MICROBIAL FUNCTIONAL GROUPS INVOLVED IN GREENHOUSE GAS FLUXES FOLLOWING SITE PREPARATION AND FERTILIZATION OF WET LOW-PRODUCTIVITY FOREST ECOSYSTEMS

by

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Abstract

Forest site preparation and fertilization can improve stand productivity, but can alter the efflux rates of greenhouse gases (GHGs), CO_2, CH_4 and N_2O, from wet soils. This study investigated the effects of these management practices on GHG fluxes (using static closed chambers), soil physico-chemical parameters, microbial community structure (using terminal-restriction fragment length polymorphism (T-RFLP) of bacterial 16S and fungal ITS targets) and microbial functional group abundance (methanogens, methanotrophs, nitrifiers, denitrifiers, sulphate-reducing bacteria, using quantitative PCR) in both forest floor and mineral soils. The research took place in British Columbia (BC), Canada, at the Aleza Lake Research Forest (ALRF), near Prince George, in a hybrid spruce stand subject to mounding and at the Suquash Drainage Trial (SDT) site near Port McNeill, Vancouver Island, in a western redcedar–western hemlock–yellow cedar stand subject to drainage. Mounding reduced CO_2 fluxes and carbon (C) concentrations, but created anaerobic hot-spots of CH_4 and N_2O fluxes. Ditch drainage increased soil C about 20% after 15 years and did not affect respiration rates, though CH_4 fluxes were reduced. Fertilization transiently increased N_2O fluxes up to a maximum of 209 $\mu g\ m^{-2}\ h^{-1}$, two months following fertilization. Bacterial and fungal T-RFLP profiles showed distinct patterns based on soil layer, and were altered by mounding, drainage and fertilization. Up to 84.4% of variation in CO_2 emissions could be explained, with almost 50% of explained variation allocated to soil temperature. CH_4 flux variation was explained by soil water content, soil temperature, methanogen (*mcrA*) and methanotroph (*pmoA*) functional gene abundance. Variation in N_2O fluxes were significantly explained by soil water content, soil pH, NH_4-N concentration, AOB *amoA*, nitrate reductase (*narG*) gene and *nirSK* gene abundance. In addition to denitrification genes, these data highlight AOB as important determinants of denitrification either by mediating nitrification or by direct nitrifier denitrification. This study elucidates the influence of different microbial functional groups on GHG flux rates in forest ecosystems.

Preface

This work is based on an initial proposal by Sue Grayston, Cindy Prescott and Susan Baldwin that identified key research questions and selected research sites. David Levy-Booth was responsible for further development of research questions and approaches relating to microbial community characteristics. Drainage treatments at the Suquash Drainage Trial were installed previously by Annette Van Niejenhuis from Western Forest Products, Inc. Melanie Karjala and Michael Jull assisted with the identification of ecozones and the installation of the mounding treatments at the Aleza Lake Research Forest based on experimental design by David Levy-Booth. All experimental design, experimentation and statistical analysis was carried out by David Levy-Booth. Several research assistants were involved in the collection of data (see acknowledgments). All chapters in this thesis are original work written solely by David Levy-Booth, with Sue Grayston providing manuscript edits.

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Chapter 1. Introduction[1]

1.1 Forest management challenges and objectives in British Columbia

Current strategies in forest management in British Columbia (B.C.), Canada seek to increase the economic potential of the province's 55 million ha of forests while maintaining or enhancing their ecological and social functions (B.C. Ministry of Forests, Mines and Lands, 2010). In B.C., 0.4% of total forest area is harvested annually, amounting to about 69 M m^3 y^{-1}, though harvest volumes are declining due to economic conditions, the increased use for non-timber practices and the effect of mountain pine beetle (MPB; *Dendroctonus ponderosae*) outbreak, which spread rapidly to affect 18.3 million ha by 2013 (Brockley and Simpson 2004; B.C. Ministry of Forests, Mines and Lands, 2010; B.C. Ministry of Forests, Lands and Natural Resource Operations, 2013). Furthermore, the outbreak of MPB has turned the affected area from a net carbon (C) sink (0.59 Mt C y^{-1} uptake) to source of atmospheric C (17.6 Mt C yr^{-1} emissions) from decomposing beetle-killed trees (Kurz et al., 2008). To meet its forestry objectives the province has proposed widespread intensive forest management including increasing annual allowable cut (AAC) area, site preparation to improve post-harvest planting success, particularly in wet forest ecosystems, and stand fertilization to fill the resulting mid-term timber supply gap (B.C. Ministry of Forests, Mines and Lands, 2010). However, the impact of these prescriptions on ecosystem functioning and greenhouse gas (GHG) emissions must be considered to adhere to provincial (B.C. Ministry of Forests, Mines and Lands, 2010; B.C. Ministry of the Environment, 2014) and national (Canadian Council of Forest Ministers. 2007; Environment Canada, 2013; Natural Resources Canada, 2013; Warren and Lemmen, 2014) policies of maintenance of forest ecosystem services and reduction of GHG emissions. Wet forests in Canada play an important role in the global carbon cycle by sequestering atmospheric C in soil (Canadian Council of Forest Ministers. 2007). There remains no clear consensus regarding the impacts of site preparation and fertilization on the soil physico-chemical characteristics including C sequestration or GHG emissions (Johnston and Curtis, 2001; Grayston, 2007), in part due to the lack of study into the impacts of forest management on the microbial communities that drive these ecosystem processes.

Canada will likely fail to meet its international commitments to reduce GHG emissions to 607 Mt CO_2-equivalents y^{-1} by 2020 (Environment Canada, 2013). In 2012, Canada's efforts to measure and

[1] Elements of this chapter have been published: Levy-Booth, D.J., Prescott, C.E., Grayston, S.J. 2014. Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. Soil Biology and Biochemistry 75, 11-25.

reduce GHG emissions took into account land-use change and forestry for the first time, a sector which is of particular importance to Canada as the country contains 10% of the world's forests, including 229 million ha of managed forests (Environment Canada, 2013), which are thought to act as a net C sink (Warren and Lemmen, 2014). Therefore, contributions by the forestry sector to reducing contributions to net GHG emissions, through practices that improve C sequestration and reduce emissions, can play an important role for Canada's ability to get closer to meeting future GHG emission reduction commitments (Warren and Lemmen, 2014). As of 2013, natural disturbances (fire, insect infestations (e.g., MPB)) were no longer used in Canada's C accounting due to their unpredictability and lack of anthropogenic control (Environment Canada, 2013). This decision means that by 2020 the projected uptake of 148.7 Mt CO_2-equivalents directly resulting from forest management will substantially contribute to the net GHG balance of Canada's C accounting framework. GHG emission reduction and C sequestration enhancement should be goals of forest ecosystem management (Brown et al., 1996).

1.2 Contribution of forest ecosystems to regulation of greenhouse gas cycles

Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are the most important GHGs, in terms of their atmospheric concentrations and radiative forcing, and have increased by about 36%, 150% and 19% in the last two centuries to about 380, 1.78 and 0.33 ppm, respectively, primarily due to anthropogenic influence (Forster et al., 2007). While mixing ratios of CH_4 and N_2O are several orders of magnitude lower than that of CO_2, their global warming potentials are 34 and 298 times that of CO_2 over a 100-year period, respectively (Forster et al., 2007; Myhre et al., 2013). Global increases in GHGs from anthropogenic sources have likely increased global average temperatures by 0.15°C to 0.3°C per decade, and will continue increase global temperatures unless reduction and mitigation efforts improve (Forster et al., 2007). Forests play a major role in regulating C dynamics and GHG fluxes globally. The world's forests contain about 1.15 x 10^{18} tons of C, with about half of that in temperate and boreal forests (Melillo, 1996; Watson et al., 2001). Temperate and boreal forests together cover about 2.4 billion ha and contain 272 and 119 Pg C, and are sequestering an additional 0.5 and 0.72 Pg C yr^{-1} respectively, of which 65% and 49% is stored in soil, respectively (Pan et al., 2011). Forest ecosystems contain more than 80% of all terrestrial aboveground C, and more than 70% of all soil organic C (Batjes, 1996; Jobbágy and Jackson, 2000), though these estimates do not take into account litter layers that comprise the forest floor (Jandl et al., 2007). Mean CO_2 efflux from soil in boreal and temperate forests is estimated to be 322 and 647 to 681 g C^{-2} y^{-1}, based on a mean net primary productivity measurements of ~ 266 and ~ 590 g C m^{-2} yr^{-1}, respectively (Raich and Schlesinger, 1992; Raich and Potter, 1995). About 44% of biogenic CH_4 emissions worldwide are from natural and cultivated wetlands, with massive potential for increased

biogenic CH_4 fluxes due to warming of arctic ecosystems (Bloom et al., 2010; Jahn et al., 2010). Globally, soil acts as a sink of CH_4, with net uptake of about 460 Tg CH_4 y^{-1}, though waterlogged boreal forests have net CH_4 emissions of about 115 Tg y^{-1} after oxidizing about 27 Tg CH_4 y^{-1} (Reeburg, 1996). Soils emit about 40% of the global annual emissions of N_2O and emission rates have increased by about 30% since 1992 (Forster et al, 2007). Natural soils, including grassland and forest soils, emit about 3.3 to 9.0 Tg N_2O-N yr^{-1}, compared to agroecosystems which emit about 1.74 to 4.8 Tg N_2O-N yr^{-1}. Temperate forest soils specifically emit between 0.1 and 2.0 Tg N_2O-N yr^{-1}, though soil N_2O sinks are largely uncalculated (Chapuis-Lardy et al., 2007). Studies on management practices in temperate and boreal forest ecosystems that alter C and N dynamics are critical due to the massive area of these ecosystems and their relative importance to global GHG budgets.

1.3 The effect of forest site preparation on soil carbon and nitrogen cycles

1.3.1 Mounding

Site preparation is the use of physical and chemical intervention to prepare post-harvest soil for planting or natural regeneration to improve tree growth and survival, particularly in boreal and cool-temperate regions (Örlander et al,. 1990; Stathers et al., 1990; Sutton, 1993; Ryans and Sutherland, 2001; Löf et al., 2012). Mechanical site preparation such as mounding and ditch drainage are used to reduce soil moisture content, improve aeration, increase soil temperature and prevent paludification (Örlander et al,. 1990; Sutton, 1993; Ballard, 2000; von Arnold et al., 2005a,b; Löf et al,. 2012). Site preparation methods that remove or incorporate soil organic matter (OM) such as harrowing, scarification and some types of mounding (e.g, mixed mounding, inverted humus mounding) can lead to increased rates of OM decomposition and soil respiration rates (Johansson, 1994; Liechty et al., 1997; Lundmark-Thelin and Johansson, 1997; Ballard, 2000; Byrne and Farrell, 2005; von Arnold et al., 2005a; Piirainen et al., 2007; Mojeremane et al., 2012). For example, Lundmark-Thelin and Johansson (1997) report that Norway spruce (*Picea abies* L. Karst.) needles in trench-mounds in an orthic podzol soil in central Sweden had 19% less mass remaining after four years compared to needles in unprepared soil, and Piirainen et al. (2007) show that dissolved organic C (DOC), dissolved organic N (DON), mineral N, and mineral P were all greater in ridges in trench-mounded plots in Norway spruce stands on a haplic podzol soil in eastern Finland, indicating higher rates of OM mineralization and nitrification within mounds. However, Smolander et al. (2000) report a decrease in C mineralization and an increase in mineral N availability in mounded soil, attributable to the removal of understory vegetation, one year after mounding of a clear-cut Norway spruce stand on a podzol soil with mor humus in south-eastern Finland.

Mojeremane et al. (2012) did not report any differences in CO_2 efflux from a mounded versus unmounded peaty gley soil in England. The increased rate of decomposition following the removal or burying of forest floor layers due to mounding (Örlander et al,. 1990; Sutherland and Foreman, 1995; Sims and Baldwin, 1996), can reduce C in soil but increase C sequestration in above-ground biomass due to improved tree growth. The loss of forest floor layers following mounding can nevertheless allow for regeneration of tree species that require mineral soil for regeneration such as Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), Jack pine (*Pinus banksiana* Lamb.) and white spruce (*Picea glauca* (Moench) Voss) (White, 2004), whereas other species such as western redcedar (*Thuja plicata* Donn. ex D. Don) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) regenerate in soils with intact forest floor layers (Wright et al., 1998).

1.3.2 Drainage

Drainage of C-accumulating peatlands and organic fen soil can promote succession towards ecosystems suitable for the growth of economically-important tree species (e.g., Norway spruce, Scots pine (*Pinus sylvestris* L.), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and black spruce (*Picea mariana*)) (Laiho and Finér, 1996; Laiho and Laine, 1997; Macdonald and Yin, 1999; Laiho et al., 2004). Drainage can lead to greater above- and below-ground biomass accumulation and litter addition to soil (Laiho and Finér, 1996; Laiho and Laine, 1997; Macdonald and Yin, 1999; Hargreves et al., 2003; Byrne and Farrell, 2005; Choi et al., 2007), nitrification (Choi et al., 2007) and CO_2 emissions (Armentano and Menges, 1986; Silvola, 1989; Glen et al., 1993; Martikainen et al., 1995). For example, CO_2 fluxes were about 21% higher in drained plots compared to undrained plots two-years following drainage of a Sitka spruce stand on a peaty-gley soil in England (Mojeremane et al., 2012). Short-term CO_2 emissions are generally expected to increase transiently following drainage of high-organic soils (Laiho, 2006), with durations between three weeks (Moore and Dalva, 1993) and two to four years (Hargreaves et al., 2003), depending on water table depth (Silvola et al., 1996b; Chimner and Cooper, 2003), decomposable OM availability (Laiho and Finér, 1996) and changes in root respiration (Silvola et al., 1996a). The increase in soil respiration in drained sites was hypothesized to be cause by increased oxidation of litter and OM sources (Glenn et al., 1993). However, rates of litter and soil OM decomposition in Finnish Scots pine stands on boreal peatlands 70 years after drainage were lower than in undrained sites (Minkkinen and Laine, 1998; Minkkinen et al., 1999; Dornisch et al., 2000), indicating that CO_2 fluxes were not entirely caused by increased heterotrophic litter and OM decomposition as hypothesized, but that up to 50% of CO_2 emissions from drained sites can be attributed to increased autotrophic root respiration (Silvola, 1989; Glen et al., 1993; Laiho and Finér, 1996; Silvola et al., 1996a,b; Minkkinen et al., 2002; Laiho, 2006).

Drainage of highly organic soils for forestry is hypothesized to a) reduce C sequestration potential of a site due to enhanced soil respiration, b) convert waterlogged sites from below-ground C sequestration to above-ground C sequestration, or c) improve both above- and below-ground C sequestration, with many of the above studies suggesting the latter scenario (Laiho, 2006). Site preparation in B.C.'s wet coastal and interior forests that exhibit poor drainage characteristics, but otherwise support tree stands, could allow for the growth and harvest of productive stands in these marginal areas, provided timely regeneration can occur.

CH_4 can either be taken up or emitted from forest soil depending primarily on soil water-table depth and temperature (Crill et al., 1994; Nykanen et al., 1995; Augustin et al., 1998; Dunfield, 2007; Kolb, 2009; Ullah et al., 2009; Shrestha et al., 2012; Hartmann et al., 2014). Waterlogged forest soil can emit up to 3000 µg CH_4-C m^{-2} h^{-1} (Nykanen et al., 1995), though overall forest soils act as a net CH_4 sink, taking up about 30 Tg y^{-1} (Le Mer and Roger, 2001; Wuebbles and Hayhoe, 2002). With atmospheric CH_4 increases of about 22 Tg y^{-1} (Forster et al., 2007), forest CH_4 fluxes are an important component of global CH_4 dynamics (Maljanen et al., 2007). Forest harvesting, particularly clear-cutting, raises the water table in forest soils due to the removal of tree evapotranspiration (Adams et al., 1991; Smethurst and Nambiar, 1995; Liblik et al., 1997; Huttunen et al., 2003; Zerva and Mencuccini, 2005), which can change forest soils from CH_4 sinks to sources of net CH_4 emission (Keller et al., 1990; Keller and Reiners, 1994; Zerva and Mencuccini, 2005; Dörr et al., 2010). Drainage of intact or harvested sites can reduce CH_4 fluxes by lowering the water table (Glen et al., 1993; Mojeremane et al., 2012), which suppresses methanogenesis and stimulates methane oxidation (Castro et al., 1995; Czepiel et al., 1995; Wang and Bettany, 1995; Le Mer and Roger, 2001).

1.4 Effect of forest fertilization on carbon and nitrogen cycles

1.4.1 Effect of fertilization on the carbon cycle in forest soil

Fertilization can improve tree growth and stand productivity in nitrogen (N)-limited forest ecosystems, which can reduce rotation times in plantation forests. In B.C., N fertilization has been shown to increase tree height and volume in economically-important tree species (e.g., Douglas-fir, lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm), western hemlock and western redcedar) (Weetman et al. 1988, 1989; Omule, 1990; McDonald et al., 1994; Swift and Brockley, 1994; Mitchell et al., 1996; Yang 1998; Canary et al., 2000; Kishchuk et al. 2002; Brockley and Simpson 2004; Brockley, 2005, 2006; Negrave et al., 2007). For example, fertilization with 200 kg ha^{-1} urea-N or N + 75 kg ha^{-1} sulphur (S) increased the net volume of six Douglas-fir stands ranging in age from 19 to 34 year by 13.5

and 16.0 m^3 ha^{-1}, respectively, compared to unfertilized stands (24% and 28% respective increases) (Brockley, 2006). Nitrogen fertilization can also increase soil C sequestration in many forest ecosystems (Johnson, 1992; Canary et al., 2001; Oren et al., 2001; Adams et al., 2005; Jandl et al., 2007; Negrave et al., 2007; Pregitzer et al., 2008), though some results are contradictory (Neff et al., 2002; Waldrop et al., 2004; Knorr et al., 2005; Allison et al., 2010). Periodic fertilization with 224 kg N ha^{-1} of 26 to 48-year old Douglas-fir stands on the (US) equivalent of a dystric brunisol and a gray-brown luvisol soil in western Washington increased tree biomass C by an average of 20% (135 and 161 Mg C ha^{-1} in unfertilized and fertilized stands, respectively) and soil C by an average of 48% (175 and 260 Mg C ha^{-1} in unfertilized and fertilized soils, respectively) (Adams et al., 2005). Nitrogen fertilization-induced C sequestration likely involves the reduction of litter and SOM decomposition rates due to the alleviation of N constraints on fungal and bacterial decomposer growth, alteration of microbial community structure (Gallo et al., 2004; Allison et al., 2007; 2008; 2010) and decrease in fungal enzyme activity, including the activity of lignin peroxidases and cellulases, (Waldrop et al., 2004; Allison and Vitousek , 2005), though effects on respiration can be inconclusive (Allison et al., 2008; 2010). For example, fertilization did not alter respiration rates from a range of forest soils in Canada, US and Finland (Prescott et al., 1993; Chappell et al., 1999; Smolander et al., 2000). Nitrogen fertilization may decrease root biomass (Mäkipää, 1995; Eriksson et al., 1996; Kurz et al., 1996; Gundersen et al., 1998) and mycorrhiza-stimulating C exudation from roots (Bowden et al., 2004), which would also lead to decreased soil respiration. However, in a meta-analysis of fertilization with 100-150 kg ha^{-1} N in Scots pine (*Pinus sylvestris* L.), Norway spruce and birch (*Betula pendula* Roth.) stands throughout Sweden, Sathre et al. (2010) demonstrated that overall, N fertilization increased root biomass by 0.78 t ha^{-1} yr^{-1} and soil OM C by 12 to 20 t CO_2- equivalents ha^{-1}. Watson et al. (2001) estimate that Canada can increase its forest C stocks by 11.9 – 69.8 t yr^{-1} through N fertilization. See Figure 1.1 for schematic depiction of N fertilization effects on soil C dynamics.

1.4.2 Effect of fertilization on forest soil CH_4 flux

CH_4 fluxes can be altered by N fertilization, though the magnitude and direction of alteration is unclear (Bodelier and Laanbroek, 2004; Mohanty et al., 2006; Bodelier, 2011). Fertilization can decrease CH_4 uptake by inhibiting CH_4 oxidation by methanotrophs (Steudler et al., 1989; Crill et al., 1994; Willison et al., 1995; Primé and Christensen, 1997; Saari et al., 1997; Maljanen et al., 2006) (Figure 1.1). For example, fertilization (100 kg NH_4-N, 100 kg NO_3-N ha^{-1}) of a Norway spruce stand on a haplic podzol soil in southern Finland decreased CH_4 uptake from 153 to 123 ug m^{-2} h^{-1} (Maljanen et al., 2006), and fertilization of a drained peatland in Finland with 100 kg ha^{-1} NH_4-N, NO_3-N or urea-N decreased

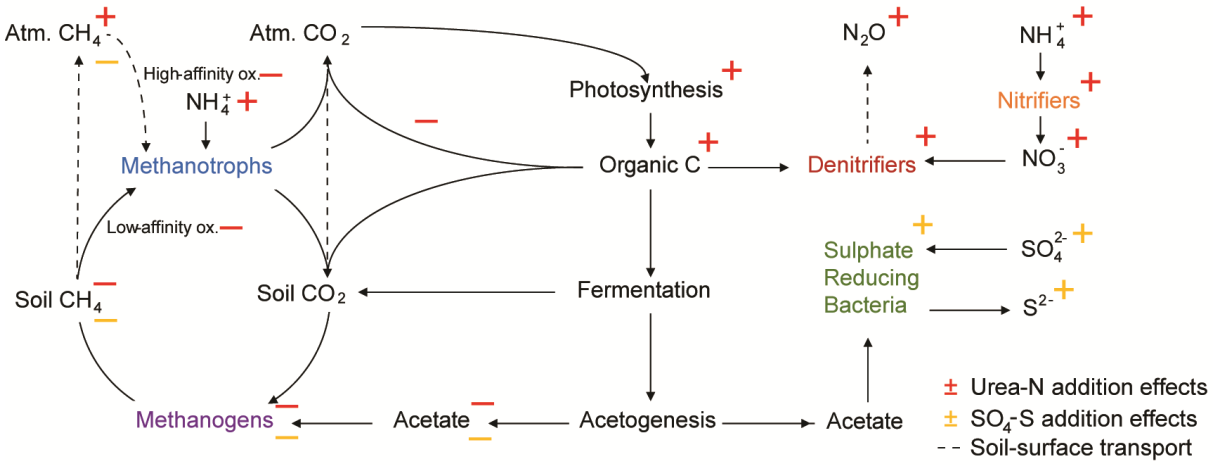

Figure 1.1. Schematic depiction of select pathways related to forest soil GHG cycles (CO_2, CH_4, N_2O) including likely effects of addition of N and SO_4-S by fertilization of forest ecosystems.

CH_4 uptake by 79.4%, 69.4% and 29.6%, respectively, indicating that urea-N has less suppressive effects on CH_4 oxidation than mineral N (Crill et al., 1994), though this is disputed (Bodelier, 2011). Nitrogen fertilization has also been shown to increase CH_4 uptake due to alleviation of N-limitations of methanotrophs (Bodelier et al., 2000; Bodelier and Laanbroek, 2004; Liu and Greaver, 2009) or have no effect on CH_4 fluxes in N-limited environments (Steinkamp et al., 2001; Basiliko et al., 2009). Nitrogen-limitation in on methanotrophic archaea appears to determine, in part, the effect of fertilization on CH_4 oxidation rates; immobilization of mineral N in these environments should prevent inhibition of the relatively unspecific particulate methane monooxygenase (pMMO) enzyme of methanotrophs by NH_4^+ (Purkhold et al., 2000). Nitrogen-addition can also decrease methanogenesis (the production of CH_4 by methanogens) by stimulation of nitrate-reducing and denitrifying organisms that out-compete methanogenic archaea for low-molecular weight organic C in soil and through production of intermediaries (NO_2^-, NO, N_2O) that are toxic to methanogens (Bodelier, 2011). Hydrogenotrophic and acetoclastic methanogenesis occur in very low redox-potential soils, as they are out-competed for acetate and H_2 by NO_3-, Fe- and sulphate (SO_4^{2-})-reducing bacteria (SRB) (Thauer et al., 1989; Achtnich et al., 1995; Muyzer and Stams, 2008), suggesting a suppressive effect of SO_4-S fertilization on methanogenesis (Abram and Nedwell, 1978; Denier Van Der Gon et al., 2001). The impact of fertilization on CH_4 fluxes from forest soil can be further elucidated by characterization of the methanogenic archaea and methanotrophic bacteria community structure and function (Mohanty et al., 2006; Freitag et al., 2010; Angel et al., 2012; Ma et al., 2012; Hartmann et al., 2014), which are discussed below.

1.4.3 Effect of fertilization on the nitrogen cycle in forest soil

The microbial cycling of nutrients affects many ecological properties of forests including tree growth, productivity, soil C sequestration and GHG emissions. Nitrogen availability is often the limiting factor in terrestrial ecosystem productivity (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008), including forest soils in western North America (Hooper and Johnson, 1999). The limit on N availability in forest soils is a result of the lack of inputs, rapid immobilization and removal by leaching and gaseous emission (Vitousek et al. 1997, 2002). However, anthropogenic N inputs to terrestrial ecosystems through fertilization and atmospheric deposition can remove these limitations, increasing reactive N availability and N loss from the soil. Nitrogen fertilization is used in forests to increase aboveground biomass production and shorten rotation times, and can enhance belowground C sequestration (Brockley and Simpson, 2004; Grayston, 2007; Van Miegroet and Jandl, 2007). Alterations to the net addition of N in forests soils are likely to have reverberating effects on the function of the soil community, including rates of decomposition (Janssens et al., 2010), N mineralization (Wallenstien et al., 2006) and the abundance

and activity of nitrifying and denitrying microorganisms (Wallenstien et al., 2006; Hallin et al., 2009). Quantification and characterization of microbial functional genes in the N-fixation, nitrification and denitrification pathways can help create informative models of N cycling process rates, reactive N availability and N_2O emissions from soil, providing predictions and mitigation strategies for GHG emissions (Bothe et al., 2000; Richardson et al., 2009; Morales et al., 2010).

The cycling of N in soil can be subdivided into (i) decomposition processes, (ii) assimilative processes and (iii) dissimilative processes (Figure 1.2). Decomposition processes include high molecular-weight soil organic N released during decomposition of plant litter, which can be further degraded to low molecular weight dissolved organic N (DON). Assimilative processes include the uptake and utilization of DON, NH_4^+, or NO_3^- by plants and microorganisms for growth and replication. Dissimilative process, which are the focus of this review, include the oxidation of NH_3 for the generation of reducing equivalents ($NADPH^+$) or the use of oxidized N products as electron acceptors during facultatively anaerobic respiration by denitrifying microorganisms. Dissimilative process rates are likely to be highest in N-rich ecosystems. Denitrification proceeds stepwise as soil redox potential decreases. Two additional dissimilative processes that will not be examined in this review are dissimilatory nitrate reduction to ammonium (DNRA) and the anaerobic oxidation of ammonium (anammox). DNRA has been measured in tropical forest soil (Silver et al., 2001) and in paddy soil (Yin et al., 2002), though DNRA is not expected to be a major source of NO_3^- loss in non-flooded soil (Silver et al., 2001). The anammox bacteria are able to combine both oxidized and reduced inorganic N compounds to produce N_2. Common in marine environments (Kuenen, 2008), anammox bacterial 16S rRNA has been detected in flooded terrestrial environments (Humbert et al., 2010; Zhu et al., 2011; Humbert et al., 2012). Long et al. (2013) have used the hydrazine oxidase (*hzo*) gene as a functional marker for the quantification of anammox bacteria in fertilized agricultural soil, though the role of these organisms in N_2 loss from non-flooded soil has yet to be resolved. Nitrification and denitrification are linked to the loss of N from forest soil through the leaching of nitrate and the emission of NO, N_2O and N_2.

The N-fixing, nitrifier and denitrifier communities will be the focus of this study due to their importance for N availability and loss in forest ecosystems and their ability to be studied using microbial functional genes. I pay particular attention to dissimilatory processes that drive N_2O emissions from forest soil. This review will describe recent advances in the use of molecular methods to relate functional gene diversity and abundance to activity of microorganisms primarily to dissimilative N cycling processes in forest soil ecosystems, with a focus on forest stand fertilization and N_2O emissions. Although several excellent reviews of the molecular biology of N-cycling microorganisms in soil exist (e.g., Bothe et al., 2000, Wallenstien et al., 2006c; Hayatsu et al., 2008), there is a lack of synthesis of the role of microbial

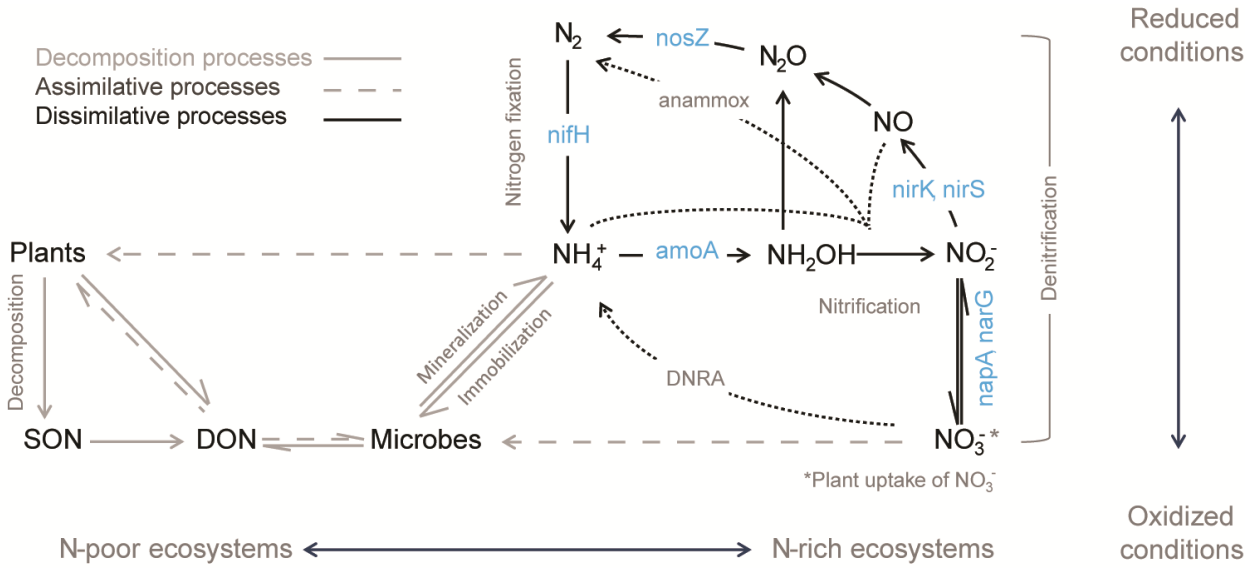

Figure 1.2. The nitrogen (N) cycle in forest soil. The cycling of N in soil can be subdivided into (i) decomposition processes, (ii) assimilative processes and (iii) dissimilative processes. Anammox, anaerobic ammonia oxidation; DNRA, dissimilatory nitrate reduction to ammonium.

functional genes in elucidating the key players in the N cycle in forest soil. Here, I focus on temperate and boreal forest ecosystems of North America and Europe where the majority of research on functional gene communities has been undertaken. Studies that link soil characteristics and N cycling dynamics to functional gene abundance and diversity can be used to identify key factors to assess the functioning of forest ecosystems, incorporate microbial dynamics into biogeochemical models, improve soil management and mitigate N loss from forest soil.

1.4.4 Effect of fertilization on forest soil N_2O flux

Soils are the source of about 70% of the N_2O emitted to the atmosphere (Conrad, 1996). Forest soil N_2O emissions are substantially less than those from industrial or agricultural sources, but are increasing due to fertilization (Grayston, 2007; Smethurst, 2010) and atmospheric deposition (Gundersen et al., 2012). At about 314 ppb, the concentration of N_2O in the atmosphere is minute, although the gas has a global warming potential (GWP) 296 times that of CO_2 over a 100-year period (IPCC, 2007). N_2O is also an important ozone-depleting molecule (Ravishankara et al., 2009). Forest soil can either be a source or sink of N_2O depending on the activity and structure of the nitrifier and denitrifier communities (Matson et al., 1992; Chapuis-Lardy et al., 2007; Dalal and Allen, 2008; Jassal et al., 2010).

Anthropogenic N inputs to forests can increase emissions of N_2O from soil (Johnson et al., 1980; Brumme and Beese, 1992; Sitaula and Bakken, 1993; Situala et al., 1995; Bateman and Baggs, 2005; Pilegaard et al., 2006; Jassal et al., 2008, 2010, 2011; Mojeremane et al., 2012; Pielegaard, 2013; Ussiri and Lal, 2013; Wu et al., 2013), with N_2O fluxes increasing with N-amendment intensity (Aronson and Allison, 2012), though not in all cases (Pang and Cho, 1984; Johnson and Curtis, 2001; Wallenstein et al. 2006a,b; Basiliko et al., 2009; Gundersen et al., 2012). Temperate forest soils can also act as sinks for N_2O, in both very wet (Chapuis-Lardy et al., 2007; Gundersen et al., 2012) and aerated soil (Martikainen et al., 1996; Chapuis-Lardy et al., 2007; Goldberg and Gebauer 2009). Static closed-chamber measurements of N_2O fluxes in a 58-year-old coastal Douglas-fir stand on a humo-ferric podzol soil in B.C. showed that N_2O fluxes increased from zero (or minute uptake of 0.06 μmol m^{-2} h^{-1}) to emissions of 26 μmol m^{-1} h^{-1} three months after fertilization with urea, with N losses totaling about 5% of added N (Jassal et al., 2008). Wet alpine ecosystem sites can emit significantly greater N_2O than dry sites following fertilization (Neff et al., 1994), and N_2O emissions are common in wet forests (Weier et al., 1993; Smith et al., 1998; Davidson et al., 2000; Bateman and Baggs, 2005; Pilegaard et al., 2006; Pilegaard, 2013). In addition to mineral N availability and soil moisture (Smith et al., 1998; Bateman and Baggs, 2005; Kool et al., 2011; Wu et al., 2013; Zhu et al., 2013), which are positively correlated with

N_2O flux, major determinants of N_2O flux rates from forest soil include soil pH (Šimek and Cooper, 2002; Pielegaard, 2013) and C:N ratios (Klemedtsson et al., 2005; Pilegaard et al., 2006; Gundersen et al., 2012), which are negatively correlated with N_2O flux. Microbial communities responsible for N_2O-producing nitrification and denitrification reactions can be characterized to resolve conflicting findings regarding N-fertilization, soil physico-chemical properties and N_2O flux rates (Hallin et al., 2009; Morales et al., 2010; Petersen et al., 2012; Harter et al., 2014).

1.5 Molecular analysis of microorganisms in forest soil

One gram of soil can contain up to about 10^9 microbial cells (Gans et al., 2005; Roesch et al., 2007). Approximations of the density of unique microbial genomes vary widely, but current estimates suggest that there are between 10^3 to 10^7 species g^{-1} soil, with forest soils being more phylum-rich and less species-rich than agricultural soils (Torsvik et al., 1990, 2002; Gans et al., 2005; Roesch et al., 2007) and distinct in terms of community structure and function from other environments such as grassland soils (Rösch et al., 2002; Morales et al., 2010). A variety of methods exist to characterize the microbial community and its function at varying levels of resolution. Each methodology has benefits and constraints; for reviews of the application of molecular methods for soil microbiology see Kirk et al. (2004), Leckie (2005), Spiegelman et al. (2005), Sharma et al. (2007) and Smith and Osborn (2009). This review focuses on data derived from PCR-based studies including quantitative PCR (qPCR) which is used to estimate functional gene abundance. DNA fingerprinting and quantification methods are currently widely adopted, and are able to provide high-resolution taxonomic information and organism abundance; the methods are highly reproducible and are suitable for high-throughput analysis. DNA-based microbial community analysis techniques are nonetheless subject to methodological bias during nucleic acid extraction from soil and amplification and are only able to resolve potential activity, as they are unable to differentiate active, dormant or dead sources of DNA.

PCR primer sequence development and protocol selection also affect the accuracy of amplification-based community analysis techniques. Primers for functional genes are unlikely to capture the full diversity of the target genes for which they are designed, due to the high divergence of nucleotide sequences at current primer sites (Green et al., 2010). Penton et al. (2013) provide a comprehensive discussion of the limitations of current functional gene primer sets, which include the absence of a complete database of functional gene sequences. This constraint results from the lack of deep sequencing studies designed to capture the full sequence diversity of target genes (Palmer et al., 2012; Palmer and Horn, 2012). Targeted functional gene studies and application of amplification-based metagenomic

surveys can be used to mine gene-sequence and metagenomic datasets for functional genes, which can identify novel sequences of known functional genes (Penton et al., 2013; Myrold et al., 2013). This improvement in coverage should be used to design more comprehensive primers for functional gene analysis. Current functional gene primer sets are likely to vastly underestimate gene abundance and may provide an inaccurate estimation of the linkages between functional genes and environmental processes. The studies presented in this review should therefore be evaluated based on these limitations. Despite this, PCR-based studies have contributed greatly to our understanding of functional genes in soil.

The majority of interest in functional gene analysis is its use in characterizing populations that drive biogeochemical cycles. Functional gene abundance can be linked to soil characteristics and process rates, for example methane-cycling gene diversity and abundance were weakly correlated with methane flux in peat soil (Freitag et al., 2010; Andert et al., 2012). It remains to be seen if this is true for N-cycling genes. In contrast to ribosomal subunit marker genes (e.g., 16S and 18S rRNA) that have long been used for phylogenetic analysis of microbial communities, functional genes allow researchers to study only those groups responsible for biochemical transformations of interest. This may prevent the masking of important relationships by non-related microbial groups and directly relate gene diversity and abundance with environmental characteristics and biological functioning (McGill et al., 2006; Sharma et al., 2007; Penton et al., 2013). The relative importance of functional gene abundance versus diversity and community composition in ecological functioning is still unclear (Hallin et al., 2009; Graham et al., 2013).

The presence of functional genes does not always indicate an active community (Wertz et al., 2009). An alternative method of linking functional communities in soil to process rates is qPCR of mRNA transcripts of functional genes, which provide an estimate of gene expression from metabolically active microbial cells. Studies examining functional gene transcript abundance and attempting to link this measure to process rates have largely taken place in laboratory incubations or microcosms (Holmes et al., 2004; Nicolaisen et al., 2008; Freitag and Prosser, 2009; Liu et al., 2010). *In situ* field estimates of functional gene activity are less common, but have provided important links between functional gene activity and process rates. For example, methanogen gene expression in the top 10 cm of soil have been positively correlated to CH_4 flux rates using *mcrA* gene:transcript abundance ratios at one of two peat bog sites, and methanotroph *pmoA* gene:transcript ratio was negatively correlated with CH_4 flux rates at a different peat bog site (Freitag et al., 2010). However, researchers have struggled to detect functional gene transcriptional activity under field conditions, including denitrification genes (Liu et al., 2010). While caution must be taken in interpreting functional gene abundance as indicating microbial activity, the low expression levels in field soil, the rapid turnover of soil microorganisms and the wide

phylogenetic distribution of microbial functional groups, make the *in situ* study of functional gene community structure and abundance suitable for relating soil functional communities to biochemical transformation rates of N in soil.

1.6 Microbial functional groups responsible for CH_4 fluxes in forest soil

1.6.1 Methanogens

Biogenic CH_4 is produced exclusively by methanogenic archaea. Methanogens comprise a monophyletic lineage of *Euryarcheota* in which the strict anaerobic reduction of organic compounds or CO_2 to CH_4 is the sole source of energy (Hedderich and Whitman, 2006; Whitman et al., 2006). Full anaerobic fermentation of photosynthates occurs with the reaction (Equation 1):

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \quad (1)$$

Methanogens are responsible for the terminal mineralization of low-molecular weight C compounds and require close syntrophy with other fermentative microbial communities: hydrolytic microorganisms that carry out hydrolysis of biological polymers into monomers (e.g., glucides, fatty acids, amino acids), fermentative microorganisms that ferment hydrolysed compounds to low-molecular weight C compounds (e.g., alcohols, organic acids, CO_2) via acidogenesis, and homoacetogenic microorganisms that further ferment these compounds to acetate (Le Mer and Roger, 2001; Hedderich and Whitman, 2006; Stams and Plugge, 2009). A limited schematic depiction of select pathways involved in CH_4 production is shown in Figure 1.1. Together, these microbial groups are responsible for anaerobic decomposition of organic compounds in soil. CH_4 can be produced from three known substrate groups: reduction of CO_2 with H_2 (hydrogenotrophic) (Equation 2), reduction of acetate (CH_3COOH) (acetoclastic) (Equation 3) and reduction of other methyl-containing compounds (e.g., methanol (CH_3OH), formate, methylamines, dimethyl sulfide, and methanethiol) (methylotrophic) (Equation 4) (Ferry, 1999, 2010; Deppenmeier, 2002, respectively):

$$CO_2 + 4H_2 \rightarrow 2H_2O + CH_4 \quad (2)$$

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (3)$$

$$4CH_3OH \rightarrow 2H_2O + CO_2 + 3CH_4 \quad (4)$$

Some species of methanogens contain multiple pathways (Le Mer and Roger, 2001; Conrad, 2005). The three unique and highly complex methanogensis pathways have an identical terminal step: the reduction

of a methyl-coenzyme M (CoM) group with a coenzyme B (CoB) sulphide-bound proton to CH_4 by the methyl-CoM reductase (MCR) enzyme (Thauer, 1998) (Equation 5):

$$MoC\text{-}CH_3 + CoB\text{-}SH + 2e^- \xrightarrow{MCR} CoM\text{-}S\text{-}S\text{-}CoB + CH_4 \quad (5)$$

The α-subunit of MCR is encoded by the *mcrA* gene (Cram et al., 1987; Thauer, 1998; Lutton et al., 2002). Nazaries et al. (2013) provide a detailed taxonomic breakdown of the methanogenic archaea including CH_4 production pathways and optimal growth conditions. Methanogens in forest ecosystems are mostly mesophiles and primarily use hydrogenotrophic or acetoclastic pathways of CH_4 production (Conrad, 1999, 2005; Le Mer and Rogers, 2001; Nazaries et al., 2013). Molecular evidence suggests that methanotrophs from the genera *Methanosarcina* and *Methanocella* are present in high abundance in most soils including upland forests, and can rapidly produce CH_4 as otherwise aerated soil becomes anoxic (Angel et al., 2012). This further supports the idea that forest management practices that raise water tables in forest soil, such as clear-cut harvest, can rapidly induce CH_4 emissions. Molecular investigation using the *mcrA* functional marker can elucidate further effects of forest management on the methanogen community (Lutton et al., 2002).

Methanotrophs are active only in low-redox potential habits, as they are out-competed for protons and low-molecular weight C compounds by other anaerobic dissimilatory reducing microorganisms including NO_3-reducers (discussed below) and SO_4-reducing bacteria (SRB) (Muyzer and Stams, 2008). The SRB can use several types of low-molecular-weight C compounds as the reducing agent in the reduction of SO_4^{2-} including acetate, propionate, butyrate and lactate; only the hydrogen ($\Delta G^{o'}$ = 151.9 kJ reaction^{-1}) (Equation 6) and acetate (-47.6 kJ reaction^{-1}) (Equation 7) pathways are shown below:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad (6)$$

$$CH_3COOH + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (7)$$

The SRB are investigated in molecular ecology studies by targeting the α- and β-subunits of the dissimilatory sulfite reductase (DSR) enzyme (encoded by the *dsrA* and *dsrB* genes, respectively), which catalyzes the terminal reduction of sulphite (Muyzer and Stams, 2008) (Equation 8):

$$SO_3^{2-} + H^+ + \xrightarrow{DSR} H_2S + H_2O \quad (8)$$

1.6.2 Methanotrophs

Methane-oxidizing bacteria (MOB) in aerated upland soil create a sink for about 5% of atmospheric CH_4 (Reeburg, 1996; Forster et al., 2007), and oxidize about 50% of CH_4 produced by methanogens in soil and sediments before the CH_4 can diffuse to the surface (Bodelier, 2011). Methanotrophs oxidize CH_4 or methanol as their sole C and energy source (Bédard and Knowles, 1989; Hanson and Hanson, 1996) in a multi-step pathway (Kalyuzhnaya et al., 2013) that can be expressed with the following reaction (Equation 9):

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \quad (9)$$

High-affinity methanotrophs are able to oxidize atmospheric concentrations of CH_4 (~ 1.75 ppm) and are thought to be the dominant MOB in upland soil, though the species responsible for high-affinity methanotrophy and their enzyme systems are yet to be clearly identified (Kolb, 2009; Bodelier, 2011). However, genomic and transcriptomic studies of upland-soil MOB (e.g., *Methylocystis* sp. Strain SC2) are attempting to resolve these knowledge gaps (Dam et al., 2012, 2014). Low-affinity methanotrophs reside primarily in wetland soils and sediments and at the soil oxic/anoxic interface and can oxidize CH_4 concentrations > 100 ppm (Bender and Conrad, 1992; Conrad, 2006). High-affinity methanotrophs are further divided into the upland soil cluster (USC)α and USCγ, which phylogenetically cluster with the families *Beijerinckaiceae* and *Methylococcaiceae*, respectively, and appear to be differentiated by pH-based niche adaptations, with USCα showing affinity for acid forest soil and USCγ found in neutral or near-neutral soil (Kolb, 2009). USCα and USCγ are loosely related to the classification system used for cultivated MOB, the type II and type I methanotrophs, respectively, a classification system revised to alphaprotobacterial (*Beijerinckaiceae, Methylocystaceae*) and gammaprotobacterial (*Methylococcaeae*) methanotrophs using phylogenetics (Holmes et al., 1999; Singh and Tate, 2007). A third major phylogenetic group, the acidophilic *Verrucomicrobia,* was recently identified (Dunfield et al., 2007; Op den Camp et al., 2009). Aerobic CH_4 oxidation is carried out by the methane monooxygenase (MMO) enzyme, of which soluble (sMMO) and particulate (pMMO) forms have been characterized (Prior and Dalton, 1985; Lipscomb, 1994; Semrau et al., 2010). MMO catalyzes the first step in the CH_4-oxidation pathway, the oxidation of one C-H bond in CH_4, forming methanol in the following reaction (Lewis et al., 2011) (Equation 10):

$$CH_4 + O_2 + NADH + H^+ \xrightarrow{MMO} CH_3OH + NAD^+ + H_2O \quad (10)$$

The pMMO enzyme is found in all MOB except those in the genera *Methylocella* and *Methyloferula* (Dedysh et al., 2000; Dunfield et al., 2003), though some MOB contain both MMO forms (Nazaries et al., 2013). The α-subunit of pMMO is encoded by the *pmoA* gene, which is widely used in molecular

investigation of MOB communities in soil (Holmes et al., 1999; Henckel et al., 2000; Kolb et al., 2003; Kolb, 2009; Frietag et al., 2010).

1.6.3 Investigating methanogen and methanotrophs dynamics using molecular methods

Little molecular data exist regarding the quantitative dynamics of the *mcrA* and *pmoA* genes in soils. In incubated peat slurries the ratio of *mcrA* gene and transcript abundance, measured using qPCR of functional gene targets, correlated positively to MCR enzyme activity and CH_4 production rates, with both *mcrA* gene:transcript ratio and CH_4 production showing optimal temperatures of 25^oC, supporting the characterization of methanogens in soil as mesophiles (Nazaries et al., 2013). In incubations of soil from a variety of terrestrial environments including desert, temperate forest and grassland soil, CH_4 production rate was also positively correlated to methanogenic 16S rRNA gene copy numbers, particularly from the genus *Methanosarcina* (Angel et al., 2012). In German grassland soil, *pmoA* gene abundance ranged from 10^5 to 10^6 copies g^{-1} soil, and correlated positively with CH_4 uptake rates of 0 to 70 μg m^{-2} h^{-1} (Shrestha et al., 2012). Freitag et al. (2010) found high *mcrA* gene and transcript abundance (2.2 x 10^9 and 4.2 x 10^9 copies g^{-1} soil (dry weight (dw)), respectively), and positive correlation between *mcrA* gene:transcript ratios and CH_4 flux rate in a Welsh peat bog. The bog had a net flux of 10.2 mg CH_4 m^{-2} h^{-1}, which was strongest in the top 10 cm of soil. Frietag et al. (2010) also found that *pmoA* gene and transcript abundances were 5.0 x 10^8 and 1.0 x 10^7, respectively, and that in a bog site showing uptake of 0.95 mg CH_4 m^{-2} h^{-1}, *pmoA* gene:transcript ratio positively correlated with CH_4 flux rates. Furthermore at a CH_4-emitting site log_2-transformed *pmoA* and *mcrA* transcripts were linearly and positively correlated, indicating that activity of low-affinity MOB is driven by the activity of methanogenic archaea in waterlogged organic soils (Freitag et al., 2010). Methanogenic archaea are thought to be most active in waterlogged soil, though Watanabe (2009) demonstrated that *mcrA* was transcribed even in drained cultivated soil, a result that has not been confirmed in upland forest soil.

