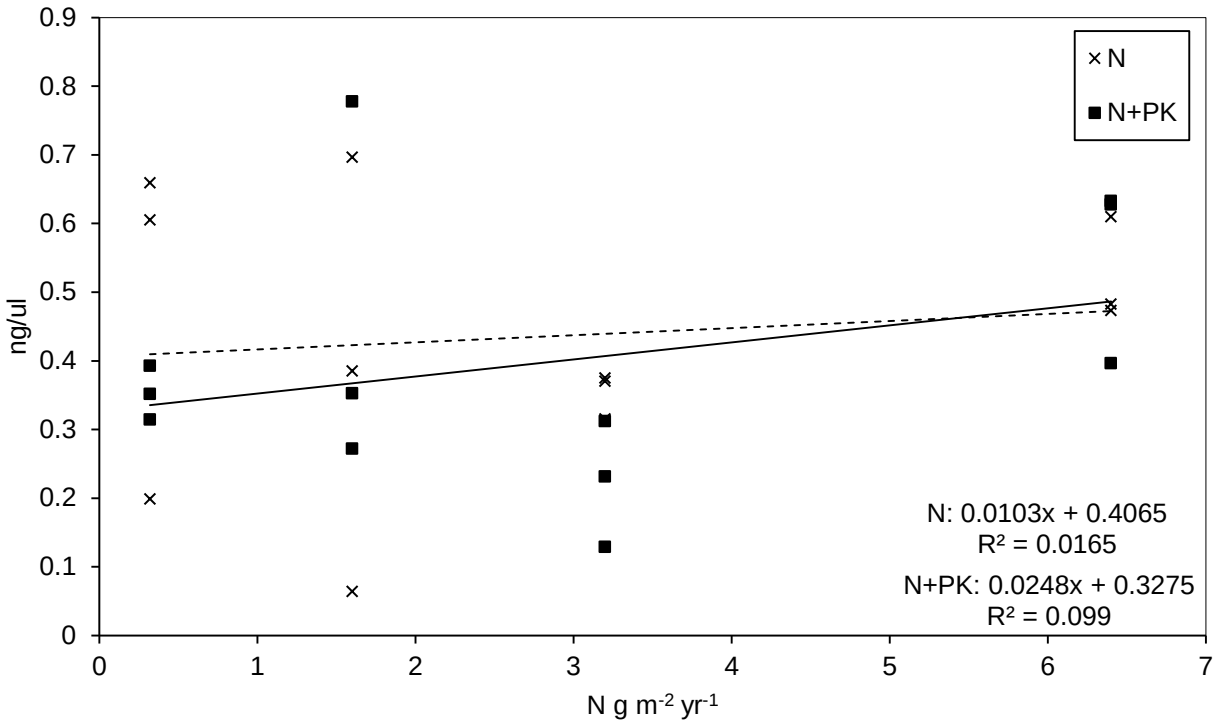


Figure 4-7- Regression plot of *mmoX* copy number vs N added with and without PK. (Regression line: N only = dashed; N+PK = solid).