Land-use can change CH_4-associated functional gene diversity and abundance. CH_4 uptake correlated linearly and positively with *pmoA* terminal-restriction fragment (T-RF) richness in a variety of land-uses, from agroecosystems to deciduous forests, and showed a clear trend of increasing *pmoA* richness as ecosystem successional age increased, with forest plots having the greatest richness, methane uptake potential and the lowest CH_4 flux variability (Levine et al., 2011). Similarly, Nazaries et al. (2011) found a positive association between *pmoA* T-RF richness, stand age and CH_4 uptake in an afforestation gradient of shrubland, *Pinus radiata* plantations and natural forestsin New Zealand. Mohanty et al. (2006) used phospholipid fatty acid (PLFA) profiles to reveal that type I MOB can be stimulated by NH_4-N

fertilizer, while type II can be suppressed by NH_4-N, and T-RFs of type I and II MOB were correlated with NH_4-N and NO_3-N fertilizer, respectively (Mohanty et al., 2006). The abundance and T-RF richness of *pmoA* copies, as well as *mcrA* abundance, in a sandy pine forest soil in the eastern U.S.A. were significantly higher in sites repeatedly fertilized with 220.88 kg ha^{-1} NH_4NO_3, but were not affected by fertilization with 16.48 kg ha^{-1} NH_4NO_3 (Aronson et al., 2013). The authors report that CH_4 fluxes were positively correlated to total C, total N, soil moisture, soil temperature and mineral N content, as well as positive correlation between *pmoA* T-RFs and total C and soil moisture.

Sulphate has been shown to have a suppressive effect on methanogenesis. Ma et al. (2012) demonstrated that *mcrA* genes and transcript abundance were negatively correlated with ferric iron (Fe^{3+}) and SO_4^{2-} in an intermittently drained rice field. However, in a river estuary marsh soil in southeast China, the abundance of methanogen *mcrA* genes and *dsrB* genes were positively correlated with each other, with NO_3^- concentrations, with organic C concentrations including acetate, and ultimately with CH_4 flux rates (Tong et al., 2013), suggesting that in non-C-limited soils SO_4^{2-} addition and SRB stimulation may not suppress methanogenesis. The relationship between methanogens, SRB and SO_4^{2-} should be used to determine the effect of SO_4-S fertilization in wet forests to suppress CH_4 fluxes. Molecular data can elucidate the complex interactions between the soil environment and microbial communities. Further investigation of *mcrA* and *pmoA* dynamics in waterlogged forest soils are required to better understand the effects of site preparation and fertilization on methanogenic and methanotrophic microorganisms and ultimately on CH_4 dynamics.

1.7 Microbial functional groups involved in nitrogen cycling and N_2O fluxes in forest soil

1.7.1 Nitrogen-fixation

Diazotrophic microorganisms are unique in their ability to fix atmospheric N_2 into a biologically useable form. The nitrogenase enzyme catalyzes this reaction. The most common form of nitrogenase contains an electron-delivery Fe protein and a catalytic MoFe protein (Hoffman et al., 2013). The nitrogenase reductase subunit of the diazotrophic *Klebsiella oxytoca* is encoded by the *nifHDKTY* operon, which clusters with operons for Fe electron transport (*nifJ* and *nifF*) and MoFe cofactor biosynthesis (*nifENXU* and *nifUSVWZM*), as well as genes whose function is currently unknown (Temme et al., 2012). The *nifH* gene is the most often used marker for the molecular analysis of N-fixing bacteria. A wide variety of PCR primer sets from multiple N-fixing bacteria have been used to characterize and quanitify the *nifH* gene in soil (Table 1.1). The most common *nifH* primer sets use a nested or semi-nested

Table 1.1. Selected primer sets for amplification of nitrogenase reductase (*nifH*) genes

Primers	**Nucleotide Location**	**Primer sequences (5'-3')**	**Ref. Species (GenBank accession no.)**	**Reference(s)**
nifH-forA	19-38	GCIWTITAYGGNAARG	*Azotobacter vinelandii* (M20568)	Widmer et al. (1999)
nifH-forB (nested)	112-131	GGITGTGAYCCNAAVGCNGA		Levy-Booth and Winder (2010)
nifHrev	463-482	GCRTAIABNGCCATCATYTC		
nifHF	34-59	AAAGGYGGWATCGGYAARTCCACCAC	*Sinorhizobium meliloti* (46285)	Rösch et al. (2002)
nifHR	466-491	TTGTTSGCSGCRTACATSGCCATCAT		
nifH-19F	19-39	GCIWTYTAYGGIAARGGIGG	*A. vinelandii* (M20568)	Ueda et al. (1995)
nifH-3	1002-1018	ATRTTRTTNGCNGCRTA		Zani et al. (2000)
nifH-11 (nested)	639-655	GAYCCNAARGCNGACTC		Yeager et al. (2005)
nifH-22 (nested)	984-1000	ADWGCCATCATYTCRCC		
nifHF	34-59	AAAGGYGGWATCGGYAARTCCACCAC	*Bradyrhizobium japonicum* USDA 110 (BA000040)	Rösch and Bothe (2005)
nifHRb	412-437	TGSGCYTTGTCYTCRCGGATBGGCAT	*S meliloti* (46285)	Yergeau et al. (2007)
				Morales et al. (2010)

IUPAC degenerate bases: B, C+G+T; D, A+G+T; H, A+C+T; K, T+G; M, A+C; N, A+C+G+T; R, A+G; S, G+C; W, A+T; V, A+C+G; Y, C+T

approach aligned with the *nifH* sequence from the non-symbiotic diazotroph *Azotobacter vinelandii* (GenBank accession number M20568) (Ueda et al., 1995; Widmer et al., 1999; Zani et al., 2000; Levy-Booth and Winder, 2010; Yeager et al., 2005) or target *nifH* sequences aligned with *nifH* from the symbiotic N-fixer *Sinorhizobium meliloti* (46285) (Rösch et al., 2002; Rösch and Bothe, 2005; Morales et al., 2010).

Major groups of diazotrophs include those in the phyla Cyanobacteria and Chlorobi (green sulfur bacteria), as well as the Proteobacterial groups *Azotobacteraceae* and *Rhizobia*, and the Actinobacteria *Frankia*. In Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) litter, the genera *Rhizobium*, *Sinorhizobium* and *Azospirillum* dominated *nifH* RFLP fragments, while *Bradyrhizobium*, *Azorhizobium*, *Herbaspirillum*, and *Thiobacillus* dominated in soil (Widmer et al., 1999). Mixed conifer soil was dominated by *nifH* clones that clustered with *Beijinckia derxii* ssp. *venezuelae*, *Frankia* sp. *Paenibacillus* sp. and *Clostridium pasteurianum* (Yeager et al., 2005). Free-living diazotrophs are thought to be the dominant form of N-fixing bacteria in bulk soil of forests. With the exception of *Frankia*-alder systems, symbiotic N-fixer interactions in temperate coniferous forests are relatively rare, unlike tropical forests where leguminous N-fixing trees are common. Although symbiotic diazotrophs can fix about 100 times more N than non-symbiotic diazotrophs (Cleveland et al., 1999), free-living strains also contribute N to soil ecosystems, particularly in temperate coniferous forests. For example, in a subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) forest, free-living diazotrophs fixed about 0.9 kg N $ha^{-1}year^{-1}$, while in a cedar–hemlock [*Thujaplicata* (Donn ex D. Don) Lindl.– *Tsuga heterophylla* (Raf) Sarg.] forest, N-fixation averaged 1.1 kg N ha^{-1} $year^{-1}$ (Jurgensen et al., 1992). In fir and lodgepole pine forests in B.C. non-symbiotic N fixation can account for 0.3 and 2.8 kg of N ha^{-1} $year^{-1}$, respectively (Cleveland et al., 1999). Symbotic and non-symbiotic N-fixation rates in boreal forest soil are 0.3 and 1.1 kg N ha^{-1} $year^{-1}$,respectively (Cleveland et al., 1999). Johnson and Curtis (2001) suggest that the presence of free-living N-fixing microorganisms accounts for significantly more soil N than fertilization, based on a meta-anaylsis of 10 temperate coniferous forests, four temperate deciduous forests and three temperate mixed forests worldwide. Free-living diazotrophs are associated with ectomycorrhizae that colonize Douglas-fir roots (Li and Hung, 1987), and improve the establishment of mycorrhizae and conifer seedlings (Cracknell and Lousier, 1988). Frey-Klett et al. (2007) detected *nifH* genes in ectomycorrhizal tissue. Endophytic N-fixers, e.g. *Paenibacillus polymyxa*, have been reported in western redcedar and lodegpole pine from N-poor sites in B.C. and have been shown to fix 36% and 68% of the nitrogen found in these two tree species, respectively (Anand and Chanway, 2010; Anand et al., 2013).

There is a dearth of studies examining the abundance of the *nifH* gene in forests (Table 2), as N-fixation is generally assumed to be of minimal importance in forest soil. However, the *nifH* gene was

Table 1.2. Selected studies of nitrogenase reductase (*nifH*) genes in forest soil

Forest Type	**Conditions**	**Major Relationships**	**Reference**
Pseudotsuga menziesii ssp. *menziesii*	Natural forest	Distinct *nifH* community structure in litter and soil	Widmer et al. (1999)
Oak-hornbeam; acid spruce	Acid forest soil	Low *nifH* species richness conserved across sites.	Rösch et al. (2002)
Pinus ponderosa - *P. menziesii*	Exposure to fire	Higher diversity after fire	Yeager et al. (2005)
P. menziesii ssp. *Menziesii*	Thinning, clear-cut	Abundance of *nifH* correlated with total C, organic C, and N conc. in LFH layer	Levy-Booth and Winder (2010)
Oak-hickory, beech-maple	Successional stage	Abundance of *nifH* correlated with organic C	Morales et al. (2010)

found in greater abundance in forest soil than agricultural soil (Morales et al., 2010). Using a targeted metagenomic approach, Wang et al. (2013) found distinct *nifH* communities between soil samples from boreal forest/taiga (AK), subtropical dry forest (FL), subtropical/lower montane wet forest (HI) and grassland/shrubland sites (UT). For example, n*ifH* sequences in AK and FL contained about 7 and 43% *Azospirillum* and about 22 and 9% Δ-Proteobacteria, respectively. The other two sites had *nifH* sequences between these two ecological extremes, with UT being more similar to FL. Community structural differences between these sites were driven most strongly by drainage class (ranging from very poor in AK to excessive in FL) and mean annual temperature (-3^{o}C in AK to 20^{o}C in FL), then by relative sunlight exposure and pH (4.6 in AK to 8.0 in UT) and finally by soil organic matter (1.2% in FL to 51.4% in HI) and mean annual precipitation (260 mm in AK to 4000 mm in HI). Highly conserved species richness of free-living and symbiotic N-fixing bacteria was found in acid forest soil, with *Herbaspirillum seropedicae*, *Burkholderia* sp., *Beijerinckia indica* ssp. *indica*, *Azorhizobium caulinodans*, *Bradyrhizobium japonicum*, *Azospirillum* sp. and *Rhodobacter sphaeroids nifH* sequences being detected in a survey of functional genes in forest soil (Rösch et al., 2002). Abundance of the *nifH* gene was correlated with organic C concentration in soil under Douglas-fir (Levy-Booth and Winder, 2010), oak-hickory and beech-maple stands (Morales et al., 2010). Because (1) fixed N can drive interlinked N- and C-cycling events, including mycorrhizal symbiosis and litter decomposition (Larson et al., 1978), (2) N-fixation in forest soil can add to C availability (Johnson and Curtis, 2001), and (3) soil C concentration can be linked to *nifH* abundance, comparing the abundance of N-fixing bacteria to fungal symbiosis and decomposition rates, particularly in high C:N ratio environments, should be the focus of future studies to help elucidate the role of N-fixer abundance in soil C dynamics.

1.7.2 Nitrification

Nitrification is the biological oxidation of NH_3 to NO_3^-. NO_3^-can leach from soil causing groundwater contamination and lead to further N loss as N_2O/N_2 (see 3.3 Denitrification). Two distinct groups of microorganisms, chemolithotrophic ammonia-oxidizers and nitrite-oxidizers are required for this process (Figure 1.3). The former is further divided between ammonia-oxidizing bacteria (AOB) and archaea (AOA). Nitrification in bacteria begins when the membrane-bound hetero-trimeric Cu enzyme ammonia monooxygenase (AMO) oxidizes NH_3 to hydroxylamine (NH_2OH) (Richardson, 2000; Bergmann et al., 2005) (Equation 11); periplasmic NH_2OH oxidoreductase (HAO) produces HNO_2 (Equation 12).

$$NH_3 + O_2 + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \quad (11)$$

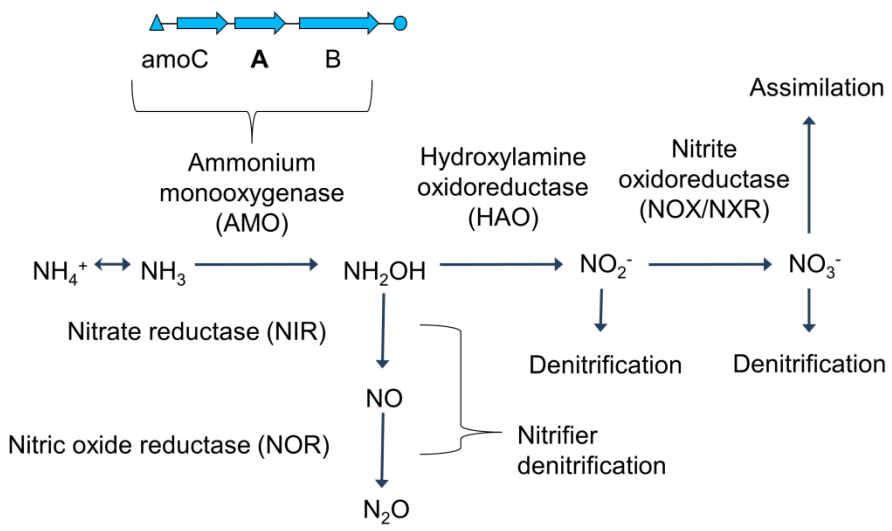

Figure 1.3. The nitrification pathway. Nitrification is the oxidation of ammonium (NH_4^+) by ammonia monooxygenase (AMO) to a variety of intermediates and final products depending on the microbial community and the characteristics of the soil environment. The AMO operon is based on the genome sequence of *Nitrosomonas europaea* (Chain et al., 2003).

$$NH_2OH + H_2O \xrightarrow{HAO} HNO_2 + 4H^+ + 4e^- \quad (12)$$

Two electrons from this reaction are shuttled to the terminal oxidase cytochrome aa3 (ferrocytochrome c:oxygen oxidoreducase with a and a3 hemes), which is likely the rate-limiting step of ammonia-oxidation (Frijlink et al., 1992). Because two of the four electrons generated from the activity of HAO are cycled back into the intital reaction in the oxidiation of ammonia, growth rate is limited by the amount of electrons availiable from this reaction to produce reducing equivilants for fixation of CO_2 via the Calvin cycle (Frijlink et al., 1992). The slow growth rate of ammonia-oxidizing bacteria (e.g., *Nitrosomonas europaea)* in soil (Verhagen and Laanbroek, 1991; Frijlink et al., 1992; Bollmann et al., 2002) is a physiological limitation that can have repercussions throughout the global N cycle. Further nitrification of NO_2^- to NO_3^- using the nitrite oxidoreductase (NOR, NXR) (Equation 13) is carried out by a separate group of autotrophic nitrite-oxidizing bacteria (NOB) (Sundermeyer-Klinger et al., 1984):

$$NO_2^- + H_2O \xrightarrow{NOR,NXR} NO_3^- + 2H^+ + 2e^- \quad (13)$$

Nitrification is primarily studied using the marker gene *amoA*, which encodes the α-subunit of the AMO enzyme. The *amoA* gene is well suited for use as a marker gene for molecular studies of AOA and AOB communities because its nucleotide sequence is strongly conserved and because of the essential role of *amoA* in the energy-generating metabolism (Norton et al., 2002). All studies of AOB *amoA* structure and abundance summarized in this review relied on primer sets that were aligned with the *N. europaea* (L08050) *amoA* sequence (Rotthauwe et al., 1997; Yeager et al., 2005; Ball et al., 2010; Onodera et al., 2010; Szukics et al., 2010; Rasche et al.; 2011; Zeglin et al., 2011; Hynes and Germida, 2012; Long et al., 2012; Petersen et al., 2012; Szukics et al., 2012) (Table 1.3). The amoA1F/2R primer set was used in the majority of reviewed *amoA* studies, allowing their results to be readily comparable. The expansion of *amoA* primer sets to include more common soil AOB strains may yet improve estimates of *amoA* structure and abundance in forest soil. The archaeal *amoA* primer sets are also constrained in their design, being based largely on a single fosmid clone (54d9, AJ627422) found in German soil and the Sargasso Sea database (Venter et al., 2004) (Table 1.3). The expansion of targeted metagenomics of AOA can help expand the number of sequences to draw from when designing further primer sets for AOA *amoA.* The use of the *amoA* functional gene from both AOB and AOA can clarify (1) the effects of soil biochemical characteristics on nitrifying microorganisms, (2) the effect of the *amoA* gene community structure and abundance on nitrification rates and N_2O flux, and (3) the response of AOB and AOA after disturbance

events, both natural (e.g., fire) and anthropogenic (e.g., N fertilization, deposition), which periodically lift restrictions on growth and activity of ammonia oxidizers (Table 1.4).

There are important differences between bacterial and archaeal nitrifiers. Crenarchaeota appear to lack recognizable HAO homologs (Schleper and Nicol, 2010) and therefore may oxidize NH_3 via nitroxyl (HNO) (Walker et al., 2010). An alternate oxidation pathway to nitroxyl (HNO) using nitroxyl oxidoreductase (NXOR) has been proposed to explain the low O_2 requirements and slow growth rate of AOA (Walker et al., 2010) (Equation 14):

$$NH_3 + O_2 + 2e^- \xrightarrow{NXOR} NHO + H_2O \quad (14)$$

This alternate pathway requires less oxygen than in bacteria, allowing archaeal ammonia-oxidation to occur in anoxic zones in soil (Schleper and Nicol, 2010). AOA growth rate and *amoA* transcription were greater than AOB in soil microcosms (Tourna et al., 2008), though AOB appear to be adapted to recover quickly to N pulses following starvation (Bollman et al., 2002). The response of AOA to O_2, pH and temporal variation in NH_3 availability may differentiate environment-specific communities of AOA, and separate AOA ecologically from AOB. Prosser and Nicol (2008) and Schleper and Nicol (2010) provide comprehensive reviews of the physiology and ecology of AOA.

The oxidation of NH_3 in bacteria is restricted to β- and γ-Proteobacteria. AOB include the genera *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira* (Koops and Möller, 1992). *Nitrosomonas* sp. make up a sizable portion of known AOB and together with *Nitrosococcus* sp. and *Nitrosospira* sp., are abundant in soil (Purkhold et al., 2000). *Nitrosospira* sp. clusters 1, 2, 3 and 4 dominate AOB *amoA* sequences in forest soil (Laverman et al., 2001; Yeager et al., 2005). The AOB *amoA* gene is closely related to the particulate methane monooxygenase (*pmoA*) gene found in methane-oxidizing bacteria (MOB) (Holmes et al., 1995; Purkhold et al., 2000) and there is evidence that MOB can also oxidize NH_3 (Bédard and Knowles, 1989). AOB have been widely studied using molecular tools, as ammonia-oxidation was previously thought to be entirely mediated by AOB (Purkhold et al., 2000; Kowalchuk and Stephen, 2001). However, growing evidence suggests that AOA may dominate ammonia-oxidation in some soils.

AOA are abundant in forest soil and Crenarchaeota groups 1.1a and 1.1b have been shown to be numerically and transcriptionally important players in the oxidation of NH_3 (Venter et al., 2004; Schleper et al., 2005; Treusch et al., 2005; Leininger et al., 2006; He et al., 2007; Adair and Schwartz, 2008; Nicol et al., 2008). AOA *amoA* transcripts ranged from statistically equivalent to AOB *amoA* transcripts in arable grassland soils (pH 5.5), to 16-fold greater in sandy grassland soil (pH 7) (Leininger et al., 2006). He et al. (2007) found AOA in 1.02 to 12.36 times greater abundance than AOB in silty clay agri-undic

Table 1.3. Selected primer sets for amplification of bacterial and archaeal ammonia monooxygenase (*amoA*) genes

Primers	**Location**	**Primer sequences (5'-3')**	**Ref. Species (GenBank accession no.)**	**Reference(s)**
		Bacterial *amoA*		
amoA1F amoA2R	322-249 802-822	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	*Nitrosomonas europaea* (L08050)	Rotthauwe et al. (1997) Yeager et al. (2005) Ball et al. (2010) Onodera et al. (2010) Szukics et al. (2010, 2012) Rasche et al. (2011) Zeglin et al. (2011) Hynes and Germida (2012) Long et al. (2012) Petersen et al. (2012)
amoA1F* amoA2R	322-249 802-822	GGGGHTTYTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	*Nitrosomonas europaea* (L08050)	Stephen et al. (1999) Laverman et al. (2001) Leininger et al. (2006)
amoA-2F amoA-5R (prior to amoA1F/2R for nested PCR)	279-298 1065- 1079	AARGCGGCSAAGATGCCGCC TTATTTGATCCCCTC	*Nitrosomonas europaea* (L08050)	Webster et al. 2002) Yeager et al. (2005)
		Crenarcheotal *amoA*		
amoA19F amoA643R	19-36 643-669	ATGGTCTGGCTWAGACG TCCCACTTWGACCARGCGGCC ATCCA	Fosmid clone 54d9 (AJ627422) Sargasso Sea database (AACY000000000)	Onodera et al. (2005) Treusch et al. (2005) Leininger et al. (2006) Bru et al. (2011)

Primers	Nucleotide Location	Primer sequences (5'-3')	Ref. Species (GenBank accession no.)	Reference(s)
Arch-amoAF Arch-amoAR	3-23 618-638	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Fosmid clone 54d9 (AJ627422) Sargasso Sea database (AACY000000000)	Francis et al. (2005) Szukics et al. (2010, 2012) Rasche et al. (2011) Zeglin et al. (2011) Petersen et al. (2012)
CrenamoA23F CrenamoA616R	7-24 611-631	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	Fosmid clone 54d9 (AJ627422) Sargasso Sea database (AACY000000000)	Könneke et al. (2005) Tourna et al. (2008) Bru et al. (2011) Long et al. (2012)

Table 1.4. Selected studies of ammonia monooxygenase (*amoA*) genes in forest soil

Forest Type	Conditions	Major Relationships	Reference
Pinus sylvestris	N-saturated acid forest soil	*Nitrosospira* sp. cluster 2 dominant AOB[a] *amoA*	Laverman et al. (2001)
P. ponderosa - *Pseudotsuga menziesii*	Exposure to fire	*Nitrosospira* sp. cluster 1,2,4 *amoA* found pre-fire; cluster 3a dominant AOB post-fire; correlated with higher NH_3	Yeager et al. (2005)
Various	Various	AOB *amoA* community structure correlated with site temp., C:N ratio; *Nitrosospira* sp. cluster 3 dominant AOB	Fierer et al. (2009)
Picea abies, P. sylvestris, Larix ssp.	3.9-6.6 soil pH	Group 1.1c Crenarchaeota *amoA* greater at lower pH	Lehtovirta et al. (2009)
P. ponderosa - *P. menziesii*	Exposure to fire	Higher AOB *amoA*, nitrification rates following fire; community shift toward *Nitrosospira* sp. cluster 3	Ball et al. (2010)
Cypress-oak	Natural forest	AOA[b] *amoA* more abundant than AOB; AOB *amoA* community vertically stratified; *Nitrosospira* sp. clusters 1 and 4 dominant	Onodera et al. (2010)
Spruce–fir–beech[c]	30-70% WFPS[d], 5-25^{o}C	Abundance of *amoA* increased with temp., decreased at 25^{o}C and 70% WFPS; AOB and AOA *amoA* equivalent in abundance and diversity	Szukics et al. (2010)
Various	Landscape scale analysis	AOA *amoA* more abundant than from AOB, correlated to total Crenarchaeota; AOB, but not AOA, abundance explained by land use and soil C; AOA:AOB ratio driven by pH	Bru et al. (2011)
Fagus sylvatica	Tree girdling	AOB, AOA *amoA* community structure influenced by seasonality, tree girdling; AOB and AOA *amoA* abundance correlated with DON[e], NH_3, temp, moisture. AOA *amoA* correlated with N_2O emissions	Rasche et al. (2011)

Forest Type	Conditions	Major Relationships	Reference
P. menziesii, Alnus rubra	Land use, forest type	N-mineralization, C:N ratios drive differences between cultivated and forest AOB *amoA* communities	Zeglin et al. (2011)
Spruce–fir–beech; beech[c]	40-70% WFPS; NH_4-N or NO_3-N incubation	AOA or AOB *amoA* response to N incubation dependent on site; no effect on nitrification rate	Szukics et al. (2012)
>60% *P. contorta* subsp. *Latifolia*	Clear-cut	*amoA* community structure related to stand age, N bioavailability	Hynes and Germida (2012)
P. taeda	Elevated CO_2, NH_4-NO_3 fertilization	N fertilization increased AOB *amoA* at ambient CO_2; AOA, AOB community structure influenced by pH	Long et al. (2012)
Picea mariana; Salix spp., *Betula* spp., other	Vegetation gradient	AOB *amoA* 8-18 times more abundant than AOA *amoA;* correlation between AOB amoA and NH_4^+, potential nitrification rate	Petersen et al. (2012)

[a]Ammonia oxidizing bacteria
[b]Ammonia oxidizing Archaea
[c]Incubated soils
[d]Water-filled pore space
[e]Dissolved organic nitrogen

ferrosol soil, and Adair and Schwartz (2008) showed that in semi-arid soils, archaeal *amoA* sequences were 17 to 1,600 times more abundant than AOB *amoA*. The abundance of Crenarchaeota *amoA* genes have been studied in a variety of forest soils, with mixed results. Onodera et al. (2010) found that AOA *amoA* had a greater abundance than AOB *amoA* genes in a natural cypress-oak forest. Bru et al. (2011) reported that at the landscape scale, AOA were 10 to 400 times more abundant than AOB at 77 sampled sites, but displayed less or equivalent abundance at nine sites. In contrast, Petersen et al. (2012) found that AOB *amoA* genes were 8-18 times more abundant than AOA *amoA* in both coniferous and deciduous forest soil. Mertens et al. (2009) found AOB *amoA* were more abundant than AOA *amoA* following disturbance of the soil ecosystem and *Nitrosospira* sp. (clusters 2, 3 and 4) remained the most abundant AOB in acidic forest soils following N fertilization (Compton et al., 2004; Horz et al., 2004). Di et al. (2010) found that the number of AOB, but not AOA, *amoA* gene copies increased under fertilization and were linearly correlated to nitrification rates. Thus it remains unclear which populations of ammonia-oxidizers are primarily responsible for nitrification in forest soils.

Ammonia availability and pH are closely related factors that influence differences in AOB and AOA *amoA* abundance in forest soil. Ammonia is largely converted to NH_4^+ in low pH environments (NH_3/NH_4^+ pKa = 9.24). Strategies to oxidize NH_3 in acidic environments include containing high-affinity AMO enzymes, regulation of the transcription for the *amoCAB* operon, active transport of NH_4^+, hydrolysis of urea, and biofilm formation. Lehtovirta-Morley et al. (2011) cultivated an acidophilic AOA *Nitrosotalea devanaterra* from pH 4.5 from an agricultural soil with high-affinity AMO that was capable of growth on low (0.18 nM) NH_3 concentrations. AOA can oxidize NH_3 in environments where concentrations are lower than the growth requirements of AOB (Olson, 1981; Yool et al., 2007). Martens-Habbena et al. (2009) indicate that AMO from AOA has an affinity for NH_3 of almost 10^5, 3-4 orders of magnitude greater substrate affinity than AOB species. This is in the range of some of the highest measured substrate affinities of organotrophic organisms.

Soil pH can drastically change AOB and AOA diversity, abundance and function. To assess the effect of pH on AOB community composition, Bäckman et al. (2003) and Nugroho et al. (2007) added lime to Norway spruce (*Picea abies* L.) and Scots pine (*Pinus sylvestris* L.) stands, respectively. Liming treatments used in Bäckman et al. (2003) of 3 and 6 t $CaCO_3$ ha^{-1} raised pH(KCl) in the top 5 cm of soil from 2.6 to 3.6 and 4.8, respectively, while Nugroho et al. (2007) used laboratory measurements of field soil incubated with 30 mg $CaCO_3$ g^{-1}, which raised soil pH(KCl) from 2.8 to 3.9. Liming treatments increased diversity of the *amoA* gene from *Nitrosospira* spp. cluster 2 and increased nitrification rates (Bäckman et al., 2003; Nugroho et al., 2007). The model AOB, *N. europaea*, cannot function at low pH due to its inability to utilize NH_4^+ (Frijlink et al., 1992). Yet nitrification occurs in acidic soil (Booth et al.,

2005). Clusters 2 and 4 of *Nitrosospira* sp. are the primary AOBs in acid forest soil (pH 4.2-5.5) (Stephan et al., 1998). *Nitrosospira* sp. are able to passively transport urea in a low-pH culture and hydrolyze it to NH_3 (Jiang and Bakken, 1999; Burton and Prosser, 2001). AOA can also hydrolyze urea to facilitate NH_3 oxidation in acidic soil. AOA *amoA* abundance in forest soil (pH~5.40) and tea orchard soil (pH~3.75) increased significantly following urea amendment (Lu and Jia, 2013). Soil pH is the principal factor in AOB and AOA community structure (Gubry-Rangin et al., 2011), which determines *in situ* nitrification potential and the ability of the community to respond to flushes of N in forest soil.

Nitrogen addition can alter AOA and AOB communities. Forms of N that lower soil pH, such as NO_3^- and $(NH_4)_2SO_4$ can significantly reduce AOA and AOB *amoA* (Hallin et al., 2009). In a NH_4NO_3-fertilized *P. taeda* stand, pH controlled *amoA* community structure (Long et al., 2012). In boreal forest soil, AOB *amoA* gene abundance was correlated with NH_4^+ abundance and potential nitrification (Petersen et al., 2012). The AOB *amoA* community shifted prior to increases in potential nitrification and soil NO_3^- concentrations in lodgepole pine (*Pinus contorta* ssp. *latifolia*) and spruce (*Picea glauca*) stands receiving annual and periodic N fertilization at a rate of 200 kg ha^{-1} (Wertz et al., 2012). Webster et al. (2005) found that increase in potential nitrification rates following fertilization were preceded by an *amoA* community shift to ammonium-sensitive *Nitrosospira* ssp. cluster 3a. Pederson *et al.* (1999) and Jordan *et al.* (2005) found that nitrification was primarily heterotrophic in N-fertilized forests, while Wertz et al. (2012) showed that 54.6–96.9% of nitrification was carried out by autotrophic bacteria following N addition. Neither Pratscher et al. (2011) or Wertz et al. (2012) found a correlation between AOA *amoA* gene abundance and nitrification rates or CO_2 fixation following fertilization.

Fire is an important and widespread disturbance in temperate forests. The AOB community is sensitive to increases in pH and ammonia availability which are common after fire in forest ecosystems. The presence of *Nitrosospira* spp. cluster 3a *amoA* sequences were positively correlated with an increase in soil pH from 5.6 to 7.5 after wildfire, demonstrating a shift from pre-fire cluster 1, 2 and 4 communities (Yeager et al., 2005). *Nitrosospira* ssp. cluster 3a is also highly sensitive to changes in soil NH_4^+ ions and its growth is suppressed at high NH_4^+ concentrations (Webster et al., 2005; Tourna et al., 2010). Ball et al. (2010) also reported that the AOB *amoA* community shifted from *Nitrosospira* spp. cluster 4 towards cluster 3 after wildfire. The post-fire community shift resulted in a greater abundance of AOB *amoA* gene copies in all soil layers and increased gross nitrification rates, yet with no difference in net nitrification. The question remains, how does the concurrent increase in pH and NH_3 in post-fire forest soil affect the ammonia-oxidizing community?

Nitrification is a key process that can lead to N_2O emission from soil. In a urea-fertilized pine forest soil, N_2O production was suggested to be caused by autotrophic ammonia oxidation at low pH

(Martikainen, 1985). Nitrifier denitrification occurs when NO_2^- produced by HAO is converted to N_2O directly, particularly in oxic soils where redox conditions for denitrification are not met (Wrage et al., 2001; Kool et al., 2010, 2011). AOB such as *N. europaea* are able to produce, but not reduce, N_2O (Schmidt et al., 2004). Nitrifier denitrification can account for up to 30% of N_2O emissions from soil (Clough et al 2004).

Plant or tree species, temperature, water content, C:N ratio and soil total N have also been linked to differences in AOB and AOA community structure (Boyle-Yarwood et al., 2008; Fierer et al., 2009; Rooney et al., 2010; Szukics et al., 2010; Zeglin et al., 2011; Rasche et al., 2011; Szukics et al., 2012). Boyle-Yarwood et al. (2008) demonstrated that tree species affect AOA and AOB community structure and nitrification rates: nitrifying potential was 2- to 12-fold greater under red alder (*Alnus rubra*) than Douglas-fir and AOB terminal-restriction fragment length polymorphism (T-RFLP) signatures differed between the two species at two sites in north-western Oregon. AOB and AOA community structure differed based on soil nutrient status (Di et al., 2010). Additionally, the capacity of AOA for heterotrophic growth (Hallam et al., 2006; Pratscher et al., 2011; Tourna et al., 2011; Pester et al., 2012) may contribute to their greater numbers in soil relative to AOB under conditions of low N availability.

Within the AOB and AOA there are distinct communities that occupy niches along physico-chemical gradients common in forest soil. Some populations of AOA appear better suited for ammonia oxidation in acidic soil due to the high affinity of their AMO enzymes for NH_3, their ability to hydrolyze urea and their potential for heterotrophic or mixotrophic growth. Some AOB communities, particularly those including *Nitrosospira* ssp. clusters 2, 3a and 4 are able to compete at low pH due to their ability to hydrolyze urea and possible mixotrophy. Other AOB may dominate NH_3 oxidation during periods of N amendment to soil or in microsites with pH approaching or exceeding 7 (Bollman et al., 2002; Webster et al., 2005; Wertz et al., 2012). The abundances of AOA *amoA* genes have been weakly but significantly correlated with N_2O emissions in a temperate beech forest (Rasche et al., 2011). These results suggest that either archaeal nitrifiers are directly producing N_2O or that nitrification is closely coupled to denitrification in this ecosystem. It remains to be seen at which spatial and temporal scales such data are useful for incorporation in statistical models of nitrification and N_2O flux.

1.7.3 Denitrification

Denitrification, the full or partial dissimilative reduction of NO_3^- by microorganisms to dinitrogen gas (N_2), is the primary pathway of N_2O emissions from soil (Colliver and Stephenson, 2000;

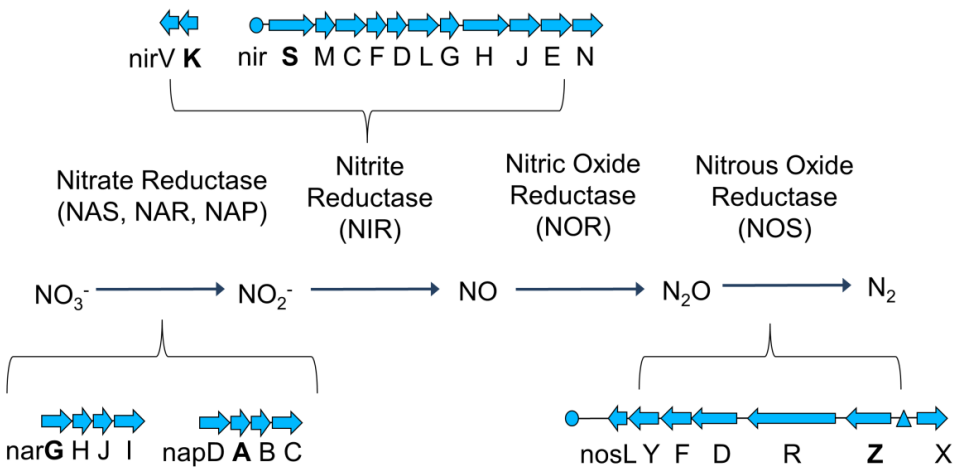

Figure 1.4. The denitrification pathway. The organization of *nap*, *nar*, and *nirS* genes are based on partial or complete operons of *Pseudomonas aeruginosa* PAO1, and the partial *nirK* operon is a consensus of cu-NIR containing organisms (Philippot, 2002). Organization of *nos* is based on partial *nos* operons (Philippot, 2002) and on conserved sequences found in contigs 878 and 1042 from a soil metagenome described by Demanèche et al. (2009).

Kowalchuck and Stephen, 2001; Shaw et al., 2006) (Figure 1.4). Denitrification is ubiquitous in bacteria, archaea and fungi (Baalsrud and Baalsrud, 1954; Carlson and Ingraham, 1983; Bollag and Tung, 1972; Shoun et al., 1992; Cabello et al., 2004; Bartossek et al., 2010). NO_3^- reduction occurs via the membrane-bound, Mo-containing nitrate reductase (NAR) enzyme, a membrane-bound, Mo-containing enzyme, which is encoded by the *nas*, *nar* and *nap* operons. The *narG and napA* genes are utilized most often in studies of NO_3^- reduction (Tavares et al., 2006; Kandeler et al., 2009; Bru et al., 2011). (Equation 15), NO_2^- reduction to NO occurs via the Cu-containing or multi-heme cytochrome cd1-containing nitrite reductase (NIR) enzyme (molecular markers: *nirK* and *nirS* genes, respectively) (Equation 16). *NirK* and *nirS* genes are examples of convergent evolution and generally do not appear in the same organism. Rare exceptions include the hot-springs bacterial strain *Thermus oshimai* JL-2, where both the *nirK* and *nirS* gene are contained within a circular megaplasmid, and *T. scotoductus* SA-01, where both genes are included within the chromosome (Murugapiran et al., 2013). NO reduction occurs via the cytochrome bc-containing nitric oxide reductase (NOR) enzyme (Equation 17) and N_2O is reduced via the multi-Cu nitrous oxide reductase (NOS) enzyme (molecular marker: *nosZ* gene) (Equation 18) (Berks et al., 1995; Chan et al., 1997; Bertero et al., 2003; Zumft, 2005; Kandeler et al., 2006; Tavares et al., 2006; Sundararajan et al., 2007):

$$NO_3^- + 2H^+ + e^- \xrightarrow{NAR} NO_2^- + H_2O \quad (15)$$

$$2NO_2^- + 4H^+ + 2e^- \xrightarrow{NIR} 2NO + 2H_2O \quad (16)$$

$$2NO + 2H^+ + 2e^- \xrightarrow{NOR} N_2O + H_2O \quad (17)$$

$$N_2O + 2H^+ + 2e^- \xrightarrow{NOS} N_2 + H_2O \quad (18)$$

The energy produced during denitrification decreases with the sequential reduction of substrates proportional to their oxidation number (Koike and Hattori, 1975). Likewise, the functional genes in each step of denitrification decrease in abundance (Bru et al., 2011), due to lower free energy available to be liberated stepwise along the pathway (Koike and Hattori, 1975).

Denitrifying microorganisms are prevalent in soil, accounting for between 0.5 and 5% of the total bacterial population (Henry et al., 2006; Demanèche et al., 2009; Bru et al., 2011). This trait is found in a wide range of both heterotrophic (e.g., *Pseudomonas stutzeri*, *P. aeruginosa*, and *Paracoccus denitrificans)* (Carlson and Ingrahm, 1983) and autotrophic bacteria (e.g., *Thiobacillus denitrificans)* (Baalsrud and Baalsrud, 1954). The polyphyletic distribution of denitrifying genes results in their co-occurrence with N-fixation and ammonia-oxidation genes in many strains. There are several hypotheses

as to the widespread taxonomic distribution of denitrification genes, including duplication/divergence and lineage sorting (Jones et al., 2008; Palmer et al., 2009) and horizontal transfer events (Philippot, 2002; Heylen et al., 2006; Alvarez et al., 2011). Archaeal *nir* and *nos* genes have also been sequenced (Bartossek et al., 2010; Cabello et al., 2004), and a significant portion of denitrification may take place in AOB and AOA (Clough et al 2004; Schmidt et al., 2004). Denitrification also occurs in fungi including Ascomycota (e.g., *Fusarium oxysporum, F. solani, Cylindrocarpon tonkinense* and *Gibberella fujiuroii*) and Basidiomycota (e.g., *Trichosporon cutaneum*) (Bollag and Tung, 1972, Shoun et al., 1992). The fungal dissimilatory N-reduction system is located in the mitochondria and contains dissimilatory *nar* genes and *nir* genes for a Cu-NOR orthologous to bacterial *nirK* (Kobayashi et al., 1995; Uchimura et al., 2002). Denitrifying fungi have a distinct cytochrome P450-*nor*, in contrast to the bacterial *bc-nor* (Nakahara et al., 1993). Fungi do not contain an enzyme orthologous to NOS, leading to hypotheses that fungi are responsible for a large portion of N_2O emissions from soil (Kobayashi et al., 1995; Laughlin and Stevens, 2002). For example, Laughlin and Stevens (2002) found that fungal denitrification produced up to 89% of N_2O in a grassland soil. In a silver birch (*Betula pendula* Roth.) plantation on drained peat soil, fungal phosopholipid fatty acids (PLFA) showed strong negative correlations with pH, and fungal:bacterial PLFA ratios were indirectly correlated to modeled N_2O emissions (Rütting et al., 2013). This study demonstrates that fungi:bacteria ratios and estimated N_2O emission (likely due to shifting N_2O:N_2 ratios, see later in this section) are higher in low pH soil. Fungal contributions to N_2O emissions from forest soil evidently require futher elucidation and could benefit from nitrite reductase primers specific to fungal *nir* sequences.

Primer sets commonly used to amplify *nirK* and *nirS* are largely derivations of the primers put forth by Hallin and Lindgren (1999), which reference the nucleotide sequences of *Alcaligenes faecalis* (D13155) and *Pseudomonas stutzeri (*X53676), respectively (Table 1.5). The current *nirK* primer sets exhibit amplification biases that preferentially target the α-Proteobacteria to the exclusion of *nirK* sequences found in other nitrite reducers, including archea (Penton et al., 2013). For example, commonly used *nirK* forward primers 517F (Chen et al., 2010) and nirK1F (Braker et al., 1998) aligned with39 and 25 of 215 unique *nirK*-containing species with 0 mismatches, respectively, while their respective reverse primers (1055R and nirK5R) aligned with only 17 and 15 unique *nirK*-containing species with 0 mismatches, respectively. Neither primer set was matched with archaeal *nirK* sequences. Depending on PCR conditions the number of targeted species could be higher as more mismatches per primer are common with less stringent amplification protocols. Other primer sets used in forest ecology reseach such as the *nirK* primer set KA15-F/KA16-R (Rösch et al., 2002) have substantially lower coverage. Penton et al. (2013) suggest that the current primers be redesigned and that alternative primer-binding regions be

Table 1.5. Selected primer sets for amplification of nitrite reductase (*nirK* and *nirS*) genes

Primers	Nucleotide Location	Primer sequences (5'-3')	Ref. Species (GenBank accession no.)	Reference(s)
		napA		
V17m napA4r	nl. (152 bp fragment)	TGGACVATGGGYTTYAAYC ACYTCRCGHGCVGTRCCRCA	*Pseudomonas aeruginosa* (AE004091)	Bru et al. (2007) Kandeler et al. (2009) Bru et al. (2011)
		narG		
1960m2f 2050m2r	1960-1961 2050-2072	TAYGTSGGGCAGGARAAACTG CGTAGAAGAAGCTGGTGCTGTT	nl. (environ. clones)	López-Gutiérrez et al. (2004) Kandeler et al. (2006)
narG-f narG-r	nl. (173 bp fragment)	TCGCCSATYCCGGCSATGTC GAGTTGTACCAGTCRGCSGAYTCSG	*Pseudomonas aeruginosa* (Y15252)	Bru et al. (2007) Kandeler et al. (2009) Bru et al. (2011)
		nirK		
KA15-F KA16-R	526-555 1075-1101	GGCATGGTACCTTGGCACGTAACCTCGGGC CATTAGATCGTCGTTCCAATCACCGGT	*Alcaligenes faecalis* (D13155)	Rösch et al. (2002)
F560-589 R906-935	560-589 906-935	GGGCATGAACGGCGCGCTCATGGTGCTGCC CGGGTTGGCGAACTTGCCGGTGGTCCAGAC	*Pseudomonas chlororaphis (*Z21945)	Chénier et al. (2003) Levy-Booth and Winder (2010)
FlaCu R3Cu	568-584 1021-1040	ATCATGGTSCTGCCGCG TTGGTGTTRGACTAGCTCCG	*A. faecalis* (D13155)	Hallin and Lindgren (1999) Kandeler et al. (2009)
nirK1F nirK5R	526–542 1023–1040	GGMATGGTKCCSTGGCA GCCTCGATCAGRTTRTGG	*A. faecalis* (D13155)	Braker et al. (1998) Szukics et al. (2009, 2010)
nirK876 nirK1040	876-893 1020-1040	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGGTT	*S meliloti* (AE006469)	Henry et al. (2006) Bárta et al. (2010) Bru et al. (2011) Petersen et al. (2012)
nirK517F nirK1055R	517-537 1035-1055	TTYGTSTAYCACTGCGCVCC GCYTCGATCAGRTTRTGGTT	*Rhizobium etli* (NC_007766.1)	Chen et al. (2010)

Primers	Nucleotide Location	Primer sequences (5'-3')	Ref. Species (GenBank accession no.)	Reference(s)
		nirS		
KA3-F KA25-R	292-314 967-990	CACGG**Y**GT**B**CTGCGCAAGGGCGC CGCCACGCGCGG**Y**TC**S**GGGTGGTA	*Paracoccus denitrificans* (U05002)	Rösch et al. (2002)
nirS1F nirS3R	763-781 1001-1019	CCTAYTGGCCGGCRCART GCCGCCGTCRTGVAGGAA	*P. stutzeri* (X53676)	Braker et al. (1998) Levy-Booth and Winder (2010)
nirSCd3aF nirSR3cd	918–935 1322-1341	AACGYSAAGGARACSGG GASTTCGGRTGSGTCTTSAYGAA	*P. stutzeri* (X53676)	Hallin and Lindgren (1999) Kandeler et al. (2006, 2009) Bárta et al. (2010) Petersen et al. (2012)
*nirS*263F nirS950R	263-285 930-950	TGCGYAARGGGGCNACBGGCAA GCBACRCGSGGYTCSGGATG	*Azoarcus* sp. (YP_157499)	Chen et al. (2010)
		nosZ		
nosZ-F nosZ-R	1211–1230 1897-1917	CGYTGTTCMTCGACAGCCAG CATGTGCAGNGCRTGGCAGAA	*P. denitrificans* (398932)	Rösch et al. (2002)
nosZ-F nosZ-R	1181-1201 1880-1900	CGCTGTTCITCGACAGYCAG ATGTGCAKIGCRTGGCAGAA	*P. stutzeri* (M22628)	Rich et al. (2003)
nosZ2F nosZ2R	1617-1640 1864-1884	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	*Pseudomonas fluorescens* (AF197478)	Henry et al. (2006) Kandeler et al. (2006, 2009) Bru et al. (2007, 2011) Petersen et al. (2012)

nl, not listed.

Table 1.6. Selected studies of nitrate reductase (*narG, napA*), nitrite reductase *(nirS, nirK)* and nitrous oxide reductase *(nosZ*) genes in forest soil

Gene	Forest Type	Conditions Tested	Major Relationships	Reference
nirS, nirK, nosZ	oak-hornbeam; acid spruce	Acid forest soil	Low *nirS, nirK* diversity, high *nosZ* diversity across sites	Rösch et al. (2002)
nosZ	*Abies procera, A. grandis, A. concolor*	Meadow-forest transect	*nosZ* community structure related to denitrification activity, vegetation type and C:N ratio	Rich et al. (2003)
nirS, nirK	*P. menziesii* ssp. *menziesii*	Thinning, clear-cut	Abundance correlated with total N conc. in Ae layer	Levy-Booth and Winder (2009)
narG, napA, nirK, nosZ	*P. abies*	N deposition level, soil depth	*napA* abundance influenced by organic C; *nirK* correlated with total N, NH_4^+, pH; increasing *nosZ*/*nirK* ratio with depth. Denitrification gene abundance not influenced by N deposition.	Kandeler et al. (2009)
nirK	Spruce–fir–beech[a]	40-70% WFPS, NH_4^+, NO_3^- additions	Abundance of *nirK* correlated with nitrate reductase activity, NO emissions at one site	Szukics et al. (2009)
nirK,	Spruce–fir–beech[a]	30-70% WFPS, 5-25^{o}C	Abundance of *nirK* increased with., WFPS until NO_3^- became limiting; community structure associated with soil water content	Szukics et al. (2010)
nirS, nosZ	Oak-hickory, beech-maple	Successional stage	*nirS* abundance correlated to NO_3^-, N_2O emissions; *nirS-nosZ* difference strong predictor of N_2O emissions	Morales et al. (2010)
nirK	*P. abies*	High acid N deposition, bark beetle infestation	*nirK* abundance correlated with available P conc., DOC[b], pH	Bárta et al. (2010)

Gene	Forest Type	Conditions Tested	Major Relationships	Reference
narG napA nirK nirS nosZ	Various	Landscape scale analysis	Soil chemistry main driver of gene abundance; pH most important parameter; soil C, Mn_{ex}[c] also contribute	Bru et al. (2011)
nirS, nosZ	*F. sylvatica*	Tree girdling	Abundance of *nirS*, *noZ* correlated with NH_3, N_2O efflux, temp, moisture. Only *nosZ* correlated with DON, NO_3^-	Rasche et al. (2011)
nirK, nirS, nosZ	*Picea mariana; Salix* spp., *Betula* spp., other	Vegetation gradient	Abundance of *nosZ* gene predicted potential denitrification rate	Petersen et al. (2012)

[a]Incubated soils
[b]Dissolved organic carbon
cExchangable manganese

investigated. In contrast, the coverage limitations of *nirS* may be alleviated by expanding the degeneracy of the current primers to encapsulate known *nirS* sequences.Functional gene analysis for the denitrification pathway reveals complex interactions between the soil environment and the denitrifying community (Table 1.6). While denitrification rates have been intensely studied, less is known about how the genes involved are influenced by the soil environment or affect process rates. The abundance of the *narG* and *napA* genes was positively correlated with soil C (Kandeler et al., 2009; Bru et al., 2011). The *napA* gene has also been positively correlated with exchangeable manganese (Mn) (Bru et al., 2011). Nitrite reduction activity is thought to have a major influence on rates of NO and N_2O production. The diversity of *nirS* and *nirK* genes were found to be low in both oak and spruce forest stands (Rösch et al., 2002), indicating that while these genes are found in diverse organisms, the community structure is restricted in acid forest soils. The *nirS* and *nosZ* sequences that were amplified clustered with N-fixing bacteria *Azospirillum* sp. and *Bradyrhizobium japonicum*, demonstrating a potential link between biological N fixation and denitrification *(*Rösch et al., 2002). *NirS* and *nirK* quantities are influenced by a range of factors, including soil moisture and temperature (Szukics et al., 2010; Rasche et al., 2011), total N concentration (Kandeler et al., 2009; Levy-Booth and Winder, 2010), NH_4^+ concentration, NO_3^- concentration in soil (Morales et al., 2010), available phosphorus (P) concentration, soil organic matter (SOM) (Petersen et al., 2012), dissolved organic carbon (DOC) (Bárta et al., 2010) and pH (Kandeler et al., 2009; Bárta et al., 2010). The *nosZ* gene was significantly more abundant than *nirS* in forest sites, while in agricultural sites *nirS* abundance was up to four orders of magnitude greater than *nosZ* (Morales et al., 2010). The number of *nosZ* copies remained consistently around 1×10^3 in both environments, but *nirS* was significantly greater in agricultural soil than in forest soil. Denitrification gene abundance was strongly influenced by soil C, particularly the response of *nirS* to percent organic C, which differentiated agricultural and forest soil samples following principle component analysis (PCA). Alternatively, in a tree girdling experiment designed to test the limitation of soil C on the microbial community, Rasche et al. (2011) found no significant difference in *nirS* and *nosZ* gene abundance between plots containing girdled and ungirdled beech trees. Dissolved organic C (DOC) correlated significantly, and positively, with *nosZ* gene abundance when C was constrained, as did soil NO_3^- and NH_4^+ concentrations. Under low soil O_2, the ability to facultatively reduce N as an alternate electron acceptor provides denitrifying organisms a selective advantage in the competition for organic C (Tiedje, 1988). Denitrification rates and end-products are influenced by soil pH, O_2, organic C, temperature and moisture (Knowles, 1982; Tiedje, 1988; Brumme and Beese 1992; Saad & Conrad, 1993; Thomsen et al., 1994; Bergaust et al., 2008, 2010). Likewise, factors that influence the abundance of denitrification functional genes include availability of terminal electron acceptors for respiration, temperature, moisture and organic C sources for heterotrophic growth.

Denitrification is the primary source of N_2O from wet (>80% water-filled pore space (WFPS)) soils (Kool et al., 2010, 2011, Zhang et al., 2013), though in field soils water saturation and low substrate concentrations can lead to uptake and reduction of atmospheric N_2O to N_2 (Davidson et al., 2000; Chapuis-Lardy et al., 2007; Goldberg and Gebaur, 2009). The metabolic source of N_2O depends on soil moisture: in moderate moisture regimes (50 and 70% WFPS) denitrification accounted for 16.1 and 20% of total N_2O, respectively; in high moisture soil (90% WFPS) denitrification accounted for 92.1% of total N_2O production, with the balance being allocated to nitrification and nitrifier denitrification (Kool et al., 2011).

It is unclear how denitrification process rates and N_2O emissions are changed by N addition to soil. Restricting N deposition in spruce forest soil for 14 years generally did not influence denitrification genes, although NH_4^+, *nosZ* abundance and NAR enzyme activity were greater in N-deposition sites (Kandeler et al., 2009). The form of N in soil and its effect on soil pH play a major role in the microbial community response to N additions and can explain some of the contradictory results observed between studies. Fertilization of a Swedish agricultural Eutric Cambisol clay loam soil with 80 kg N ha^{-1} y^{-1} as $(NH_4)_2SO_4$ lowered soil pH and significantly reduced *narG*, *nirK*, *nirS* and *nosZ* abundance, while organic fertilizers with near-neutral pH increased *narG*, *nirK*, and *nosZ* abundance (Hallin et al., 2009). Moisture status and organic C can also influence the denitrifier community response to N addition. Following the amendment of forest soil with NH_4^+-N or NO_3^--N, Szukics et al. (2009) incubated soil at 40% and 70% water-filled pore space (WFPS). Soils that had higher WFPS and high initial organic C (16.0%) demonstrated higher *nirK* copies g^{-1} soil, which correlated significantly with increased NIR enzyme activity and NO emissions regardless of N addition. Soil with lower initial organic C (3.8%) showed no significant difference in *nirK* gene abundance following N addition, although amended soils did emit significantly more NO. Unamended soil with low organic C took up NO when incubated at 70% WFPS. These data suggest that *napA*, *nirS* and *nirK* and *nosZ* abundance increases when total N and NH_4^+ concentrations are raised through external inputs, if moisture, pH and organic C levels are favorable for denitrification to occur.

The abundance of denitrification genes can be correlated with denitrification rates and N_2O flux. NO emissions have been positively correlated with *nirK* gene abundance (Szukics et al., 2009) and N_2O emissions have been correlated with *nirS* gene abundance (Morales et al., 2010; Rasche et al., 2011). Girdling of beech trees provided more support for the correlation of *nirS* and *nosZ* gene abundance to soil N_2O emissions (Rasche et al., 2011). Path analysis of potential nitrification and denitrification in boreal ecosystems suggest that complete dissimilatory reduction of nitrate to N_2 is governed by *nosZ* gene abundance, which is in turn influenced by *nirS/K* abundance (Petersen et al., 2012). The difference

between genes that produce N_2O (*nirS/K*) and remove it (*nosZ*) can provide a useful measure to predict N_2O emissions.

$N_2O:N_2$ emission ratios decrease as soil pH increases (Richardson et al., 2009; Liu et al., 2010; Rütting et al., 2013). For example, the relative $N_2O:N_2$ production was about 28, 23 and 16% in soils with pH 5.5, 6.8 and 7.7 (Čuhel et al., 2010). While N_2O emission rates remained relatively constant across pH treatments, N loss as N_2 increased from ~50 to ~95 and ~280 mg N m^{-2} h^{-1} in the aformentioned acidic, natural and alkaline soils. This can result from increased activity of the NOS enzyme, or decreased abundance or activity of the preceding enzymes in the denitrification pathway (Hütsch et al., 2001; Richardson et al., 2009; Čuhel et al., 2010; Liu et al., 2010). Mitigation strategies to reduce N_2O emission from forest soil can benefit from a focus on the potential of the soil microbial community to impact the $N_2O:N_2$ ratio (Richardson et al., 2009). Soil conditions that stimulate organisms without *nosZ* (i.e., AOA, AOB and fungal denitrifiers) may lead to increased N_2O emissions, due to the increased genetic ability to produce the gas, without the ability to reduce it via NOS. For example, Zhu et al (2013), incubated soil with either urea or $(NH_4)_2SO_4$ and exposed the soil to between 21 and 0.5% O_2, and found that the majority of N_2O emissions came from ammonia oxidizers (Zhu et al., 2013). Ammonia oxidizers were responsible for most of the N_2O emissions in the presence of O_2: between 150- 1000 ng g^{-1} N_2O in a 0.5% O_2 headspace. However in the complete absence of O_2, heterotrophic denitrification was responsible for the emission of 2000-5000 ng g^{-1} N_2O. This study did not estimate functional genes for denitrification or calculate nitrous oxide reduction, which would further aid in the understanding of how the shift from N_2O emissions via heterotrophic denitrifiers to autotrophic nitrifiers in aerated soil might affect the $N_2O:N_2$ ratio. Studies involving denitrification genes in soil indicate that soil pH, moisture, C and NH_4^+ correlate with *napA*, *nirS*/*K* and/or *nosZ* genes. Denitrification genes and rates are most abundant as soil pH and moisture increases. Denitrification rates appear to decline as conditions become too anoxic for nitrifying microorganisms to produce NO_3^-. A study of nitrification and denitrification functional gene and transcript abundance along a moisture gradient would clarify the relationships between the activity of nitrifiers and denitrifiers in waterlogged soil. Because NIR has been considered as the most important enzyme in the study of the denitrification pathway due to its role in gas formation, *nirS*/*K* genes have been used to link the denitrifying community to NO and N_2O emissions from soil (Braker et al., 2000; Bothe et al., 2000). New evidence linking *nosZ* abundance to N_2O shows that further study of nitrous oxide reducing genes and activity is needed to better understand the ecology of N_2O reduction and uptake in forest soil. To achieve this goal, studies that catalogue both gross N_2O production and N_2O reduction from forest soil are required and should be coupled with measurements of soil C (including DOC), pH, soil moisture and quantification of *napA*/*narG*, *nirS/K* and *nosZ* genes.

1.8 Conclusions

Studies using *in situ* functional gene measurements can be used to determine relationships between microbial communities and ecosystem functions, and hold the potential to resolve inconclusive or contradictory relationships between forest management, soil characteristics and microbial community function. While few studies have used functional genes from methanogen and methanotrophs communities to link forest soil physico-chemical parameters and management practices to CH_4 flux rates, the data that do exist show positive relationships between these functional targets, their relative transcription and CH_4 emissions and uptake (Freitag et al., 2010; Levine et al., 2011; Shrestha et al., 2012). Forest soil has a greater capacity for CH_4 uptake than agricultural soil, and land-use changes that result in re- or afforestation can increase methanotrophs functional gene richness and abundance. These studies clearly show the capacity to resolve uncertainties regarding the effect of drainage and fertilization on CH_4 fluxes through the quantification of *pmoA* and *mcrA* genes. However, current datasets suggest that the additional value gained from adding functional genes to models of N cycling process rates may be minimal due to the overwhelming impact of edaphic factors. For example, Graham et al. (2013) found that models of nitrification and N_2O flux relied only on soil pH and were not improved statistically by the inclusion of functional genes during linear regression. Functional genes did improve statistical models of N cycling when broken into seasonal (Graham et al., 2013) and site (Freedman et al., 2013) specific datasets. Therefore, researchers must determine the temporal and spatial scales at which the inclusion of functional genes may improve statistical models. In this review, quantitative analysis of genes involved in N-fixation, nitrification and denitrification were particularly useful in correlating microbial functional-group abundance with changes in soil characteristics and process rates. The synthesis of these studies allowed us to draw several conclusions about N-cycling functioning of the soil microbial community and recommend future research. The application of quantitative *nifH* gene analysis provided evidence that the diazotrophic community plays an important role in soil C availability, as increased N availability from N-fixation can stimulate the degradation of soil organic matter. The quantitative analysis of the *amoA* gene suggests that pH and NH_3 availability create niche separation between communities within the AOA and AOB. AOA play an important role in nitrification and, therefore, require greater study in forest ecosystems. The role of nitrifier denitrification in N_2O flux from forest soil also requires further research. Genes for denitrification are ubiquitous in soil microorganisms. The abundance of denitrification genes was linked to soil pH, soil moisture, concentrations of various N species that act as electron-acceptors and organic C concentration. The studies reviewed herein agree with the model of facultative denitrification in low redox environments and microsites as an adaptation to compete for organic C. N_2O emissions from

forest soil following N addition was linked to the nitrifying and denitrifying communities through the quantitation of *amoA, napA*/*narG*, *nirS/K* and *nosZ*. The role of *nosZ* gene abundance in regulating N_2O uptake or emissions to or from forest stands is supported by several studies, but requires further examination to be used as a predictor of the effect of soil management on GHG emissions. Specific attention to the dynamics of populations without *nosZ* (i.e., AOA, AOB and fungal denitrifiers) and those that contain *nosZ* (i.e., bacterial nitrous-oxide reducers) will contribute to our knowledge of how the soil community composition affects N_2O flux. The difference between *nosZ* and *nirS/K* abundance may prove to be a more accurate predictor of N_2O flux than using single genes. The studies reviewed here provide a framework for the use of microbial functional gene analysis to fill gaps in our knowledge of soil ecosystem functions such as C and N cycling processes.

1.9 Objectives and hypotheses

This study investigated the effect of site preparation (mounding and drainage) and fertilization of low-productivity forest ecosystems on bacterial and archaeal microbial communities, CO_2, CH_4 and N_2O emissions and functional genes involved in these GHG fluxes. The overarching objective of this study was to evaluate the theory that forest management can alter biogeochemical processes that effect soil-atmosphere fluxes of greenhouse gases (GHGs) through alterations in the populations of soil microorganisms. There is a lack of data regarding of the influence of community structure, abundance and activity of the microbial community on GHG fluxes. The links between soil physico-chemical characteristics and the populations of GHG mediating-microorganisms are also poorly understood. Therefore, to meet the primary objective, several specific sub-objectives were required. For visual representations of specific site preparation treatments and hypotheses related to greenhouse gas fluxes see Figure 1.5.

Objective 1: Quantify the response of forest soil to mounding, drainage and fertilization, including soil water content and soil physico-chemical characteristics. The specific hypotheses tested are that i) drainage and mounding result in areas of lowered soil moisture suitable for establishment of economically-important and climactically-suitable tree species; iia) mounded plots will have removed or reduced forest-floors (orgainic layers), but enhanced C and N concentrations in the mineral soil due to layer mixing, iib) drained plots will have reduced forest floor and mineral soil C and N concentrations due to enhanced aerobic decomposition; iii) plots subjected to NPK-S fertilization will have elevated mineral nitrogen (NH_4-N, NO_3-N) and sulphate (SO_4-S) concentrations compared to unfertilized control plots.

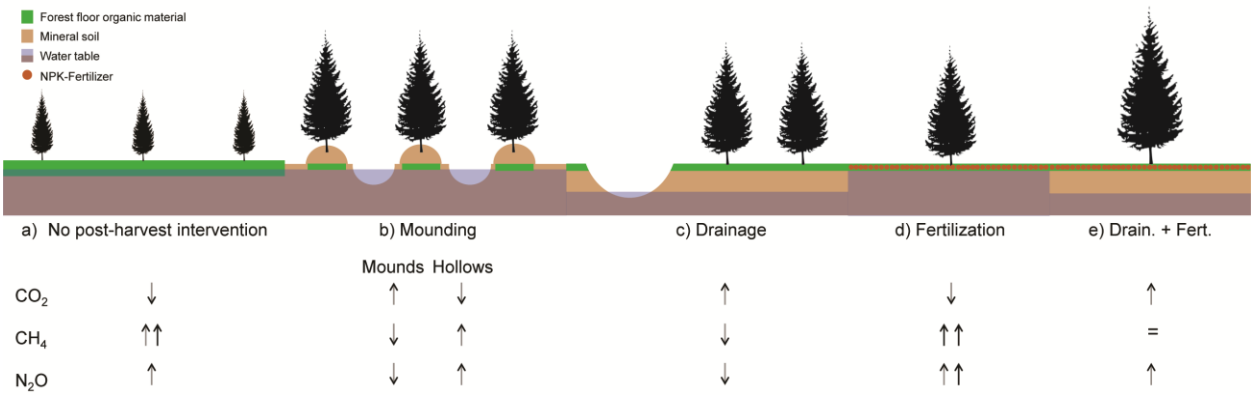

Figure 1.5. Schematic depiction of effects of site preparation and fertilization and hypotheses related to greenhouse gas fluxes. a) Post-harvest paludification, b) mounding, c) drainage, d) NPK fertilization and e) drainage with fertilization.

Objective 2: Quantify GHG flux rates following mounding, drainage and fertilization of waterlogged forest stands. Hypotheses tested are: i) locations with reduced soil water content following site preparation (drained sites, mound tops) will have reduced CH_4 and N_2O emissions and increased CO_2 emissions, and that locations with increased water content (mound-associated hollows) will have elevated CH_4 and N_2O emissions and decreased CO_2 emissions; iii) fertilization will have reduced CO_2 emissions, decreased CH_4 emissions due to the presence of SO_4-S and increased N_2O emissions in locations of elevated soil moisture.

Objective 3: Quantify the effect of mounding, drainage and fertilization on the soil microbial community structure and functional group abundance. The hypotheses tested to meet this objective are: i) total bacterial and fungal community structure will be shifted by all treatments: ia) drainage will increase diversity due to enhanced growth of aerobic microorganisms, ib) mounding will alter community structure due to removal of key niches (i.e., forest floors) and the creation of anaerobic microsites (i.e., mound hollows) and ic) fertilization will reduce fungal diversity by suppressing decomposition, ii) methanogen gene (*mcrA*) abundance will be decreased by all site preparation methods and will be higher in waterlogged areas and soil layers (i.e., mineral soil, mound hollows); iii) methanotroph gene (*pmoA*) abundance will be elevated by mounding (in mound tops) and drainage, and will be greater in aerated forest floors relative to wetter mineral soil; iv) SRB gene (*dsrB*) abundance will be decreased by mounding (in mound tops) and drainage and elevated by NPK-S fertilization, and will be greater in mineral soil relative to forest floors; v) nitrifying bacterial and archaeal gene (*amoA*) abundance will be elevated by all site preparation methods and vi) denitrifying gene (*narG*, *nirK*, *nirS*, *nosZ*) will be decreased following mounding and drainage, but increased by fertilization and will be higher in areas of high organic C (e.g., forest floors, only if water content and mineral N concentrations are sufficient to provide anaerobic environment and a substrate for respiration, respectively.

Objective 4: Determine relationships between soil physico-chemical characteristics, soil microbial functional groups and CH_4 and N_2O fluxes. For the CH_4 pathway, hypotheses include: i) soil moisture will directly increase and decrease methanogen and methanotroph gene abundances, respectively, ii) methanogen gene abundance will be positively correlated with CH_4 flux rates, while methanotroph gene abundance will be negatively correlated with CH_4 flux rates, iii) methanogen gene abundance will be negatively correlated with SO_4 availability. For the N_2O pathway, hypotheses are i) nitrifier gene abundance will be positively correlated to NH_4-N availability and soil pH, ii) denitrifier gene abundance will be positively correlated with soil C, soil N, pH and soil moisture; iii) nitrifier and denitrifier gene abundance will be positively correlated with N_2O emissions.

Chapter 2. Effect of mounding, drainage and fertilization on soil physico-chemical parameters, CO_2 emissions and microbial community structure in wet forest ecosystems

2.1 Introduction

Mechanical site preparation methods and fertilization are used to manage post-harvest forest stands to increase the growth and survival of planted seedlings. The use of site preparation techniques are expected to increase in British Columbia (B.C.), Canada, as low-productivity wet forests may be harvested to fill the mid-term timber supply gap caused by a large-scale outbreak of mountain pine beetle (*Dendroctonus ponderosae*) (Brockley and Simpson 2004) and interventions to improve site productivity are increased to respond to increased international demand for wood products while maintaining or enhancing the ecological and social functions of forested areas (B.C. Ministry of Forests, Mines and Lands, 2010). Changes in physical, chemical and biological properties of soil following mechanical preparation methods, e.g., excavator mounding and ditch drainage, and chemical preparation methods, e.g., fertilization, are complex and inter-related. Site preparation can alter greenhouse gas (GHG) flux rates (Smolander et al., 2000; von Arnold et al., 2005a; Jandl et al., 2007; Liu and Greaver, 2009; Mojeremane et al., 2012). Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are potent GHGs affected by these practices. CO_2 is the main driver of anthropogenic climate change and CH_4 and N_2O have 100-year global warming potentials (GWP) of about 23 and 298 times that of CO_2, respectively (Forster et al., 2007). Determining the impact of forest management practices on soil environment and GHG flux is needed to further our understanding of the impacts of these practices relative to their potential economic benefits. Maintenance of soil biodiversity and reduction or mitigation of GHG emissions must be considered to adhere to provincial (B.C. Ministry of Forests, Mines and Lands, 2010; B.C. Ministry of the Environment, 2014) and national (Canadian Council of Forest Ministers. 2007; Environment Canada, 2013; Natural Resources Canada, 2013; Warren and Lemmen, 2014) forest management policies.

Edaphic constraints on seedling survival and growth in northern forests include competition from endemic vegetation, low soil temperature in the root zone, elevated soil moisture in wet sites or insufficient soil moisture in dry sites and nutrient deficiencies in the root zone (Sutton, 1993). Timber harvest can exacerbate these constraints by raising the water table due to the absence of evapotranspiration, and by inducing peat accumulation and paludification, leading to the domination of the forest floor by mosses including Sphagnum (Paavilainen and Päivänen 1995; Paré and Bergeron 1995;

Roy et al., 2000; Lavoie et al., 2005). Excavator mounding can relieve post-harvest constraints on tree growth by removing surrounding vegetation, increasing soil temperature, reducing soil moisture on the mounds and adding organic material to the root zone through mechanical mixing of soil layers (Sutton, 1993; Ballard, 2000). However, forest floor inputs to the mineral soil can elevate organic matter decomposition and soil respiration rates (Johnson, 1992; Johansson, 1994; Burgess et al., 1995; Örlander et al., 1996; Schmidt et al., 1996; Liechty et al., 1997; Lundmark-Thelin and Johansson, 1997; Mallik and Hu, 1997; Giasson et al., 2006). Scarification, a practice of soil turnover similar to mounding, increased growth of white pine and white spruce stands by 20.8%, but decreased soil organic C by 64% and N by 57.2% (Burgess et al., 1995). These soil C losses can be offset by increases in aboveground biomass (Johansson et al., 2012). Hollows created by mounding can also produce anoxic microenvironments that can act as a hotspot for emissions of GHG from anaerobic processes, e.g., denitrification and methanogenesis (Ballard, 2000). This study seeks to determine the effect of mounding on the physico-chemical properties of a poorly-drained soil following harvest of a mature, second-growth hybrid spruce stand in the interior of B.C.

Ditch drainage following harvest can also be used to prevent paludification in wet forests (Hillman et al., 1992). Drainage to draw down the soil water table and improve tree productivity originated in Nordic countries and Russia, and drainage trials have been conducted in Canadian forests throughout the latter half of the 20th century (Päivänen, 1997; Lavoie et al., 2005). There are currently about 15 million ha of drained forest wetlands globally, with the majority of this area occurring in Finland and Russia (Päivänen, 1997; Lavoie et al., 2005). Drainage lowers the soil water table, which can improve soil aeration and temperature (von Arnold et al., 2005a), stimulating aerobic decomposers (Jaatinen et al., 2007), decreasing soil carbon (C) stocks by increasing C mineralization and respiration (Byrne and Farrell, 2005; von Arnold et al., 2005a; Mojeremane et al., 2012) and ultimately reducing the thickness of the forest floor (von Arnold et al., 2005a). The loss of belowground C following drainage can be compensated for by improved tree root metabolism and growth (Laiho and Finér, 1996; Laiho and Laine, 1997; Sajedi et al., 2012), which can result in significantly greater aboveground biomass C stocks compared to undrained stands (Laiho and Laine, 1997; Macdonald and Yin, 1999; Hargreves et al., 2003; Byrne and Farrell, 2005). Coastal forests in B.C. are subject to high precipitation, which can lead to waterlogging on poorly-drained sites that would otherwise exhibit high productivity. This study investigates the use of drainage to prevent paludification following harvest of a coastal western redcedar stand on Vancouver Island, B.C., and determine how this site preparation technique affects soil properties and GHG emissions.

Nitrogen (N) fertilization also affects soil chemical factors through increases in bio-available N, which can alter C and N cycles including rates of litter and OM decomposition (Micks et al., 2004; Gallo et al., 2005; Jassal et al., 2010). N fertilization is often performed at the time of stand establishment in western Canadian forest stands (Stienbrenner, 1968; Chappell et al., 1992). For example, early-stand fertilization of a Douglas-fir stand on Vancouver Island with 20-10-5 N-phosphorus (P)-potassium (K) fertilizer at a rate of 8.4 to 16.8 g seedling^{-1} resulted in a 12-31% increase in height growth response over a 3 to 6 year period (van den Driessche, 1988). Lodgepole pine (Brockley 1996, 2001) and interior spruce (Brockley and Simpson 2004; Brockley 2006) have also shown positive responses to fertilization with N and sulphur (S). Sulphur fertilization can reduce S deficiency in lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) in the interior regions of B.C. (Sanborn et al., 2005). Positive growth responses following N and/or N+S fertilization of growing stands have also been demonstrated in some species, including Douglas-fir, Sitka spruce and western hemlock (Miller, 1986; Chappell et al., 1992; Blevins and Prescott, 2002), while N fertilization of a western redcedar stand seven years after planting did not significantly improve growth after four years (Blevins and Prescott, 2002).

Nitrogen fertilization can reduce net soil respiration rates and increase the accumulation of belowground C (Allison et al., 2010 ; Jassal et al., 2010; Bodelier et al., 2011; Mojeremane et al., 2012; Lemprière et al., 2013). Nitrogen fertilization led to a shift in the fungal decomposer community within one year of fertilization, leading to about 6% C losses in the organic soil layers, yet decreased soil CO_2 flux rates by about 50% (Allison et al., 2010). Nitrogen fertilization can initially increase decomposition rates and soil respiration due to stimulation of the decomposer community (Parker et al., 2001; Micks et al., 2004; Gallo et al., 2005; Jassal et al., 2010) or stimulation of fine root growth (Raich et al., 1994; Cleveland and Townsend, 2006). Fertilization can alternatively decrease soil respiration (Haynes and Gower, 1995; Allison et al., 2010), or have no effect when added N was rapidly immobilized by microorganisms (Prescott et al., 1993; Chapell et al., 1999). Knorr et al. (2005) found that N addition decreased respiration rates when the ratio of addition to N deposition was >20, but stimulated respiration above this threshold. Fertilization can reduce mineralization of soil C > 4-years-old and increase humificiation (Hagedorn et al., 2003). Humification of C in soil following decomposition increases due to the retardation of low-quality litter, i.e., litter with a high proportion of lignin, cellulose and hemi-cellulose, resulting from biological (e.g., suppression of lignolytic enzyme activity) or chemical (e.g., condensation reactions) effects (Knorr et al., 2005; Prescott, 2010). Nitrogen fertilization has been shown to increase the emission of other GHGS. For example, N fertilization can increase N_2O emissions (Johnson et al., 1980; Brumme and Beese, 1992; Sitaula and Bakken, 1993; Situala et al., 1995; Bateman and Baggs, 2005; Pilegaard et al., 2006; Jassal et al., 2008, 2010, 2011; Mojeremane et al., 2012;

Pielegaard, 2013; Ussiri and Lal, 2013; Wu et al., 2013) or have no effect (Basiliko et al 2009). Fertilization has also been shown to decrease CH_4 uptake, resulting in increases in CH_4 emissions (Steudler et al., 1989; Crill et al., 1994; Willison et al., 1995; Primé and Christensen, 1997; Saari et al., 1997; Maljanen et al., 2006), but not always (Basiliko et al 2009).

Understanding how the soil microbial community responds to management practices including site preparation and fertilization can elucidate the biological effect of changes in the physico-chemical environment. Bacterial and fungal communities can be altered by management practices including fertilization (Frey et al., 2004; Hallin et al., 2009; Ramirez et al., 2010). Community structure can be related to ecosystem process such as organic matter decomposition, e.g., fungal community structure and function (Voříšková et al., 2014), though bacterial community structure was not related to soil functioning including respiration following fertilization of an agricultural soil (Hallin et al., 2009). Little research has been conducted on the effect of forest management including site preparation and fertilization on bacterial and fungal community structure, abundance and diversity.

Linking C sequestration and GHG fluxes to site preparation techniques can be improved by understanding the effect of site preparation on soil conditions and microbial communities as a method of quantifying treatment effects. In this study the effects of mechanical site preparation and fertilization on soil moisture and soil chemistry are measured throughout one growing season, as this time period is crucial to understanding the transient effects of fertilization treatments on the soil environment and microorganisms. The study was conducted in two forest management systems: a newly-initiated mounding trial in an interior hybrid spruce stand subject to fertilization at planting, and a 15-year-old drainage trial in a coastal western redcedar-western hemlock plantation fertilized at 11 years. The objective of this study is to quantify the physico-chemical and microbial community response of forest soil to mounding, drainage and fertilization. The specific hypotheses tested are that i) drainage and mounding result in areas of lowered soil moisture; iia) mounding mixes C, N and S from forest floor layers into mineral soil, elevating concentrations in mineral soil but lowering concentrations overall; iib) forest floor and mineral soil in drained plots have lower C, N and S due to enhanced aerobic decomposition; iii) plots subjected to fertilization have elevated mineral N and S (NH_4-N, NO_3-N, SO_4-S) concentrations compared to unfertilized control plots; v) mounding reduces CO_2 fluxes and drainage increases CO_2 fluxes and v) site preparation and fertilization leads to shifts in bacterial and fungal communities due to the potential for reduction of litter-specific operational taxonomic units (OTUs) (mounding), anaerobic OTUs (drainage) and N-limited decomposer OTUs (fertilization).

2.2 Materials and methods

2.2.1 Field sites

2.2.1.1 Aleza Lake Research Forest (ALRF)

The effects of mounding and fertilization on the soil physico-chemical environment were studied at the Aleza Lake Research Forest (ALRF) located near Prince George, B.C. at coordinates 54°5'31"N, 122°3'53" W. The Prince George region has a continental climate, with average monthly temperatures $<0^{o}$C in the winter (October to March) and $>10^{o}$C in the summer (June to August) (Figure 2.1). Climate data were measured at a weather station located 3 km south-west of the study area (Jull, M., personal communication). The mean monthly temperatures in 2012 reflected the 20-year averages at this site. Mean annual precipitation is 900 mm at ALRF, although in 2012 annual precipitation was 419 mm. Summer and winter precipitation was reduced relative to the 20-year average, although major precipitation events were recorded in April and June of 2012.

The ALRF installation is located within the wet-cool (wk1)-variant of the sub-boreal spruce (SBS) biogeoclimatic zone, in a transitional area between dry interior plateau forests and the wetter Interior Cedar Hemlock (ICH)/Engelmann Spruce-Subalpine Fir (ESSF) subzone as described by Meidinger and Pojar (1991). Soils at this site are fine-textured and fall between the Orthic Gleyed Luvisols and the Orthic Luvic Gleysols subgroups. The soil subgroup Ortho Humo-Ferric Podzol is also found at ALRF where coarser textures are observed. Mean soil pH (1:1 H_2O) at ALRF was 4.7 ± 0.1. The canopy was dominated by interior hybrid spruce (*Picea engelmannii* x *glauca*) with large amounts of subalpine fir (*Abies lasiocarpa*) in the regeneration layer. Waterlogged areas within the wk1 zonal site series 08, 09 and 10 containing the diagnostic herbaceous layer species oak fern (*Gymnocarpium dryopteris*), devil's club (*Oplopanax horridus*) and lady fern (*Athyrium filix-femina*) were identified for harvest in October 2010. Such areas are subject to paludification following harvest; therefore stand regeneration is limited without mechanical site preparation to increase soil aeration at planting sites. The northern-most area of the stand had abundant standing water on heavy clay soil and was dominated by horsetail (*Equisetum* sp.) in the herbaceous layer. Due to the high water table and potential for soil compaction during mechanical site preparation this area was deemed inappropriate for harvest and site preparation and left in reserve.

Winter harvesting of the 70-year-old second-growth stand took place in February 2011, with debris removal and slash burning taking place in May 2011. The site was divided into eight 1080 m^2 plots (60 m x 18 m, 10m buffer between) on June 23, 2011 for the mounding and fertilization trials. Mounding took place on August 22, 2011 using an excavator with a custom-built rotary head that turned over soil

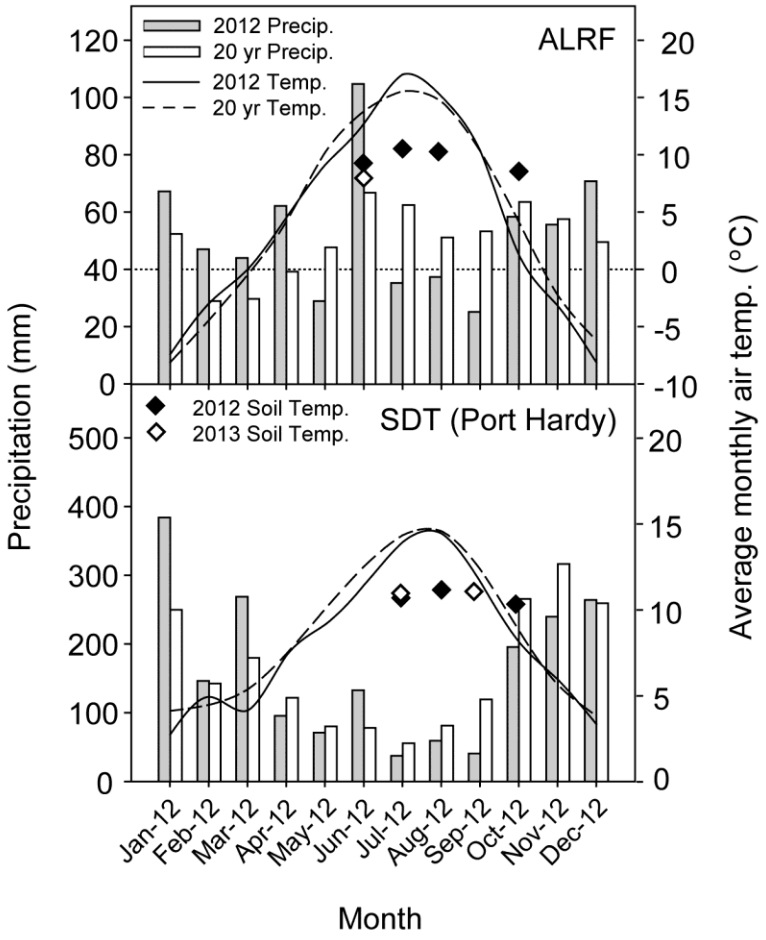

Figure 2.1. Mean monthly air temperature and total precipitation for 2012 at Aleza Lake Research Forest (ALRF) and Port Hardy near the Suquash Drainage Trial (SDT) compared to 20-year means. Mean soil temperature are shown for 2012 (black diamond) and 2013 (white diamond) sampling dates.

layers. Mounds were spaced 2 m apart and were 0.25-1 m in height. Plots were re-planted with interior hybrid spruce at an operational density of 1400 seedlings ha^{-1} on June 6, 2012. Seedlings were placed mid-mound slope in mounded plots and about 2 m apart in control plots. ALRF plots were organized in two blocks in a complete-block design, with each block incorporating each of the four treatments (unmounded/unfertilized control (C), unmounded/fertilized (C+F), mounded/unfertilized (M, mound; H, mound hollow), mounded/fertilized (M+F, H+F)) (Figure 2.2). Shell Thiogro^{TM} Fertilizer (15-15-15-15S) (Shell Canada Ltd., Calgary) was amended with Urea (40-0-0) and NPK (20-10-10) fertilizer (Evergro Canada Inc., Delta) applied manually using a rotary spreader at a final formulation of 200 kg N, 100 kg P, 100 kg K, and 50 kg S ha^{-1} on June 26, 2012 (See Appendix A for full fertilizer formulation). Sampling for soil chemistry and water content took place on June 28, 2012 (Jun-12), July 17, 2012 (Jul-12), August 24, 2012 (Aug-12), October 18, 2012 (Oct-12) and June 13, 2013 (Jun-13). Aug-12 soil samples were further sub-sampled for microbial community analysis.

Effect of excavator mounding at ALRF was determined by estimating the number of natural or created soil mounds equal or greater to 25 cm in height. Mound densities were measured in three circular sub-plots 8 m in diameter in each of the eight treatment plots. The subplots were set 20 m apart and 10 m from the top and bottom of the plot boundary. Untreated plots had 617 natural mounds ha^{-1} while mounded plots had an average of 1783 natural and created mounds ha^{-1}.

2.2.1.2 Suquash Drainage Trial (SDT)

The Suquash Drainage Trial is located near the Salal Cedar Hemlock Integrated Research Program (SCHIRP) research site installed by Western Forest Products Inc. between the towns of Port Hardy and Port McNeill on northern Vancouver Island, B.C., coordinates 50°37'49" N, 127° 14' 21" W. The area has a cool maritime climate with mild, wet winters and cool moist summers. Temperatures recorded at the nearest Environment Canada weather station in Port Hardy, BC, during the 2012 growing season (April to October) were similar to the 20year average, with monthly mean temperature in August of 14.4^{o}C and 14.5^{o}C, respectively (Figure 2.1) (Environment Canada, http://weather.gc.ca/). The winter of 2012 had higher than average precipitation, with a total of 797 mm between January and March 2012, compared to a 20-year average of 570 mm for these months. Summer and fall precipitation was lower than the 20-year monthly average. The SDT site is located in the Sub-montane Very Wet Maritime (vm1)-subzone of the Coastal Western Hemlock ecozone (CWH). (Green and Klinka 1994). The original site vegetation consisted of western redcedar (*Thuja plicata*) and shore pine (*Pinus contorta* var. *contorta*), with an understory dominated by sphagnum (*Sphagnum* spp.) and skunk cabbage (*Lysichiton americanum*).

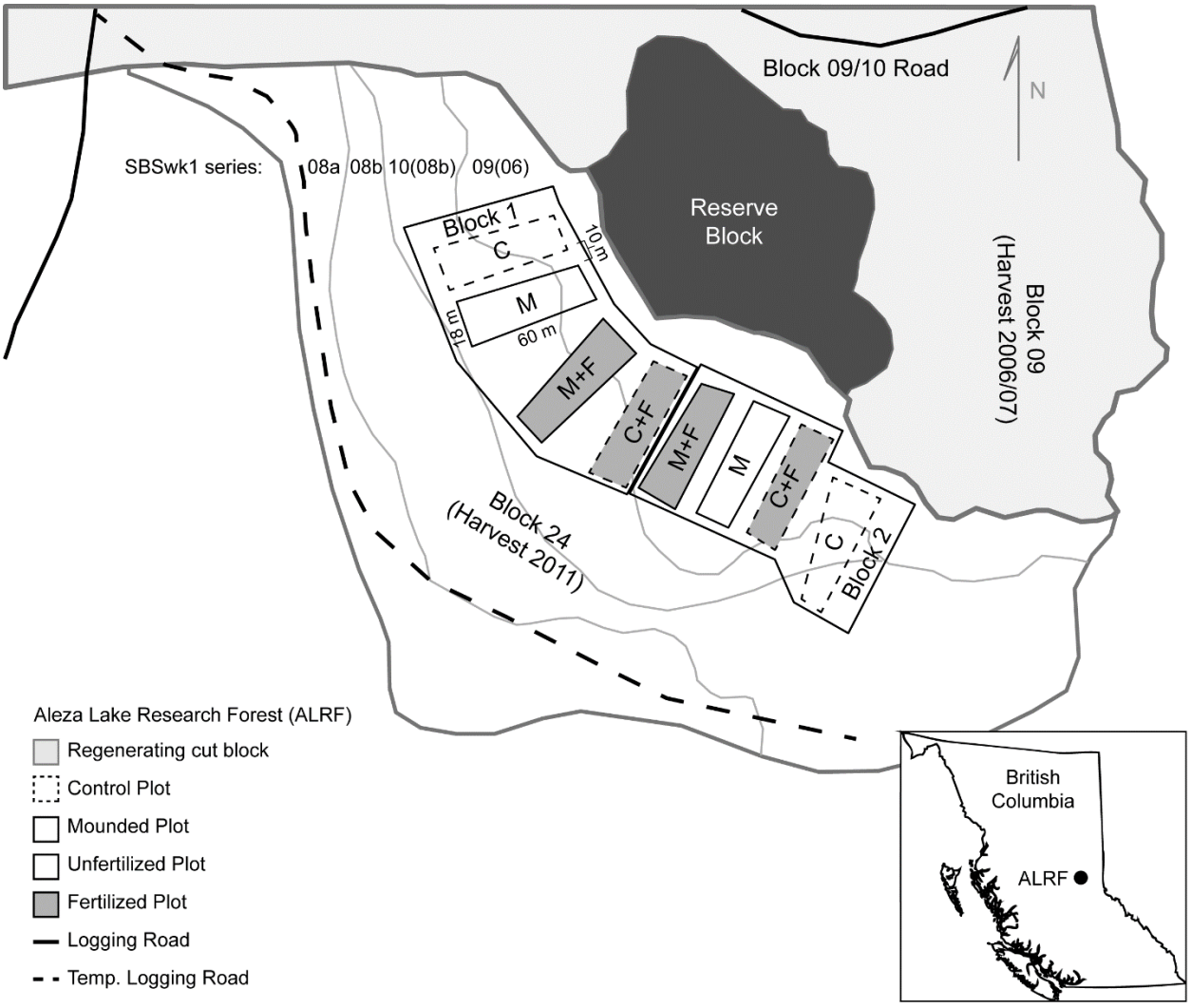

Figure 2.2. Map of Aleza Lake Research Forest (ALRF) block 24 showing locations of control (C), mounding (M) and fertilization (F) treatment plots. Insert: position of the ALRF site within British Columbia, Canada.

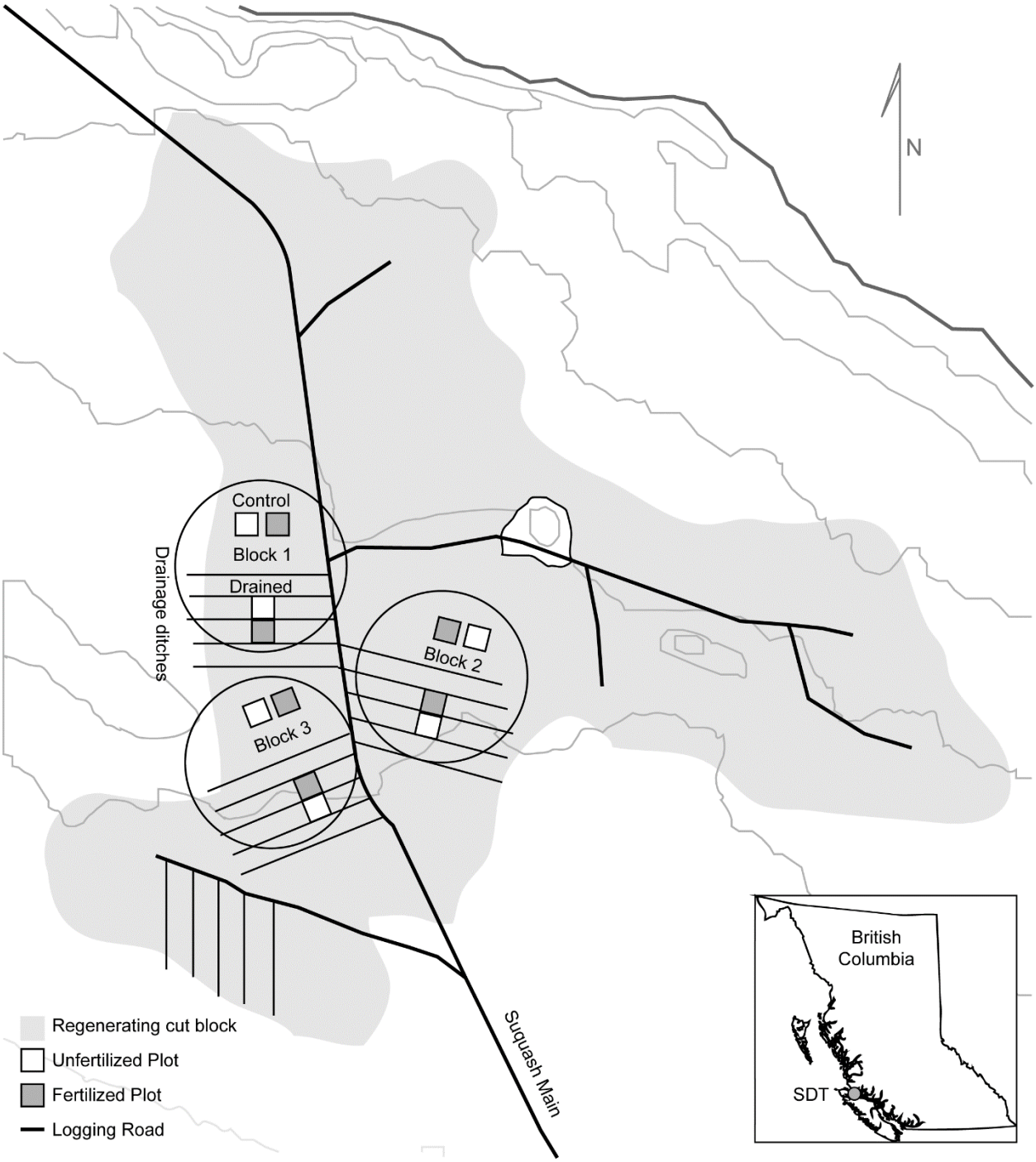

Figure 2.3. Map of Suquash Drainage Trial (SDT) showing the locations of control, drainage and fertilization plots. Insert: position of the SDT site within British Columbia, Canada.

Sphagnum and skunk cabbage are diagnostic of zonal site series 13 and 14 within the CWHvm1. Skunk cabbage is indicative of nutrient-rich stands that are too wet to support productive stands. The stand had characteristics of a productive forest site, but had excess moisture throughout much of the year, which was assessed as a factor limiting stand regeneration, making the area ideal for mechanical drainage (Sajedi et al., 2012). Drainage of the stand shifted the site series towards 03, as indicated by the domination of the shrub layer by the ericaceous shrub salal (*Gaultheria shallon*) (Meidinger and Pojar, 1991).

The SDT site is situated within the Suquash basin, a coal-bearing sub-basin located within the greater Georgia Basin. Parent material is sandstone, shale, conglomerate and coal (Clapp, 1912). The soils are Humo-Ferric Podzols with mor humus, and include low-lying areas of poorly-drained mucky organic soil and marine silty clays. Raised hummocks of organic matter and rotting wood are common on this site. Mean soil pH (1:1 H_2O) at SDT was 3.6 ± 0.1.

Harvesting and slash-burning of the 22-ha stand took place in 1993 and 1994, respectively. The site was planted with western redcedar in 1995. In 1997, five open-channel ditches were installed at 30 m intervals in four 120 m x 45 m drainage plots using a V-notch bucket. Additional planting of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and yellow cedar (*Chamaecyparis nootkatensis* (D. Don) Spach) occurred in March of 1998. In 2006 the entire cutover was operationally fertilized with 225 kg N and 75 kg P ha^{-1}. Only three drainage areas were used for this study as one of the treatment plots had become inaccessible and waterlogged due to beaver activity. Undrained control plots were selected at least 30 m away from each ditched area to avoid the effects of ditching on subsurface drainage, which extended 15 m from each drainage ditch (van Niejenhuis and Barker, 2002).

Each treatment and control plot was subsequently divided in two for the fertilization experiment. Two 30 x 30 m transects were identified in each drainage plot and one assigned a fertilized treatment. Fertilizer was applied on July 25, 2012 at the same formulation as in ALRF. Plots were organized in complete-block design and included the following treatments: undrained/unfertilized controls (C), undrained/fertilized (C+F), drained/unfertilized (D), drained/fertilized (D+F) (Figure 2.3). There were three plots per treatment, with two unpooled replicate samples in each. Soil water content at SDT was measured on July 27, 2012 (Jul-12), August 29, 2012 (Aug-12), October 25, 2012 (Oct-12), July 3, 2013 (Jul-13) and September 12, 2013 (Sep-13). Soil was sampled for chemical analysis on all dates except Sep-13, and CO_2 fluxes are unavailable for Oct-12. Sampling for microbial community analysis took place in Aug-12.

2.2.2 Field sampling

The surface organic (o) layers (forest floor F and H horizons) and the mineral (m) soil (A and B horizons; ~ 0-5 cm) were sampled from three and two randomly chosen locations in each treatment plot at ALRF and SDT, respectively. ALRF had two plots per treatment while SDT had three, leading to n-values of six for each treatment at both ALRF and SDT. Volumetric soil moisture was measured in the field using a TH_2O^{TM} portable moisture probe (Dynamax Inc., Houston, U.S.A.) by taking the mean of three readings around each sampling point. Laboratory measurements of gravitational soil moisture were conducted by oven-drying field moist soil samples to determine water content as a percent of the mass of field-moist soil. Field soil was homogenized by removing roots, grinding and was then oven-dried at 50^{o}C to avoid DNA degradation.