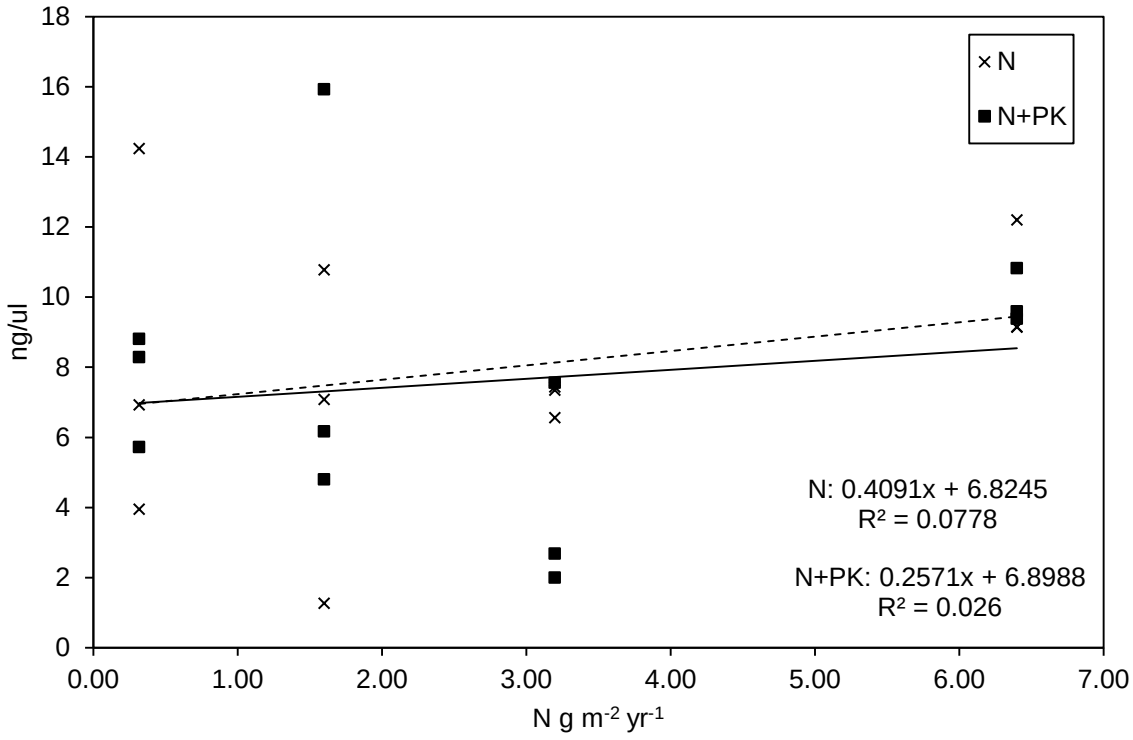


Figure 4-8 - Regression plot of *pmoA* copy number vs N added with and without PK. (Regression line: N only = dashed; N+PK = solid).

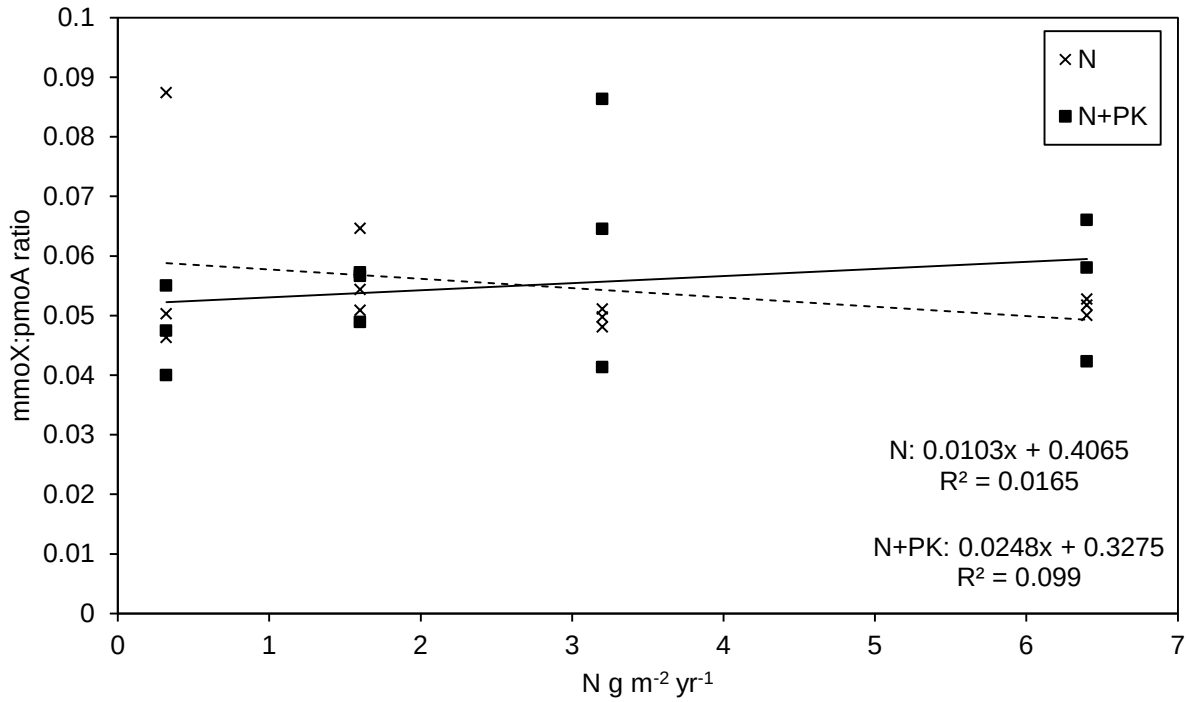


Figure 4-9 - Regression plot of *mmoX*:*pmoA* gene copy ratio vs N added with and without PK. (Regression line: N only = dashed; N+PK = solid).