2.2.3 Soil chemistry

Ten g dry soil was analyzed for pH (1:1 H_2O), total C, total N, available NH_4-N and available NO_3-N by the British Columbia Ministry of Forests, Lands and Natural Resources Operation Analytical Laboratory (Victoria). Briefly, samples analyzed for total C and N analysis were ground to -100 mesh (0.149 mm) on a Rocklabs Ring Grinder (Rocklabs Ltd. Onehunga, New Zealand) and run on a Thermo Flash 2000 combustion NCS analyzer (Thermo Fisher Scientific Inc. Waltham, U.S.A.). Soil for available mineral N were sieved to 2 mm. Available NH_4-N and NO_3-N were extracted by mixing soil in 2M KCl at a ratio of 1:10 soil:KCl for mineral soils and shaking on an oscillating shaker for 60 minutes. The extracts were immediately centrifuged and the filtrate analyzed on an OI-Analytical Alpkem FSIV segmented flow automated chemistry analyzer (OI Analytical College Station, U.S.A.). Samples for total S analysis were ground to -100 mesh (0.149 mm) on a Rocklabs Ring Grinder (Rocklabs Ltd. Onehunga, New Zealand). Forest floor samples were run on a Thermo Flash 2000 combustion NCS analyzer (Thermo Fisher Scientific Inc. Waltham, U.S.A.). Total S analysis for the mineral soils was conducted with a Leco Truspec combustion S analyzer (Leco Corp., St. Joseph, U.S.A). SO_4-S was extracted from the soil using 500 mg L^{-1} PO_4-P extractant, at a ratio of 1:10 soil:extractant for mineral soils, or 1:20 soil:extractant for high organic soils and shaking for 60 minutes. The extracts were immediately centrifuged and the filtrate analyzed for SO_4-S using a Waters HPLC system (Waters Corp., Milford, U.S.A.) configured for non-supressed ion chromatography. Peak detection was by conductivity.

2.2.4 Field measurement and gas chromatography analysis of CO_2 fluxes

Each treatment plot at ALRF was divided into three equal segments lengthwise. At SDT treatment plots were divided in two segments. Within each segment a closed static PVC chambers (Basiliko et al., 2009) were installed at randomly selected locations to measure the net surface exchange of GHG. For mounded plots at ALRF, chambers were installed mid-slope on the mound and hollow closest to the randomly chosen location. Upon installation, the chambers were allowed to settle for at least two hours before sampling. Prior to chamber headspace sampling, 6 ml of air was inserted and the headspace mixed by plunging a 20 ml plastic syringe three times. Six ml of chamber headspace were removed and inserted into pre-evacuated 5 ml Exetainers® (Labco Ltd., Lampeter, UK) every 15 minutes for one hour. Gas samples were measured on an Agilent 5890 series II gas chromatograph (Agilent Technologies, Santa Clara, U.S.A.) equipped with a flame ionisation detector (FID) set at 300°C. The FID carrier gas was helium with a flow rate of 14 ml min^{-1}. Standards for gas chromatography used 1800, 900, 450 and 300 ppm CO_2. Standard curves were constructed with simple linear regression.

2.2.5 DNA extraction, PCR and qPCR of bacterial 16S rRNA and fungal ITS

DNA was extracted from 0.25 g homogenized mineral soil or 0.1 g ground forest floor material using the MoBio PowerClean soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, C.A., U.S.A.). DNA concentrations we calculated with spectrophotometry using the Quant-iT™ PicoGreen® dsDNA assay (Life Technologies Corp., Carlsbad, U.S.A.) and quality was checked using electrophoresis in agarose gels (1% w/v in TAE). DNA was stored at -20°C prior to PCR.

PCR for T-RFLP was performed for bacterial 16S and fungal internal transcribed spacer (ITS) region targets were in triplicate and used a total volume of 50 µl reaction mixture containing 10 ng template DNA, 0.4 mM dNTPs (Applied Biosystems) 2 mM $MgCl_2$, 10 µl PCR-buffer (Life), 0.5µl 100x BSA, 2 U Amplitaq® 360 DNA polymerase (Applied Biosytems) and 0.2 µM of each primer. The bacterial 16S rRNA forward primer (519f, 5'-GCC AGC AGC CGC GGT AAT-3') was modified with a 5' 6-6-Carboxyfluorescein (FAM) fluorophore and the reverse (907r, 5'-CCG TCA ATT CCT TTG AGT TT-3') with a 5' Hexachlorofluorescein (HEX). Bacterial 16S PCR used an initial denaturation step of 7 min at 95°C and 30 cycles of 94°C denaturation for 1 min, 50°C annealing for 1 min and 72°C extension for 1 min, with a final extension step of 10 min at 72 °C. The fungal ITS forward primer (ITS-1F, 5'-TCCTCCGCTTATTGATATGC-3') was modified with 5' 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) and the reverse (ITS4, 5'- TCCGTAGGTGAACCTGCGG-3') with 5' 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) (Gardes and Bruns, 1993). Fungal ITS PCR used

an initial denaturation step of 5 min at 94^{o}C and 30 cycles of 94^{o}C denaturation for 30 s, 55^{o}C annealing for 30 s and 72^{o}C extension for 1 min, with a final extension step of 10 min at 72^{o}C. Post-PCR amplicons were purified using QIAquick PCR purification kits (Qiagen, Venlo, Netherlands) to remove excess salts and unbound primers.

Quantitative PCR (qPCR) was used to determine bacterial 16S and fungal ITS target abundance. All qPCR was carried out in 20 µl reactions with 1 µl of template DNA (~5 ng) added to a 19 µl qPCR mixture containing 10 µl Power SYBR® Green PCR Master Mix (Life Technologies Corp., Carlsbad, U.S.A.). Bovine serum albumin (BSA, 200 ng µl^{-1}) was added to increase PCR efficiency. Reactions were carried out with an Applied Biosystems® StepOnePlus™ real-time PCR system using 10x dilutions of soil DNA extracts to reduce PCR-inhibiting humic substances. Gene copy numbers were expressed as copy number g^{-1} soil (dry weight (dw)). The ~338 bp bacterial 16S rRNA fragment was amplified as above. Standard curves were constructed from 10x serial dilutions of linearized plasmids containing 16S rRNA fragments from *Pseudomonas aeruginosa* (ATCC 17933) *Methylococcus capsulatus (*ATCC 19069), *Desulfomicrobium baculatum* (DSM 4028), *Nitrosospira multiformis* (NCIMB 11849) from 10^9 to 10^4 16S copies with an amplification efficiency of 88% (R^2 = 0.99).

Quantification of a ~300 bp amplicon of the fungal ITS region for measurement of total fungal abundance was conducted using the ITS-1F and 5.8s (5'-CGC TGC GTT CTT CAT CG-3'; Vilgalys and Hester, 1990.) primers as in Fierer et al. (2005) with modification. Amplification reactions were carried out in 20 µl reactions with 1-5 ng µl^{-1} of template DNA added to a 19 µl qPCR mixture containing 10 µl Power SYBR® Green PCR Master Mix (Life Technologies Corp., Carlsbad, CA), 0.2 µM of each primer (Integrated DNA Technologies Inc., Coralville, IA) and 200 ng µl^{-1} bovine serum albumin (BSA). PCR conditions were 10 min at 95^{o}C, followed by 40 cycles of 95^{o}C for 1 min, 30 s at 53^{o}C, 50 s at 72^{o}C and 10 s at 80^{o}C. Fluorescence was read at 80^{o}C to reduce the formation of non-target and primer self-complementation structures. All qPCR was run in duplicate. Standard curves for ITS qPCR were developed using linearized plasmids containing ITS amplified from environmental samples as well as from ITS isolated from *Aspergillus citrisporus* genomic DNA. Standards made from amplification of environmental DNA contained ITS sequences that aligned with *Venturia* sp (97-99%). Restricted plasmids were measured for concentration using spectrophotometry and subject to 10 x serial dilution with a range of 10^9 to 10^3 ITS copies. Standard curves showed an amplification efficiency of 101.3% (R^2 = 0.99).

2.2.6 T-RFLP of bacterial 16S rRNA and fungal ITS

Terminal-restriction fragment length polymorphism (T-RFLP) profiles were constructed following DNA extraction from forest floor and mineral soil from Aug-12 samples. Restriction enzymes *BamHI, EcoRI, MspI, HaeIII, HhaI,* Taq$^{\alpha}$I were tested individually and in pairs. For bacterial 16S rRNA digestion a combination of *MspI* and *HhaI* gave the highest number of terminal restriction fragments with the best separation between fragments. Fungal ITS restriction used the enzymes *Taq$^{\alpha}$I* and *HaeIII*. Digestions were carried out in a total volume of 10 µl containing 5 µl of PCR product (about 1µg per reaction), 2 U of each restriction enzyme (New England Biolabs, Ipswich, U.S.A.) in 1x NEB 4 buffer. Bacterial 16S rRNA restriction reactions were incubated for 3 h at 37^{o}C and 20 min at 65^{o}C. Fungal ITS restriction occurred in two steps, with the first *Taq$^{\alpha}$I* incubation occurring for 3 h at 65^{o}C and 20 min at 80^{o}C, and the *HaeIII* incubation for 3 h at 37^{o}C and 20 min at 65^{o}C. Incubations were purified and sent to the University of British Columbia Nucleic Acid Protein Service Unit (UBC-NAPS) for analysis where 1 µl of each restriction digest was mixed with the ROX500 internal size standard (Applied Biosystems) separated on an Applied Biosystems 3730S DNA Analyzer (Applied Biosystems) equipped with a 50 cm capillary and POP-7 polymer. Peak signals were converted to numeric data for fragment size and peak height using PeakScanner 1.0 (Applied Biosystems). Bacterial 16S T-RFLP was conducted on eight samples per treatment, divided between forest floor and mineral soil for unmounded plots and mound top and hollows for mounded plots. One T-RFLP peak profile that failed the quality check in PeakScanner 1.0 was discarded from a mounded unfertilized hollow. Fungal ITS T-RFLP at ALRF was conducted on 12 samples per treatment, divided as above. All profile peaks met quality standards. SDT T-RFLP of bacterial and fungal targets was conducted on eight samples from each treatment, divided between forest floor and mineral samples. All SDT T-RFLP peaks met quality checks.

2.2.7 Statistical analysis

Statistical analysis was performed using R v. 2.15.3 (R Core Team, 2013). Data were tested for normality using Q-Q plots and the Shapiro–Wilk test. Homoscedasticity was tested using Levene's test. Soil moisture data combined forest floor and mineral soil. Moisture and CO_2 data were fitted with the linear mixed-effects model and subject to two-factor ANOVA (main effects: mounding/drainage × fertilization) using the *lme* and *Anova* functions in the *nlme* and *car* packages, respectively, in order to test treatment effects. Soil chemistry and gene abundance data were subject to fractional factorial ANOVA with three main effect terms (mounding/drainage, fertilization, soil layer) and two interactions

(mounding/drainage × fertilization and mounding/drainage × fertilization × soil layer). Single-factor ANOVA was performed using the *aov* function in R with Tukey's honestly significant difference test to determine significance of sampling location. The *lme* function used fertilization and mounding or drainage as fixed effects and blocking as a random effect. T-RFLP profiles for bacterial 16S and fungal ITS were analyzed using non-metric multidimensional scaling (NMDS) with the *metaMDS* function in the *vegan* package for R (Oksanen et al., 2007). Profiles were binary transformed after removing T-RF peaks with an area less than 5% (Rees et al., 2004) and a dissimilarity matrix was calculated using Bray-Curtis distance measure (Bray and Curtis, 1957; Legendre and Legendre, 1998). Optimal NMDS configuration was determined using 999 permutations and the configuration with the smallest stress value was produced. Soil parameters including bacterial and fungal abundances were combined in a secondary matrix and parameters with $p < 0.05$ following 999 permutations were plotted as vectors on the T-RF ordination using the *envfit* function in *vegan*. Surface fitting of total C concentration to ordination scores was performed using *ordisurf* in *vegan*. Treatment effects on T-RFLP structure were determined by analysis of similarity (ANOSIM) on Bray-Curtis dissimilarity matrices using the *anosim* function in *vegan*. Shannon–Weaver Diversity Indices (H') were calculated from T-RFLP profiles using the *diversity* function in *vegan* and subject to one-way ANOVA. Cannonical variation partitioning of bacterial and fungal OTU distributions from T-RFLP analysis were conducted with *vegan*. Catagorical treatment and site variables were converted to numerical "dummy" variables prior to analysis.

2.3 Results

2.3.1 Soil water content

Soil water content of mounded plots was compared to that of mineral soil layers in control plots. Soil water content at ALRF was significantly greater in unmounded plots compared to mounded plots when all sampling dates were combined, but significantly lower in mounded plots compared to unmounded plots only in Aug-12 and Oct-12 ($p > 0.0001$) (Figure 2.4). Soil water content in untreated control plots was consistently around 40% during the study period. Soil from mound hollows contained about 80% water by mass in Jun-12 and Jul-12 samples, and had significantly greater soil moisture than unmounded samples or mound top samples in during these months. The water content of mound hollows declined in Aug-12 and Oct-12 with moisture measurements in mound hollow soil becoming statistically equivalent to unmounded controls. The mound tops, however, became significantly drier than unmounded controls during these dates. Soil moisture at SDT was higher than at ALRF and was significantly lower in

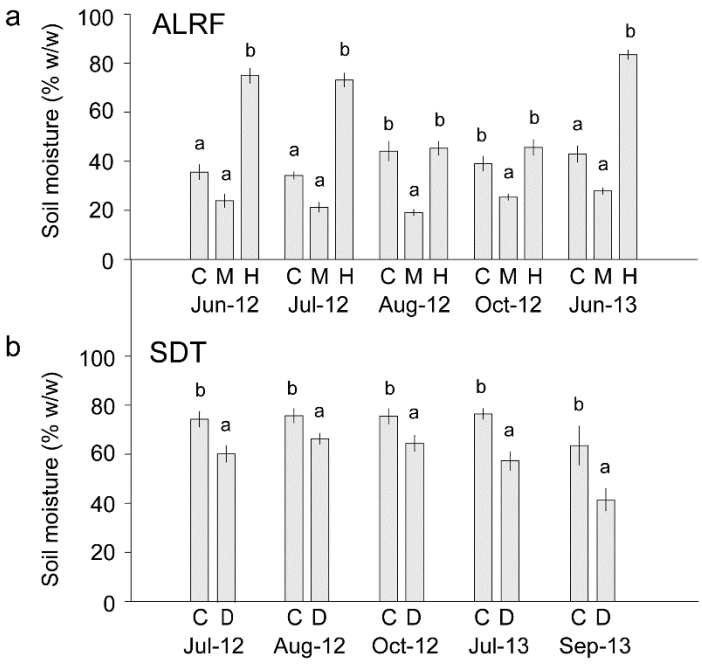

Figure 2.4. Mineral soil moisture percentage by mass from a) Aleza Lake Research Forest (ALRF) and b) Suquash Drainage Trial (SDT). (C, control; M, mound top; H, mound hollow; D, drained). Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA. ±SEM, n=12.

drained plots compared to undrained controls over the course of this study (p = 0.002) (Figure 2.4). In 2012 undrained soil had about 80% water content by mass, with a 20% decline in soil moisture attributable to drainage. In Sep-13 soil moisture in undrained plots was about 60%, and about 40% in drained plots.

2.3.2 Soil chemistry

2.3.2.1 C and N

Mounding led to difference in total C between sampling locations (Table 2.1), as well as between treatment plots (Table 2.2). "Locations" in this study refer to separate sample areas to which single-factor ANOVA was applied including the organic and mineral soil layers in unmounded plots and the topographic microsites (i.e., mound tops and mound hollows) created by mounding (Table 2.1). As the organic layer was largely removed following mounding, the effect of mounding on soil chemical factors was tested only in the mineral soil in these plots. At ALRF, mounding significantly reduced total soil C concentration from mound tops and hollows compared to unmounded controls in Jul-12, Aug-12 and Oct-12 and Jun-13 (Tables 2.1, 2.2). The majority of the mounding effect on soil C is attributable to the loss or mixing of organic C from forest floor material, though differences in mineral soil C from unfertilized unmounded plots and mounded plots was shown in Aug-12 (Table 2.1). Forest floor material had mean total C concentrations between 282 and 471 g kg^{-1} through the course of this study regardless of fertilization status, while mineral soil and mixed mound soil C concentrations were between 13.6 and 79.8 g kg^{-1}. Total C was not greater in any single location following fertilization, but following multi-factor ANOVA, total C was greater in fertilized forest floor and mineral soil. Total C concentrations were greater in fertilized plots compared to unfertilized plots in Jun-12, Oct-12 and Jun-13 (Table 2.1), most prominently in the forest floors (Table 2.2), leading to significantly interactive effects between mounding, fertilization and layer during these sampling dates (Table 2.1).

Total C at SDT was greater in drained plots relative to undrained plots in both forest floor and mineral samples in Aug-12, Oct-12 and Jul-13 (Tables 2.3, 2.4). Total C was greater in forest floor than mineral soil, and higher in mineral soil in drained plots than mineral soil from undrained controls (Table 2.3). Total C concentrations in fertilized plots were also greater than unfertilized plots in Jul-13, mostly due to higher concentrations of total C in the mineral layer in fertilized plots relative to mineral soil in unfertilized plots (Table 2.3), leading to significant interaction between drainage, fertilization and month within the mineral layer (Table 2.4).

Table 2.1. Soil C, N and S concentrations and pH in Aleza Lake Resarch Forest (ALRF) treatment plots.

	Jun-12	Jul-12	Aug-12	Oct-12	Jun-13	Jun-12	Jul-12	Aug-12	Oct-12	Jun-13
	Total C (g kg^{-1})					Total N (g kg^{-1})				
C_o	433.4±11.2b	411.3±9.3b	432.3±7.3c	282.5±13.9b	289.2±9.3b	13.0±0.0b	15.3±0.6b	16.8±0.5d	11.0±1.3b	9.7±1.0c
C_m	42.1±13.6a	39.3±4.2a	138.9±40.5b	78.1±32.9a	48.4±17.6ab	2.7±0.0a	2.6±0.3a	7.9±1.9c	4.6±2.3a	2.3±0.3b
C_o+F	471.7±4.7b	386.6±41.7b	417.4±18.5c	405.8±35.6c	380.4±26.6c	16.6±0.4c	13.6±1.2b	17.9±0.2d	13.9±2.3b	14.1±1.5c
C_m+F	57.0±11.4a	46.0±9.2a	79.8±14.8ab	48.6±17.4a	39.8±19.6ab	3.5±0.6a	2.8±0.5a	5.5±1.0bc	3.0±0.9a	3.1±1.4b
M	24.1±7.0a	30.5±5.2a	37.5±6.0a	46.6±17.5a	25.4±8.4ab	1.5±0.4a	1.7±0.2a	2.3±0.3ab	2.6±1.0a	1.9±0.5a
M+F	36.3±10.2a	25.9±2.9a	33.6±11.9a	35.6±8.5a	24.7±14.0ab	2.3±0.7a	1.7±0.2a	1.8±0.6ab	2.1±0.4a	1.8±0.4a
H	31.8±9.7a	28.3±4.8a	16.2±2.3a	13.6±2.4a	13.8±4.6a	2.0±0.6a	1.8±0.3a	1.1±0.1a	0.9±0.2a	0.9±0.3a
H+F	42.7±9.2a	19.4±3.7a	14.8±2.7a	22.6±5.6a	18.7±6.9ab	2.8±0.7a	1.3±0.2a	1.0±0.1a	1.3±0.3a	12.2±0.5c
	NO_3-N (mg kg^{-1})					NH_4-N (mg kg^{-1})				
C_o	10.8±3.9ab	1.0±0.7a	5.4±.13a	0.8±0.1a	3.4±3.0a	62.7±4.2a	48.2±2.9a	88.7±6.7a	39.7±4.4ab	41.5±7.8b
C_m	1.2±0.4a	0.9±0.4a	10.0±2.0a	17.1±11.5a	22.3±14.5b	21.0±3.9a	53.1±16.6a	36.1±8.1a	18.4±5.4a	21.3±6.5b
C_o+F	16.1±3.2b	6.2±1.8b	149.7±41.9b	45.0±17.2a	68.3±14.2c	2233±111c	382.7±183.3b	475.3±170.7b	66.7±21.2b	89.9±11.9c
C_m+F	6.1±3.4ab	1.3±0.7a	26.8±7.9a	17.3±13.0a	21.5±17.4b	65.4±12.5a	59.6±11.9a	28.7±8.3a	15.3±8.1a	18.3±10.7ab
M	2.5±0.7a	2.0±0.6a	3.1±1.2a	10.7±10.1a	0.7±0.3a	7.3±0.6a	8.8±1.1a	13.5±3.7a	17.0±11.0a	6.1±0.4a
M+F	7.8±4.3ab	4.2±0.5ab	12.9±5.8a	5.8±1.4a	7.0±1.0ab	113.1±41.3ab	47.3±15.5a	67.9±18.3a	15.7±4.6a	17.8±5.7ab
H	2.4±1.3a	2.0±1.0a	0.7±0.6a	6.1±5.5a	0.6±0.1a	13.6±2.2a	41.7±14.0a	31.7±8.7a	6.9±1.3a	6.2±1.7a
H+F	4.3±3.0ab	0.5±0.2a	10.0±5.8a	1.7±0.7a	2.1±0.9a	351.7±125.6b	52.8±7.9a	88.4±38.6a	5.9±0.7a	6.0±1.0a
	Total S (g kg^{-1})					SO_4-S (mg kg^{-1})				
C_o	0.1±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	17.6±0.4c	13.6±1.0c	34.6±3.9c	22.4±6.0b	24.9±3.2b
C_m	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	4.7±1.6a	3.2±0.8ab	9.6±2.5b	7.9±4.0a	8.0±2.9a
C_o+F	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	246.9±3.2d	52.8±12.6d	80.5±14.8d	60.3±5.4c	35.1±6.6b
C_m+F	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	5.5±1.1ab	3.7±0.9ab	10.0±1.9b	7.8±1.7a	6.2±0.3a
M	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.9±0.5a	1.8±0.3a	2.4±0.4a	3.6±1.3a	4.1±2.8a
M+F	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	9.1±3.4bc	5.0±0.7b	7.2±2.4b	4.0±0.4a	4.2±2.6a
H	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.1±0.7a	2.2±0.5ab	1.7±0.2a	4.1±1.7a	4.7±1.5a
H+F	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	16.5±4.2c	3.0±0.6ab	12.4±2.3b	5.4±2.0a	7.8±3.1a

C, control; o, organic; m, mineral; +F, fertilizer; M, mound; H, hollow. Letters following mean+SEM denote statistical difference in columns after ANOVA at $p = 0.05$. n=6.

Table 2.1. Cont. Soil C, N and S concentrations and pH in ALRF treatment plots.

	Jun-12	Jul-12	Aug-12	Oct-12	Jun-13	Jun-12	Jul-12	Aug-12	Oct-12	Jun-13
			C:N ratio					pH (1:1 H2O)		
C_o	33.5±0.9c	27.0±0.6ab	25.9±1.3b	26.3±1.9ab	27.0±1.2b	5.0±0.1ab	4.7±0.3ab	4.6±0.1abc	4.7±0.1a	4.7±0.0b
C_m	15.7±0.9a	15.4±0.6a	17.0±1.5a	18.2±2.2ab	19.2±2.7ab	4.7±0.1a	4.8±0.0ab	4.6±0.1ac	4.4±0.1a	4.5±0.2ab
C_o+F	28.5±0.5b	32.6±9.3b	23.3±0.8b	34.0±10.5b	26.6±0.8b	5.5±0.1b	4.7±0.1a	4.6±0.1ac	4.6±0.1a	4.7±0.3b
C_m+F	15.9±0.9a	15.8±0.7a	14.8±0.5a	15.7±1.0ab	15.2±1.2a	4.8±0.1a	4.8±0.1ab	4.3±0.2a	4.5±0.1a	4.4±0.0a
M	15.3±0.6a	18.2±1.8ab	15.7±0.7a	18.2±1.4ab	18.4±2.0ab	4.9±0.1ab	4.8±0.0ab	4.6±0.1ac	4.6±0.1a	4.4±0.1a
M+F	16.8±1.1a	15.5±0.4a	17.6±1.4a	16.2±1.2ab	15.7±1.5a	5.1±0.2ab	4.8±0.2ab	4.5±0.2a	4.6±0.1a	4.4±0.1a
H	15.7±0.5a	15.7±0.7a	14.6±0.5a	14.6±0.6a	14.7±0.8a	5.0±0.1ab	5.0±0.1ab	5.1±0.1b	4.8±0.2a	4.6±0.1b
H+F	15.8±1.1a	14.1±1.1a	14.5±1.2a	16.6±0.8ab	15.9±0.5a	5.2±0.2ab	5.0±0.0b	5.0±0.1bc	4.7±0.1a	4.6±0.2b

C, control; o, organic; m, mineral; +F, fertilizer; M, mound; H, hollow. Letters following mean+SEM denote statistical difference in columns after ANOVA at $p = 0.05$. n=6.

Table 2.2. *F* and *p* statistics following ANOVA of mounding, fertilization and interactions on C, N and S concentrations and pH at Aleza Lake Research Forest (ALRF)

Model term	df	Total C		Total N		NO_3-N		NH_4-N		Total S		SO_4-S		C:N		pH	
Forest floor		*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Fert.	1	6055.5	<0.001	51.8	<0.001	93.8	<0.001	116.6	<0.001	2.0	0.167	49.9	<0.001	0.0	0.862	9.1	0.004
Date	4	64.4	<0.001	504.4	<0.001	82.8	<0.001	54.7	<0.001	14.8	<0.001	7.2	<0.001	0.9	0.474	28.1	<0.001
F×D	4	2132.9	<0.001	39.7	<0.001	95.4	<0.001	53.1	<0.001	2.6	0.047	8.1	<0.001	0.7	0.618	4.8	0.002
Mineral soil																	
Mound.	1	57.8	<0.001	0.0	0.905	24.3	<0.001	2.5	0.114	10.6	0.001	3.3	0.069	0.1	0.763	31.6	<0.001
Fert.	1	5.5	0.020	8.9	0.003	3.2	0.074	16.2	<0.001	5.3	0.023	23.9	<0.001	3.6	0.059	0.8	0.379
Date	4	107.8	<0.001	36.8	<0.001	5.6	<0.001	6.7	<0.001	2.0	0.100	4.5	0.002	0.7	0.599	20.8	<0.001
M×F	1	0.3	0.592	1.8	0.182	1.0	0.329	5.9	0.017	10.6	0.001	10.4	0.002	0.7	0.417	2.0	0.161
M×D	4	17.5	<0.001	1.1	0.357	6.2	<0.001	2.2	0.066	4.0	0.004	2.2	0.068	0.1	0.982	3.0	0.021
F×D	4	3.4	0.011	9.6	<0.001	3.8	0.006	8.3	<0.001	2.0	0.100	4.1	0.004	1.4	0.223	2.1	0.085
M×F×D	4	0.4	0.820	1.5	0.208	0.2	0.944	2.0	0.091	4.0	0.004	1.6	0.180	1.6	0.181	0.9	0.480

Table 2.3. Soil C, N and S concentrations and pH in Suquash Drainage Trial (SDT) treatment plots.

	Jul-12	Aug-12	Oct-12	Jul-13	Jul-12	Aug-12	Oct-12	Jul-13
	Total C (g kg^{-1})				Total N (g kg^{-1})			
C_o	419.4±0.5a	439.7±0.0ab	446.2±40.7b	443.3±54.4b	12.0±0.1ab	12.3±0.1ab	10.5±0.6ab	16.4±8.3ab
C_m	416.7±115.5a	430.3±111.7ab	249.5±64.6a	113.6±11.4a	10.5±4.4a	8.5±1.7a	7.8±2.1a	11.5±2.3a
C_o+F	523.9±0.0a	487.4±0.0b	486.7±5.9b	456.5±9.9b	18.0±0.0c	21.1±0.1b	18.4±2.4b	18.5±3.8b
C_m+F	373.2±101.8a	212.2±13.8a	218.6±30.3a	230.4±20.4ab	12.0±3.3ab	7.5±0.2a	6.8±1.3a	6.2±1.8a
D_o	564.1±0.2a	537.1±0.5b	498.6±46.1b	473.2±15.1b	15.1±0.1bc	18.2±0.1b	16.8±2.1b	9.0±1.6a
D_m	491.1±63.8a	407.6±94.1ab	447.2±50.2b	430.2±23.4b	13.7±0.1b	12.6±2.1ab	13.9±0.3ab	16.0±4.6ab
D_o+F	502.7±0.0a	555.3±0.7b	526.1±3.1b	477.4±20.9b	18.3±0.0c	18.2±0.2b	18.7±0.3b	18.7±2.9b
D_m+F	487.9±47.7a	470.0±46.4b	432.5±35.1ab	460.9±27.2b	15.9±0.4bc	13.5±0.1ab	12.5±0.2ab	16.1±5.7ab
	NO_3-N (mg kg^{-1})				NH_4-N (mg kg^{-1})			
C_o	41.3±0.8c	2.6±0.0a	4.5±1.8a	32.4±1.6c	213.9±0.1a	106.5±0.5a	32.8±3.0a	20.7±9.3a
C_m	0.4±0.2a	1.1±0.5a	15.0±8.1a	8.5±3.5a	22.8±5.7a	78.4±32.0a	86.1±34.4a	48.1±4.4ab
C_o+F	48.7±2.0c	421.4±0.0c	426.3±106.9b	103.2±18.5d	811.4±0.0b	3122.8±90.4c	1336.6±354.2b	197.0±71.9b
C_m+F	2.1±1.4a	7.0±6.6a	1.7±0.8a	29.4±8.6ab	164.4±127.5a	44.5±19.4a	30.8±8.8a	45.2±9.3ab
D_o	3.2±0.0a	3.7±0.0a	46.1±40.1a	30.1±3.9c	76.9±4.5b	358.7±46.2ab	75.9±17.8a	66.7±9.0ab
D_m	1.0±0.8a	0.4±0.1a	9.1±3.9a	17.0±7.5ab	30.9±13.5a	75.4±21.1a	48.2±7.3a	54.9±1.4ab
D_o+F	18.0±0.0b	536.0±0.0c	420.7±101.8b	211.1±8.2e	1004.2±163.1b	1496.5±28.8b	500.8±72.4a	285.7±16.5c
D_m+F	0.7±0.5a	166.0±82.9b	10.1±1.7a	15.7±1.1b	178.2±66.1a	630.5±293.7b	131.2±19.3a	83.2±16.9b
	Total S (g kg^{-1})				SO_4-S (mg kg^{-1})			
C_o	1.6±0.0a	1.5±0.0b	1.3±0.1a	1.3±0.0b	68.8±0.0b	39.5±0.0a	21.1±4.0a	35.0±6.1a
C_m	1.2±0.8a	0.9±0.4ab	1.0±0.6a	0.4±0.1a	20.1±3.0a	16.3±4.0a	51.7±21.6a	23.1±6.3a
C_o+F	2.1±0.0a	3.0±0.0c	3.7±1.0b	3.1±0.0d	234±14.2c	546.5±27.2d	425.7±56.8b	400.9±89.3b
C_m+F	1.5±0.8a	0.0±0.0a	0.5±0.3a	1.0±0.5b	58.8±38.1b	35.5±24.5a	21.3±6.0a	28.8±10.7a
D_o	1.6±0.0a	1.9±0.0b	1.3±0.5a	1.3±0.0b	19.4±7.4a	56.4±0.0ab	34.9±8.2a	47±3.0a
D_m	2.2±0.2a	0.9±0.8	1.2±0.7a	1.4±0.2b	9.9±0.7a	23.8±7.7a	18.8±3.0a	31.2±9.1a
D_o+F	2.1±0.0a	2.3±0.0c	2.6±0.3ab	2.3±0.0c	205.7±5.0c	269.9±11.1c	234.0±82.5a	244.1±30.3a
D_m+F	2.2±0.1a	1.1±0.6b	1.1±0.7a	1.1±0.5b	36.7±6.9b	90.0±35.2b	44.9±8.0a	53.7±14.2a

C, control; D, drained; o, organic soil; m, mineral soil; +F, fertilizer. Letters following mean+SEM denote statistical difference in columns following ANOVA at $p = 0.05$. n=4.

Table 2.3. Cont. Soil C, N and S concentrations and pH in Suquash Drainage Trial (SDT) treatment plots.

	Jul-12	Aug-12	Oct-12	Jul-13	Jul-12	Aug-12	Oct-12	Jul-13
	C:N ratio				pH (1:1 H_2O)			
C_o	35.0±0.0a	35.6±0.0b	43.6±5.6b	42.0±6.6b	4.0±0.0a	3.9±0.0ab	4.1±0.1ab	4.2±0.2c
C_m	48.0±17.8a	48.8±4.0b	33.3±1.7b	33.9±1.1b	3.8±0.3a	3.8±0.3ab	4.0±0.1ab	4.0±0.2b
C_o+F	29.0±0.0a	23.1±0.0a	28.3±2.8a	28.3±3.7a	4.5±0.0a	4.7±0.0b	4.5±0.2b	4.7±0.3d
C_m+F	31.5±2.4a	28.4±2.4ab	34.3±3.6b	35.2±2.1b	3.8±0.2a	3.9±0.1ab	3.9±0.2ab	4.0±0.3b
D_o	37.4±0.0a	29.5±0.0ab	30.5±3.7b	26.3±4.5a	4.4±0.6a	4.1±0.0b	4.7±0.4b	4.5±0.2d
D_m	35.9±4.3a	31.8±2.0ab	32.4±4.3b	31.7±3.0ab	3.5±0.0a	3.5±0.1a	3.8±0.0a	3.6±0.2ab
D_o+F	27.5±0.0a	30.4±0.0ab	28.2±0.2a	28.3±1.6a	4.2±0.0a	4.1±0.0b	4.0±0.1ab	4.2±0.2c
D_m+F	30.6±2.3a	34.9±3.4b	34.9±3.5b	36.6±2.4b	3.6±0.1a	3.4±0.1a	3.6±0.1a	3.4±0.1a

C, control; D, drained; o, organic soil; m, mineral soil; +F, fertilizer. Letters following mean+SEM denote statistical difference in columns following ANOVA at $p = 0.05$. n=4.

Table 2.4. *F* and *p* statistics following ANOVA of drainage, fertilization and interactions on C, N and S concentrations and pH at Suquash Drainage Trial (SDT)

Model term	df	Total C		Total N		NO_3-N		NH_4-N		Total S		SO_4-S		C:N		pH	
Forest floor		*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Drain.	1	4.3	0.045	16.3	<0.001	1.7	0.200	17.5	<0.001	55.7	<0.001	6.9	0.012	1.4	0.247	1.9	0.172
Fert.	1	2.0	0.164	3.7	0.059	173.8	<0.001	130.5	<0.001	597.5	<0.001	22.2	<0.001	6.5	0.014	0.6	0.437
Month	3	68.4	<0.001	1207.1	<0.001	54.5	<0.001	41.3	<0.001	2431.5	<0.001	3.3	0.028	0.6	0.619	1.9	0.138
D×F	1	1.1	0.307	1.7	0.202	0.1	0.807	20.7	<0.001	54.9	<0.001	6.7	0.013	26.9	<0.001	15.2	<0.001
D×M	3	1.0	0.401	16.8	<0.001	3.0	0.042	5.2	0.004	25.0	<0.001	3.3	0.029	7.9	<0.001	0.3	0.845
F×M	3	4.9	0.005	4.8	0.005	96.7	<0.001	40.8	<0.001	308.1	<0.001	3.6	0.021	9.0	<0.001	1.3	0.278
D×F×M	3	0.1	0.959	1.4	0.267	5.5	0.003	11.1	<0.001	25.4	<0.001	3.5	0.022	4.5	0.007	1.0	0.411
Mineral soil																	
Drain.	1	6.6	0.013	751.5	<0.001	0.0	0.860	0.4	0.509	12.5	0.001	0.9	0.340	0.9	0.348	0.3	0.581
Fert.	1	0.4	0.531	73.5	<0.001	0.1	0.784	1.3	0.253	0.2	0.646	13.0	0.001	5.6	0.022	0.4	0.519
Month	3	29.9	<0.001	3980.5	<0.001	1.0	0.399	2.4	0.082	117.4	<0.001	0.2	0.866	0.9	0.467	1.7	0.177
D×F	1	4.7	0.035	30.3	<0.001	4.7	0.035	7.2	0.010	12.0	0.001	3.9	0.054	6.4	0.015	0.3	0.615
D×M	3	4.8	0.005	759.7	<0.001	3.3	0.029	4.1	0.011	6.7	0.001	4.9	0.005	0.7	0.577	5.8	0.002
F×M	3	1.8	0.156	73.7	<0.001	3.1	0.034	1.9	0.142	0.3	0.820	2.6	0.064	1.1	0.351	0.6	0.625
D×F×M	3	1.5	0.218	30.7	<0.001	1.4	0.242	1.7	0.184	18.3	<0.001	2.1	0.107	1.3	0.296	0.4	0.779

Total N concentrations were not significantly different following mounding at ALRF. As with total C, total N was greatest in the forest floor (Table 2.1). At SDT, total N was equivalent in drained and undrained soil in Jul-12 but was significantly greater in drained soil overall (Table 2.4). Total N was significantly lower in mineral layers that had been drained during this time period (Table 2.3). A significant fertilization effect on N concentration was observed at ALRF and SDT throughout the study, except in the forest floor at SDT. Locational effects in fertilized samples were found at SDT (Table 2.3) with drained and fertilized mineral soil having the largest contribution to the interactions between factors (drainage, fertilization and date) in the mixed ANOVA model used in this study (Table 2.4).

Soil C:N ratio was greater at SDT than at ALRF; SDT generally had higher soil C and N than ALRF. There were no effects of treatment on soil C:N ratios at ALRF (Tables 2.1, 2.2). Soil layer contributed most prominently to soil C:N ratios, as these ratios were consistently greater in forest floor samples compared to mineral soil at ALRF. Drainage did not significantly affect C:N ratios at SDT. Fertilization effects on C:N ratios were observed at SDT, with fertilized plots having lower C:N ratios than unfertilized plots (Tables 2.3, 2.4).

2.3.2.2 NH_4-N and NO_3-N

Fertilization increased NH_4-N and NO_3-N concentrations over the course of this study at both ALRF (Tables 2.1, 2.2) and SDT (Tables 2.3, 2.4). At ALRF, NH_4-N was greatest during Jun-12, immediately following fertilization of unmounded plots, where concentrations of 2233 mg kg^{-1} were measured in the forest floor (Table 2.1). This is an order of magnitude greater than fertilized mound tops or hollows (where forest floor and mineral soil layers were mixed), and two orders of magnitude greater than mineral soil from unmounded plots or samples from unfertilized mound plots (Table 2.1). By percentage, the concentration of NH_4-N in the forest floor or fertilized unmounded, mineral mound top and mound hollow samples from ALRF decreased 82.7%, 58.2% and 85.9% between Jun-12 and Jul-12, respectively, and had exponential loss rates of -0.80 (R^2=0.90), -0.45 (R^2=0.83) and -0.93 (R^2=0.87) over the first five months of the study, respectively, after which time the concentration remained relatively constant until the following year (fittings not shown). NH_4-N concentrations were significantly greater in forest floor and mineral layers in fertilized plots compared to unfertilized plots at ALRF (Table 2.2). At SDT, NH_4-N was higher in fertilized relative to unfertilized plots throughout the study, though concentrations peaked in Aug-12 at 1496 and 3122 mg kg^{-1} in drained and undrained forest floors, respectively. Drained plots at SDT had greater NH_4-N in the forest floor compared to undrained plots

(Tables 2.3, 2.4). There were significant interactions between drainage, fertilization and sampling date, with NH_4-N concentrations in forest floor responding more to treatments than mineral soil.

NO_3-N concentrations at ALRF were not significantly greater in fertilized plots relative to unfertilized plots in Jun-12 or Jul-12, but increased sharply by Aug-12, after which time they dissipated but remained significantly greater in fertilization plots relative to unfertilized plots (Tables 2.1, 2.2). Peaks in NO_3-N concentration at ALRF occurred in the same unmounded fertilized plots that had the highest concentrations of NH_4-N following fertilization (Table 2.1). Concentrations of NO_3-N were lower than NH_4-N, with a maximum of 149.7 mg kg^{-1} in a fertilized forest floors in Aug-12. Higher concentrations of NO_3-N in the forest floor was observed in Jul-13, and mineral soil in undisturbed control plots had a higher NO_3-N concentration than mounded soil (Table 2.1). A significant fertilization and sampling date interaction was observed due to the changes in NO_3-N concentration over the growing season in fertilized plots (Tables 2.1, 2.2). At SDT NO_3-N concentrations also peaked in Aug-12, with higher concentrations than at ALRF (Table 2.3). Forest floors in fertilized plots at SDT had greater NO_3-N concentrations than unfertilized plots, and the change in NO_3-N concentrations over time led to significant interactions between date and fertilization for both forest floor and mineral samples (Table 2.4).

2.3.2.3 Total S and SO_4-S

Trace concentrations of total S (0.1g kg^{-1}) were measured at ALRF in the forest floor, and were doubled by fertilization (Table 2.1). This led to significantly greater total S concentrations in fertilized mineral samples and significant interaction between fertilization and date in forest floor and mineral samples over the sampling period (Table 2.2). Total S concentrations were significantly lower in mounded mineral soil compared to unmounded mineral soil, and interactions between mounding and fertilization, as well as between mounding, fertilization and sampling date indicate the complex changes to total S concentrations occurring over this period (Table 2.2). In contrast, SO_4-S was measured in almost all of the mineral samples taken from ALRF (Table 2.1). SO_4-S concentrations were significantly greater in fertilized plots at ALRF relative to unfertilized plots (Table 2.2). In contrast to ALRF, there were measurable amounts of total S at SDT in both fertilized and unfertilized plots (Table 2.3). Forest floors had higher total S concentrations than mineral soil. Control plots had higher total S concentrations than drained plots, with the greatest differences between undrained and drained plots being observed in the forest floors. The greatest SO_4-S concentrations at SDT were measured in the forest floor in fertilized plots in undrained controls (Table 2.3). SO_4-S concentrations were significantly greater in fertilized plots

compared to unfertilized plots, and were greater in drained forest floors compared to undrained forest floors (Table 2.4).

2.3.2.4 pH

Soil pH was significantly greater in mounded plots at ALRF relative to unmounded controls (Tables 2.1, 2.2). These differences resulted from the higher pH of the highly-moist soil found in the mound hollows, which had pH > 5.0 in Jun- and Aug-12 (Table 2.1), and from the higher pH in forest floors relative to mineral soil at ALRF. Following the application of fertilizer consisting mostly of urea and NH_4-N in Jun-12, significantly higher pH was measured in fertilized plots relative to unfertilized controls. There was no initial fertilization effect on soil pH during Jul-12 at SDT, though by Aug-12 fertilized plots had a higher pH than unfertilized plots, an effect that was most predominant in the undrained plots (Tables 2.3, 2.4). Drained plots at SDT had higher soil pH in Aug-12 and Jul-13 than undrained controls. This led to interactive effects between drainage and sampling date in both forest floors and mineral soil.

2.3.3 CO_2

ALRF soil CO_2 fluxes were measured for one year after fertilization using static closed chambers (Figure 2.5a). CO_2 emissions in undisturbed control plots were higher than the mounded sites in Jun-12, having mean (± standard error of the mean, SEM) rates of 771.7 ± 89.9 and 375.6 ± 43.1 mg CO_2 m^{-2} h^{-1}, respectively. Significant locational differences in CO_2 efflux were detected between the fertilized unmounded plots and all mounded plots, as well as between the unfertilized unmounded plots and the mound hollows in the unfertilized mounding treatments. No effects of fertilization on CO_2 emissions were measured within 24 hours. In Jul-12 there were no significant treatment effects, although the trend of lower CO_2 flux in mounded plots continued. This led to significant locational differences between unfertilized unmounded plots (1300.9 ± 120.4 mg CO_2 m^{-2} h^{-1}) and fertilized mound tops (814.0 ± 95.9 mg CO_2 m^{-2} h^{-1}). The mound hollows had the lowest CO_2 efflux at this date, 658.0 ± 12.0 and 740.0 ± 58.5 mg CO_2 m^{-2} h^{-1} for unfertilized and fertilized hollows, respectively. Fertilized plots were statistically equivalent to unfertilized plots in Aug-12. Locational differences were found in Aug-12, with unmounded plots (426.9 ± 53.9 mg CO_2 m^{-2} h^{-1}) and mound tops (442.2 ± 59.8 mg CO_2 m^{-2} h^{-1}) of unfertilized plots having significantly higher CO_2 emission rates relative to fertilized mound hollows (191.2 ± 54.2 mg CO_2 m^{-2} h^{-1}). Significant interactive effects of mounding and fertilization were seen in Jun-13. Mounding decreased mean CO_2 emission rate by 55.0 mg CO_2 m^{-2} h^{-1}, with the lowest values measured in the mound

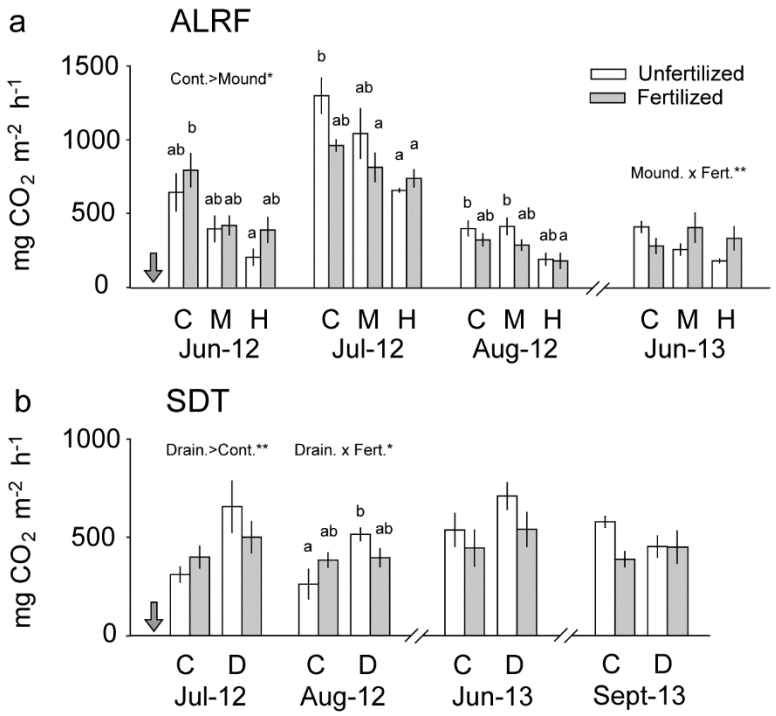

Figure 2.5. CO_2 fluxes from a) undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF), and b) undisturbed control (C) and drained plots (D) subject to fertilization at Suquash Drainage Trial (SDT). Shaded arrow shows time of fertilization. Error bars, SEM. n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

hollows. However, fertilization increased CO_2 emissions in the mounded plots by an average of 160.6 mg CO_2 m^{-2} h^{-1} but had no significant effect on CO_2 flux from soil in the unmounded plots. The mean CO_2 emissions at ALRF in the Jun-13 sampling were 328.8 ± 29.3 mg CO_2 m^{-2} h^{-1}. Soil CO_2 emission rates at SDT were in the same range as those measured at ALRF. Drainage significantly increased CO_2 emissions (Figure 2.5b). Following sampling at SDT in July 2012, the mean CO_2 emissions mg m^{-2} h^{-1} were 577.9 ± 76.8 and 354 ± 35.8 in undrained control plots and plots subject to mechanical ditch drainage, respectively. This trend was also observed one month after fertilization where there was a significant interactive effect between drainage and fertilization. While not statistically different, fertilized undrained plots had a greater mean CO_2 efflux than unfertilized plots, a trend that was reversed in the drained plots. Significant locational differences within the unfertilized treatment was observed between drained and undrained plots, which had mean CO_2 emission rates of 513.5 ± 32.3 and 260.0 ± 75.4 mg m^{-2} h^{-1}, respectively. There were no treatment or location effects measured one year and 14 months following fertilization. These sampling dates had mean emission rates of 552.9 ± 44.1 and 465.1 ± 30.2 mg m^{-2} h^{-1}, respectively.

CO_2 made up the majority of GHG flux from the ALRF and SDT sites. When other measured GHGs (CH_4 and N_2O) (following chapters) were converted to CO_2-equivalants based on their 100-year GWP, CO_2 accounted for between 91.0 and 100.4% of total GHG CO_2-equivalants (see Appendix B for table of GHG CO_2-equivilants), with the exception of fertilized mound hollows in Aug-12, where N_2O accounted for 24.0% of total CO_2-equivilants. Soil at ALRF and SDT frequently acted as a sink for CH_4 and N_2O, reducing the effect of CO_2 on GWP. For a complete discussion of CH_4 and N_2O flux at ALRF and SDT refer to Chapters 3 and 4, respectively.

2.3.4 Bacterial and fungal abundance

Estimates of total bacterial 16S rRNA copies at ALRF were distributed between 10^8 and 10^{13} copies g^{-1} soil (dw) (Figure 2.6a). In Jun-11 neither mounding nor fertilization had taken place, and gene quantification should reflect the abundances in harvested stands without the application of site preparation techniques. Soil from the plots to be mounded was sampled only from the mineral layers in Jun-11 to facilitate comparisons with post-mounded gene abundances from the mounded plots. Bacterial abundance was generally higher in the forest floor relative to mineral soil, though not significantly so in Jun-11. Control plots had a significantly higher abundance of bacterial 16S abundance than mounded plots (Table 2.5). In forest floor samples there was an initial increase in bacterial 16S abundance after fertilization, leading to significant interactions between fertilization and sampling date. Ranging between 10^9 and 10^{12}

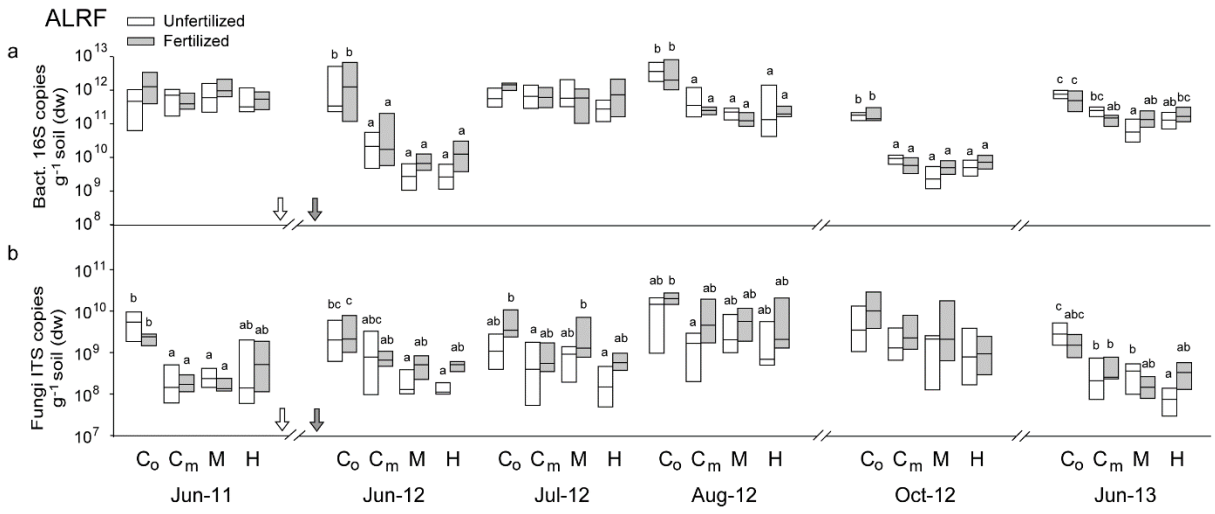

Figure 2.6. Abundance of a) total bacterial 16S rRNA and b) fungal ITS genes in soil from undisturbed control organic material (C_o), control mineral soil (C_m) and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA.

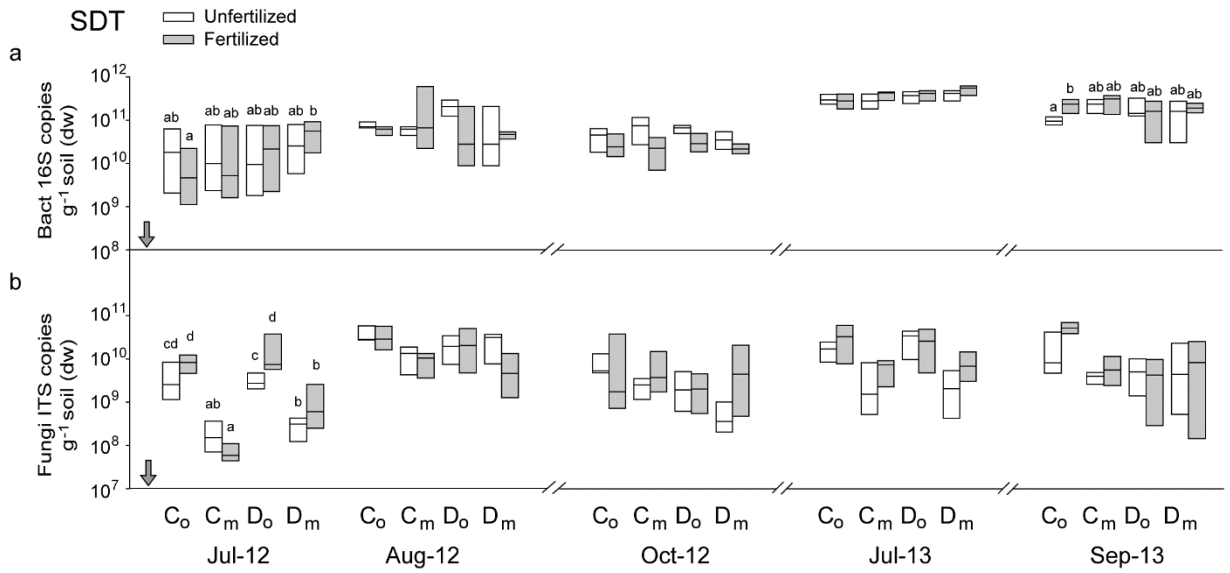

Figure 2.7. Abundance of a) total bacterial 16S rRNA and b) fungal ITS genes in organic (o) and mineral (m) soil from undrained control (C) and drained (D) plots subject to fertilization at Suquash Drainage Trial (SDT). Shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA.

Table 2.5. *F* and *p* statistics following ANOVA of mounding, fertilization and interactions on bacterial and fungal abundance at Aleza Lake Research Forest (ARLF)

Model term	df	Bacterial 16S		Fungal ITS	
Forest floor		*F*	*p*	*F*	*p*
Fert.	1	0.0	0.873	2.8	0.097
Date	4	10.6	<0.001	3.3	0.011
D×F	4	0.5	0.753	3.0	0.017
Mineral soil					
Mound.	1	15.4	<0.001	1.6	0.202
Fert.	1	1.0	0.325	19.6	<0.001
Date	4	148.8	<0.001	22.4	<0.001
M×F	1	4.5	0.035	0.1	0.770
M×D	4	1.7	0.160	1.3	0.251
F×D	4	2.1	0.082	1.1	0.359
M×F×D	4	0.4	0.774	0.4	0.869

Table 2.6. *F* and *p* statistics following ANOVA of drainage, fertilization and interactions on bacterial and fungal abundance at Suquash Drainage Trial (SDT)

Model term	df	Bacteria 16S		Fungi ITS	
Forest floor		*F*	*p*	*F*	*p*
Drain.	1	0.8	0.377	8.9	0.004
Fert.	1	0.1	0.786	0.5	0.480
Month	4	36.9	<0.001	12.4	<0.001
D×F	1	1.0	0.310	0.7	0.424
D×M	4	0.2	0.926	3.1	0.023
F×M	4	4.3	0.004	1.1	0.376
D×F×M	4	1.4	0.232	1.2	0.319
Mineral soil					
Drain.	1	0.8	0.367	0.2	0.675
Fert.	1	0.8	0.386	1.6	0.215
Month	4	26.2	<0.001	18.5	<0.001
D×F	1	2.6	0.114	0.3	0.602
D×M	4	3.9	0.007	1.8	0.142
F×M	4	1.8	0.151	1.8	0.141
D×F×M	4	1.2	0.315	0.7	0.569

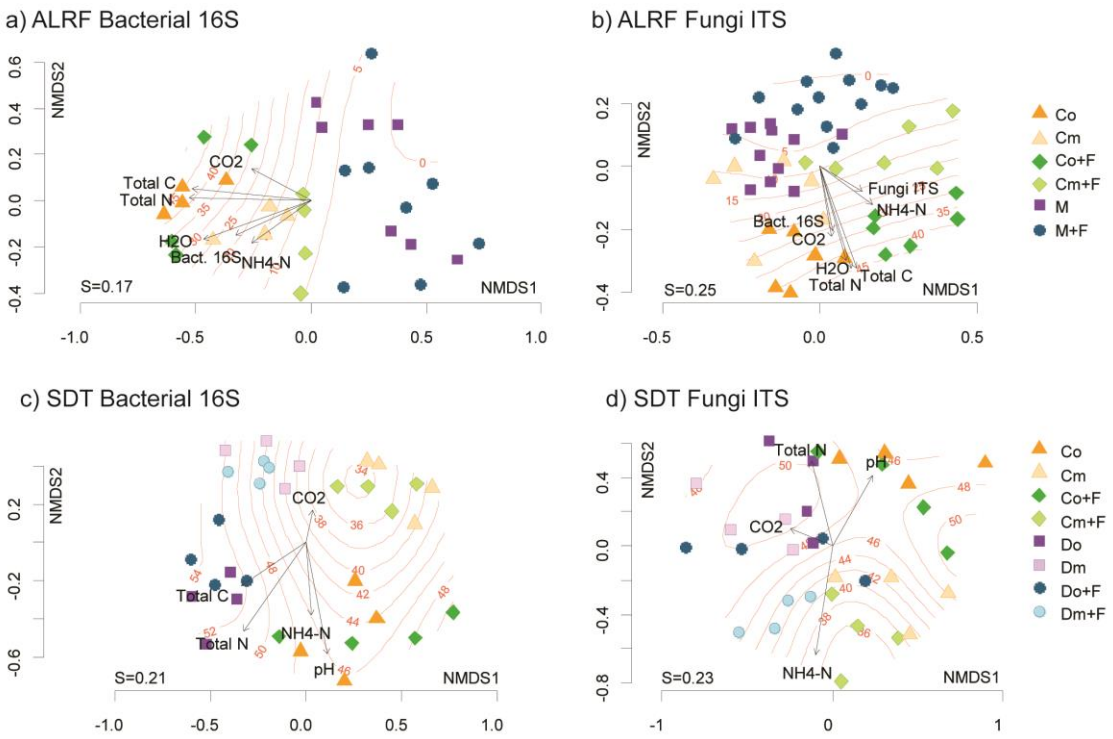

Figure 2.8. Non-metric multidimensional scaling (NMDS) analysis of bacterial and fungal T-RFLP profiles at Aleza Lake Research Forest (ALRF) and Suquash Drainage Trial (SDT) from Aug-12 samples. a) Bacterial 16S at ALRF, b) fungal ITS at ALRF, c) bacterial 16S at SDT, d) fungal ITS at SDT. A secondary matrix of significant ($p < 0.05$) soil physico-chemical parameters and CO_2 flux rate was imposed on the matrix following 999 permutations, with arrow length showing strength of correlation. Contours show the total C concentration fit to sample ordination scores. S, stress.

Table 2.7. Mounding, drainage, fertilization and soil layer effects on community structure and diversity of bacteria and fungi at Aleza Lake Research Forest (ALRF) and Suquash Drainage Trial (SDT). Treatment effects on community structure were determined by analysis of similarity (ANOSIM) on Bray-Curtis dissimilarity matrices of bacterial 16S and fungal ITS T-RFLP profiles, with the ANOSIM *R* statistic and *p*-value provided. Treatment effects on diversity were determined by analysis of variance (ANOVA) on Shannon–Weaver Diversity Indices (H'), with the *F*-statistic and *p*-value provided.

	Bacterial 16S				Fungal ITS			
ALRF	ANOSIM (*R*)	Structure	H' (*F*)	Diversity	ANOSIM (*R*)	Structure	H' (*F*)	Diversity
Mound.	0.63^{***}	C ≠ M	21.65^{***}	C > M	0.29^{***}	C ≠ M	31.44^{***}	C > M
Fert.	0.11^{NS}	C = F	0.02^{NS}	C = F	0.27^{***}	C ≠ F	0.15^{NS}	C = F
Layer.	0.46^{***}	O ≠ M	1.93^{NS}	O = M	0.52^{***}	O ≠ M	13.05^{***}	O > M
SDT								
Drain.	0.57^{***}	C ≠ D	2.11^{NS}	C = D	0.36^{***}	C ≠ D	0.65^{NS}	C = D
Fert.	0.29^{***}	C ≠ F	0.62^{NS}	C = F	0.08^{NS}	C = F	0.41^{NS}	C = F
Layer.	0.54^{***}	O ≠ M	6.53^{*}	O > M	0.29^{***}	O ≠ M	0.04^{NS}	O = M

Treatments: C, control; M, mounded; D, drained; F, fertilized; Soil layers: O, organic layer; M, mineral layer (NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

16S rRNA copies g^{-1} soil (dw), bacteria at SDT had a higher mean abundance than at ALRF (Figure 2.7a). Bacterial abundance was statistically equivalent in organic (forest floor) material and mineral soil, in contrast to differences observed at ALRF. No drainage effects were evident for estimates of total bacterial 16S rRNA abundance at SDT in mineral soil, but drained forest floor samples had a higher abundance of bacterial 16S than undrained samples (Table 2.6).

Abundance of fungal ITS at ALRF ranged from 10^7 to 10^{11} copies g^{-1} soil (dw), lower than bacterial 16S (Figure 2.6). Fungal ITS was significantly greater in forest floor samples than in mineral soil throughout the sampling period. Fungal ITS was higher in fertilized mineral samples relative to unfertilized mineral samples, and there was a significant fertilization and sampling date interaction (Table 2.5). At SDT, fungal ITS and bacterial 16S abundance was similar (Figure 2.7). Fungal ITS gene abundance was greater in forest floor samples relative to mineral soil, significantly so in Jul-12 (Figure 2.7b). Drainage significantly reduced total fungal ITS abundance in the forest floor, though this reduction was not observed in all months, leading also to a significant interaction between drainage and sampling date (Table 2.6).

2.3.5 Bacterial and fungal community structure

Bacterial 16S T-RFs from ALRF showed significant separation between samples from control and mounded plots following ANOSIM, with no separation between unfertilized and fertilized samples (Figure 2.8a, Table 2.7). Soil variables fitted to the NDMS ordination with $p > 0.05$ were shown. CO_2 flux rate was always plotted regardless of significance. The bacterial T-RFs in forest floors of unmounded plots was positively correlated with total C, total N and CO_2 flux. Soil water content, NH_4-N concentration and bacterial abundance were also positively correlated with the ordination scores of bacterial 16S T-RFs in unmounded samples. T-RF diversity as calculated using H' (Shannon-Weiner index) was significantly greater in control plots compared to mounded plots (Table 2.7). Fungal community structure at ALRF, assessed using ITS, was significantly affected by mounding to the same extent as observed in bacterial 16S, but no distinct clustering based on presence of fertilizer were observed (Figure 2.8b, Table 2.7). The soil physico-chemical parameters that significantly correlated with ordination scores were NH_4-N, which positively correlated with fungal ITS abundance and T-RFs from mounded and fertilized plots. Bacterial abundance, soil water content, total C concentration, total N concentration and CO_2 flux rate positively correlated with fungal T-RFs in unmounded plots. T-RF diversity was significantly greater in control versus mounded samples as well as forest floor versus mineral soil (Table 2.7).

At SDT, significant separation of bacterial 16S T-RFs was primarily related to soil layer (forest floor versus mineral soil) along the second ordination axis (NMDS2), while significant separation between bacterial T-RFs from drained and undrained plots occurred along NMDS1 (Figure 2.8c, Table 2.7). There was also significant separation between samples from fertilized and unfertilized plots. NH_4-N concentrations and pH were positively correlated with forest floor bacterial 16S T-RFs. Total N and total C concentrations were positively correlated with drained and fertilized forest floors. CO_2 fluxes were associated with OTUs in drained, unfertilized samples. Diversity of bacterial T-RFs was significantly higher in forest floor compared to mineral soil (Table 2.7). Fungal ITS T-RFs at SDT separated by drainage along NMDS1, and were not differentiated by fertilization in undrained controls, but exhibited separation by fertilization in drained plots along NMDS2 (Figure 2.8d). Soil pH and total N were positively correlated with OTUs in forest floors, with higher pH associated with communities in undrained forest floor samples and total N with drained forest floors. NH_4-N concentrations were significantly correlated with fungal community structure in drained, fertilized forest floors. CO_2 flux rate was positively correlated with fungal community structure from drained, unfertilized plots. There were no significant correlations between bacterial and fungal diversity and abundance.

Canonical variation partitioning was used to determine the primary sources of variation in the bacterial and fungal OTU distribution (Figure 2.9). ALRF and SDT T-RFLP profiles were combined for this analysis, converted to Bray-Curtis dissimilarity matrices and their variation partitioned into three variable groupings: soil factors (See Tables 2.1, 2.3), categorical treatment variables (mounding, drainage, fertilization) and site, the significance of which was determined with Monte-Carlo permutations. Following multivariate regression of bacterial 16S OTU distribution (R^2-adjusted: 0.72, $p = 0.005$), soil factors uniquely explained about 8% of variation, treatment variables explained about 15% of variation and site differences explained about 6% of variation. The largest sources of variation were the overlap between these categories. Variation in fungal ITS OTU distribution (R^2-adjusted: 0.80, $p = 0.005$) was explained primarily by site (16%), with soil factors (1%) and categorical treatment variables (3%) uniquely explaining small but significant portions of the overall variation.

2.4 Discussion

2.4.1 Mounding effects on soil moisture and chemistry

Mounding is used to create raised planting sites in wet forest soils that increase temperature and decrease soil moisture for optimum seedling survival and growth (Sutton, 1993; Hallsby and Örlander, 2004). In this study mounding decreased soil water content on mound tops relative to unmounded

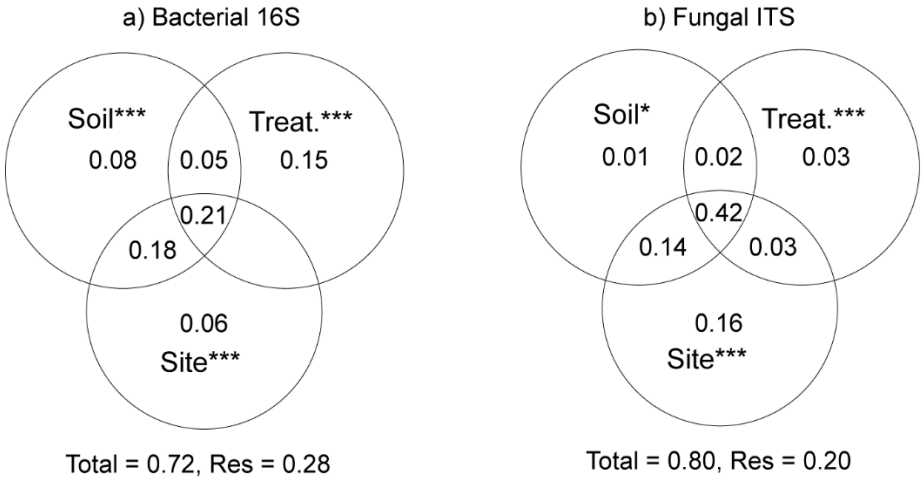

Figure 2.9. Canonical variation partitioning of a) bacterial 16S and b) fungal ITS OTU distribution into groupings of soil factors, categorical treatment variables and site differences following multivariate regression.

controls, though the process reduced total C and N due to the removal or reduction of forest floor (L, F and H) layers. Following excavator mounding the treatment plots at ALRF contained freshly-exposed mineral Ae and Bt horizons that settled and became colonized by pioneer vegetation as the study progressed. Turning the soil using an excavator created mounds about 50 cm in height, causing the majority of forest floor organic material to be buried beneath the rooting zone of the hybrid spruce seedlings that were planted the following year. Mounding treatments at ALRF created about 2.9 times the number of elevated planting sites compared to untreated plots. This disturbance to the physical structure of the soil removed the vegetation and forest floor layers and led to the creation of about 1166 hollows, or pits, ha^{-1}. Due to rainfall events and subsequent effects on water-table depth, these hollows contained standing water during the Jun-12 and Jul-12 sampling dates. The data presented in this study show that mounding treatments can reduce soil moisture on mound tops during summer months at the cost of elevated soil moisture and standing water in the hollows created by soil turnover (Sutton, 1993).

Soil C and N were reduced by mounding. Soil turnover can bury organic material in the forest floor, which can remove C and N from the planting zone and accelerate its decomposition (Johansson, 1994; Liechty et al., 1997; Lundmark-Thelin and Johansson, 1997; Paul et al., 2002). For example, in a mixed hardwood boreal stand in Ontario, Canada, mounding treatments following harvest that mixed the soil forest floor and mineral layers had the highest rate of respiration (~ 1.0 g CO_2 m^{-1} h^{-1}) during the growing season relative to non-harvested or unmounded controls (~ 0.8 g CO_2 m^{-1} h^{-1}), and had lower SOM (23% compared to 35% and 26%, respectively), which were caused by soil layer mixing and elevated levels of soil temperature and aeration (Mallik and Hu, 1997). Increased total C and N mineralization in mineral soil of a Finnish Norway spruce stand following mounding has also been reported by Smolander et al. (2000) and Smolander and Heiskanen, (2007) as well as elevated microbial biomass C. Mounding resulted in decreased soil total N concentrations in mounded plots in this study. This was also due to physical removal of the forest floor, although the long-term effect of mounding on C and N mineralization requires further long-term study to follow tree growth and forest floor regeneration. Microbial nitrification and denitrification may be responsible for this N loss from mounded areas as nitrification- and denitrification-related N_2O losses following fertilization are common following N addition to forest soil (Szukics et al., 2009, 2010, 2012). This possibility should be investigated as a possible source of N loss following mounding. Changes in soil respiration following mounding can be used to determine the rate of organic matter oxidation following vegetation removal and the burying of the forest floor in mounded plots. Mounding methods that more homogenously mix the forest floor throughout the mound may be superior at providing seedlings with higher concentrations of organic C and N in the rooting zone than the mounding method used in this study.

Soil pH was also affected by mounding, with values greater in mound hollows (Table 2.1). The higher pH in mounded plots is likely due to the reduction of oxidized compounds in waterlogged mound hollows by microorganisms, primarily the reduction of Fe(III) to Fe(II) (Ponnamperuma, F.N., 1984; Smolander and Heiskanen, 2007). The loss of organic C from the mound tops can account for the reduction of pH relative to control plots and mound hollows (Ponnamperuma, 1984), for example in Aug-12 and Jun-13.

2.4.2 Drainage effects on soil moisture and chemistry

SDT had a higher annual precipitation (Figure 2.1) and poorer drainage (Figure 2.4) than ALRF. Undrained areas of SDT can be considered a "cedar swamp" due to the presence of western red-cedar throughout this wetland, which is composed of deep organic soils. Standing pools of water in the pre-drainage and undrained areas are the result of restricted drainage due to an underlying undulating hardpan (van Niejenhuis et al. 2003). Drainage improves productivity in wet forests (Laiho and Laine, 1997; Macdonald and Yin, 1999; Hargreves et al., 2003; Byrne and Farrell, 2005; Sajedi et al., 2012). The lowering of the soil water table following drainage can increase soil aeration, seedling survival, organic matter decomposition, N mineralization and pH as well as decrease forest floor depth and C:N ratio (von Arnold et al., 2005a). Drainage significantly reduced soil moisture throughout the year by about 20% at SDT. Previous research conducted at SDT demonstrated a 100% decrease in soil moisture content, 22% increase in tree height and 29% increase in tree diameter 10 years following drainage (Sajedi et al., 2012). Previous measurements of soil pH (H_2O) at SDT showed a decrease from 3.75 to 3.10 following drainage (Sajedi et al., 2012). Our results indicate slightly higher soil pH in the same plots two years following the original measurements and an increase in forest floor and mineral soil pH following drainage, possibly as a result of increased N mineralization and nitrification (Tietema et al., 1992). Further study to verify the cause of N loss in drained plots is required.

The data presented in this study indicate that drainage can lead to an accumulation of total C in the mineral layer compared to undrained controls. This result has been demonstrated at SDT in a prior study (Sajedi et al., 2012), which also showed an increase in the depth of the forest floor and total N and C ha^{-1}. von Arnold et al. (2005a) showed a decrease in soil C and forest floor depth in an Irish peatland 30 years after drainage. Byrne and Farrell (2005) showed a decrease in peat depth and increase in forest floor depth in a Swedish birch stand 3 to 39 years after drainage. The elevated soil C at SDT could result from enhanced root- and litter-derived organic matter to soil, and due to incomplete drainage that maintains a suboxic zone (e.g., redox potential $\leq$ 300 mV) in the rooting zone (Sajedi et al., 2012). Drainage can

enhance both above- and below-ground C stocks, which may lead to suggestions to use site preparation as a method to enhance C sequestration. However, anoxic or suboxic root zones with high organic C inputs are ideal microhabitats for methanogenesis and methane oxidation, respectively (Brune et al., 2000). Therefore, soil respiration, CH_4 flux and presence of archaeal methanogens and methane-oxidizing bacteria should be investigated in these areas to ensure that drained sites are not hotspots for GHG production and release, which would diminish the benefits of drainage on C sequestration.

2.4.3 Fertilization effects on soil moisture and chemistry

Fertilization with N and S can improve growth response of many economically important tree species, e.g., lodgepole pine, Douglas-fir, western redcedar and western hemlock (Miller, 1986; van den Driessche, 1988; Chappell et al., 1992; Brockley, 1996, 2001, 2006; Blevins and Prescott, 2002; Brockley and Simpson, 2004). Fertilizer is generally applied following stand establishment, although increases in tree growth and volume have also been documented following fertilization several years after planting (Blevins and Prescott, 2002). In this study the ALRF hybrid spruce stand was fertilized with NPKS at time of planting, while the cedar-hemlock stand at SDT was fertilized with NP 11 years after planting, then with NPKS 17 years after planting, the latter application as part of this study. NH_4-N concentrations were greatest in August at SDT, demonstrating that the release and mineralization of urea fertilizer peaked during mid-growing season. The same samples with high NH_4-N at both ALRF and SDT had high concentrations of NO_3-N within one month of fertilization, indicating nitrification activity in these fertilized plots. The increase in NO_3-N was much more pronounced at SDT compared to ALRF, possibly indicating a greater potential for nitrification at this site (Tables 2.1, 2.3). NO_3-N comprised less than 1% of the fertilizer formulation, with an applied concentration of about 0.8 kg ha^{-1}, while 87%, or 174 kg ha^{-1}, of the N applied in this study was in the form of urea (Appendix A). It is, therefore, likely that higher NO_3-N concentrations in fertilized plots are due to mineralization of urea and nitrification of fertilizer-derived NH_4-N. Further research is required to determine the rates of nitrification and denitrification in these treatments and the links between nitrifying and denitrifying organisms and N loss from fertilized forests.

2.4.4 Factors influencing CO_2 flux

2.4.4.1 Mounding and drainage

The CO_2 emissions measured in this study (Figure 2.5) are within the range measured in conifer forests in north-western Europe and North America, including those subject to a variety of site preparation techniques and fertilization regimes (von Arnold et al., 2005b; Basiliko et al., 2009; Jassal et al., 2010; Mojeremane et al., 2012). For example, a lodgepole pine site located near ALRF, a western hemlock site near SDT and a Douglas-fir site located on a boundary between the coastal Douglas-fir (CDF) and cedar-western hemlock (CWH) BEC zones all had CO_2 flux rates between 100 and 1000 mg m^{-2} h^{-1}, with the warmer, drier interior lodgepole pine site generally having higher maximum respiration rate than the coastal sites (Basiliko et al., 2009). Similar patterns were observed at our sites. The mounded plots at the ALRF site were lacking the shrub layer and forest floors, which can remove sources of CO_2 from autotrophic respiration and heterotrophic decomposition of organic matter, respectively (Tam et al., 2008; Jassal et al., 2010). Forest floor removal can significantly decrease total C and N, microbial biomass C and enzyme activity, such as was shown in a boreal aspen stand in B.C. (Tan et al., 2008). At ALRF, mean mineral soil total C, N and mineral N were significantly lower in mounded plots compared to unmounded control plots one year following the mounding treatment, though these variables were not significantly correlated to CO_2 efflux rates. CO_2 flux was lower in the mounded plots than unmounded plots at the outset of the study, but became equivalent in late 2012 and early 2013. Over this time primary successional grasses, herbs and mosses similar to those found in the post-fire SBS stands (Driscoll et al., 1999) colonized the mounded plots.

CO_2 emissions were inhibited in waterlogged soil, such as undrained soil at SDT in Jul-12. Mojeremane et al. (2012) found that drainage increased CO_2 flux by about 18.5% over two years following drainage in a peaty gley soil in England, due to increased soil oxygen and temperature. In a previous study at SDT, drainage was also shown to reduce soil moisture, resulting in a 22% increase in tree height, increased forest floor thickness and increased forest floor total C, without altering microbial biomass soluble organic carbon or CO_2 fluxes (Sajedi et al., 2012).

2.4.4.2 Fertilization

There was no clear effect of fertilization on CO_2 flux at ALRF. At SDT CO_2 flux was higher in fertilized, drained plots than in unfertilized, drained plots, while the reverse occurred in undrained plots in Aug-12. Mojeremane et al. (2012) reported that fertilization increased CO_2 emissions about 23.1% from peat soil two years after fertilization. The stimulation of soil respiration by N fertilization occurs

frequently in N-limited environments such as ALRF (Raich et al., 1994; Micks et al., 2004; Gallo et al., 2005; Cleveland and Townsend, 2006; Jassal et al., 2010) Soil N concentrations at ALRF were significantly lower than at SDT. In this study, the positive interactions between mounding and fertilization following fertilization indicate that in low-N soil fertilization can increase CO_2 emissions. However, interaction between drainage and fertilization on CO_2 flux four months after fertilization at SDT suggests that in higher productivity, previously fertilized sites, fertilization may not alter CO_2 emissions.

2.4.5 Global warming potential

GWP is used in the Kyoto Protocol to the United Nations Framework Convention on Climate Change to directly compare the impact of different GHGs based on their radiative forcing over a unit of time (the standard is 100 years, with GWP values of ~23 and ~293 for CH_4 and N_2O on a unit mass basis) (Shine et al., 2005). In this study, CO_2 was the overwhelming contributor to total GWP, accounting for more than 90% of total GHG emissions in CO_2-equivalents, with a single exception of fertilized mound hollows in Aug-12 at ALRF, where N_2O contributed about 24% of the plots total GWP (Chapter 4 and Appendices B, C). The highest measured CH_4 flux was 26.9 mg CO_2-equivalents m^{-2} h^{-1}, making up about 3.5% of the GWP of the fertilized mound-hollow plot in which it was measured in Jul-12, also at ALRF (Chapter 3). These events were relatively rare, as both CH_4 and N_2O values were two to three orders of magnitude lower than CO_2 when converted to CO_2-equivalents. The relative amounts of GHGs emitted from forest soil are equivalent to those measured by Basilko et al. (2009), as CH_4 and N_2O flux rates were never significantly different from 0, with a single exception of a N_2O in a fertilized plot at a single date in a Douglas-fir stand one month following fertilization. These data indicate that forest researchers and managers investigating the effects of site preparation can use CO_2 flux as a useful measure of overall site GWP and soil-atmosphere C flux. However, for a complete understanding of GWP, CH_4 and N_2O flux rates should be considered particularly when using interventions that can disproportionality alter the flux rate of these gases, such as fertilization of wet soil.

2.4.6 Site preparation and fertilization effects on bacterial and fungal abundance and community structure

Unmounded plots had higher CO_2 flux rates than mounded plots, possibly due to higher total C, intact forest floors and higher water content, which could be correlated to shifts in the bacterial and fungal communities at ALRF (Figure 2.8, Table 2.7). Fungal community shifts at ALRF as a result of mounding

are implicated in decreased CO_2 fluxes from soil, as were total soil C removal and alterations to soil water content in mounds and hollows. Forest floors were intact following drainage, and the major delineation between bacterial and fungal communities at SDT was between forest floors and mineral soil. The microbial community structure data are from a single date and therefore do not capture potentially-important temporal fluctuations. Further work is required to determine if the treatment differences shown in this study are consistent or transient. Fungal enzyme activity and ITS pyro-sequencing showed clear forest-floor specific fungal community functioning and structure between forest floors and mineral soil in a *Quercus petraea* forest soil in the Czech Republic (Voříšková et al., 2014). However, Chow et al. (2002) did not report reduction of bacterial 16S diversity following forest floor removal at *Pinus contorta* sites throughout B.C.

The effects of fertilization on the microbial community are complex and appear to depend greatly on the magnitude of N application, litter quality, stand age and the characteristics of the microbial community (Allison et al., 2010; Janssens et al., 2010). Fertilization had inconsistent effects on bacterial or fungal community structure in this study, with fertilization not significantly altering community structure of bacteria at ALRF or fungi at SDT, and had no statistical effect on bacterial abundance or T-RF diversity. This is in contrast to results from Hallin et al. (2009) that showed significantly different bacterial 16S T-RFs in a clay-loam Eutric Cambisol agricultural soil following application of a variety of mineral fertilizers at 80 kg N ha^{-1}. In a temperate hardwood forest dominated by *Quercus velutina* and *Quercus rubra* and in a *Pinus resinosa* forest fertilization with up to 150 kg ha^{-1} as NH_4NO_3 altered microbial community catabolic response profiles and the suppression of fungal ligninolytic enzyme activity (Frey et al., 2004). Allison et al. (2010) also showed a clear separation between fungal OTUs between fertilized and nonfertilized Alaskan black spruce forest soil recovering from fire. Fungi in the mineral soil at ALRF, though lower in abundance than in the forest floor, were more affected by fertilization, which significantly increased fungal ITS abundance (Figure 2.6). This increase in fungal abundance was associated with increased NH_4-N concentration and fungal OTUs from fertilized mineral soil (Figure 2.8b). The increase in fungal abundance (Figure 2.6) while CO_2 fluxes were generally decreased by fertilization (Figure 2.4) is reflected in the finding of Kaštovská et al. (2010), which showed that fertilization of a Czech grassland soil initially increased microbial biomass while decreasing CO_2 fluxes. Following fertilization with urea-N, decomposition is severely retarded by a decrease in N-releasing enzyme production (Allison, 2005). OM degradation via extracellular oxidative enzymes is therefore likely an N scavenging strategy. The recalcitrance of soil OM pools appears to be the result of a stoichiometric C:N balance resulting in a community-wide strategy of suppression of oxidative enzyme production in favor of N-mineralization enzyme production (e.g., n-acetylglucosamine, proteases, urease). Thus, the fungal community under N fertilization can increase in size as nutrient limitation is alleviated,

with net CO_2 fluxes decreasing decomposition of recalcitrant organic material is no longer required to meet N needs. However, the community size is expected to fall once labile C sources are used up, requiring community and physiological shifts towards the initial state. Since fungal community structure was only measured on one sampling date in this study it remains to be seen how resilient the fungal community is to fertilization-driven changes.

Partitioning bacterial community structure into sources of unique variation indicate that treatment explained the highest proportion of unique variation in the OTU distribution, showing that there is a clear relationship between site preparation and fertilization, the effect on the soil environment and the community structure of bacteria (Figure 2.9a). Site accounted for little variation in this model, indicating that OTU presence is mediated more by niche availability in this model than by site limiations such as dispersal or broad climactic patterns. In contrast, site was the main source of fungal ITS OTU distribution, suggesting these site-specific mechanisms were important determinants of fungal community structure. For both groups of organisms, the overlap between categorical sources of variation was the largest proportion of overall variation. This study demonstrates that bacterial and fungal community structure is altered by site preparation, though variably by fertilization, and that these shifts can be linked to forest floor-specific OTUS and to changes in total C, soil water content and CO_2 fluxes. Though for fungal comminty structure these shifts are mediated by site on which these treatments are conducted.

2.5 Conclusions

This study met its objectives by quantifying the physico-chemical response of forest soil to mounding, drainage and fertilization. Gravimetric soil moisture was reduced 15-20% in the mound tops and 20% in the drained plots, supporting hypotheses i. Hypothesis iia was only partially supported for mounding at ALRF as mounding decreased total C, total N and total S at ALRF overall, including in mineral soil, suggesting that organic material mixed into the mounds was too deep to increase concentrations in the rooting zone. Hypothesis iib was not supported as drainage increased total C, total N and total S in both organic and mineral soil at SDT, suggesting that any stimulation of decomposition by drainage is offset by increased biomass litter entry into the soil. Fertilization increased NH_4-N, NO_3-N and SO_4-S concentrations at both sites, supporting hypothesis iii. CO_2 fluxes were reduced by mounding and increased by drainage at ALRF and SDT, respectively, at a single sampling date each (Figure 2.2), supporting hypothesis iv for drainage, but not for mounding. Mounding shifted bacterial and fungal community structure and reduced OTU diversity in plots where forest floors were removed or reduced, supporting hypothesis v. Drainage shifted bacterial and fungal community structure but did not reduce T-RF diversity, suggesting that aerobic OTUs become dominant in post-drainage soil, supporting hypothesis

v. There existed distinct T-RFs related to forest floor and mineral soil at both sites (Figure 2.6). Fertilizer had inconsistent effects on bacterial and fungal community structure between ALRF and SDT and but did not reduce T-RF diversity at any site, suggesting either that decomposers were unaffected by fertilization, or that communities responding to fertilization largely replaced those that were suppressed by abundant mineral N. In wet forests, the incorporation of forest floor into the soil during mounding can improve root nutrition for seedlings. Yet this study demonstrated that mounding can reduce total C and N due to the deep mixing of the forest floors, leading to potential nutrient deficiencies in the rooting zone. In addition, the potential for desiccation of mounds during peak summer temperatures, means seedling mortality may be heightened instead of relieved by mounding. Therefore, I recommend mounding for sites where moisture constraints are compounded by low soil temperatures and competition from vegetation, and in sites with high clay content and poor drainage potential, or slopes where ditch drainage is not viable. Thorough soil layer mixing should be a goal of mounding treatments to increase nutrients in the rooting zone, and operational mounds should be inspected for proper forest floor dispersal through the mound. Fertilization may be necessary to ameliorate the effects of mounding on nutrient availability. However, prudence in the use of fertilizers is needed as N fertilization may increase GHG emissions from the site (See Chapters 3 and 4). While drainage did not reduce soil moisture to the same levels seen in mounding trials, the resulting soil aeration has been shown to be enough to improve tree biomass accumulation in previous work. Drainage conserved and even enhanced soil C and N concentrations. Therefore, drainage may be a viable method of improving site productivity while enhancing site C sequestration. The use of mechanical site preparation and fertilization can optimize planting sites for the regeneration of economically important tree species on sites that would otherwise be subject to paludification, though these practices can lead to shifts in GHG fluxes, soil phyisco-chemical properties and soil microbial community structure.

Chapter 3. Effect of mounding, drainage and fertilization on methane fluxes and functional genes in wet forest ecosystems

3.1 Introduction

Elevated atmospheric concentrations of greenhouse gases (GHGs) are of major concern worldwide. Carbon dioxide (CO_2) and methane (CH_4) are the two most important GHGs in terms of radiative climate forcing, and their concentrations have increased by about 36% and 150% in the last two centuries to about 380 ppm and 1780 ppb, respectively (Forster et al., 2007). CH_4 has a 100-year global warming potential (GWP) about 34 times that of CO_2 (Myhre et al., 2013). Exchange of CO_2 and CH_4 with forest soil is a major component of the global carbon (C) cycle (Raich and Schlesinger, 1992; Raich and Potter, 1995; Reeburg, 1996; Watson et al., 2001; Pan et al., 2011). Boreal (1372 Mha) and temperate forests (1038 Mha) contain 272 and 119 Pg C in total, and are sequestering an additional 0.5 and 0.72 Pg C yr^{-1} respectively, of which 65% and 49% is stored in soil (Pan et al., 2011). Forest site preparation such as drainage (Laiho and Finér, 1996; Laiho and Laine, 1997; Laiho et al., 2004), mounding (Örlander et al,. 1990; Stathers et al., 1990; Sutton, 1993; Ryans and Sutherland, 2001; Löf et al., 2012) as well as nitrogen (N) fertilization (Weetman et al. 1988, 1989; Omule, 1990; McDonald et al., 1994; Swift and Brockley, 1994; Mitchell et al., 1996; Yang 1998; Canary et al., 2000; Kishchuk et al. 2002; Brockley and Simpson 2004; Brockley, 2005, 2006; Negrave et al., 2007) are used to enhance seedling establishment and growth in wet forest ecosystems, and can increase site C sequestration through the accumulation of aboveground biomass and/or soil C (Laiho and Finér, 1996; Sims and Baldwin, 1996; Laiho and Laine, 1997; Macdonald and Yin, 1999; Canary et al., 2000; Smolander et al., 2000; Johnson and Curtis, 2001; Oren et al., 2001; Bond-Lamberty et al., 2002; Hargreaves et al., 2003; Choi et al., 2007; Jandl et al., 2007; Negrave et al., 2007; Pregitzer et al., 2008; Blaško et al., 2013). However, site C sequestration due to site preparation and fertilization could be offset by increasing GHG fluxes through alterations to the microbial community (Jandl et al., 2007; Allison et al., 2010; Mojeremane et al., 2012). This study examines the microbial communities involved in CO_2 and CH_4 fluxes in wet forest soil ecosystems.

Sources of soil-to-surface CO_2 efflux in forests include heterotrophic respiration, the biological oxidation of soil organic C (SOC) by microorganisms, as well as autotrophic respiration, the oxidation of photosynthesis-derived C compounds partitioned between actual root respiration and respiration by ectomycorrhizal fungi and other rhizospheric organisms (Subke et al., 2011). In drained forest soil autotrophic respiration can account for up to 50% of total soil respiration (Silvola et al., 1996a). Environmental conditions affecting CO_2 efflux are complex and include abiotic (temperature and

moisture) and biotic factors (tree species, stand age, amount and quality of litter, root growth and exudation rates) (Raich, 1992; Bowden et al., 1993; Bauhus et al., 1998; Subke and Bahn, 2010).

Methanogens from phylum *Euyarchaeota* produce nearly all biogenic CH_4 using a variety of metabolic pathways, though methanogenesis in terrestrial ecosystems is primarily hydrogenotrophic, the reduction of CO_2 with H_2, or acetoclastic, the reduction of acetate (Thauer et al., 1989; Thauer, 1998; Conrad, 1999, 2005). Molecular characterization of methanogens target the *mcrA* gene, which encodes the methyl coenzyme M reductase enzyme common to all known methanogensis pathways (Luton et al., 2002). Acetoclastic methanogensis is responsible for about two-thirds of methane production in soil, though some species of acetoclastic methanogens can utilize multiple substrate pathways, e.g., archaea from the genus *Methanosarcina* that are abundant in upland forest soil (Le Mer and Roger, 2001; Conrad, 2005). Studies of methanogen community structure, including *mcrA* and 16S rRNA characterization, suggest that both hydrogenotrophic methanogens, such as those from the genus *Methanobacterium* (Kanokratana et al., 2011) and aceticlastic methanogens such as those from the genus *Methanomicrobium* (Kemnitz et al., 2004; Frey et al., 2011) are common in waterlogged forest soil. Methanogen diversity, methanogenesis and net CH_4 fluxes in forest soil are weakly but positively influenced by soil temperature (Fey and Conrad, 2000; Krause et al., 2013), and strongly and positively correlated with soil water content (Ullah et al., 2009; Hartmann et al., 2014). Management practices that change these soil parameters can greatly alter CH_4 flux from forest soil (Fey and Conrad, 2000; Watanabe et al., 2009; Ma et al., 2011; Angel et al., 2012; Hartmann et al., 2014). Methanogensis has very low energy yields ($\Delta G^{o'}$ = -131 and -136 kJ for hydrogenotrophic and acetoclastic pathways, respectively) and generally occurs in soils with very low redox potential, as methanogens are generally out-competed for acetate and protons by other biological reducers, e.g., sulphate-reducing bacteria (SRB) ($\Delta G^{o'}$ = -152.2 kJ) (Thauer et al., 1989; Muyzer and Stams, 2008). It is, therefore, predicted that SO_4-S fertilization of waterlogged soil will stimulate the SRB (characterized using the dissimilatory sulfite reductase β-subunit (*dsrB*) gene) and suppress aceticlastic CH_4 production (Abram and Nedwell, 1978; Thauer et al., 1989; Achtnich et al., 1995; Denier Van Der Gon et al., 2001; Muyzer and Stams, 2008; Ma et al., 2012).

Methane-oxidizing bacteria (MOB) in temperate upland forests soil provide a net sink of atmospheric CH_4 (Adamsen and King, 1993; Dutaur and Verchot, 2007; MacDonald et al., 1996; Krause et al., 2013), and CH_4-uptake in soils can account for between 15 and 45 Tg CH_4 uptake yr^{-1} (Wuebbles and Hayhoe, 2002). The MOB contain either soluble or particulate methane monooxygenase (MMO) enzymes to oxidize CH_4, which are encoded by the *mmoX* and *pmoA* genes, respectively. Nearly all MOB (except genera *Methyloferula* and *Methylocella*) contain *pmoA*, the structure and abundance of which is influenced negatively by soil water content and weakly but positively by soil temperature, pH and forest

type (Dunfield, 2007; Kolb, 2009; Shrestha et al., 2012). The use of molecular markers for methanogens and MOB can elucidate effects of site preparation and management on the organisms driving CH_4 fluxes in forest ecosystems.

Mechanical site preparation (i.e., ditch drainage and excavator mounding) can alter soil moisture and temperature, creating planting sites ideal for economically-important tree species. Drainage leaves soil structure and stand vegetation relatively undisturbed, while mounding can disrupt soil structure, bury forest floor layers and remove competing vegetation (Åkerström and Hånell, 1996; Örlander et al., 1998). Alterations to the soil environment following site preparation can enhance litter decomposition, increase N mineralization and nitrification, increase soil respiration and reduce CH_4 emissions (Martikainen et al., 1995; Lundmark-Thelin and Johansson, 1997; Smolander et al., 2000; Minkkinen et al., 2002; von Arnold et al., 2005b; Mojeremane et al., 2012).

Fertilization can increase soil organic C and N concentrations (Smolander et al., 2000; Johnson and Curtis, 2011; von Arnold et al., 2005a,b; Jandl et al., 2007), and can increase (Hasselquist et al., 2012; Mojeremane et al., 2012) or decrease (Liu and Greaver, 2009; Janssens et al., 2010; Krause et al., 2013) CO_2 fluxes from forest soil. Nitrogen fertilization can increase soil respiration due to stimulation of the decomposer community (Micks et al., 2004; Gallo et al., 2005; Jassal et al., 2011; Hasselquist et al., 2012) or stimulation of fine root growth (Raich et al., 1994; Cleveland and Townsend, 2006). Fertilization can alternatively decrease soil respiration from forest soils, as root growth and decomposition rates are retarded by low availability of inorganic N (Haynes and Gower, 1995; Bowden et al., 2004; Liu and Greaver, 2009; Janssens et al., 2010; Krause et al., 2013). In other studies, no effect of fertilization on soil respiration were observed in stands where added N was rapidly immobilized by microorganisms (Prescott et al., 1993; Chapell et al., 1999; Smolander et al., 2000). Decreases in CH_4 uptake in upland soil or increases in CH_4 emissions from wetland soil following N deposition or fertilization can result from NH_4^+ saturating the binding sites for CH_4 in PMO, suppressing CH_4 oxidation (Butterbach-Bahl et al., 2002; Basiliko et al., 2009; Jassel et al., 2011), possibly due to evolutionary similarities between PMO and ammonium monooxygenase (AMO) enzymes from nitrifying bacteria (Bédard and Knowles, 1989; Holmes et al., 1995; Purkhold et al., 2000; Bodelier and Laanbroek, 2004). Nitrogen fertilization has been shown to increase CH_4 flux rates as a result of decreased CH_4 uptake (Castro et al., 1994; Liu and Greaver, 2009; Mojeremane et al., 2012), suggesting that the effect of N addition to forest soils on CH_4 flux is not yet fully understood (Gundersen et al., 2012). Characterization of the soil environment and microbial community in a variety of forest ecosystems can identify the underlying causes of CO_2 and CH_4 flux differences following site preparation and N fertilization.

This study used quantitative PCR (qPCR) of the 16S rRNA, *mcrA*, *pmoA* and *dsrB* genes to estimate the response of the total bacterial, methanogen, MOB and SRB communities respectively in two regenerating wet forests subject to mounding, drainage and fertilization. We attempt to link these functional groups to CO_2 and CH_4 fluxes measured using static closed chambers to better understand the importance of the microbial community in determining GHG fluxes from managed forests. Hypotheses tested were: i) mounding will decrease CH_4 fluxes, but waterlogged hollows can act as hot-spots of methanogensis, ii) drainage will reduce CH_4 fluxes and methanogen genes, iii) aerobic methanotrophs will be more abundant in forest floors and anaerobic methanogens and SRB will be more abundant in mineral soil, iv) fertilization will decrease CH_4 fluxes and methanogens due to SO_4-S, but also methanotrophs due to N, v) soil moisture will be the primary factor influencing CH_4 fluxes and microbial functional gene abundances.

3.2 Materials and methods

3.2.1 Field sites

The effects of fertilization and site preparation (mounding and drainage) on GHG flux, and on the abundance of methanogenic archaea, MOB and SRB was investigated at two wet forest sites in British Columbia (B.C.), Canada. Field site descriptions are provided in Chapter 2. Briefly, mounding treatments were installed at Aleza Lake Research Forest (ALRF), near Prince George, B.C. The ALRF installation is located in the wk1 variant of the sub-boreal spruce (SBS) biogeoclimatic zone. Soils at ALRF are Orthic Gleyed Luvisols, Orthic Luvic Gleysols and Ortho Humo-Ferric Podzols. The 70-year-old second-growth stand of interior hybrid spruce (*Picea engelmannii* x *glauca*) and subalpine fir (*Abies lasiocarpa*) were harvested in Feburary 2011 and replanted with interior hybrid spruce on June 6, 2012. Excavator mounding took place in August 22, 2011. Fertilizer was applied at a final formulation of 200 kg N, 100 kg P, 100 kg K, and 50 kg S ha^{-1} on June 26, 2012. Treatment plots were organized in a complete-block design, with two blocks containing each of the four treatments (unmounded/unfertilized, unmounded/fertilized, mounded/unfertilized, mounded/fertilized). In mounded plots, the tops of mounds as well as the hollows were sampled. Soil sampling for functional gene characterization took place on June 23, 2011 (Jun-11), June 28, 2012 (Jun-12), July 17, 2012 (Jul-12), August 24, 2012 (Aug-12), October 18, 2012 (Oct-12) and June 13, 2013 (Jun-13). Sampling of CH_4 fluxes took place Jun-12, Jul-12, Aug-12 and Jul-13). Sampling for soil chemistry took place Jun-12, Jul-12, Aug-12, Oct-12 and Jun-13.