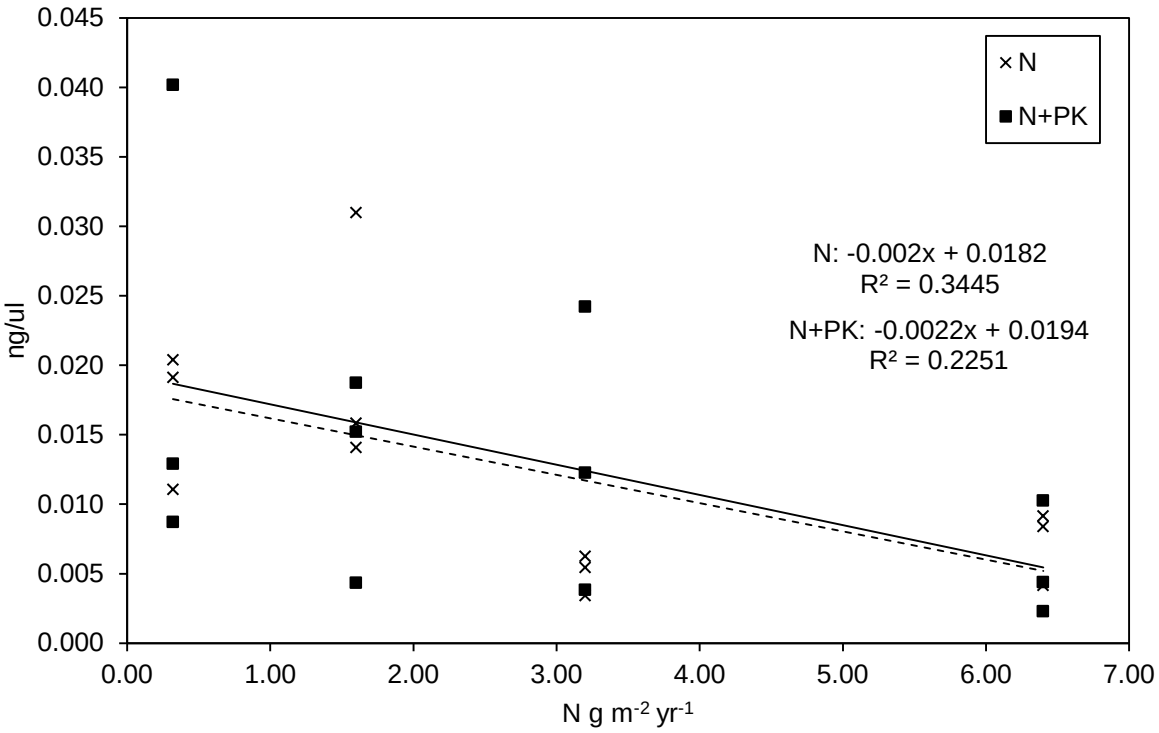


Figure 4-10 - Regression plot of *mcrA* copy number vs N added with and without PK. (Regression line: N only = dashed; N+PK = solid).

4.7 Tables

Table 4-1 - Primers for CH_4 dynamics functional genes

Target gene	Primer name	Sequence	Annealing (°C)	Citation
pmoA	A189F	GGN GAC TGG GAC TTC TGG	65.5	(Costello and Lidstrom, 1999)
	mb661R	CCG GMG CAA CGT CYT TAC C		
mmoX	mmoXLF	GAA GAT TGG GGC GGC ATC TG	68.0	(Rahman et al., 2011)
	mmoXLR	CCC AAT CAT CGC TGA AGG AGT		
mcrA	mlasF	GGT GGT GTM GGD TTC ACM CAR TA	48.0	(Steinberg and Regan, 2008)
	mcrA-revR	CGT TCA TBG CGT AGT TVG GRT AGT		

5 Thesis conclusions and future directions

The original questions of my thesis centred around whether and how increased N and nutrient additions in an ombrotrophic bog would impact microbial community structure, and in turn how any changes would link to microbial greenhouse gas cycling. The ultimate goal is to help understand the role microbes play in the climate change feedbacks in northern peatlands. With the continued increase of global anthropogenic inorganic N and potential negative impacts on peatland C storage, these are valid question to consider.