The Suquash Drainage Trial (SDT) is located near the Salal Cedar Hemlock Integrated Research Program (SCHIRP) research site installed by Western Forest Products Inc. between the towns of Port

Hardy and Port McNeill on northern Vancouver Island, B.C. The SDT site is located in the vm1 subzone of the Submontane Very Wet Maritime Coastal Western Hemlock ecozone (CWHvm1). (Green and Klinka 1994). Soil is Humo-Ferric Podzols with mor humus. The original 22-ha western redcedar (*Thuja plicata*) and shore pine (*Pinus contorta* var. *contorta*) stand was harvested and slash-burned in 1993 and 1994, respectively, and planted with western redcedar (*Thuja plicata*) in 1995. Three 120 m x 45 m treatment plots containing five drainage ditches from a 1997 installation were used in this study. Operational fertilization of 225 kg N and 75 kg P ha^{-1} was conducted in 2006. Undrained control plots were selected at least 60 m away from each ditched area to avoid the effects of ditching on subsurface drainage, which extended 15 m from each drainage ditch (van Niejenhuis and Barker, 2002). One of two 30 x 10 m transects in each drained or undrained plot was fertilized on July 25, 2012 using the same formulation used in ALRF. Plots were organized in complete-block design and included the following treatments: drained/fertilized, undrained/fertilized, drained/unfertilized and undrained/unfertilized. Soil sampling at SDT for microbial gene analysis took place on July 27, 2012 (Jul-12), August 29, 2012 (Aug-12), October 25, 2012 (Oct-12), July 3, 2013 (Jul-13) and September 12, 2013 (Sep-13). GHG measurements did not occur in Oct-12 and soil chemical factors were not measured in Sept-13 (See Appendix D for full sampling dates).

3.2.2 Soil sampling and preparation

At ALRF, three 10-cm-deep sub-samples of soil were removed with a 5-cm-diameter soil core in each of the two plots per treatment. Soil from control plots comprised the organic forest floor F and H layers (C_o) and the mineral A and B horizons (C_m). Mounding plots did not contain a forest floor layer so the top 10 cm of mineral-forest floor mix were pooled into a single sample from either mound tops (M) or mound hollows (H). At SDT two sub-samples of soil in each of the three plots per treatment were removed with the same soil core, and also divided into organic and mineral fractions. Locations of gene abundance estimation were control organic (C_o), control mineral (C_m), drained organic (D_o) and drained mineral (D_m). Volumetric soil moisture was measured using a TH_2O^{TM} portable moisture probe (Dynamax Inc., Houston, U.S.A.) and gravitational soil moisture was measured by oven drying field moist soil. Field soil was homogenized and partitioned for DNA extraction and soil chemical analysis. Results of Total C, Total N, Total S, NO_3-N, NH_4-N, SO_4-S and pH analysis are described in Chapter 2.

3.2.3 Field measurement and gas chromatography analysis of CH_4 fluxes

In situ GHG fluxes at ALRF and SDT were measured as described in Basiliko et al. (2009) and in Chapter 2. Briefly, three closed PVC chambers were installed on collars buried about 5 cm in the soil in each of two treatment plots at ALRF, and two chambers were installed in each of three treatment plots at SDT. Prior to chamber headspace sampling, 6 ml of air was inserted and the headspace mixed by plunging a 20 ml plastic syringe three times. Six ml of chamber headspace were removed and inserted into pre-evacuated 5 ml Exetainers® (Labco Ltd., Lampeter, UK) every 15 minutes for one hour. Gas samples were measured on an Agilent 5890 series II gas chromatograph (Agilent Technologies, Santa Clara, U.S.A.) equipped with a flame ionisation detector (FID) set at 300°C. The FID carrier gas was helium with a flow rate of 14 ml min^{-1}. Standards for gas chromatography used 4, 2, 1 and 0.67 ppm CH_4. Standard curves were constructed with simple linear regression.

3.2.4 Nucleic acid extraction and quantitative PCR

Sub-samples of 0.25 g dry soil were removed from each sample for DNA extraction. DNA was extracted from using the MoBio PowerClean soil DNA isolation kit. DNA concentrations were calculated with spectrophotometry of fluorescence emission using the Quant-iT™ PicoGreen® dsDNA assay (Life Technologies Corp., Carlsbad, U.S.A.).

All qPCR was carried out in 20 µl reactions with 1 µl of template DNA added to a 19 µl qPCR mixture containing 10 µl Power SYBR® Green PCR Master Mix (Life Technologies Corp., Carlsbad, U.S.A.). Bovine serum albumin (BSA, 200 ng μl^{-1}) was added to increase PCR efficiency. Reactions were carried out with an Applied Biosystems® StepOnePlus™ real-time PCR system using 10x dilutions of soil DNA extracts to reduce PCR-inhibiting humic substances. Gene copy numbers were expressed as copy number g^{-1} soil (dry weight (dw)).

McrA qPCR forward (ML-F, 5'- GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and reverse (ML-R, 5'- TTCATTGCRTAGTTWGGRTAGTT-3') primers (Luton et al., 2002) were each added at 0.5 µM. qPCR was modified from Freitag et al. (2010) using an initial denaturation step of 5 min at 95°C and 40 cycles of 95°C denaturation for 30 s, 56°C annealing for 45 s and 68°C extension for 45 s, followed by fluorescence quantification at the end of a 82°C step for 10 s. Standard curves for calibration of *mcrA* qPCR were created using triplicate 10-fold dilutions from 10^3 to 10^8 copies of a 420-bp *mcrA* amplicon from *Methanolinea mesophila*, amplified from soil near the SDT site. Briefly, *mcrA* sequences from waterlogged soil with positive methane flux near SDT were cloned and sequenced. The dominant sequences of *mcrA* aligned with *M. mesophila*, a hydrogenotrophic methanogen. Primer

sequences (Mm-mcra-245-f: AGATCTGGCTCGGCTCCTAC; Mm-mcra-743-r: TAGTTGGGTCCACGGAGTTC) outside of the ML-F and ML-R region were aligned within the M. *mesophila mcrA* gene sequence (AB496719) using PRIMER3 (Untergrasser et al., 2012) software. The sequence resulting from amplification with the *M. mesophila mcrA* primers and used for the *mcrA* standard curve was deposited in NCBI GenBank (ascension: KF306340).

PmoA qPCR used forward (A189F, 5;-GGNGACTGGGACTTCTGG-3') and reverse (Mb661r, 5'-GGTAARGACGTTGCNCCGG-3') primers from Bourne et al. (2001) using a protocol modified from Freitag et al. (2010) using primer concentrations of 0.5 µM each. The qPCR run included an initial denaturation step of 5 min at 95^{o}C and 40 cycles of 95^{o}C denaturation for 30 s, 64^{o}C annealing for 45 s, 68^{o}C extension for 45 s, followed by fluorescence quantification at the end of a 86.5^{o}C step for 16 s. Standard curves for *pmoA* were created using triplicate 10-fold dilutions from 10^3 to 10^8 copies of *Methylococcus capsulatus* genomic DNA.

DsrB qPCR used forward (Dsr2060f, 5′-CAACATCGTYCAYACCCAGGG-3′) and reverse (Dsr4r, 5′-GTGTAGCAGTTACCGCA-3′) primers from Geets et al. (2006) at concentrations of 0.5 µM each. QPCR included an initial denaturation of 5 min at 95^{o}C and 40 cycles of 95^{o}C denaturation for 30 s, 55^{o}C annealing for 45 s, 72^{o}C extension for 45 s, after which fluorescence was measured. Standard curves for *dsrB* were created using triplicate 10-fold dilutions from 10^2 to 10^8 copies of *Desulfosporosinus orientis* and *Desulfomicrobium baculatum* genomic DNA.

3.2.5 Statistical analysis

Statistical analysis was performed using in R v. 2.15.3 (R Core Team, 2013). For parametric tests, data were tested or normality using Q-Q plots and the Shapiro–Wilk test. Homoscedasticity was tested using Levene's test. CH_4 data were fitted with the linear mixed-effects model and subject to two-factor ANOVA (main effects: mounding/drainage × fertilization). Gene abundance data were subject to multi-factor fractional factorial analysis of variance (ANOVA) using the *lme* and *Anova* functions in the *nlme* and car packages, respectively, in order to test treatment effects. Single-factor ANOVA was performed using the *aov* function in R with Tukey's honestly significant difference test to determine significance of sampling location. The *lme* function used fertilization, mounding /drainage and soil layer as fixed effects and blocking as a random effect. qPCR data were analyzed as $\log_{10}$ values. CH_4 data were logarithmically transformed prior to ANOVA. Data was visualized using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). qPCR data were presented graphically using 25%-75% quartile boxplots. Unconstrained, exploratory ordination was carried out using principal component analysis (PCA) on scaled parameters

with the *prcomp* function for ordination by singular value decomposition. The *FactoMineR* package for R was used to calculate variable coordinates within this ordination, including treatment variables imposed on the ordination as constrained dummy variables. The *prcomp* and *FactoMineR pca* functions differ in how coordinates are calculated: *prcomp* normalized using *n* in the denominator while *pca* uses *n*-1. Therefore *prcomp* coordinates were adjusted to match the output of the *pca* function. PCA plots were visualized using the *ggbiplot* package in R. Constrained ordination using redundancy analysis (RDA) was used to investigate relationships between biotic and abiotic soil parameters with the *rda* function in *vegan* for R. Forward selection of significant soil parameters was performed using permutational testing with 1000 permutations. Principal coordinate of a neighbour matrix (PCNM) analysis (Borcard and Legendre, 2002; Borcard et al., 2004) was used to generate variables for spatial structure using the *PCNM* function in the *PCNM* package in R. The geographic locations of sample sites were transformed to Cartesian coordinates using *geoXY* function in the *SoDA* package in R prior to PCNM. PCNM variables were tested using Moran's I statistic to select significant and positive variables, which were then forward selected against spatially detrended dependent variables or matrices using permutational testing with 1000 permutations of the reduced model, ensuring that R^2-adjusted of the forward-selected models did not exceed the R^2-adjusted of the non-selected models. Canonical variation partitioning using RDA (Borcard et al., 1992; Ramette and Tiedje, 2007; Bru et al., 2011) was carried out to allocate dependent variable or matrix variance uniquely explained by each soil parameter, or groupings of parameters, by constraining linear partial regression by all other variables. Venn diagrams for variation partitioning were created using the *venneuler* package in R.

3.3 Results

3.3.1 CH_4 flux

One-third of chambers measured for CH_4 flux in Jun-12 at ALRF displayed CH_4 uptake in these samples, including 100% of chambers in the top of unfertilized mounded plots (Figure 3.1a). However, high rates of CH_4 efflux were measured in Jul-12. The wettest areas, which were the hollows in the mounded plots, had the greatest efflux measured during this study, with rates of 422.4 ± 176.6 and 1171.3 ± 368.8 μg CH_4 m^{-2} h^{-1} in unfertilized and fertilized plots, respectively. CH_4 emissions measured in chambers from fertilized mounded plots were significantly higher than those from chambers installed in unmounded and the tops of mounded plots. In contrast to the high emission rates observed in Jul-12, 79% of chambers in the driest month (Aug-12) demonstrated CH_4 uptake. Unfertilized mound tops had

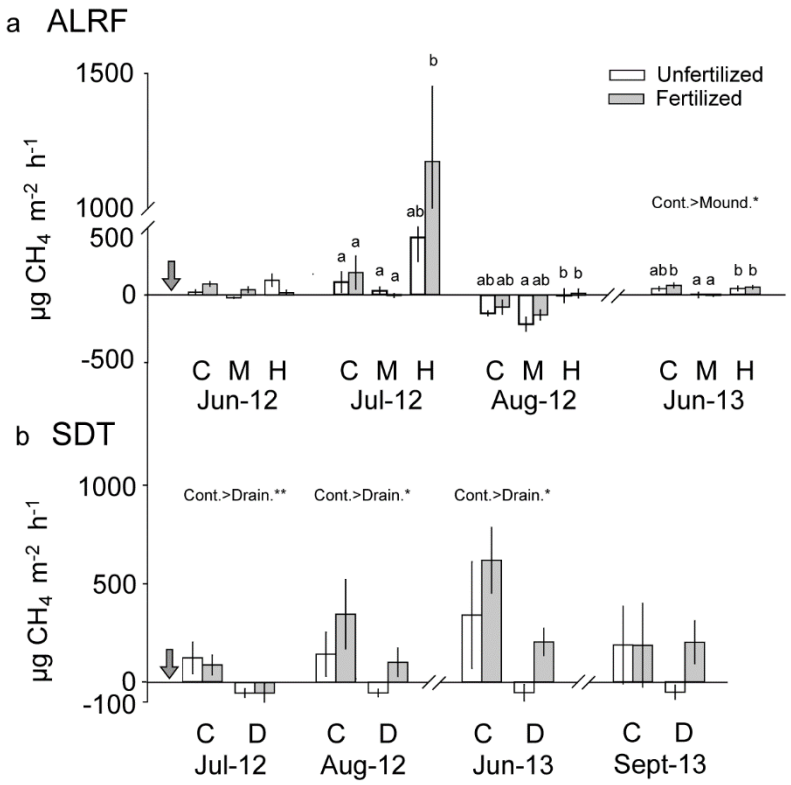

Figure 3.1. CH_4 fluxes emissions from a) undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF) and b) undrained control (C) and drained (D) subject to fertilization at Suquash Drainage Trial (SDT). Shaded arrow shows time of fertilization. Error bars: SEM. N = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA. Treatment effects and interactions at each date following two-way ANOVA (e.g., C > D) are provided if significant (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

significantly greater CH_4 uptake rates than the mound hollows. There was a significant mounding effect only in Jun-13, two years following mounding and one year following fertilization. Control plots had significantly greater CH_4 emission rates than mounded plots. This is likely the result of mound tops being a location of greatest CH_4 uptake, while emissions were measured in water-saturated or near-saturation soil (i.e., mound hollows, unmounded plots). There was consistent CH_4 uptake measured in the drained plots at SDT, particularly in chambers located in unfertilized plots (Figure 3.1b). While there was CH_4 uptake in all plots throughout the course of the field measurements, the undrained control plots had significantly greater emissions throughout the experiment (i.e., Jul-12, Aug-12 and Jul-13).

3.3.2 Gene abundance

3.3.2.2 *McrA*

Pre-mounding (Jun-11) *mcrA* abundances were around 10^5 copies g^{-1} soil (dw) (Figure 3.2b). Following mounding and fertilization *mcrA* gene abundance varied considerably at ALRF between samples, with minimum and maximum values between 10^4 and 10^7 log copies g^{-1} (dw), respectively. Between Jun-12 and Aug-12 a non-significant trend of elevated *mcrA* in the bottom of mounded sites was observed, as hollows were water-saturated during these sampling dates. In Oct-12 *mcrA* abundance in the unmounded plots was significantly greater than in mounded sites (Table 3.1), in part due to desiccation of the mounded sites. In Jun-13, as the mounded plots once again became waterlogged, fertilized hollows and unmounded plots had significantly greater *mcrA* abundance than mounds (Figure 3.2b), which contributed to a trend of significantly greater *mcrA* in fertilized plots than in unfertilized plots in Jun-13 (Table 3.1). In Jul-12 *mcrA* copies had a mean of 3.8 ± 0.2 log copies g^{-1} soil (dw) in the undrained fertilized plots at SDT, the lowest recorded during this study (Figure 3.3b). The maximum mean *mcrA* abundance, 6.9 ± 0.4 log copies g^{-1} soil (dw) was observed in Sep-13. A consistent drainage effect on *mcrA* abundance was observed at SDT (Table 3.2). Undrained control plots had significantly greater *mcrA* abundance than drained plots in Aug-12, Oct-12, Jul-13 and Sep-13.

3.3.2.3 *PmoA*

PmoA abundance at ALRF was higher in the organic material in Jun-11, Jun-12 and Aug-12 (Figure 3.2c, Table 3.1). There was a significantly greater abundance of *pmoA* in unmounded plots relative to mounded plots in Oct-12 (Table 3.1). In Aug-12 there was significantly higher *pmoA* abundance in unfertilized organic material relative to fertilized organic soil in unmounded plots (Figure

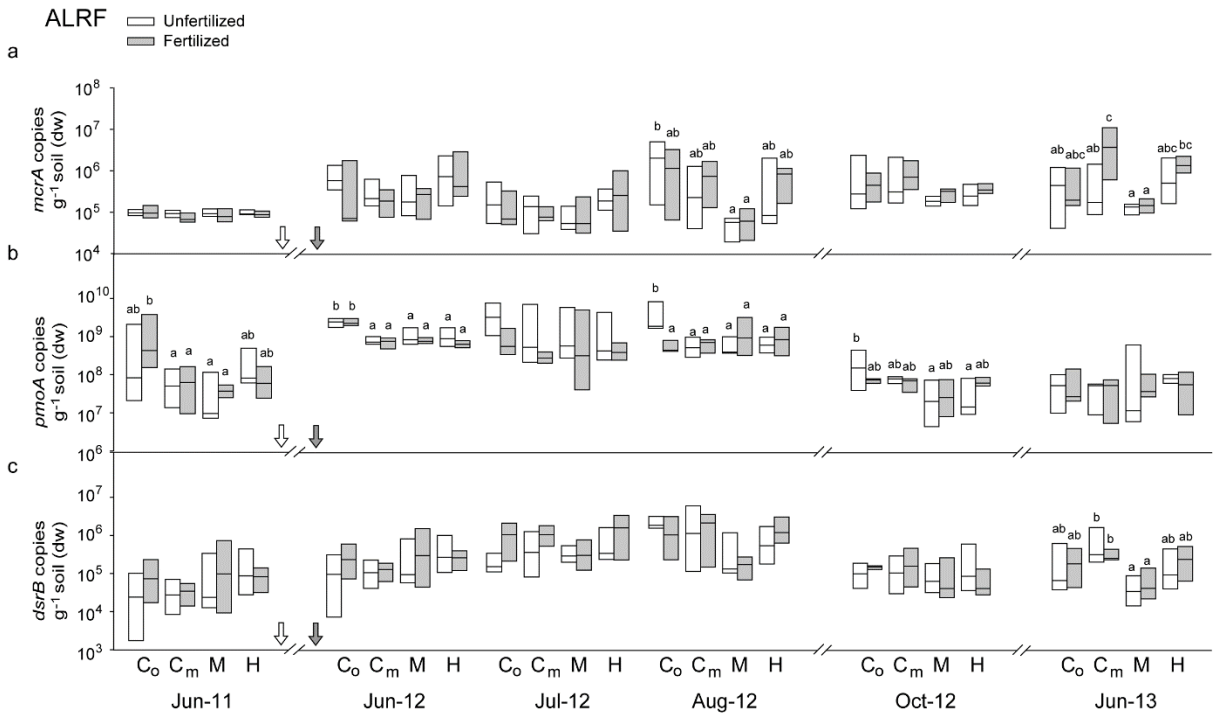

Figure 3.2. Abundance of a) *mcrA* genes, b) *pmoA* genes and c) *dsrB* genes in soil from undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA.

Table 3.1. *F* and *p* statistics following fractional factorial ANOVA on *mcrA*, *pmoA* and *dsrB* gene copy g^{-1} soil (dw) at Aleza Lake Research Forest (ALRF)

Gene	Model term	df	Jun-11		Jun-12		Jul-12		Aug-12		Oct-12		Jun-13	
			F	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*
	Mound.	1	1.0	0.329	0.6	0.435	0.4	0.536	1.9	0.171	**4.3**	**0.044**	2.9	0.094
	Fert.	1	1.6	0.212	0.8	0.380	0.1	0.794	0.2	0.631	1.4	0.248	**4.7**	**0.036**
mcrA	Layer.	1	2.4	0.132	0.9	0.339	0.5	0.481	1.8	0.191	0.4	0.524	3.6	0.065
	M×F	1	0.0	0.996	1.3	0.266	0.3	0.619	0.6	0.429	0.1	0.826	0.9	0.356
	M×F×L	1	1.0	0.316	0.4	0.530	0.1	0.772	1.0	0.314	1.0	0.334	2.2	0.145
	Mound.	1	0.2	0.697	0.1	0.829	0.1	0.809	1.5	0.224	**4.6**	**0.038**	1.5	0.225
	Fert.	1	0.6	0.434	0.4	0.548	3.9	0.055	**4.4**	**0.043**	0.3	0.576	0.1	0.816
pmoA	Layer.	1	**10.5**	**0.002**	**89.7**	**<0.001**	1.5	0.227	**19.5**	**<0.001**	1.9	0.172	0.5	0.473
	M×F	1	0.8	0.378	0.3	0.569	0.1	0.847	**15.3**	**<0.001**	3.8	0.060	0.4	0.529
	M×F×L	1	1.4	0.240	0.1	0.737	0.1	0.841	**29.07**	**<0.001**	0.7	0.457	0.1	0.799
	Mound.	1	2.4	0.129	2.4	0.129	0.3	0.561	1.7	0.201	1.2	0.279	**13.9**	**<0.001**
	Fert.	1	1.3	0.266	1.3	0.266	**5.7**	**0.022**	0.3	0.595	0.0	0.962	0.1	0.717
dsrB	Layer.	1	0.0	0.956	0.0	0.956	2.0	0.168	0.1	0.769	0.0	0.998	**4.2**	**0.047**
	M×F	1	1.0	0.324	1.0	0.324	1.6	0.209	0.4	0.543	3.7	0.062	0.3	0.583
	M×F×L	1	1.3	0.258	1.3	0.258	0.2	0.673	1.7	0.204	0.1	0.836	0.4	0.521

Bolding denotes statistical significance at $p<0.05$

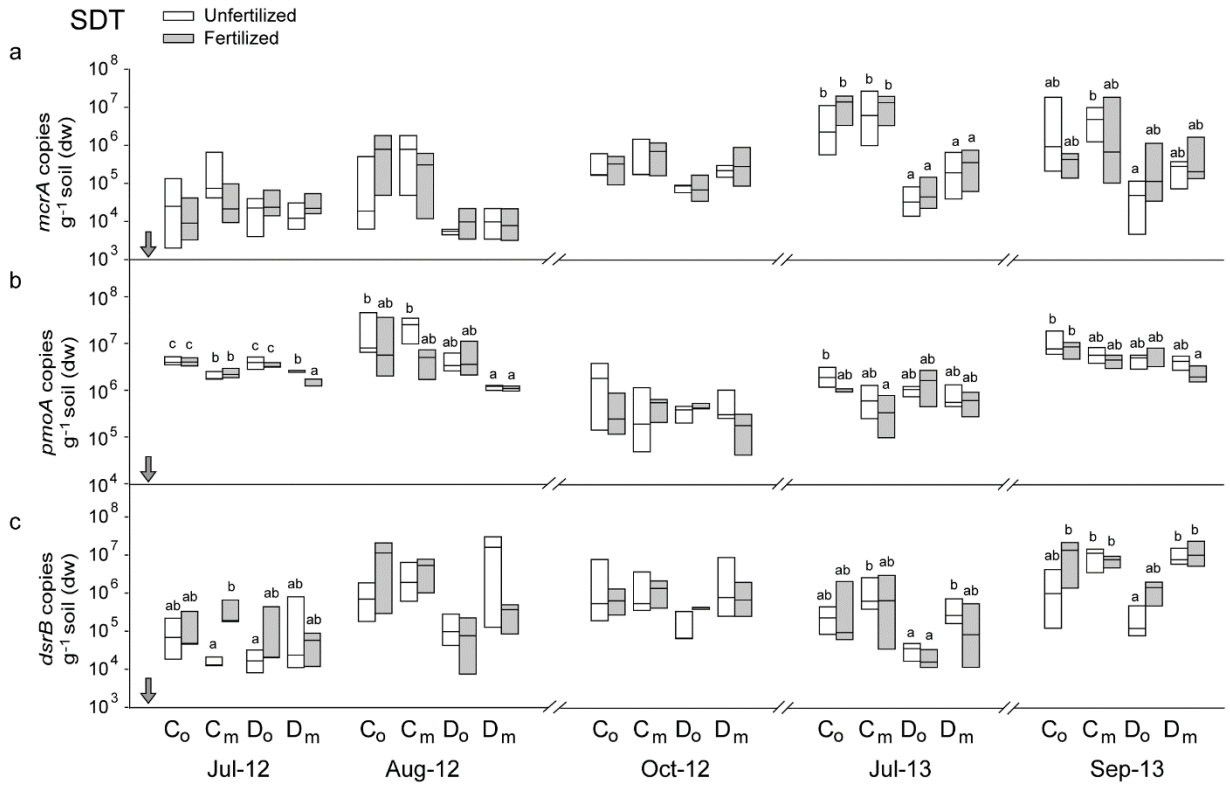

Figure 3.3. Abundance of a) *mcrA* genes, b) *pmoA* genes and c) *dsrB* genes in soil from undrained control (C) and drained (D) subject to fertilization at Suquash Drainage Trial (SDT). Shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA.

Table 3.2. *F* and *p* statistics following fractional factorial ANOVA on bacterial 16S, *mcrA*, *pmoA* and *dsrB* gene copy g^{-1} soil (dw) at Suquash Drainage Trial (SDT)

Gene	Model term	df	Jul-12		Aug-12		Oct-12		Jul-13		Sep-12	
			F	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*
	Drain.	1	0.4	0.537	**20.1**	**<0.001**	**6.9**	**0.015**	31.2	**<0.001**	**11.2**	**0.003**
	Fert.	1	0.1	0.818	1.1	0.307	0.1	0.798	0.0	0.929	0.0	0.997
mcrA	Layer.	1	2.8	0.109	0.0	0.923	**6.1**	**0.021**	1.2	0.294	3.9	0.060
	D×F	1	3.3	0.081	0.0	0.949	0.0	0.867	0.1	0.707	3.0	0.095
	D×F×L	1	1.2	0.332	0.6	0.610	0.5	0.708	0.1	0.974	0.1	0.947
	Drain.	1	1.4	0.241	**24.9**	**<0.001**	0.2	0.698	0.1	0.710	**8.3**	**0.008**
	Fert.	1	2.5	0.129	**4.9**	**0.037**	0.6	0.446	2.1	0.162	1.6	0.225
pmoA	Layer.	1	**265.5**	**<0.001**	**6.7**	**0.016**	1.8	0.194	**13.0**	**0.001**	**8.1**	**0.009**
	D×F	1	**5.3**	**0.030**	**5.1**	**0.033**	0.1	0.713	1.4	0.250	0.0	0.843
	D×F×L	1	1.3	0.301	0.8	0.500	1.4	0.266	0.7	0.542	0.9	0.474
	Drain.	1	2.4	0.138	**8.5**	**0.008**	1.7	0.202	**11.7**	**0.002**	3.3	0.081
	Fert.	1	0.9	0.361	0.2	0.692	0.3	0.611	3.6	0.069	**4.5**	**0.045**
dsrB	Layer.	1	0.1	0.710	**4.6**	**0.042**	3.6	0.069	**7.4**	**0.012**	**29.5**	**<0.001**
	D×F	1	3.5	0.074	4.0	0.058	0.3	0.620	0.6	0.431	0.2	0.669
	D×F×L	1	2.7	0.070	0.8	0.517	1.1	0.380	0.5	0.668	**3.2**	**0.040**

Bolding denotes statistical significance at $p<0.05$

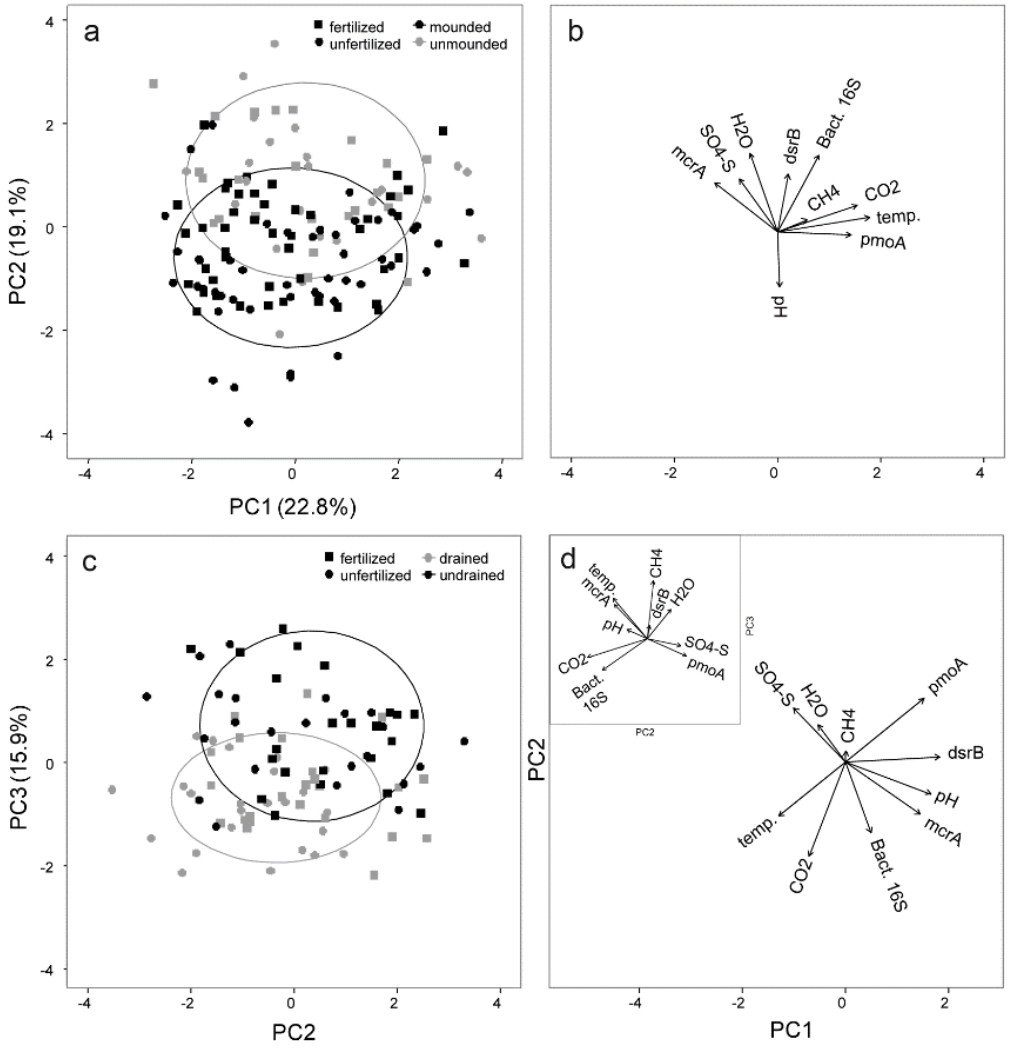

Figure 3.4. Principal component analysis (PCA) of mounding, drainage and fertilization treatments on microbial gene abundance, GHG emission rates and soil characteristics. a) Sample coordinates at Aleza Lake Research Forest (ALRF) by treatment, b) unconstrained loading plot for ALRF samples with treatments and sampling date correlations imposed as constrained supplementary variables`, c) sample coordinates at Suquash Drainage Trial (SDT) by treatment and d) unconstrained loading plot for SDT samples with treatments and sampling date correlations imposed as constrained supplementary variables (blue). Ellipses indicate one standard deviation of mean PCA coordinates grouped by site preparation.

3.2c), leading to a significantly lower *pmoA* abundance due to fertilization overall for this date (Table 3.1). This also led to significant layer effects, low-order interactive effects between mounding and fertilization and high-order interactive effects between all three main effect factors in Aug-12 at ALRF. Seasonal effects related to *pmoA* abundance were observed at SDT (Table 3.4c). Organic material had higher *pmoA* abundance than mineral soil for all dates except Oct-12 (Table 3.2). Control plots had significantly greater *pmoA* abundance than drained plots in Aug-12 and Sep-13. A fertilization effect at SDT was observed in Aug-12 (Table 3.2), with fertilized plots having lower *pmoA* abundance than unfertilized plots (Figure 3.3c). There was also significant interaction between drainage and fertilization in Aug-12.

3.3.2.4 *DsrB*

DsrB genes, found in SRB, were significantly more abundant at ALRF in plots treated with mineral fertilizer (including SO_4-S) in Jul-12 (Figure 3.2d, Table 3.1). A significant effect of mounding on *dsrB* abundance was observed in Jun-13. Mounded plots had significantly lower *dsrB* than unmounded plots. The effect of mounding was due to the low abundance of *dsrB* in the tops of mounds relative to the associated hollows, which had mean *dsrB* log copies g^{-1} soil (dw) of 4.6 ± 0.1 and 5.2 ± 0.2, respectively. The *dsrB* gene ranged in abundance between 10^4 and 10^8 copies g^{-1} soil (dw) at SDT (Figure 3.3d), demonstrating a larger variation and maximum abundance than *dsrB* values from ALRF. Treatment effects related to drainage were observed for *dsrB* abundance. *DsrB* abundance was lower in drained plots relative to undrained plots in Aug-12 and Jul-13 (Table 3.2). In Sept-13, *dsrB* abundance was greater in mineral soil relative to the organic material (Figure 3.3d), leading to a significant layer effect (Table 3.2), as well as being higher in fertilized plots relative to unfertilized plots at this date. This resulted in significant interactions between the three main effect factors in this study (Table 3.2).

3.3.3 Influence of site preparation and fertilization on CH_4 fluxes, soil physico-chemical parameters and functional gene abundance

The overall influence of site preparation and fertilization on microbial gene abundance (*mcrA*, *dsrB*, *pmoA*, bacterial 16S rRNA (See Chapter 2)), CH_4 emissions, soil parameters associated with CH_4 flux and affected by site preparation (CO_2 fluxes, SO_4-S, pH, water content, temperature (see Chapter 2)) was investigated using unconstrained ordination with PCA (Figure 3.4). PCA of ALRF samples accounted for 22.8% of variation along the first principal component (PC1) and 19.1% of variation along PC2 (Figure 3.4a). There was no significant separation of sample coordinates based on site preparation or

fertilization status, though mounded and unmounded sample coordinates were distinct but overlapping along PC2. The loading plot for the ALRF PCA showed that soil physico-chemical parameters separated samples along the second PC, along which with soil water content and pH were negatively correlated (Figure 3.4b). The mounding and fertilization treatments, as well as sample month, were encoded as a "dummy" numerical variables and added to the PCA as constrained supplementary variables, which did not affect the original ordination. Of these, mounding was negatively associated with PC2. Temperature was the dominant soil factor correlating with PC1, and was also correlated to CO_2 flux, bacterial 16S rRNA abundance and *pmoA* abundance.

Following PCA of the GHG flux, soil parameters and functional genes from SDT PC1 explained 26.5% of the variation while PC2 explained 20.1% of the dataset variation (Figure 3.4c). Site prepration and fertilization did not lead to significantly different sample coordiants based on the measured soil, gas and gene parameters. The abundance of *mcrA*, *pmoA* and *dsrB* were loaded towards the positive coordinates on PC1 (Figure 3.4d), which was associated with drained samples. Soil pH clusted with functional gene abundance measures on PC1. PC2 was associated positively with bacterial 16s rRNA abundance and CO_2 flux, and negatively with the fertilization treatment.

3.3.4 Relationship between soil physico-chemical parameters, CH_4 fluxes, functional gene abundance and spatial structure

Exploratory PCA analysis indicated that samples from ALRF and SDT did not significantly differ when grouped by site preparation and fertilization treatments, though functional genes, GHG flux and chemical, physical and climactic soil parameters were shown to be correlated (Figure 3.4). At ALRF, the abundance of the *mcrA* gene was positively correlated with CH_4 flux, CO_2 flux, SO_4-S concentration, soil water content, soil temperature and *pmoA* gene abundance (Pearson correlation coefficients for variables used in PCA of ALRF samples can be found in Table 3.3a). The abundance of *pmoA* genes was correlated with temperature and CO_2 flux. The *dsrB* gene was positively correlated with temperature. CO_2 flux was positively correlated with total bacterial 16S, *pmoA* abundance, soil temperature and CH_4 flux.

At SDT, *mcrA* abundance correlated positively with *dsrB* abundance, CH_4 emissions and pH (Pearson correlation coefficients for variables used in PCA of SDT samples can be found in Table 3.3b). The abundance of *pmoA* abundance was positively correlated to *dsrB* abundance and temperature, pH and CH_4 flux. CH_4 was also positively correlated with abundance and soil moisture. CO_2 flux was positively correlated with bacterial 16S, *dsrB* and temperature. Soil pH was negatively correlated with soil water content and SO_4-S.

Table 3.3a. Pearson correlation coefficients between measured variables at Aleza Lake Research Forest (ALRF)

	Microbial genes				GHG emissions		Soil characteristics			Climate
	mcrA	*pmoA*	*dsrB*	Bact.16S	CO_2	CH_4	SO_4-S	pH	H_2O	Temp.
mcrA		*			*	*	***		***	***
pmoA	-0.26				***					
dsrB	0.09	-0.05		*			*			***
Bact.16S	0.01	-0.04	0.25		***			***		*
CO_2	-0.20	0.32	-0.14	0.50		*	*			***
CH_4	0.21	0.18	0.01	0.09	0.20					
SO_4-S	0.31	0.05	0.22	0.02	-0.19	-0.06			***	***
pH	-0.01	0.15	-0.13	-0.39	-0.04	0.13	-0.01			
H_2O	0.41	0.00	0.14	0.19	0.06	0.00	0.37	-0.16		
Temp.	-0.34	0.58	0.37	0.20	0.38	0.15	-0.10	0.05	-0.11	-0.11

(*, p<0.05, **, p<0.01, ***, p<0.001)

Table 3.3b. Pearson correlations between measured variables at Suquash Drainage Trial (SDT).

	Microbial genes				GHG emissions		Soil characteristics			Climate
	mcrA	*pmoA*	*dsrB*	Bact.16S	CO_2	CH_4	SO_4-S	pH	H_2O	Temp.
mcrA			***			***	**	***		
pmoA	0.03		**	***		**		**		***
dsrB	0.61	0.26			**			***		**
Bact.16S	0.09	0.38	0.01		***	*			**	***
CO_2	-0.01	0.01	-0.25	0.67		*				***
CH_4	0.32	-0.28	0.13	-0.21	-0.21				***	
SO_4-S	-0.25	-0.03	0.14	-0.06	-0.10	0.09		-0.43		
pH	0.48	0.27	0.32	0.13	-0.03	0.02	-0.43		*	
H_2O	0.18	-0.12	-0.02	-0.25	-0.15	0.42	0.05	-0.18		
Temp.	-0.05	0.33	-0.28	0.55	0.52	-0.13	-0.01	-0.03	-0.14	

(*, p<0.05, **, p<0.01, ***, p<0.001)

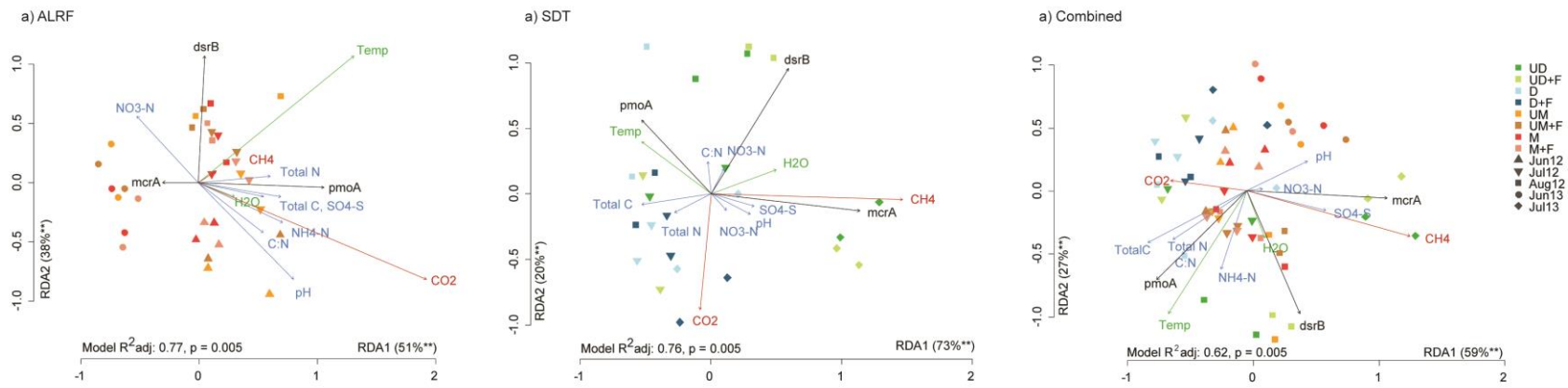

Figure 3.5. Redundancy analysis (RDA) of *mcrA*, *pmoA* and *dsrB* gene abundance (black vectors) constrained by soil physical (green) and chemistry (blue) factors, with CO_2 and CH_4 flux rates fit to model (red) for a) Aleza Lake Research Forest (ALRF), b) Suquash Drainage Trial (SDT) and c) combined ALRF and SDT measurements. Model and axis significance determined using Monte-Carlo permutation tests.

RDA was used to test the relationships between the microbial community associated with CH_4 fluxes and soil physico-chemical parameters (Figure 3.5). Models were developed by constraining the abundance of genes quantified in this study (*mcrA*, *pmoA* and *dsrB*) to a matrix of soil parameters and forward-selecting the variables that significantly explained the variation in the gene abundance measurements. For ALRF samples, the gene abundance variation was explained significantly by all soil variables included in the analysis (R^2-adjusted: 0.77) (Figure 3.5a). CO_2 and CH_4 flux rates were fit to the model, not included in the constrained model itself. Abundance of *pmoA* was positively correlated with soil temperature and CO_2 flux rates, and was assocated with the month with the highest soil temperatures (Jun-12 and Jul-12). The abundance of *mcrA* clusted with Jun-13 samples, where they were greatest in unmounded fertilized mineral soil. RDA of variables measured at SDT revealed a separation of anerobic and aerobic processes into positive and negative RDA1 coordinates, respectively, which were assocated with undrained control and drained plots, respectively (Figure 3.5b). Abundance of *mcrA* genes was associated with undrained control plots in Jul-13, at which date *mcrA* genes were most abundant and were significantly greater in undrained plots relative to drained plots. The *mcrA* and *dsrB* genes were postitively correlated with each other and with soil moisture. *PmoA* abundance and soil temperature were postiviely correlated and assocated with the drained sites at SDT. RDA of genes and soil factors when ALRF and SDT sites were combined revealed little separation between the sites (Figure 3.5c), although CH_4 fluxes primarily clustered with the samples from undrained plots from SDT in the month of Jul-13, as shown in Figure 3.5b. These fluxes were positively correlated with the abundance of the *mcrA* gene. CO_2 fluxes clusted with drained samples from SDT, and were positively correlated with *pmoA* abundance and total C and N concentrations.

PCNM of ALRF and SDT sampling locations resulted in 192 and 128 PCNM variables, or spatial relationships, respectively, between nearest-neighbor sampling locations. Of these, four and three PCNMs had significant Moran's I statistics for ALRF and SDT, respectively, representing increasingly fine levels of spatial structure. The abundance of microbial functional genes at ALRF was significantly explained only by the first PCNM axis following RDA, which showed that gene abundance data were similar in adjacent plots (See Appendix E for PCNM axes 1 and 2 values of treatment plots). The abundance of microbial functional genes at SDT was not significantly explained by PCNM variables. When ALRF and SDT samples were combined for between-site analyses, the first two PCNM variables significantly explained gene abundance data.

When each microbial gene and GHG variable at ALRF was regressed independently against partialized explanatory factors with RDA, 18.4% of bacterial 16S variation could be explained with the measured variables (Table 3.4a). Soil chemistry (total C, total N, CN ratio, NO_3-N) was the only

Table 3.4a. Canonical variation partitioning of functional gene and greenhouse gas parameters from Aleza Lake Research Forest (ALRF)

Model	df	N	F-Ratio	Total Variance (%)	Space	Physics/ Climate	Chemistry	Genes
Bacterial 16S	9	144	5.78***	18.4	1.5NS	1.0NS	9.5***	
Methane cycling genes								
mcrA	3	144	18.25***	26.5		16.4***	3.6***	
pmoA	3	144	26.47***	34.8		29.9***	1.5*	
dsrB	3	144	15.75***	23.6		15.8***	7.6***	
Greenhouse gases								
CO_2	5	144	11.89***	27.6	11.1***	2.2*	2.6*	
CH_4	4	144	4.36**	8.6			4.0*	6.2**

Table 3.4b. Explanatory variables in canonical variation partitioning models for Aleza Lake Research Forest (ALRF)

Model	df	Individual Variables			
Bacterial 16S	9	Total C 6.2**	NO_3 3.2**	Total N 2.8**	CN 2.4*
Methane cycling genes					
mcrA	3	temp 8.1***	H_2O 7.0**	NO_3 3.6**	
pmoA	3	temp 29.9***	mcrA 1.5*		
dsrB	3	temp 16.2***	NO_3 7.6**		
Greenhouse gases					
CO_2	5	temp 13.1***	NO_3 2.9*		
CH_4	4	*mcrA* 5.5**	bact 4.6**	NO_3 4.2*	

Table 3.5a. Canonical variation partitioning of functional gene and greenhouse gas parameters from Suquash Drainage Trial (SDT)

Model	df	N	F-Ratio	Total Variance (%)	Space	Physics/ Climate	Chemistry	Genes
Bacterial 16S	3	48	12.77***	21.8		22.4***	1.8NS	
Methane cycling genes								
mcrA	5	48	13.22***	33.9	1.4NS		33.8***	0.1NS
pmoA	6	48	28.86***	58.4		39.4***	3.6**	5.7***
dsrB	5	48	18.79***	42.8	0.5NS			30.3***
Greenhouse gases								
CO_2	8	48	81.71***	84.4		42.7***	0.7NS	5.2***
CH_4	4	48	12.52***	42.2	0.6NS	5.8*	1.6NS	34.3***

Table 3.5b. Explanatory variables in canonical variation partitioning models for Suquash Drainage Trial (SDT)

Model	df	Individual Variables			
Bacterial 16S	3	temp 22.4***			
Methane cycling genes					
mcrA	5	*dsrB* 15.1***	NH_4 3.5**		
pmoA	6	Temp 39.1***	*dsrB* 6.2***	pH 2.6*	CN 1.5*
dsrB	5	*mcrA* 25.9***	*pmoA* 5.2**		
Greenhouse gases					
CO_2	8	Temp 42.7***	*pmoA* 2.4***	Total C 0.7*	
CH_4	7	*mcrA* 34.2***	H_2O 5.8**	dsrB 3.9*	

significant variable group to explain bacterial 16S abundance, uniquely accounting for 9.5% of variation, with total soil C making up the largest component of explained variation with 6.2%. The unique contribution of individual soil factors to variation partitioning models at ALRF is provided in Table 3.4b. 26.5% of *mcrA* variation was explained with RDA, with soil physico-climactic (soil water content and temperature) and chemical (NO_3-N) parameters explaining 16.4% and 3.6% of variation, respectively. 34.8% of *pmoA* variation was explained, with soil physico-climactic (soil temperature) and chemical (NO_3-N) parameters explaining 29.9% and 1.5% of variation, respectively. 23.5% of *dsrB* variation was explained, with soil physico-climactic (soil water content and temperature) and chemical (NO_3-N) parameters explaining 15.8% and 7.6% of variation, respectively. For the GHGs, CO_2 flux (reported in Chapter 2) was explained by fine-scale spatial structure (PCNM4, 11.1%) and by soil physico-climactic (soil water content and temperature, 2.2%) and chemical (NO_3-N, 2.6%) parameters, explaining 27.6% of variation. Little variation of CH_4 flux could be explained by measured parameters at ALRF; with soil chemistry (NO_3-N, 4.0%) and microbial genes (*mcrA*, bacterial 16S rRNA, 6.2%) explaining only 8.6% of total variation explained. The abundance of *mcrA* was the explanatory variable with the single largest contribution to ALRF CH_4 flux variation with 5.5%.

Variation partitioning of individual dependent variables at SDT was calculated using RDA (Table 3.5a). The variation of bacterial 16S abundance was significantly explained by one soil parameter, temperature (21.8%). The contribution of single independent soil variables to dependent microbial gene and GHG variables variance partitioning for SDT is described in Table 3.5b. 58.4% of *pmoA* variation is explained by measured variables, including physico-climactic parameters (temperature, 39.4%), soil chemistry (total C, C:N ratio, pH, 3.6%) and microbial functional genes (*dsrB*, 5.7%). 42.8% of *dsrB* variation was explained, 30.3% of which was by *mcrA* and *pmoA* abundance. 84.4% of CO_2 variation was explained by physico-climactic parameters (temperature, 42.7%), soil chemistry (total C, NO_3-N, NH_4-N, 3.6%) and microbial genes (*pmoA*, *dsrB*, Bacterial 16S, 5.7%). 42.2% of CH_4 variation was explained by the reduced RDA model, with physico-climactic parameters (soil water content, 5.8%) and microbial genes (*mcrA*, *dsrB*, 34.4%) explaining significant amounts of this variation. The *mcrA* gene was the largest single contributor to CH_4 flux variation at SDT, explaining 34.2% of variation, with soil water content explaining the next-largest proportion of CH_4 flux variation at SDT.

3.4 Discussion

3.4.1 CH_4 fluxes

3.4.1.1 Effect of mounding and drainage

CH_4 flux varies widely in forests associated with different climatic, vegetation and management parameters. For example, in a meta-analysis of CH_4 flux from UK soils, Levy et al. (2012) report that peat depth (~76% variance explained), C content (~73%), volumetric water content (~30%) and vegetation cover (~34%) were the primary site-specific determinants of CH_4 flux following univariate linear regression. Both CH_4 emission and uptake were measured at ALRF and SDT (Figure 3.1) and these fluxes were equivalent to drained and undrained peat fens containing alder in northeast Germany (Augustin et al., 1998) and Quebec, Canada (Ullah et al., 2009). Drained organic forest soils that exhibit a net uptake of CH_4 can turn into a net CH_4 source when water level increases coincide with maximum annual temperatures (Nykanen et al., 1995; Augustin et al., 1998). Forested wetlands can emit up to up to 3000 µg CH_4-C m^{-2} h^{-1}, about two times more than the highest flux rates at ALRF and SDT (Nykanen et al., 1995), while forests with well-aerated soil emit less (Ullah et al., 2009). At ALRF, mounding led to significant differences in CH_4 flux in Jun-13. Drainage had a consistent inhibitory effect on CH_4 emissions at SDT and led to a decrease in CH_4 emissions. Undrained control plots had greater CH_4 emissions than drained plots on three of four sampling dates. Variability in CH_4 flux was lower at SDT than at ALRF, potentially owing to the more-stable water table depth and temperatures. Mojeremane et al. (2012) found that drainage of peatland reduced CH_4 emissions by 57-76%, owing to increased soil temperature and soil aeration. Our data also show a consistent drop in soil moisture due to drainage. For example, by Sep-13, the mean soil water content in drained plots and undrained plots was 70.8% and 31.6%, respectively (Chapter 2), and soil water content was significantly correlated to CH_4 emissions (Figure 3.4). Soil water content was the abiotic soil variable that explained the highest percentage of variation in CH_4 flux at SDT (Table 3.5b). The positive correlation between CO_2 and CH_4 at ALRF and negative correlation between the flux rate of these gases at SDT was likely due to direct and indirect influences of temperature, respectively. Ullah and Moore (2011) show a positive influence of soil temperature on CH_4 flux rates in deciduous forest soils in eastern Canada, indicating that high soil temperature and moisture can lead to "hot spots" of CH_4 emissions, such in waterlogged mound hollows at ALRF during the warmest sampling date, Aug-12, where maximum emission rates of 1171±738 µg CH_4 m^{-2} h^{-1} were measured (though this rate was also from a fertilized plot, potentially compounding the effect. See next section). PCA more-clearly illustrates the role that drainage played in separating soil factors. Separation of drained and undrained plots along the third PC (Figure 3.4c) was driven by differences in CH_4 emissions, temperature, soil moisture and *mcrA* gene abundance (Figure 3.4d). These

data indicate that the drainage treatment creates a soil environment that is unfavourable for CH_4 emissions due to physiological constraints on the methanogenic community. Drainage-related alterations in soil physico-chemical and biological factors are less influential than variation due to seasonal and annual climate differences. These data partially support the idea that the physical soil environment is the primary driver of CH_4 emissions from soil (Bowden et al., 1998; Levy et al.,. 2012), though soil organic C and mineral N availability may also play an important role in regulating CH_4 flux both in terms of production and oxidation (Adamsen and King, 1993; Krause et al., 2013; Zhuang et al., 2013).

3.4.1.2 Effect of fertilization

Despite fertilized plots having up to 28 times more NO_3-N (Chapter 2) and 18 times the SO_4-S concentrations than unfertilized plots at ALRF (Figure 3.2), there were no significant effects of fertilization on CH_4 flux. While it was hypothesized that SO_4-S may reduce CH_4 emissions by stimulating SRB to outcompete methanogens for acetate (Abram and Nedwell, 1978; Thauer et al., 1989; Achtnich et al., 1995; Denier Van Der Gon et al., 2001; Muyzer and Stams, 2008), there were no correlations between SO_4-S and CH_4 (Figure 3.4). While Ma et al. (2012) showed that abundance of *mcrA* genes and transcripts correlated negatively with and SO_4^{2-} concentration in an intermittently drained rice field, Tong et al. (2013) showed that the abundance of *mcrA* and *dsrB* genes positively correlated with each other, NO_3^- concentrations, organic C concentrations and CH_4 flux rates, suggesting that the methanogens present in these systems are hydrogenotrophic or that in non C-limited soil SO_4-S addition may not alter methanogen abundance or CH_4 flux rates. There was no evidence from this study that SO_4-S fertilization at a concentration of 50 kg ha^{-1} can be used to reduce CH_4 emissions in waterlogged soils. CH_4 oxidation generally decreases and CH_4 emission increases following N fertilization in an NPK formulation (Steudler et al., 1989; Bodelier and Laanbroek, 2004; Aronson and Helliker, 2010; Gundersen et al., 2012). Fertilization with 37 and 120 kg N ha^{-1} in pine and hardwood stands decreased CH_4 oxidation by 15-33% (Steudler et al., 1989). Alternately, urea fertilizer can significantly increase CH_4 emissions (Bodelier, 2011). Basiliko et al. (2009) suggest that in N-limited ecosystems urea-N fertilization can stimulate CH_4-oxidizing bacteria. Following meta-analysis of wetland and upland soil, CH_4 emissions were found to be increased by about 38% following N fertilization between 10 and 560 kg N ha^{-1} yr^{-1} (Liu and Greaver, 2009). This may explain the potentially fertilization-related CH_4 "hot spots" observed in this study. The use of N fertilization levels >100 kg N ha^{-1} yr^{-1} or continuous N deposition can reduce CH_4 fluxes, potentially by overwhelming the CH_4-binding sites in the PMO enzymes in MOB, while levels below this may stimulate CH_4 fluxes if N is a limiting factor for the growth and metabolism of MOB (Purkhold et al., 2000; Basiliko et al., 2009; Bodelier, 2011; Gundersen et al., 2012; Zhuang et al., 2013).

In addition to changes in soil water content, mounding and fertilization treatments at ALRF altered soil chemical factors by removing C stocks from soil and adding mineral N, respectively (See Chapter 2). While differences in CH_4 flux between mounded and unmounded plots, specifically the significantly lower CH_4 flux from mound tops compared to unmounded plots and mound hollows in Aug-12 and Jun-13 (Figure 3.1), are likely due primarily to water content (Figure 3.4); variation partitioning (Table 3.4a) suggested that there may be a positive relationship between NO_3-N concentrations and CH_4 flux at ALRF, particularly as NO_3-N also explained a small but important amount of variation in *mcrA* abundance.

3.4.1.3 Effect of soil parameters

While soil climate and microbial community abundance played a role in CH_4 flux, there is a great deal of unexplained variation in the CH_4 emission data. Soil temperature and moisture were contributing factors in the large positive flux in Jul-12 at ALRF. It is unclear what led to this large efflux of CH_4, although the Jul-12 sampling date had a confluation of factors such as high temperature (27.4^oC) and standing water throughout the mounded areas that create ideal conditions for high CH_4 emissions. Such peaks in CH_4 efflux are not uncommon in waterlogged forest soils subject to fertilization (Augustin et al., 1998; von Arnold et al., 2005a). High soil water table (-30 cm) and temperature (>20^oC) in July and August were shown to cause an otherwise CH_4-oxidizing drained fen to emit upwards of 375 µg CH_4-C m^{-2} h^{-1} (Augustin et al., 1998). Another fen site in Finland emitted between 800 and 3000 µg CH_4-C m^{-2} h^{-1} during periods of high temperature and water-table depth (Nykanen et al., 1995). Increases in volumetric soil water content and soil temperature turned CH_4-oxidizing boreal spruce and aspen sites towards net CH_4 emission (Ullah et al., 2009). The authors were able to explain 32% of the variability of CH_4 fluxes using soil moisture and temperature. Gundersen et al. (2012) show that CH_4 oxidation is most-strongly influenced by soil moisture and C:N ratio. We did not observe any correlation between C:N and GHG emission in this study (data not shown). Our data indicate that seasonal fluctuations between CH_4 uptake and emission are likely in poorly drained forest stands and that the use of drainage and mounding to create aerated sites for planting can push a soil towards CH_4 uptake possibly due to reduced soil moisture and a resulting decline in methanogen functional genes. However, waterlogged soil exposed by mounding will act as a "hotspot" of large, seasonal CH_4 flushes, particularly in the presence of inorganic N additions.

Positive correlations between *mcrA* abundance and CH_4 emissions suggest that measuring *mcrA* gene quantity can be at least partially useful for predicting CH_4 flux (Figure 3.4). Freitag et al. (2010) suggest that while *mcrA* genes correlate with CH_4 emissions from a peat soil, calculating the

gene:transcript ratio generates a stronger relationship between the methanogen community and CH_4 flux, as *mcrA* gene:transcript abundance ratios explained 94% and 51% of variation of CH_4 flux at two fens in North Wales, UK. The relationship between *mcrA* gene:transcript ratio and CH_4 flux decreased with depth. The abundance of *pmoA* was negatively correlated with CH_4 at SDT, which indicates CH_4 fluxes are likely regulated by the methanotroph community, which in turn regulates the abundance of methanotrophs (Freitag et al., 2010). The ability of the methanotroph community to oxidize CH_4 was likely overwhelmed at SDT, leading to net emissions. Low-affinity MOB oxidize CH_4 at soil concentrations typical ofbiological sources, and tend to increase as CH_4 concentrations in soil undergo moisture-dependent increases, until oxygen availability becomes limiting. High-affinity MOB that can oxidize atmospheric concentrations of CH_4 are also prevalent in forest soil (Kolb, 2009). It is unclear whether the negative correlation between *pmoA* and CH_4 fluxes is caused by *pmoA* community dominated by high-affinity or low-affinity MOB at SDT compared to ALRF. The use of affinity-specific *pmoA* qPCR primer sets can help elucidate the importance of these MOB groups, a task made difficult by the still-unclear phylogenetic differentiation of these groups (Martineau et al., 2014). At ALRF, CH_4 flux was correlated with *mcrA* gene abundance (Figure 3.4). The abundances of *mcrA* and *pmoA* were positively and negatively correlated to CH_4 flux rate at SDT, respectively.

RDA was used to examine the relationships between the soil CH_4-cycling community and soil abiotic parameters. The resulting models showed that *mcrA* clustered with *dsrB* and NO_3-N at ALRF, indicating that methanogens were not suppressed by SRB abundance or mineral N concentrations as hypothesized (Figure 3.5a). The separation of RDA plots between aerobic and anaerobic communities and soil variables suggests that the effect of water content and O_2 supply can differentiate the size of communities responsible for CH_4 cycling. At SDT, there was a similar clustering of aerobic and anaerobic factors (Figure 3.5b), although CH_4 fluxes were positively correlated to *mcrA* abundance at this site. CH_4 fluxes were, with few expections, greater at SDT than at ALRF and the highest recorded fluxes at SDT were assocated with the highest abundance of methanogen functional genes. Between sites, the cluster of anaerobic organisms and process appears to be positively assocated with undrained SDT samples, while variation of aerobic organisms and process was assocated with drained SDT samples, with ALRF samples between these highly differentiated environments (Figure 3.5c). This separation suggests that drainage at SDT had a large effect on soil factors, CH_4 cycling genes and ultimately CH_4 fluxes, while mounding at ALRF did not shift the functioning of the CH_4-associated community to the same extent.

The amount of CH_4 flux variation explained at ALRF was extremely low (8.2%), indicating that variables not measured in this experiment contribute significantly to CH_4 fluxes at this site. The growth of algae in the standing water in fertilized mound hollows indicated eutrophication and could have removed

dissolved oxygen from these locations and contributed algal-biomass organic C for methanogenesis that was unmeasured in this study. In contrast, an important amount of CH_4 flux variation at SDT could be explained. The abundance of the *mcrA* gene explained the highest percentage of variation in CH_4 at both ALRF (Table 3.4a, Table 3.4b) and SDT (Table 3.5a, Table 3.5b). These data suggest measuring methanotroph abundance is an important component to determining the drivers of CH_4 flux from wet forest ecosystems. It remains to be seen if quantifying *mcrA* expression can further increase the explanatory power of molecular analysis of the microbial community to elucidate CH_4 flux in waterlogged forest soil (Freitag et al., 2010).

CH_4 fluxes were largely unexplained by measured variables. Multiple comparison tests in this study were applied to individual samples. Levy et al. (2012) report that plot- and site-means provided a higher degree of correlation with these factors and CH_4 flux following univariate linear regression than data from individual samples, due to the noise in the raw data. While the use of plot and site means for correlative studies can be useful for large-scale, multi-site analysis, there is a cost of lowering the degrees of freedom. RDA of gene abundances was performed on plot mean data, which greatly improved the multivariate regression models. The use of gas chromatography of samples collected from closed-static chambers can also produce sizable errors in CH_4 flux estimation. Pihlatie et al. (2013) demonstrate that CH_4 flux measurements using static closed chambers in conjunction with offline gas chromatography, as in this study, can significantly under- or over-estimate flux rates and add substantial sources of error to CH_4 flux calculations. When flux-rates are statistically indistinguishable from zero, as is common in upland forest soils (Basiliko et al., 2009), improvements to chamber methods (e.g., fan-mixing instead of syringe mixing) (Christiansen et al., 2010) or alternative analysis methods with greater sensitivity than offline gas chromotography e.g., portable laser spectroscopy (Junkunst et al., 2006; Kapitanov et al., 2007; Hillebrand, 2008), may allow for greater accuracy in allocating variation of CH_4 to soil and climate parameters.

3.4.3 Factors influencing microbial functional genes

3.4.3.1 *mcrA*

PCA was used to determine how site preparation and fertilization altered the soil parameters measured in this study including functional gene abundances. The abundance of *mcrA* was positively correlated with soil water content at ALRF (Figure 3.4b, Figure 3.5a, Table 3.3a) suggesting that waterlogged soil environments exposed by mounding led to significantly greater *mcrA* abundance in mounded plots, for example as seen in Oct-12. Soil moisture and *mcrA* abundance were also linked at

SDT (Figure 3.4d, Figure 3.5c, Table 3.3b), illustrating that the impact of drainage on methanogen populations is due in part to soil aeration, which creates an inhospitable environment for low-redox-favouring organisms. Following RDA, variation of *mcrA* abundance was explained primarily by soil physico-climactic factors at ALRF (Table 3.4a) and by soil chemical factors at SDT (Table 3.5a). Individually, temperature and water content positively influenced *mcrA* abundance at ALRF (Table 3.4b). Methanogenic archaea have been correlated with CH_4 production and are abundant and transcriptionally active in anoxic soil environments, including waterlogged upland soil (Angel et al., 2012). The abundance of *mcrA* functional genes is lower in forest soil (Frey et al., 2011) than in rice paddy soil (Watanabe et al., 2009; Ma et al., 2012). The *mcrA* copy numbers in this study are in the range reported for soil under Swiss beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst) stands. The abundance and transcription of *mcrA* declines markedly following drainage of rice paddy soil (Watanabe et al., 2009) and in soil subject to frequent drying and re-wetting relative to consistently anoxic soil (Ma et al., 2012). Soil drainage-influenced *mcrA* abundance reduction was followed by an 80-95% decrease in CH_4 emissions that did not return to pre-drainage levels upon re-wetting (Ma et al., 2012). Soil aeration can suppress methanogen abundance and CH_4 production.

3.4.3.2 *pmoA*

The mean abundance of the *pmoA* gene from MOB was between 10^5 to 10^7 copies g^{-1} (dw) soil, equivilant to the range of *pmoA* copies in a non-grazed grassland in Germany (Shrestha et al., 2012), and about an order of magnitude lower than in soils under German beech and Norway spruce stands (Degelmann et al., 2010). Abundance of *pmoA* can exhibit patterns of seasonal fluctuation linked to changes in soil CH_4 fluxes, which are primarily due to water-table depth (Shrestha et al., 2012). Although CH_4 uptake decreased during periods where soil moisture exceeded 50% by volume, *pmoA* abundance was greatest, suggesting disengagement between methane oxidation rates and MOB abundance. Soil water content had no effect on *pmoA* abundance at ALRF and SDT. While Shrestha et al. (2012) found no links between temperature and *pmoA* abundance, these two factors were strongly correlated at ALRF and SDT. Abundance of *pmoA* was correlated to pH at SDT, but only weakly at ALRF. Soil under different tree species can have significantly different pH, C:N, ratios and NH_4^+ concentration, all of which can result in alteration to the MOB community, although these changes may not necessarily effect CH_4 oxidation rates (Menyailo et al., 2010). For example, Norway spruce stands generally had lower pH and *pmoA* abundance than beech stands, although differences in pH were not consistent (Degelmann et al., 2010). Positive associations between *pmoA*, temperature and CO_2 further suggest that these soils were influenced by the MOBs in terms of microbial control of C loss from soil as GHGs. *PmoA* abundance was

generally higher at SDT under cedar/hemlock than at ALRF under hybrid spruce. SDT had a lower C:N ratio, lower pH and higher soil NH_4^+ concentration (Chapter 2).

3.4.3.3 Relationships between *mcrA*, *pmoA* and *dsrB* genes

McrA and *pmoA* abundance were negatively correlated at ALRF (Figure 3.4, Table 3.3a). While Freitag et al. (2010) found that *mcrA* and *pmoA* gene:transcript abundance ratios had a positive relationship in a methane-emitting site, there was a negative correlation in a site considered a methane sink. Quantifying *mcrA* and *pmoA* transcripts will resolve differences in methanogen and MOB activity that can more-clearly link CH_4-cycling organisms with CH_4 fluxes. With the exception of Jul-12, non-waterlogged soil at ALRF generally acted as a methane-sink. Similar to the relationships between *pmoA* and CH_4 flux, the dominance of methanotrophy in such environments can explain the negative relationship between *pmoA* and *mcrA* abundance. There was no relationship between these genes at SDT. The abundance of *mcrA* was negatively correlated with SO_4-S at SDT, which was also shown in rice paddy soil (Ma et al., 2012). However, the *mcrA* and *dsrB* gene abundances were positively correlated at both ALRF and SDT (Figure 3.4), and *dsrB* was the factor that explained the greatest amount of variation in *mcrA* abundance at SDT, while *dsrB* variation was explained by *mcrA* and *pmoA* abundance (Table 3.5b). This finding may be the result of both communities being most abundant in similar low-redox microsites within the soil, or potentially engaging in a syntrophic relationship, as hydrogen-consuming methanotrophs can remove the excess hydrogen that results from oxidation of low-molecular-weight organic compounds to acetate by SRB groups (Bryant et al., 1977). *DsrB* abundance was positively correlated with soil moisture, SO_4-S and *mcrA* in soil from ALRF, though variation in *dsrB* abundance was allocated primarily to temperature and NO_3-N concentration at ALRF (Table 3.4b). The cause of the lack of positive correlation between *dsrB* abundance and SO_4-S concentration at SDT is currently unknown. The *mcrA* and *dsrB* genes were also positively correlated with pH at SDT. Soil pH is considered a "master variable" that strongly influences the microbial community. The negative correlations of pH with SO_4-S and soil water content suggest that as water content increases and redox potential decreases, reduction of oxidized compounds such as SO_4^{2-} and NO_3^- take place, removing these strong acids from the soil matrix and increasing pH. Many microbial communities are pH-sensitive. With increasing pH the abundance of the functional genes measured in this study also increased; these data indicate that low pH can have a suppressive effect on the abundance of *mcrA*, *pmoA* and *dsrB* genes.

3.5 Conclusions

Mounding at ALRF reduced CH_4 fluxes on one date (Jun-13), though high flux rates were measured in mound hollows on Jul-12. These data support hypothesis i. Drainage at SDT significantly reduced CH_4 fluxes with minimal effect on total soil C concentrations or CO_2 emissions, indicating that at SDT drainage was not likely to significantly reduce soil C content, supporting hypothesis ii. While fertilization significantly increased SO_4-S concentrations at ALRF, there was no effect on methane rates indicating that SO_4-S fertilization in the formulation used in this study does not adequately reduce CH_4 fluxes, which does not support hypothesis iii. This may be due to the failure of SO_4-S to stimulate the SRB to outcompete acetoclastic methanogens in a way that would significantly alter CH_4 fluxes, as well as the lack of suppression of hydrogenotrophic methanogens. More study is needed to understand the relationship between SO_4-S fertilization, SRBs, acetoclastic versus hydrogenotrophic methanogens and CH_4 fluxes under field conditions. Seasonal spikes in CH_4 efflux rates at ALRF can be explained by concurrently elevated soil temperature and moisture levels, which can turn a CH_4-oxidizing soil into a CH_4 source. Hypothesis iv was supported for methanotrophs and SRB as the *pmoA* and *dsrB* genes were higher and lower in organic soil relative to mineral soil. Layer effects on methanogens were less clear, as *mcrA* was generally lower in aerated organic soil, but associated with organic sources of C found in forest floor layers. The abundance of methanogenic archaea is one driver of correlations between soil climate and CH_4 fluxes at ALRF and SDT, though the negative correlation of *pmoA* with *mcrA* abundance and CH_4 efflux rate show that fluxes are also controlled by MOB at ALRF. Hypothesis v, that soil water content would influence the regulation of CH_4 fluxes and microbial gene abudnances, is partially supported but requires revision due to knowledge gained by variation partitioning analysis. This study was unique to my knowledge in its i) measurement of the seasonal abundance of *mcrA*, *dsrB* and *pmoA* functional genes following site preparation and mounding, ii) measurement of the effects of site preparation techniques on soil-atmosphere fluxes of GHGs including CH_4, iii) comparison of *mcrA*, *dsrB* and *pmoA* genes following NPKS fertilization partially resolving the response of these communities to N and S addition to soil and iv) the partitioning of variation of CH_4 fluxes and CH_4-cycling functional genes. This study demonstrates that mounding can lead to disturbances in the soil environment that transiently reduce CO_2 emissions (Chapter 2), but create potential hot-spots for CH_4 emissions, dependent on soil temperature, water content and the population dynamics of GHG-emitting microbial communities. Drainage has a lasting effect on soil moisture levels that can inhibit methanogens and CH_4 emissions, while increasing total soil C. Therefore, drainage is recommended for site preparation of waterlogged soils over mounding, when hydrological conditions are suitable for drainage to occur. The use of microbial functional genes can help resolve how the complex changes to the soil community following site preparation can result in alterations to GHG fluxes in wet forest ecosystems.

Chapter 4. The effect of soil mounding, drainage and fertilization on nitrifying and denitrifying microbial functional groups and N_2O flux in wet forest ecosystems

4.1 Introduction

Along with carbon dioxide (CO_2) (see Chapter 2) and methane (CH_4) (see Chapter 3), N_2O is an important driver of global climate change. While the atmospheric mixing ratio of N_2O is minute relative to CO_2 (319 ppb compared to 379 ppm, respectively, in 2005), its radiative forcing is 9.6% of that of CO_2, owing to a 298 time greater global warming potential over a one-hundred year period (Forster et al., 2007). Alterations to the atmospheric N_2O mixing ratio have disproportionally large effects on the global climate. Emissions from natural and managed soils, have increased about 30% since 1992 estimate to 3.3-9.0 Tg N_2O-N yr^{-1} and 1.7-4.8 Tg N_2O-N yr^{-1} respectively, and are the primary contributors to increases in atmospheric N_2O mixing ratios (Forster et al., 2007). It is therefore important to elucidate the drivers of N_2O flux from natural and managed ecosystems including forests managed to improve productivity and biomass production (Butterbach-Bahl et al., 1997, 2013).

Post-harvest forest management can alleviate constraints on survival and growth of planted seedlings, such as competition from vegetation, inadequate soil temperature and water-saturated soil (Sutton, 1993). The management of forests for enhanced productivity is currently being investigated to fill timber-supply deficiencies in British Columbia (BC), Canada (Brockley and Simpson 2004). However, site preparation can lead to physical and chemical alterations to the soil that alter its N_2O budget. There is a dearth of information related to the impact of site preparation methods, including mounding, drainage and fertilization on soil process and associated microbial communities.

Mounding is the mechanical creation of raised planting locations. It can significantly reduce N_2O efflux due to improved soil aeration of the mounds (Mojeremane et al., 2012), though it has the potential to leave waterlogged pits, or hollows, that can act as a significant "hotspot" for N_2O flux (Ballard, 2000), leading to a net increase in emissions when these locations are factored into the N_2O budget of the site (Pearson et al., 2012). Ditch installation is an alternative to mounding for improved soil aeration that enhances drainage with less disruption of soil structure and stratification, though drainage is not recommended for high-clay soils with poor natural drainage potential. Ditch drainage did not affect N_2O flux in a Canadian boreal forest soil (Schiller and Hastie, 1996) or a peaty gley soil in England (Mojeremane et al., 2012). However, in a minerotrophic sedge fen and pine bog in Finland drained 50 years prior to GHG flux measurements (Martikainen et al., 1995), and a peat fen in Sweden drained 70 years prior to GHG flux measurement (von Arnold et al., 2005a), N_2O emissions were significantly

greater in drained stands than in undrained stands, suggesting that soil carbon availability and carbon-to-nitrogen (C:N) ratio play important roles in regulating N_2O fluxes from drained soil (Klemedtsson et al., 2005; Pilegaard et al., 2006; Ernfors et al., 2007). Soil water content is a key regulatory parameter of N_2O fluxes. In lab studies, nitrification was the primary source of N_2O in aerated soil (20% to 50% water filled pore space (WFPS)), peaking at 60% WFPS, while denitrification was minimal source of N_2O until soil WFPS reached 60%, after which point it rapidly increased until saturation (Bateman and Baggs, 2005). Field measurements show that as water content increases N_2O flux rates peak at about 90% WFPS or about -5 kPa soil water potential (Smith et al., 1998)

Fertilization can increase aboveground tree biomass, as many forests ecosystems in North America are N limited (Swift and Brockley, 1994; Mitchell et al., 1996; Kishchuk et al., 2002). Addition of N to forests can also increase N_2O emission from soil, due to up-regulation of rates of N-cycling processes such as N mineralization, nitrification and denitrification (Johnson et al., 1980; Brumme and Beese, 1992; Sitaula and Bakken, 1993; Situala et al., 1995; Pilegaard et al., 2006; Jassal et al., 2008, 2010, 2011; Mojeremane et al., 2012). However, several studies did not report an increase in N_2O flux following N fertilization of forest stands (Pang and Cho, 1984; Johnson and Curtis, 2001; Basiliko et al., 2009; Gundersen et al., 2012). Forest soil can also act as a sink for N_2O, depending upon N availability and soil water content (Chapuis-Lardy et al., 2007; Goldberg and Gebaur, 2009). The role of the microbial communities involved in regulating N_2O flux from soil, including nitrifying and denitrifying microorganisms, is often overlooked and can help resolve these conflicting findings (Hallin et al., 2009; Morales et al., 2010; Petersen et al., 2012; Harter et al., 2014).

A trait-based approach to studying ecosystem functioning begins with the microbial groups that carry out key processes (Weiher and Keddy, 1995; Green et al., 2008). Nitrification is the complete chemolithoautotrophic oxidation of NH_3^- to NO_3^-. The first step in the nitrification pathway is carried out by ammonia-oxidizing archaea (AOA) and bacteria (AOB). Studies of AOA and AOB in forest soil using the ammonia monooxygenase α-subunit marker (*amoA*) have shown complex relationships between these organisms and soil water content, soil pH, N availability and N_2O emissions (Szukics et al., 2010; Bru et al., 2011; Rasche et al., 2011; Long et al., 2012; Petersen et al., 2012). Nitrification products can be used by denitrifying microorganisms for the production of N_2O, though ammonia-oxidation and nitrifier denitrification can contribute significantly to N_2O fluxes from soil (Sahrawat and Keeney, 1986; Wrage et al., 2001; Shaw et al., 2006; Zhu et al., 2013; Levy-Booth et al., 2014). Denitrification is the stepwise reduction of NO_3^- to N_2 and is frequently studied using molecular markers for membrane-bound dissimilatory nitrate reductase (*narG*), Cu-containing and cytochrome *cd1* nitric oxide reductases (*nirK* and *nirS*) and nitrous oxide reductases (*nosZ*) (Levy-Booth et al., 2014). Denitrification community

structure, gene abundance, activity and ultimately N_2O production in forest soil is understood to be strongly influenced by organic C concentration, soil water content, pH and NO_3-N availability (Kandeler et al., 2009; Bárta et al,. 2010; Levy-Booth et al., 2010; Liu et al., 2010; Szukics et al., 2010; Rasche et al., 2011; Zhu et al., 2013; Harter et al., 2014). Yet there are few studies of forest management effects on N_2O flux using microbial functional markers for nitrification and denitrification. Furthermore, determining how functional microbial communities respond to changes in climate, soil physico-chemical parameters and other microbial groups is a key challenge for microbial ecologists that can further elucidate how forest management can alter microbially-mediated ecosystem processes (Bru et al., 2011).

The objectives of this study were to a) quantify N_2O flux rates following mounding, drainage and fertilization of two wet forests using the static closed chamber method, b) quantify the effect of mounding, drainage and fertilization on soil nitrifying and denitrifying functional genes and transcripts using quantitative PCR (qPCR), c) evaluate the effect of mounding, drainage and fertilization on potential denitrification rates and d) determine relationships between soil physico-chemical characteristics, soil microbial functional groups and N_2O fluxes using multivariate ordination and canonical variation partitioning. Specific hypotheses tested were: i) locations with reduced soil water content following site preparation (drained sites, mound tops) will have reduced N_2O emissions and that locations with increased water content (mound-associated hollows) will have higher N_2O emissions; ii) fertilization will increase N_2O emissions in locations of elevated soil moisture; iii) nitrifying bacteria and archaea will be elevated by mounding, drainage and fertilization, and will be higher in the forest floor than in mineral soil, due to increased soil aeration and available mineral N;iv) genes from denitrifying organismswill be decreased following mounding and drainage, but increased by fertilization. Denitrification genes will likely be lowest in aerated soil layers but highest in wet forest floor layers due to anaerobic conditions and availability of organic C; v) denitrification potential will be greatest in the soils with optimal moisture organic C and mineral N for denitrification to occur (i.e., unmounded soil, mound bottoms, undrained soil and fertilized plots) and will be mediated by abundance and transcription of denitrifying genes, vi) nitrifier populations will be positively correlated to NH_4 availability and soil pH; denitrifier populations will be positively associated with soil carbon, soil nitrogen, pH and soil moisture; and N_2O emissions will be positively correlated with soil C, NO_3-N and denitrifier gene abundance.

4.2 Materials and methods

4.2.1 Field sampling

The effect of soil mounding and fertilization on nitrifying and denitrifying microbial functional groups and N_2O flux were studied in interior spruce stands at the Aleza Lake Research Forest (ALRF) near Prince George, B.C., and the effect of drainage and fertilization on these parameters measured in western hemlock/western redcedar/yellow cypress stands at the Suquash Drainage Trial (SDT) near Port McNeill, B.C., on Vancouver Island. For a complete description of the ALRF and SDT sites refer to Chapter 2. Briefly, ALRF was located in the Sub-Boreal Spruce (SBS) zone in the Biogeoclimatic Ecosystem Classification (BEC) system of B.C., and the field study was conducted in the wk1 (wet cool) subzone. Soil at ALRF was classified as Orthic Gleyed Luvisols, Orthic Luvic Gleysols with minor amounts of Ortho Humo-Ferric Podzol. Soil had a very fine texture, which results in poor drainage throughout the site, though upper slope soil towards the south west of the site had a clay loam texture due to increasing sand content. The site encompasses several site series categories within the SBSwk1 dependant on slope position, with SBSwk1 *08 Sxw* – Devil's club and *09 Sxw* – Horsetail Site Series/*10 Sxw* – Devil's club – Lady fern Site Series occupying up-slope and toe-slope positions respectively. Areas of the ALRF site that were classified as SBSwk1 *09 Sxw* and *10Sxw* and were subject to seasonal flooding were deemed unsuitable for operational mounding and were left in reserve. The remaining block consisting primarily of 70-year-old second-growth interior hybrid spruce (*Picea engelmannii* x *glauca*) and subalpine fir (*Abies lasiocarpa*) was clear-cut harvested in February 2011, slash burnt in May 2011, subject to mounding in August 2011 and re-planted with interior hybrid spruce in June 2012. Excavator mounding used a rotary head to turn soil over to create mounds up to 1 m in height with 2 m spacing at a final density of about 1800 mounds ha^{-1}. Eight 0.11 ha plots were spaced 10 m apart with 0.06 ha buffer areas, and were assigned the following treatments using a complete random block experimental design: unmounded control (C), unmounded with fertilization (C+F), mounding (M) and mounding with fertilization (M+F). Within the mounded plots the tops of mounds (M) and the adjacent pits, or hollows (H), were also sampled to differentiate locational effects of mounding. Fertilizer (Shell Canada Ltd., Calgary; Evergro Canada Inc., Delta) was applied using rotary spreaders at a final formulation of 200 kg N, 100 kg P, 100 kg K, and 50 kg S ha^{-1} on June 26, 2012. Most applied N (87%) was in the form of urea, with small amounts of NH_4-N (2.8%) and NO_3-N (<1%) (See Chapter 2 for details of fertilizer formulation). Soil was samped for microbial gene quantification and chemical parameters on June 23, 2011 (Jun-11), June 28, 2012 (Jun-12), July 17, 2012 (Jul-12), August 24, 2012 (Aug-12), October 18, 2012, (Oct-12) and June 13, 2013 (Jun-13), respectively. Soil N_2O fluxes were measured at the same time

as soil sampling, but were unavailable on Jun-11 and Oct-12. Soil chemistry and water content was not measured on Jun-11.

Drainage trials were installed in the Suquash basin by Western Forest Products Inc. in 1997, following clear-cut harvesting and slash-burning of a 22 ha western redcedar (*Thuja plicata*) and shore pine (*Pinus contorta* var. *contorta*) stand between 1993 and 1994. The site was planted with western redcedar (*Thuja plicata*) in 1995. Soils at SDT were Humo-Ferric Podzols with mor humus and subsurface drainage was appropriate for the use of ditch drainage to lower the water table within the treatment plots (van Niejenhuis et al. 2002). Four 0.54 ha treatment plots containing five drainage ditches were installed in 1997, three of which were used in this study due to re-flooding of the fourth plot. Undrained areas at least 60 m from the nearest drainage ditch were used as control plots. Further planting with western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and yellow cedar (*Chamaecyparis nootkatensis* (D. Don) Spach) occurred in 1998. In each drained and undrained area, two 0.03 ha subplots were identified as unfertilized control plots or were subject to fertilization treatments. In addition to site-wide operational fertilization in 2006 using 225 kg N and 75 kg P ha^{-1}, fertilizer in the same formulation as at ALRF was applied in the fertilized subplots in July 2012. The drainage and fertilization study at the SDT site was installed as a random complete block design. Soil was sampled at SDT for microbial gene analysis and chemical analysis on July 27, 2012 (Jul-12), August 29, 2012 (Aug-12), October 25, 2012 (Oct-12), July 3, 2013 (Jul-13) and September 12, 2013 (Sep-13). GHG measurements were undertaken at the same time as soil sampling, but were not available in Oct-12. Soil chemical factors were not measured in Sept-13.

At ALRF, three 10-cm-deep sub-samples of soil were removed with a 5-cm-diameter soil core in each of the two plots per treatment. Soil from control plots comprised organic forest floor F and H layers (C_o) and the mineral Ae horizon (C_m). Mounding plots did not contain a forest floor layer so the top 10 cm of mineral-forest floor mix were pooled into a single sample from either mound tops (M) or mound hollows (H). At SDT two sub-samples of soil in each of the three plots per treatment were removed with the same soil core, and also divided into organic and mineral fractions. Locations of gene abundance estimation were control organic (C_o), control mineral (C_m), drained organic (D_o) and drained mineral (D_m). Soil was dried at 50^oC to prevent DNA degradation and homogenized prior to partitioning for nucleic acid extraction and chemical analysis. Samples from fertilized plots from both ALRF and SDT were designated as +F. Soil chemisty was analyzed at the British Columbia Ministry of Forests, Lands and Natural Resources Operation Analytical Laboratory (Victoria), the results of which are summarized in Chapter 2. Briefly, total C and N were analyzed using a Thermo Flash 2000 combustion NCS analyzer (Thermo Fisher Scientific Inc. Waltham, U.S.A.). Available NH_4-N and NO_3-N were extracted by mixing

soil in 2M KCl at a ratio of 1:10 soil:KCl and shaking for 60 minutes. Extracts were centrifuged and analyzed on an OI-Analytical Alpkem FSIV segmented flow automated chemistry analyzer (OI Analytical College Station, U.S.A.).

4.2.2 Field measurement of N_2O flux

Within each treatment plot at ALRF and SDT, three or two PVC chambers, respectively, were installed to measure the net soil surface exchange of N_2O. Chamber collars were inserted 5 cm in the soil and left for an hour to equilibrate prior to the installation of 2.5 L, open-bottom plastic chambers on top of the collars as in Basiliko et al. (2009). To collect chamber air samples, 6 ml were removed from the outside air using a plastic syringe and inserted into the chamber through a butyl rubber septum to maintain headspace pressure. The head space was mixed three times by plunging the syringe and then 6 ml of chamber air was removed and inserted into pre-evacuated 5 ml Exetainers® (Labco Ltd., Lampeter, UK). Each chamber was sampled 0, 15, 30, 45 and 60 min following installation.

4.2.3 Potential denitrification rates

Potential denitrification rate (PDR) enzyme activity assay was performed as in Groffman et al. (1999) with modification. Three randomly selected mineral soil samples per treatment from ALRF and SDT were sieved to 2 mm, and 25 g fresh soil was weighed into 250 ml air-tight glass containers with butyl rubber septa epoxied into the lid. 25 ml of solution containing 1 mM glucose and 1 mM KNO_3 in ultra-pure water were added and mixed into slurry. Soil slurries in control containers were made with 25 ml ultra-pure water without nitrate or glucose. Container headspace was evacuated for 2 min and then filled with N_2 for 2 min while a venting needle in the septa kept pressure at 1 atm. This process was repeated three times to ensure air was removed from the chambers. Despite these efforts, it is likely that minute volumes of O_2 remained in the chambers. To calculate both gross and net N_2O production from soil the samples were split and either received 23.3 ml acetylene (10% headspace volume) to suppress N_2O reduction, or an equal volume of N_2, respectively. Samples were kept on a 150 RPM rotary shaker at room temperature (~20^{o}C) and 3 ml container headspace was sampled with an air-tight plastic syringe at 0, 30, 60, 90 and 120 min. The sample volume was not replaced before or after sampling to prevent additional O_2 from entering the container. Gas samples were analyzed for N_2O concentration using gas chromatography. 10% acetylene was added to N_2O standards to account for the deleterious effects of acetylene on the gas chromatography measurements. DNA and RNA were extracted from post-incubation soil slurries for functional gene and transcript quantification.

4.2.4 Gas chromatography

N_2O samples were measured with gas chromatography (GC) on an Agilent 5890 series II chromatograph (Agilent Technologies, Santa Clara, U.S.A.) equipped with electron caption device (ECD) set at 350^oC, respectively. The carrier gas was P5 (argon-methane mix) with a flow rate of 35 ml min^{-1}. Standard curves were constructed with simple linear regression of 0.8, 0.4, 0.2 and 0.13 ppm N_2O standards. Changes in N_2O concentrations measured using GC for field fluxes and PDR were linear over the sampling time and fitted with linear regression to calculate rates.

4.2.5 Nucleic acid extraction

In total 288 and 240 soil samples from ALRF and SDT, respectively, were extracted for DNA using PowerClean soil DNA isolation kits (MO BIO Laboratories, Inc., Carlsbad, CA). The mass of homogenized soil used for extraction was 0.1 g for forest floor material and 0.25 g for mineral soil. DNA concentrations were calculated with spectrophotometry of fluorescence emission using the Quant-iT™ PicoGreen® dsDNA assay (Life Technologies Corp., Carlsbad, U.S.A). For RNA extractions from soil following PDR incubations, two g soil was removed from LifeGuard™ solution, extracted using the MoBio RNA PowerSoil® kit and immediately reverse transcribed using the Applied Biosystems high-capacity cDNA reverse transcription kit for cDNA template formation. RNA quantity and quality was determined by measuring absorbance at 260/280 and 260/230 nm.

4.2.6 Quantification of functional communities

Real-time quantitative PCR (qPCR) of nitrification (AOB *amoA*, AOA *amoA*), and denitrification (*narG*, *nirS*, *nirK* and *nosZ*) the effect of mounding, drainage and fertilization on genes was performed using qPCR in 20 µl reactions with 1 µl of template DNA (~5 ng) added to a 19 µl qPCR reaction mixture containing 10 µl Power SYBR® Green PCR Master Mix (Life Technologies Corp., Carlsbad, U.S.A.). BSA (200 ng μl^{-1}) was added to increase PCR efficiency. AOB *amoA* (amoA-1f, GGG GTT TCT ACT GGT GGT; amoa-2r, CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997) and AOA *amoA* (CrenamoA23f, ATG GTC TGG CTW AGA CG; CrenamoA616r, GCC ATC CAT CTG TAT GTC CA) (Tourna et al., 2008) primers were added at 0.5 µM each. AOB *amoA* qPCR to amplify a 490 bp fragment a was carried out with an initial denaturation step of 5 min at 95^oC and 40 cycles of 95^oC denaturation for 1 min, 59^oC annealing for 1 min and 72^oC extension for 1 min. Fluorescence values were measured at

80.5°C for 10 s to dissociate primer dimers and remove the presence of non-target amplification. The standard curve for AOB *amoA* were created with C_t values of 10-fold serial dilutions ranging from 10^2 to 10^7 copies of *amoA* from genomic DNA of *Nitrosospira multiformis* NCIMB 11849 and AOB *amoA* contained within a linearized pCR® 2.1-TOPO® plasmid (Life Technologies Corp., Carlsbad, U.S.A.) that was amplified from field soil. AOA *amoA* qPCR amplified a 593 fragment and was carried out with an initial denaturation step of 5 min at 95°C and 40 cycles of 95°C denaturation for 30 s, 57°C annealing for 30 s and 72°C extension for 1 min. Fluorescence quantification occurred during annealing. The standard curve for AOA *amoA* were created with C_t values of 10-fold serial dilutions ranging from 10^2 to 10^9 copies of *amoA* contained within a linearized pCR® 2.1-TOPO® plasmid (Life Technologies Corp., Carlsbad, U.S.A.) that was amplified from field soil. The *narG*, *nirK*, *nirS* and *nosZ* forward and reverse primers were added at a final concentration of 0.5 µM each. The *narG* primer set (narG-f, TCG CCS ATY CCG GCS ATG TC; narG-r, GAG TTG TAC CAG TCR GCS GAY TCS G) amplified a 173 bp fragment (Bru et al., 2007). The *nirK* primers (nirK1F, GGG CAT GAA CGG CGC GCT CAT GGT G; nirK1R, CGG GTT GGC GAA CTT GCC GGT GGT C) amplified a 375 bp fragment (Braker et al., 1998). The *nirS* primers (nirS1F, CCT AYT GGC CGG CRC ART; nirS3R, GCC GCC GTC RTG VAG GAA) amplified a 256 bp fragment (Chénier et al., 2003). The *nosZ* primer set (nosZ2F, CGC RAC GGC AAS AAG GTS MSS GT; nosZ2R, CAK RTG CAK SGC RTG GCA GAA) amplified a 267 bp fragment (Henry et al., 2006). qPCR for *narG*, *nirK*, *nirS* and *nosZ* was carried out as in Levy-Booth and Winder (2010) using an initial denaturation step of 5 min at 95°C and 40 cycles of 95°C denaturation for 1 min, 60°C annealing for 1 min and 72°C extension for 1 min. Fluorescence quantification occurred during extension. The standard curve for *nirK* used a triplicate 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas chlororaphis* genomic DNA. Standard curves for *narG*, *nirS* and *nosZ* qPCR were developed using triplicate 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas aeruginosa* genomic DNA. DNA was diluted 10 x prior to PCR to prevent possible inhibition by humic and fulvic substances in soil that can co-extract with DNA.

4.2.7 Statistical analysis

Data were checked for normality and homoscedasticity using quantile-quantile (Q-Q) plots, the Shapiro–Wilk test and Levene's test before being fitted with the linear mixed-effects model and subject to multi-factor fragmented factorial ANOVA using the *lme* function in the *nlme* package in R v. 2.15.3 (R Core Team, 2013). N_2O data were subject to two-way full factorial ANOVA using *lme*. One-way ANOVA used the *aov* function and Tukey's honestly significant difference test to determine significance of sampling location. qPCR data were analyzed as $\log_{10}$ values to meet assumptions of normality. PCA

was performed using the *FactoMineR* package in R. PCA was performed using a sub-set of samples where all measured soil factors (climate, GHG flux, soil chemistry, functional genes) were available (ALRF: Jun-12, Jul-12, Aug-12, Jun-13; SDT: Jul-12, Aug-12, Jul-13). A secondary overlay of treatment factors (mounding, drainage, fertilization) and sampling dates was used in PCA to investigate correlations of soil factors to treatment regimes. Pearson correlation coefficients were calculated with the *rcorr* function in the *Hmisc* package for R, and *p* values were assessed against a Bonferroni-corrected threshold for multiple comparisons. Principal coordinate of a neighbour matrix (PCNM) analysis (Borcard and Legendre, 2002; Borcard et al., 2004) was conducted using the *PCNM* package in R. The geographic coordinates of sample sites were transformed to Cartesian coordinates prior to PCNM. Positive PCNM variables with significant Moran's I were forward selected against spatially-detrended dependent variables or matrices using permutational testing with 1000 steps to test for spatial structure. The R^2-adjusted of the forward-selected models did not exceed the R^2-adjusted of the non-selected models. RDA and canonical variation partitioning (Borcard et al., 1992; Ramette and Tiedje, 2007; Bru et al., 2011) were conducted using the *vegan* package for R. Prior to RDA and variation partitioning, forward selection of significant soil parameters was performed using permutational testing with 1000 steps. Venn diagrams of variation sources were created in R using the *venneuler* function.

4.3 Results

4.3.1 *In situ* N_2O flux

Measurement of the soil-to-atmosphere flux of N_2O directly following fertilization at ALRF demonstrated a net positive flux of 1.8 μg m^{-2} h^{-1} across all treatment plots, despite 25% of samples exhibiting uptake (Figure 4.1a). One month following fertilization (Jul-12) positive fluxes were measured in both the unmounded control (C) and mounded (M) samples, with a peak of 164.1 ± 0.16 μg N_2O m^{-2} h^{-1} (mean and standard error of the mean (SEM)) occurring in the mounded and fertilized (M+F) samples. ALRF had a significantly greater N_2O flux than all other measured locations and contributed to the significant fertilization effect. Waterlogged hollows in mounded plots exhibited N_2O uptake. By Aug-12 the fertilized plots had significantly larger N_2O fluxes than unfertilized plots. At this sampling date the N_2O emissions from the mounds had dropped while fertilized control (C+F) and mound hollow (H) plots were strong emitters of N_2O, though these peaks were driven by large fluxes in two out of six "hotspot" samples at this date for each of these locations. One year after fertilization at ALRF N_2O flux showed no locational or treatment effects with a mean positive flux of 0.2 μg m^{-2} h^{-1} across all treatment plots. A significant drainage effect was measured at SDT at the initiation of the study, with chambers in drained

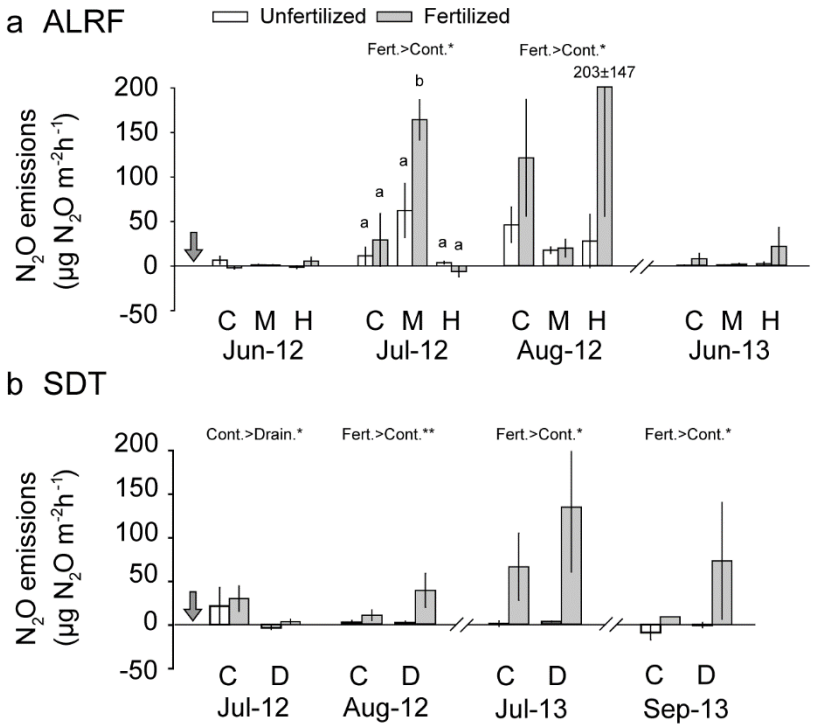

Figure 4.1. N_2O fluxes from a) undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF) and b) undisturbed control (C) and drained plots (D) subject to fertilization at Suquash Drainage Trial (SDT). Shaded arrow shows time of fertilization. Error bars: SEM; n = 6. Treatment locations identified by different letters were significantly different at p = 0.05 following one-way ANOVA. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

plots displaying either a low flux rates or uptake of N_2O, with a mean of -0.1 ± 2.2 μg m^{-2} h^{-1}, while undrained control plots had mean N_2O emissions of 25.7 ± 11.8 μg m^{-2} h^{-1} (Figure 4.1b). At SDT in Aug-12, Jul-13 and Sept-13 the fertilized plots had significantly higher N_2O emissions than unfertilized plots, with the highest values being recorded in the drained plots.