To answer these questions, I began with broad fingerprinting approaches to characterize microbial community structure (i.e. using T-RFLP and qPCR for fungi–bacteria ratios). My data have indicated changes in microbial communities following N and nutrient addition; and both bacterial and fungal communities have changed. These patterns were perhaps not surprising considering the impacts additions have had on the vegetation community and even on broad physical characteristics such as peat depth and relative water table position. In control plots, *Sphagnum* mosses were able to suppress microbial activities via the production of anti-microbial chemicals and by maintaining the strongly acidic conditions in surface peat. *Sphagnum* mosses perhaps were also successful at keeping numbers and biomass of higher trophic plants lower; including less rich and labile organic substrates and nutrients to the soil through leaf litter and root exudates. Additional and more labile organic substrates could explain the increased microbial diversity. As microbial communities diversify, microbial decomposition increases simultaneously, thus increasing C export. This pattern has been observed in previous studies, including a weakened ability to store C at the ecosystem level (Basiliko et al., 2006; Larmola et al., 2013).

My data presented in this thesis do not contain detailed taxonomic information on the type of microbes at hand. Obtaining microbial "species-level" data could help provide an idea of what functional group have appeared as a result of N deposition. With current technology and the booming –omics research approaches, next-generation high throughput sequencing might be a logical next step. Alternatively, metagenomics approaches might allow a functional profile of each treatment and answer many other questions concerning fluxes of GHG, carbon, and nutrient cycling.

The second chapter of my thesis looked at N and nutrient addition effects on microbial CH_4 dynamics, including methanotrophs and methanogens. Peatlands are typically net C sinks but also generate more than 10% of atmospheric CH_4, which is 25 times more effective as a greenhouse gas compared to CO_2. It was initially expected that understanding how communities of the key microbial players affect CH_4 dynamics was essential in answering one of my main thesis question: what is the impact of N and nutrient addition on CH_4 cycling, methanotroph community and methanogens in an ombrotrophic bog. However my data indicated N and nutrient addition had no significant impact on methanotroph communities in contrast to prior expectations. NMDS with pyrotag sequence data and *mmoX* and *pmoA* qPCR data have shown that were no substantial community structure differences or quantities of methanotroph functional genes. I was expecting a change in methanotroph considering the change in the physical and plant communities changes in the high N + PK plots observed, as vegetation often helps shape the underlying microbial community. Moreover, CH_4 oxidation potential also did not change with N deposition. I hypothesized that ammonia might have competed with CH_4 for MMO actives site, thus decreasing CH_4 oxidation. Observations of CH_4 oxidation increase and decrease have been observed in other studies.

The other key microbial players in CH_4 cycling are the methanogens. In past studies, N deposition has been associated with increases in atmospheric CH_4 release at Mer Bleue. However, in my work, I have observed a significant decrease in *mcrA* gene abundance with fertilization. Additionally, data indicated no significant effect on CH_4 production potential. One explanation might have been that the vascular plants increasing in abundance provided more O_2 to the rhizosphere via aerenchyma. In doing so, the strictly anaerobic methanogens, were inhibited, consistent with the reducing *mcrA* copy numbers.

Methanotroph communities and activities are directly dependent on the activities of methanogens via supply of CH_4. CH_4 oxidizing bacteria are therefore situated in the oxic-anoxic transition soil layer where concentrations of both O_2 and CH_4 converge. As CH_4 production grows, logically one would have expected to observe an increase in CH_4 oxidation. In my case however, data is consistent with this logic. However, additional study of these microbial communities would be important to further test this hypothesis. My sampling year was atypically wet and could have affected methanotrophs; my data showing little effect could be an "outlier" in a broader temporal context.

As mentioned in Roulet et al (2012), Mer Bleue bog has shown inter-annual variability of NEE due to changes in water table levels. Both sampling years in my thesis had significant different water tables and my data in the first research chapter indicated significant changes in broad-spectrum microbial communities between years; this important factor of inter-annual variability could also have affected CH_4 cycling communities (i.e. there might have been effects in the more normal hydrologic year of 2013 when methanotrophs were not characterized).

Microbial communities are very dynamic and can adapt to environmental changes quickly. My data showed a number reasons to believe that anthropogenic N and nutrient deposition impact peatland microbial biogeochemistry and might alter the natural processes of C and GHG cycling in particular. Although they constitute only a small proportion of the Earth's land, peatlands are important C reservoirs and sources of CH_4. With the data I have presented in this thesis, I have shown that N and nutrient addition is changing peatland microbial communities potentially related to broad scale mineralization and C release that is consisted with prior in situ studies of C exchange Those changes are likely linked to a positive feedback loop whereby increased N associated with fossil fuel burning will lead to enhanced C loss from peatlands, warmer climate, enhanced N mineralization in peatlands, etc... However expected changes in CH_4 cycling microbial communities were not observed in this study. Although this is tentatively good news, further work across a broader range of weather conditions and water table positions is warranted, as past and ongoing measurements of in situ methane fluxes indicated that nutrient additions may be impacting net methane exchange.

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