4.3.2 Potential denitrification

The potential denitrification rate (PDR) of soil from ALRF and SDT was measured to determine the influence of site preparation treatments, soil physico-chemical parameters and microbial functional communities on N_2O fluxes under ideal conditions. At ALRF, PDR was significantly greater in soil from mounding and fertilization plots than from soil in unmounded control plots or unfertilized plots (Figure 4.2a). In incubations using soil from unmounded, unfertilized controls there was a mean PDR of 13.2 ± 1.0 μg N_2O-N kg hr^{-1}, or about 0.1% of the 14 mg N kg^{-1} added as KNO_3 h^{-1}. Soil from unmounded plots subject to fertilization did not have a significantly greater PDR than their unfertilized counterparts. In contrast, fertilized soil from the mound hollows evolved significantly more N_2O during the PDR assay than unfertilized soil from these locations. Soil from the fertilized mound hollows had a mean PDR of 73.2 ± 25.8 μg N_2O-N kg hr^{-1}. There was a significant interaction between mounding and fertilization treatments. The addition of 10% acetylene to the chamber headspace prevented N_2O reduction and allowed for the estimation of N_2O reduction to N_2 and gross N_2O production. The acetylene-PDR was significantly greater in soil from fertilized plots than from unfertilized plots at ALRF, as well as in mounded plots relative to the unmounded controls (Figure 4.2a). Unlike PDR without acetylene there was no significant interaction between the treatments. Soil from the mound hollow areas had the acetylene-PDR: 33.9 ± 5.5 and 98.3 ± 31.3 μg N_2O-N kg hr^{-1} for fertilized and unfertilized soil, respectively. The later treatment released 0.7% of added NO_3-N h^{-1} as N_2O.

Soil from the SDT site had greater *in situ* N_2O emissions than soil from ALRF (Figure 4.2b). PDR at SDT ranged from a minimum of 3.7 ± 0.7 μg N_2O-N kg hr^{-1} in soil from undrained unfertilized plots to 10.2 ± 1.2 μg N_2O-N kg hr^{-1} in soil from drained and fertilized plots. PDR of soil from drained, fertilized plots was significantly greater than soil from other locations following one-way ANOVA. Two-way ANOVA showed significantly higher PDR in soil from fertilized plots than from unfertilized controls. Upon addition of 10% acetylene, the total PDR increased by 2.2 μg N_2O-N kg hr^{-1} or about 29.9% in unfertilized, undrained controls and 2.7 μg N_2O-N kg hr^{-1} or about 21.7% in fertilized, drained soil.

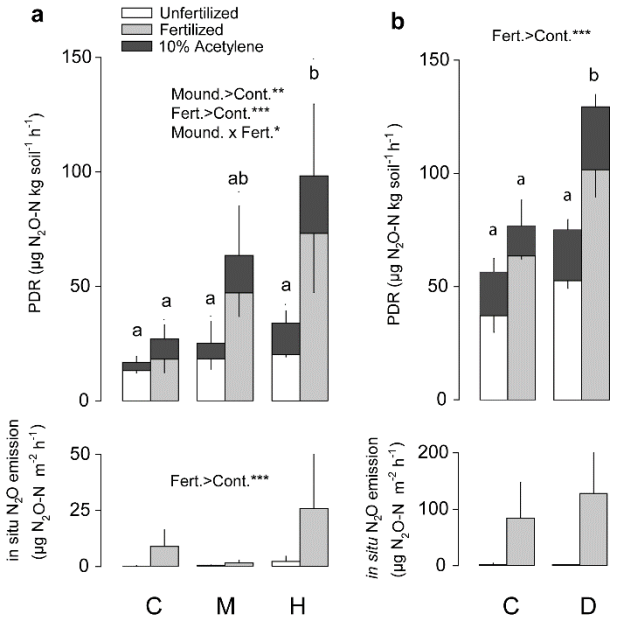

Figure 4.2. Potential denitrification rate (PDR, ng N_2O-N kg $soil^{-1}$ h^{-1}) and PDR with 10% acetylene to suppress N_2O reduction (provides estimate of gross N_2O production) from a) undisturbed control and mounded plots (mound tops and mound hollows) subject to fertilization at Aleza Lake Research Forest (ALRF) from Jun-13 samples, and b) undisturbed controls and drained plots subject to fertilization at Suquash Drainage Trial (SDT) from Jul-13 samples. I*n situ* N_2O emissions from Jun-13 (ALRF) and Jul-13 (SDT) provided for comparison. Downward vertical error bars, standard error of the mean (SEM) of PDR; upwards error bars, SEM of acetylene-PDR; n = 3. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA. Treatment effects following two-way ANOVA are provided if significant (*, $p<0.05$;**, $p<0.01$; ***, $p<0.001$).

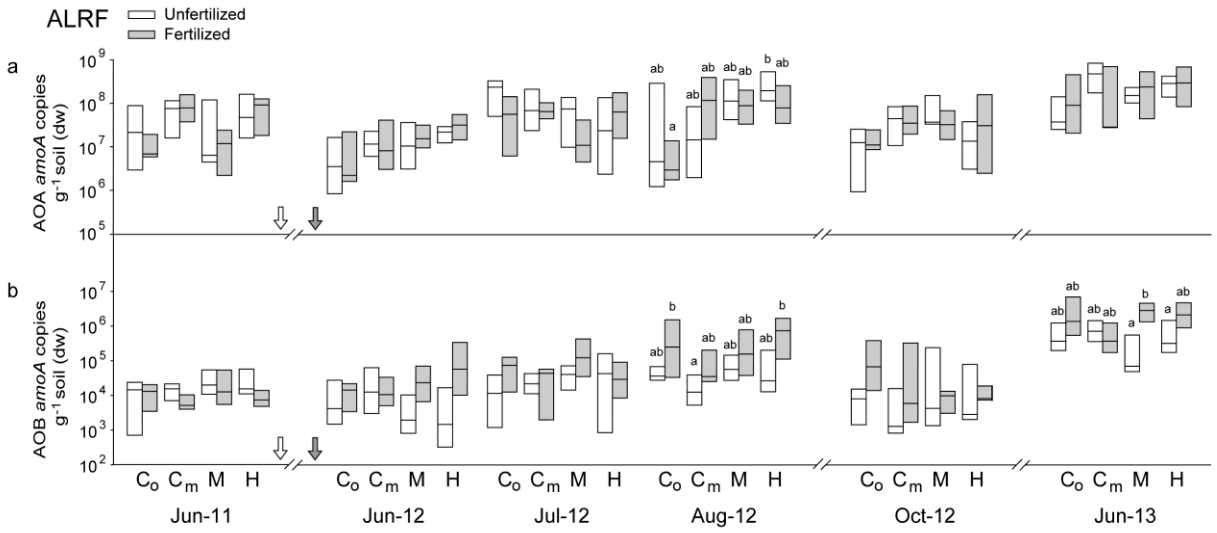

Figure 4.3. Abundance of a) AOA *amoA* genes and b) AOB *amoA* genes in forest floor (C_o) and mineral (C_m) soil from undisturbed control (C) and soil from mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA.

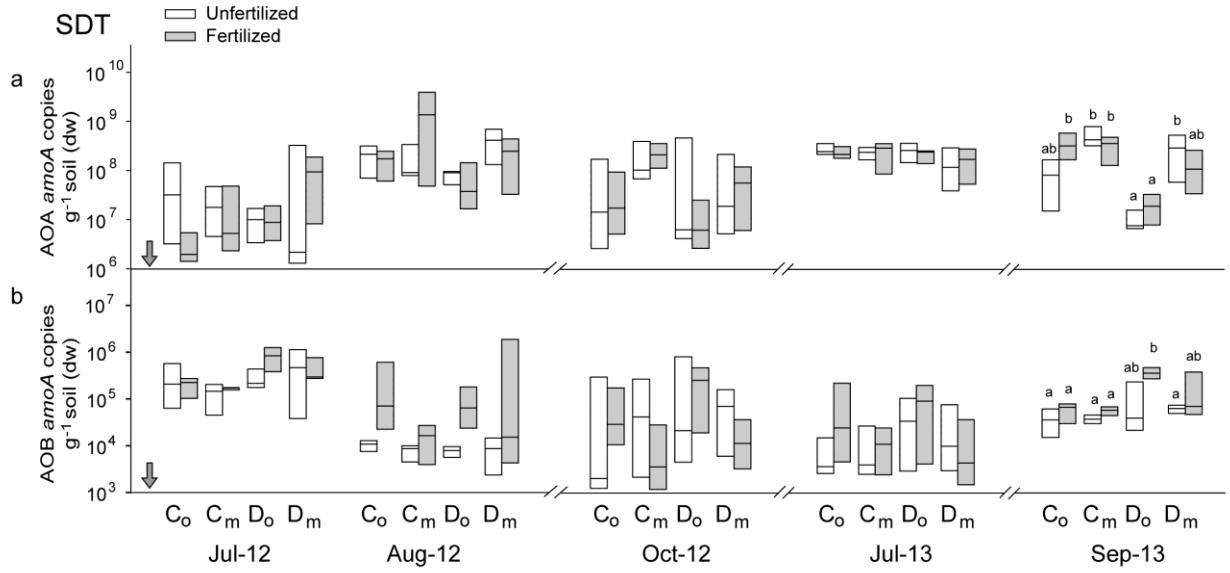

Figure 4.4. Abundance of a) AOA *amoA* genes and b) AOB *amoA* genes in forest floor and mineral soil from undisturbed control (C_o and C_m respectively) and drained plots (D_o and D_m respectively) subject to fertilization at Suquash Drainage Trial (SDT). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA.

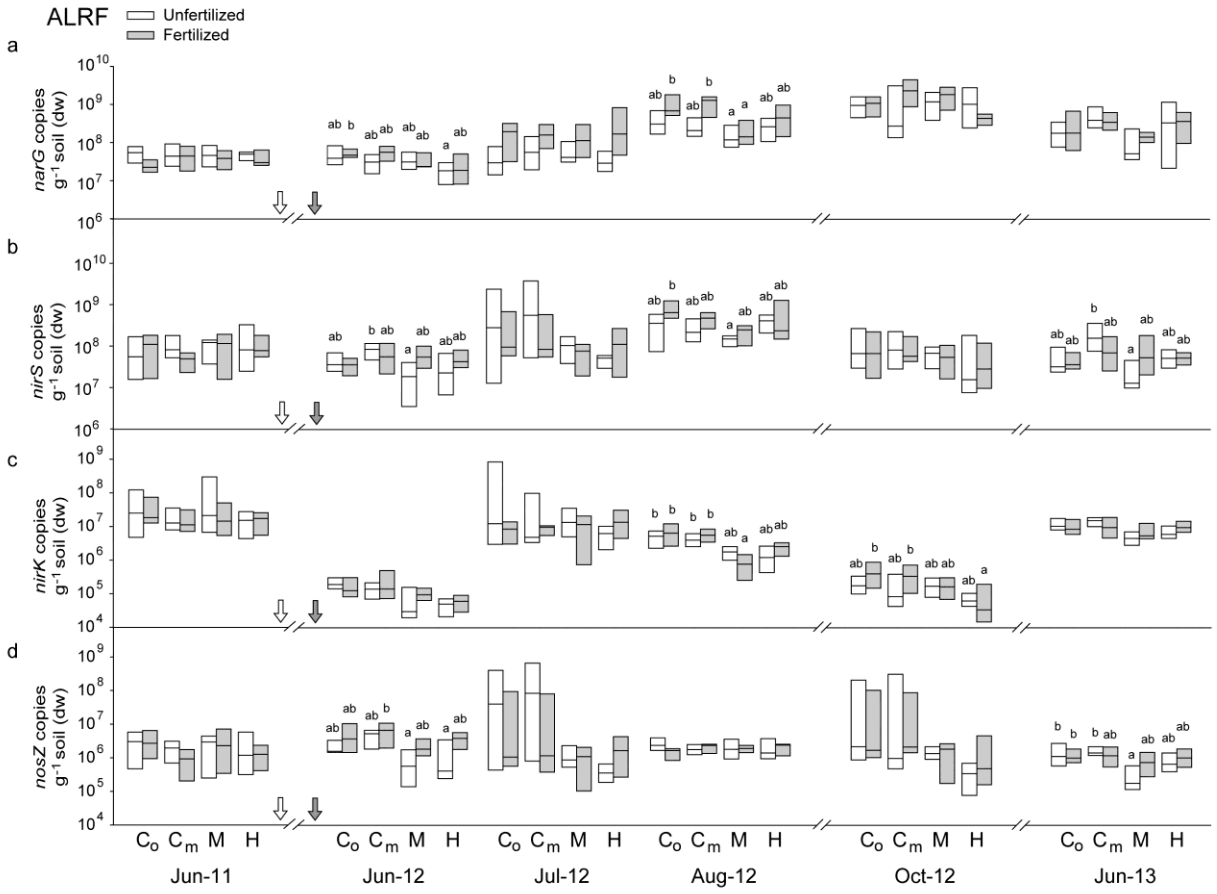

Figure 4.5. Abundance of a) *narG*, b) *nirS*, c) *nirK*, d) *nosZ* genes in forest floor (C_o) and mineral (C_m) soil from undisturbed control and mounded plots (M, mounds; H, hollows) subject to fertilization at Aleza Lake Research Forest (ALRF). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA.

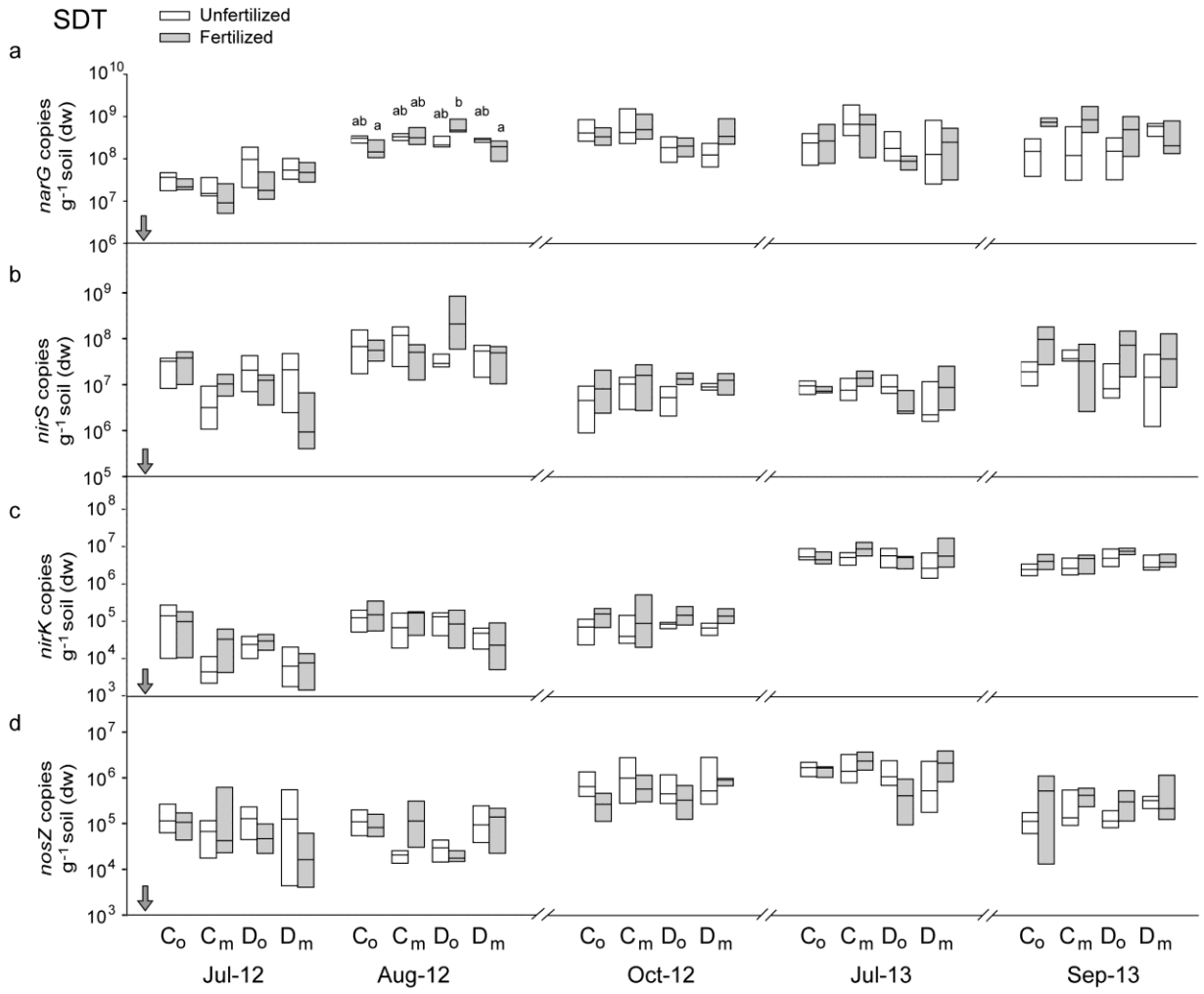

Figure 4.6. Abundance of a) *narG*, b) *nirS*, c) *nirK*, d) *nosZ* genes in organic forest floor and mineral soil from undisturbed control (C_o and C_m respectively) and drained soil (D_o and D_m respectively) subject to fertilization at Suquash Drainage Trial (SDT). White arrow shows time of mounding, shaded arrow shows time of fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 6. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA.

Table 4.1. *F* and *p* statistics following fractional factorial ANOVA on AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS* and *nosZ* gene copy g^{-1} soil (dw) at Aleza Lake Research Forest (ALRF)

		Jun-11		Jun-12		Jul-12		Aug-12		Oct-12		Jun-13	
Gene	Factor	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*
AOA *amoA*	Mound.	3.1	0.084	1.4	0.240	1.4	0.247	**6.8**	**0.013**	0.0	0.851	0.6	0.432
	Fert.	0.0	0.826	0.9	0.344	0.1	0.703	0.1	0.813	0.3	0.607	0.3	0.560
	Layer.	**8.4**	**0.006**	1.3	0.260	0.0	0.881	1.9	0.172	3.8	0.058	2.4	0.127
	M×F	0.1	0.749	0.4	0.539	0.4	0.546	1.3	0.259	0.9	0.359	1.5	0.226
	M×F×L	0.6	0.425	0.2	0.673	0.6	0.449	2.6	0.118	0.1	0.703	3.5	0.068
AOB *amoA*	Mound.	1.8	0.185	0.0	0.858	0.9	0.343	**9.5**	**0.004**	1.3	0.262	1.2	0.271
	Fert.	1.3	0.252	**6.8**	**0.013**	2.4	0.131	**16.0**	**<0.001**	**5.1**	**0.030**	**6.6**	**0.014**
	Layer.	0.1	0.763	0.5	0.489	0.0	0.897	**5.4**	**0.026**	1.6	0.211	0.5	0.463
	M×F	0.7	0.409	**5.2**	**0.028**	0.1	0.783	0.1	0.711	2.0	0.168	**9.2**	**0.004**
	M×F×L	1.6	0.209	0.0	0.952	1.6	0.216	0.1	0.724	0.2	0.667	3.0	0.092
narG	Mound.	0.0	0.860	3.7	0.061	0.2	0.678	**8.5**	**0.006**	0.3	0.572	**5.7**	**0.021**
	Fert.	2.8	0.101	1.7	0.193	**19.2**	**<0.001**	**11.3**	**0.002**	0.0	0.848	0.6	0.444
	Layer.	0.8	0.366	0.8	0.363	0.6	0.453	0.1	0.749	0.0	0.979	3.6	0.063
	M×F	0.2	0.683	0.5	0.477	0.0	0.976	2.8	0.101	1.1	0.300	0.0	0.943
	M×F×L	1.6	0.217	0.6	0.460	0.1	0.707	0.4	0.550	0.7	0.410	0.4	0.516
nirK	Mound.	0.0	0.867	**11.8**	**0.001**	0.1	0.727	**12.6**	**0.001**	2.8	0.101	2.6	0.116
	Fert.	0.2	0.661	1.0	0.330	1.1	0.303	0.0	0.911	0.2	0.694	0.4	0.538
	Layer.	0.6	0.440	0.1	0.790	0.3	0.597	0.1	0.738	0.5	0.467	0.2	0.688
	M×F	0.1	0.748	1.1	0.304	0.3	0.587	0.9	0.359	**4.5**	**0.041**	**7.4**	**0.010**
	M×F×L	0.0	0.863	1.3	0.269	0.7	0.407	0.1	0.744	0.2	0.698	0.2	0.662
nirS	Mound.	0.0	0.925	**4.7**	**0.035**	**5.4**	**0.025**	0.7	0.409	3.2	0.079	**10.7**	**0.002**
	Fert.	0.1	0.799	2.2	0.144	0.7	0.405	4.0	0.052	0.3	0.590	0.0	0.848
	Layer.	0.0	0.928	1.9	0.178	0.3	0.601	1.0	0.328	0.0	0.911	**7.0**	**0.011**
	M×F	0.2	0.651	**7.6**	**0.008**	0.0	0.887	1.7	0.194	0.0	0.897	**4.8**	**0.035**
	M×F×L	0.8	0.390	0.0	0.887	0.3	0.564	0.7	0.412	0.1	0.817	1.9	0.173
nosZ	Mound.	0.2	0.682	**9.6**	**0.003**	**7.2**	**0.010**	0.3	0.582	**6.4**	**0.015**	**9.3**	**0.004**
	Fert.	0.6	0.440	**7.6**	**0.009**	1.5	0.225	0.0	0.835	0.0	0.900	1.5	0.231
	Layer.	1.8	0.181	1.9	0.178	0.0	0.874	0.7	0.396	0.0	0.840	0.2	0.643
	M×F	0.0	0.867	3.1	0.086	1.3	0.268	0.3	0.563	0.2	0.683	2.8	0.105
	M×F×L	1.0	0.315	0.0	0.845	0.1	0.809	1.1	0.304	0.0	0.828	0.1	0.725

Bolding denotes statistical significance at $p<0.05$

Table 4.2. *F* and *p* statistics following fractional factorial ANOVA on AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS* and *nosZ* gene copy g^{-1} soil (dw) at Suquash Drainage Trial (SDT)

		Jul-12		Aug-12		Oct-12		Jul-13		Sep-13	
Gene	Factor	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*	*F*	*Pr(>F)*
AOA *amoA*	Drain.	0.4	0.559	1.5	0.227	**5.5**	**0.027**	2.1	0.158	**24.7**	**<0.001**
	Fert.	0.2	0.693	0.1	0.762	0.2	0.642	0.1	0.729	0.6	0.457
	Layer.	1.1	0.313	3.9	0.061	**4.7**	**0.041**	2.8	0.105	**24.6**	**<0.001**
	D×F	4.1	0.055	1.9	0.177	0.0	0.904	0.1	0.709	1.0	0.318
	D×F×L	0.7	0.567	0.7	0.568	0.4	0.752	0.4	0.776	**4.0**	**0.020**
AOB *amoA*	Drain.	**5.7**	**0.025**	0.0	0.940	0.0	0.894	0.6	0.460	**13.0**	**0.001**
	Fert.	2.6	0.117	**7.5**	**0.012**	0.3	0.570	0.5	0.490	**11.2**	**0.003**
	Layer.	0.9	0.348	2.2	0.155	1.2	0.276	1.7	0.206	0.8	0.392
	D×F	0.6	0.454	0.2	0.625	0.1	0.786	0.8	0.378	2.2	0.153
	D×F×L	0.1	0.943	0.5	0.682	1.2	0.334	0.5	0.696	2.1	0.131
narG	Drain.	2.0	0.174	0.1	0.788	**8.1**	**0.009**	**4.4**	**0.048**	0.1	0.773
	Fert.	0.0	0.898	0.3	0.601	0.0	0.875	0.6	0.452	**9.6**	**0.005**
	Layer.	3.7	0.066	0.2	0.644	2.1	0.162	1.5	0.236	1.1	0.310
	D×F	0.2	0.643	2.5	0.127	**5.3**	**0.030**	0.0	0.866	**5.4**	**0.030**
	D×F×L	1.1	0.379	**9.1**	**<0.001**	1.1	0.372	0.7	0.551	1.4	0.275
nirK	Drain.	1.6	0.224	2.2	0.150	0.0	0.854	1.9	0.180	**5.6**	**0.026**
	Fert.	0.5	0.505	0.2	0.698	**6.1**	**0.021**	1.2	0.287	3.5	0.073
	Layer.	**10.4**	**0.004**	3.3	0.081	0.1	0.819	0.1	0.792	1.7	0.206
	D×F	0.2	0.625	0.6	0.452	0.4	0.547	0.1	0.821	0.0	0.885
	D×F×L	0.4	0.739	0.1	0.951	0.2	0.869	1.5	0.232	0.9	0.474
nirS	Drain.	0.8	0.382	0.0	0.997	0.8	0.375	**4.7**	**0.040**	0.7	0.408
	Fert.	0.5	0.491	0.3	0.598	**5.1**	**0.033**	0.1	0.756	2.4	0.132
	Layer.	**6.4**	**0.018**	1.7	0.209	3.6	0.071	0.0	0.887	0.6	0.452
	D×F	**4.9**	**0.037**	1.0	0.328	0.1	0.727	0.3	0.564	2.1	0.158
	D×F×L	0.5	0.689	0.9	0.438	1.3	0.300	**3.2**	**0.041**	1.0	0.409
nosZ	Drain.	1.7	0.205	1.5	0.239	0.0	0.972	**5.7**	**0.025**	0.5	0.505
	Fert.	0.9	0.348	0.0	0.949	2.9	0.100	0.0	0.862	0.9	0.360
	Layer.	2.6	0.120	0.0	0.999	**4.4**	**0.047**	1.6	0.224	2.8	0.105
	D×F	1.2	0.287	1.9	0.181	1.2	0.281	0.4	0.517	0.0	0.906
	D×F×L	0.2	0.909	1.5	0.232	0.8	0.506	3.0	0.052	0.3	0.837

Bolding denotes statistical significance at $p<0.05$

4.3.3 Effect of mounding, drainage and fertilization on *in situ* functional gene abundance

4.3.3.1 AOA *amoA*

AOA *amoA* was one of the most abundant functional genes quantified in this study (Appendix F) and ranged between 10^6 and 10^9 at ALRF (Figure 4.3a). In Jun-11 forest floors in unmounded plots had significantly greater AOA *amoA* genes than mineral soil in control and mounded plots (Table 4.1). In Aug-12 the mounded plots also contained significantly more AOA *amoA* genes than control plots (Table 4.1). AOA *amoA* abundance at SDT was equivalent to ALRF, with copy numbers ranging from 10^6 to 10^9 (Figure 4.4a). No fertilization effects or interactions were observed for AOA at SDT. Drainage effects were observed in Oct-12 and Sept-13 (Table 4.2), with undrained control plots being more abundant in AOA *amoA* than drained plots (Figure 4.4). In Sept-13 these effects were largely driven by location differences, as the AOA *amoA* abundance in drained forest floor soil was significantly lower than surrounding samples.

4.3.3.2 AOB *amoA*

AOB *amoA* had the lowest abundance of the functional genes quantified in this study, with a median of about 10^4 copies g^{-1} soil (dw) (Figure 4.3b). AOB *amoA* copies were two to three orders of magnitude lower than AOA *amoA* copies at ALRF across all sampling dates. AOB *amoA* abundance was significantly higher in plots receiving fertilization compared to unfertilized plots in Jun-12, Aug-12, Oct-12 and Jun-13 (Table 4.1). In Aug-12, the fertilized forest floor samples from control plots and the mound hollow samples under fertilization had significantly greater abundance of AOB than the mineral soil from fertilized unmounded plots. Similarly, the fertilization effect in Jun-13 was observed in the fertilized mound top samples, which had significantly greater AOB abundance than the unfertilized mound top samples without fertilization. Mounding effects were also observed during Aug-12 sampling, where the mounded plots had significantly greater AOB abundance than unmounded controls. Interactive effects between mounding and fertilization were observed in Jun-12 and Jun-13, as AOB abundance was greater in mounded plots relative to control plots. AOB abundance at SDT was also about three orders of magnitude lower than AOA abundance, with AOB *amoA* ranging from 10^3 to 10^7 at this site (Figure 4.4b). Drainage effects were noted in Jul-12 and Sept-13, with AOB *amoA* abundance being significantly greater in drained plots than control plots (Table 4.2). Fertilization effects were observed in Aug-12 and Sept-13, as AOB *amoA* abundance was significantly greater in fertilized plots relative to unfertilized controls.

4.3.3.3 *narG*

The *narG* gene can be used to estimate the population of microorganisms that can reduce NO_3^- to NO_2^- during denitrification. The median abundance of *narG* throughout this study was about 10^8 copies g^{-1} soil (dw), making it the most abundant of the denitrification genes (Figure 4.5a). In Aug-12 and Jun-13 moun ded plots at ALRF had significantly lower abundance of *narG* genes than unmounded plots (Table 4.1). Mound hollow samples had significantly lower *narG* gene abundance than forest floor samples from fertilized unmounded plots for Jun-12, while unfertilized and fertilized mound tops had significantly lower *narG* abundance than fertilzied unmounded samples samples for Aug-12. This latter difference also contributed to a significant fertilization effect, as fertilized samples had higher *narG* abundance than unfertilized samples during Jul-12 and Aug-12. *NarG* abundance at SDT was significantly higher in unmounded plots relative to mounded plots in Oct-12 and Jul-13 (Table 4.2). *NarG* abundance was also significantly higher in fertilized plots relative to unfertilized plots in Sept-13. At SDT, interactions between drainage, fertilization and soil layer were observed in Aug-12 and between drainage and fertilization in Oct-12 and Jul-13 (Figure 4.6a, Table 4.2). The interactions were caused by the greater *narG* in fertilized plots compared to controls in drained plots.

4.3.3.4 *nirK* and *nirS*

The *nirK* and *nirS* genes provide an estimation of the genetic potential of microorganisms to produce NO and N_2O from NO_2^-. There is a consistent trend of significantly greater *nirK* (Figure 4.5b) and *nirS* (Figure 4.5c) abundance in unmounded plots relative to mounded plots at ALRF: for *nirK* in Jun-12 and Aug-12, and *nirS* in Jun-12, Jul-12 and Jun-13 (Table 4.1). No effect of fertilization on *nirK* and *nirS* abundance was observed at ALRF. There were several instances of interactions between mounding and fertilization (*nirK*: Oct-12 and Jun-13; *nirS*: Jun-12 and Jun-13). For *nirK* in Jun-13 and *nirS* in Jun-12 the fertilization effect was only observed in the mounding plots, while for *nirK* in Oct-12 the trend was reversed. Following quantification of *nirK* from Aug-12 samples the unmounded control soil had significantly greater than fertilized mound top samples and for Oct-12 samples fertilized control soil had significantly greater abundance than fertilized mound hollow soil, contributing to the interactive effects at these dates. The *nirS* gene also displayed locational differences, with unfertilized mound top soil being significantly lower than soil from control plots at three dates (Jun-12, Aug-12 and Jun-13). At SDT *nirK* had a greater abundance in the 2013 samples than in the 2012 samples (Figure 4.6b), whilst *nirS* abundance peaked only during the warmest sampling dates (Aug-12 and Sept-13, Figure 4.6c). There were two instances of a significant drainage effect for these genes, with *nirK* being significantly more abundant in Sept-13 in drained plots compared to undrained controls and *nirS* being significantly more

abundant in control plots than in drained plots in Jul-13 (Table 4.2). During Oct-12 sampling, both *nirK* and *nirS* were significantly greater in fertilized plots than in unfertilized plots. Drainage and fertilization interactions were observed in Jul-12 and drainage-fertilization-layer interactions were observed in Jul-13. No locational differences were found for these genes over the course of the study.

4.3.3.5 *nosZ*

With a median of about 10^5 copies g^{-1} dw soil, the *nosZ* gene had the lowest abundance of the denitrification genes quantified in this study (Figures 4.5d, 4.6d). At ALRF, there was significantly more *nosZ* abundance in control plots than in mounded plots throughout the field study (Jun-12, Jul-12, Oct-12, and Jun-13) (Table 4.1). The fertilized plots were significantly higher in *nosZ* than unfertilized plots in Jun-12. Jun-12 samples had significantly lower *nosZ* in mound top and hollow samples than in mineral soil from unmounded fertilized samples. The Jun-13 samples displayed locational differences as fertilized and unfertilized forest floors from unmounded plots and mineral soil from unmounded and unfertilized samples had significantly greater *nosZ* abundance than unfertilized M samples. At the SDT site, *nosZ* was significantly greater in the control plots than in the drained plots in Jul-13 and in mineral soil relative to forest floor layer in Oct-12 (Figure 4.6d, Table 4.2).

4.3.4 Functional gene abundance following potential denitrification incubation

The log *amoA* copy number g^{-1} soil (dw) in the ALRF soils following the PDR incubation experiment ranged from 5.6-6.8 for AOA and 4.4-5.8 for AOB (Figure 4.7a). AOB were significantly greater in soil from fertilized plots than from unfertilized. No effects on AOA or AOB *amoA* gene abundance was found after incubation in soils originating from mounded or unmounded plots at ALRF. At SDT, *amoA* ranged from 6.4-7.8 log gene copies to 5.6-6.8 log gene copies g^{-1} soil (dw) for AOA and from 3.3-4.5 log gene copies g^{-1} soil (dw) for AOB (Figure 4.8a). AOB *amoA* were significantly greater after -incubation in soil from fertilized plots at SDT. AOB *amoA* transcripts were not detected in the RNA extracted after the PDR incubation, although trace (<100) copies of AOA *amoA* transcripts were measured in several samples following PDR incubation (data not shown). No effect of drainage on AOA or AOB *amoA* abundance was found at SDT. AOB *amoA* gene abundance was significantly correlated with PDR at both ALRF ($r = 0.55$) and SDT ($r = 0.76$) following simple linear regression (Appendix G).

NarG ranged from log 8.5-9.0 gene copes g^{-1} soil (dw) at ALRF (Figure 4.7b), while expression of *narG* during PDR incubation resulted in between 6.4-8.0 log copies of transcripts (Figure 4.7c).

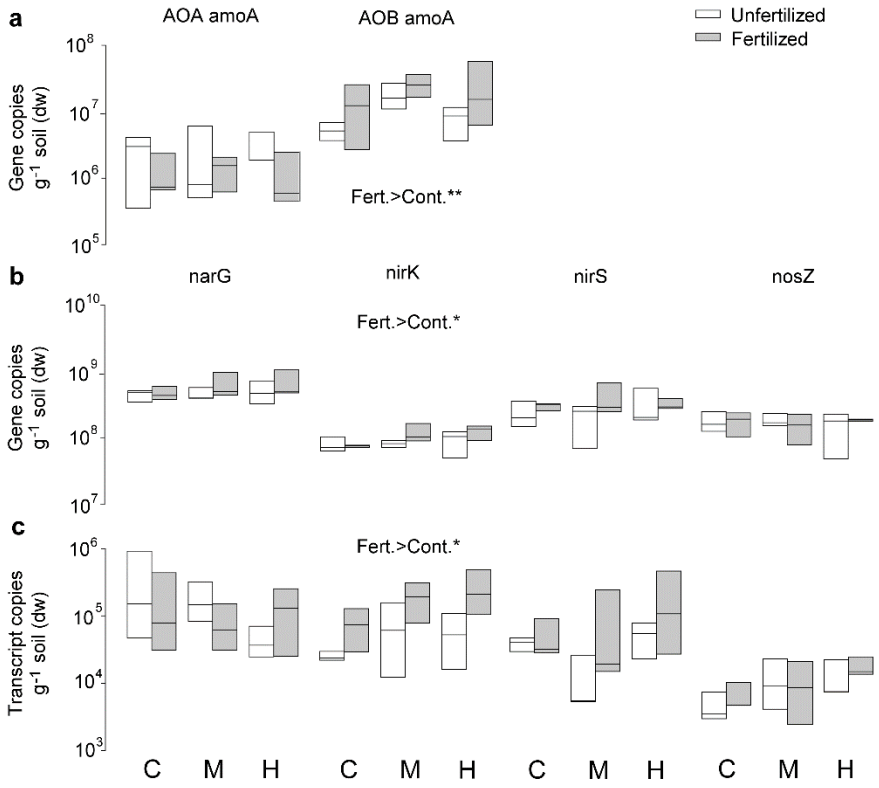

Figure 4.7. Nitrification and denitrification gene abundances following potential denitrification incubations from Jun-13 Aleza Lake Research Forest (ALRF) mineral soil samples. a) Nitrifying gene (AOA *amoA*, AOB *amoA*) abundance and b) denitrification gene (*narG*, *nirK*, *nirS*, *nosZ*) and transcript abundance from undisturbed control (C) and mounded plots (mound tops (M) and mound hollows (H)) subject to fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 3. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

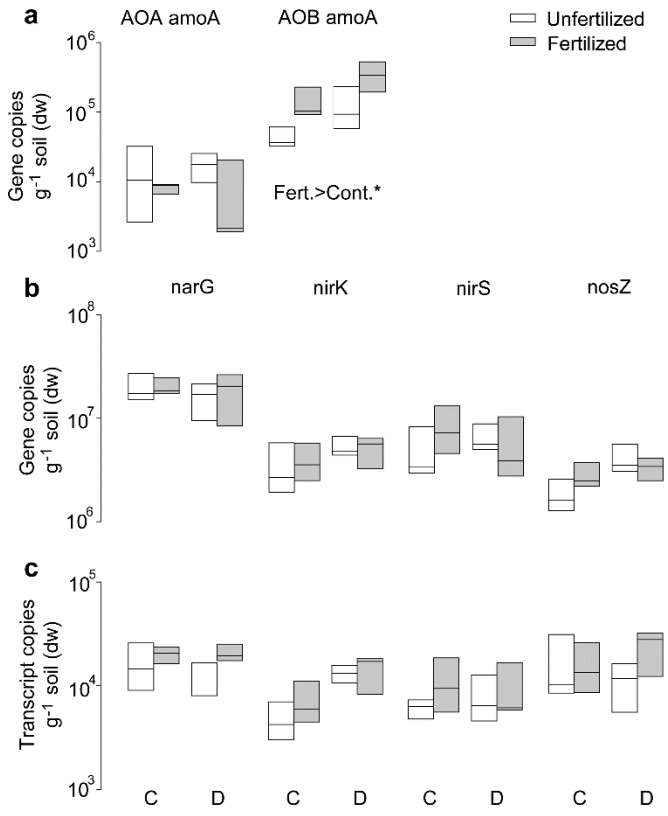

Figure 4.8. Nitrification and denitrification gene abundances following potential denitrification incubations from Jul-13 Suquash Drainage Trial (SDT) samples. a) Nitrifying gene (AOA *amoA*, AOB *amoA*) abundance and b) denitrification gene (*narG*, *nirK*, *nirS*, *nosZ*) and transcript abundance from undisturbed control (C) and drained (D) plots subject to fertilization. Boxplots show median, 25% quartile and 75% quartile; n = 3. Treatment locations identified by different letters were significantly different at $p = 0.05$ following one-way ANOVA. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

Transcript abundance was lower than gene copy number for all targets. At SDT, *narG* genes and transcripts ranged from 6.9-7.4 and 3.9-4.4 log copies g^{-1} soil (dw), respectively (Figure 4.8b,c). Treatment effects were not shown for *narG* following PDR incubation. At ALRF, *nirK* and *nirS* gene abundance ranged from 6.7-7.2 and 8.3-8.9 log copies g^{-1} soil (dw), respectively (Figure 4.7b), while gene transcripts ranged from 3.1-4.7 and 3.2-5.7 log copies, respectively (Figure 4.7c). The abundance of both *nirK* genes and transcripts were significantly greater in soil samples from fertilized ALRF plots relative to unfertilized soil following PDR incubation. At SDT, *nirK* and *nirS* genes ranged from 6.3-6.8 and 6.4-7.1 log copies g^{-1} soil (dw), respectively (Figure 4.8b,c), while transcripts ranged from 3.5-4.3 and 3.7-4.3 log copies g^{-1} soil (dw), respectively. No effects of mounding or drainage on *nirK* or *nirS* abundance were found following PDR incubation. The abundance of *nirS* transcripts ($r = 0.64$, $p = 0.002$), *nirK* transcripts ($r = 0.49$, $p = 0.019$) and *nirK* genes ($r = 0.59$, $p = 0.004$) were positively correlated with PDR at ALRF (Appendix G). *NosZ* abundance was the lowest of the measured denitrification targets, ranging from 7.7-8.4 and 2.8-4.9 log copies g^{-1} soil (dw) for genes and transcripts, respectively at ALRF (Figure 4.7b,c), and from 6.1-6.7 and 3.7-4.5 log copies g^{-1} soil (dw) for genes and transcripts, respectively at SDT (Figure 4.8b,c). No treatment effects were found following two-way ANOVA of *nosZ* gene and transcript abundances, but *nosZ* transcription likely influenced PDR as the ratio of *nirS*:*nosZ* and *nirK*:*nosZ* transcripts correlated positively to PDR ($r = 0.83$, $p < 0.001$ and $r = 0.73$, $p < 0.001$, respectively) at SDT (data not shown).

4.3.5 Relationships between site preparation, fertilization, soil physico-chemical parameters and microbial gene abundances

Exploratory analysis was performed using PCA to determine relationships between measured variables and treatment parameters following field sampling. Soil water content and chemical factors used in this analysis were described in Chapter 2 and total bacterial 16S rRNA abundance was described in Chapter 3. Ordination did not differentiate between locations within treatments (e.g., between O and M soil layers or between M and H locations in mounded plots) to focus solely on potential treatment effects on soil parameters. For all ALRF samples, the first two principle components (PCs) explained 36.1% of the variation of the dataset; about 80% of the variation was contained within the first seven PCs (Figure 4.9a). The mean of sample coordinates along PC1 and PC2 grouped by mounding and fertilization treatments separated mounded and control plots along PC2 with little difference between fertilization treatments. The loading plot of all measured variables at ALRF (Figure 4.9b) revealed relationships between factors at ALRF. N_2O flux varied along PC1, along with total N, NO_3-N, nitrification genes and denitrification genes. AOB were significantly and positively correlated with N_2O flux (Appendix H).

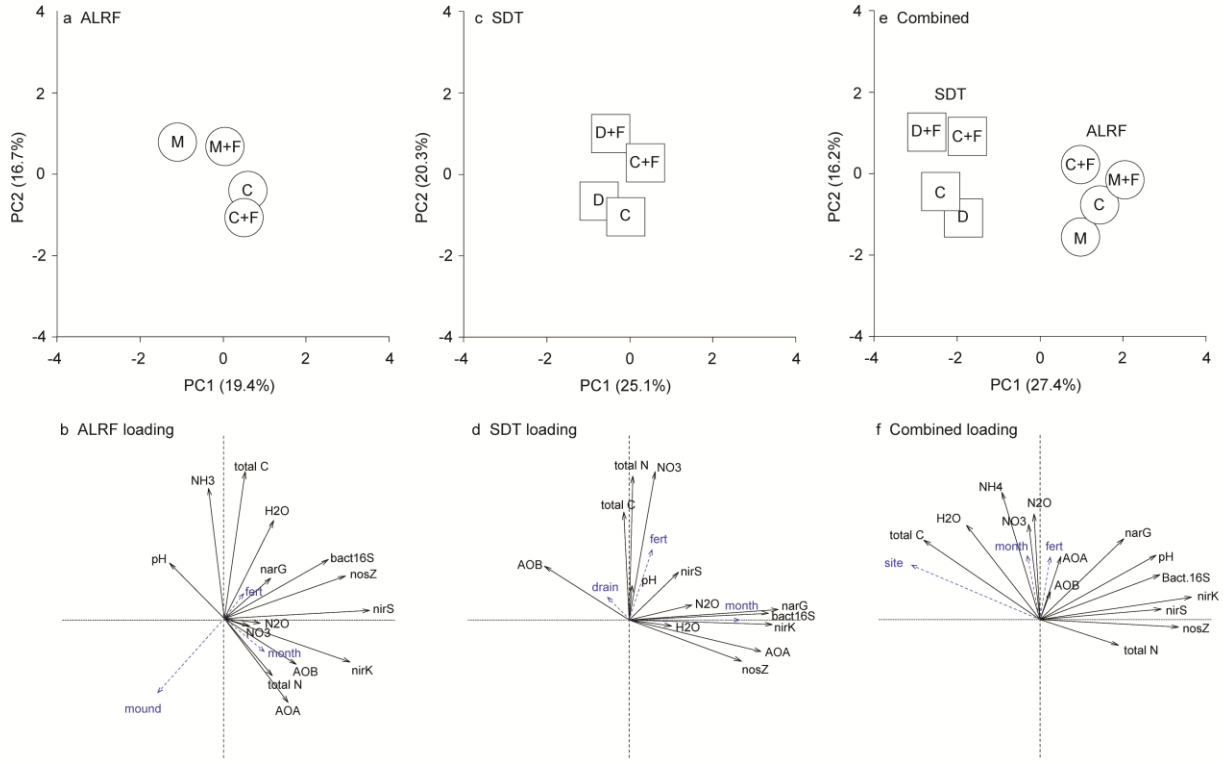

Figure 4.9. Principal component analysis (PCA) of microbial gene abundance, N_2O flux and soil characteristics at Aleza Lake Research Forest (ALRF) and Suquash Drainage Trial (SDT) showing a) mean sample coordinates grouped by treatment for ALRF (circles), b) factor loading plot for ALRF with secondary overlay of treatments (dashed arrows), c) sample coordinates for SDT (squares) showing treatment groupings, d) factor loading plot for SDT with treatment overlay (dashed), e) combined coordinates for both sites showing treatment groupings and f) factor loading plot for combined ALRF and SDT samples with treatment overlay (dashed). M; mounded; D, drained; C, control; +F, fertilized. Refer to Appendix L for the distribution of individual sampling points and treatment standard deviations following PCA.

Total C, NH_4-N and soil water content varied along PC2. AOA and AOB were significantly and positively correlated, and both nitrification genes were positively correlated with *nirK* gene abundance. The *nosZ* gene was positively correlated with total bacterial 16S rRNA, *nirK* and *nirS* abundance. Soil pH was related to soil nitrogen concentrations, specifically total N (negatively), NH_4-N and NO_3-N. Treatment factors were added to the PCA plot as supplementary dummy variables that did not influence ordination, with fertilization correlating positively with PC1 and associated factors, including N_2O flux. Mounding was negatively correlated with soil water content, NH_4-N and total C concentration. The supplementary "month" term represented the numerical value of the month of sampling (i.e., "6" for both Jun-12 and Jun-13 at ALRF). N_2O flux, NO_3-N concentration, AOA *amoA*, AOB *amoA* and *nirK* gene abundance displayed distinct seasonal patterns related to soil temperature and moisture at ALRF. Contributions of these parameters to the variation in functional gene abundances are presented in the following section.

PCA of SDT factors was able to explain 45.4% of variation along the first two PCs (Figure 4.9c). It took six PCs to explain about 80% of variation at this site. The scatterplot of PC coordinates showed no separation of samples by drainage treatment, but a separation along PC2 based on fertilization. The loading plot for SDT soil factors show that the differences that exist between drained and undrained plots are driven by soil water content on PC1, and that fertilization influenced total C, total N and NO_3-N concentrations along PC2 (Figure 4.9d). N_2O flux was significantly and positively correlated with soil water content along PC1 (Appendix I), but also had a positive correlation with bacterial 16S, *nirK* and AOA *amoA* abundance. AOA *amoA* was positively correlated with *nirK* abundance; *narG* was positively correlated with *nirK*, *nirS* and *nosZ*, while bacterial 16S was positively correlated with *nirK* and *nosZ*. Soil pH was negatively correlated with total C and positively with NH_4-N. N_2O flux, bacterial 16S, *narG* and *nirK* abundance all displayed seasonal patterns at SDT.

Following PCA of combined samples from both the ALRF and SDT sites to examine site-related effects of site preparation and fertilization on soil factors, PC1 and PC2 cumulatively explained 42.7% of dataset variation, with an additional five factors needed to explain over 80% of variation (Figure 4.9e). There was a distinct separation of soil parameters by site and treatments, with site differences exhibiting the largest separation along PC1 and fertilization providing weak separation along PC2. Loading plots of soil factors from combined samples and supplementary treatment variables show that site differences were positively correlated with total C and soil water content and negatively with total N (Figure 4.9f). Fertilization varied along PC2 with NO_3-N, NH_4-N and N_2O flux. N_2O flux was significantly and positively correlated with soil water content and pH across sites (Appendix J). Total bacterial 16S rRNA was positively correlated with *nirK*, *nirS* and *nosZ*, while AOA *amoA* and *narG* were also positively

correlated. Soil water content, in addition to significantly positively correlating with N_2O flux, was positively correlated to *narG*, though negatively to other denitrification genes. Total C was also significantly and positively correlated with *narG* and negatively with other denitrification genes. Total N was positively correlated with denitrification genes as was pH. Soil pH was positively correlated with several factors in addition to N_2O flux: total bacteria, denitrification genes and mineral N availability (NO_3-N, NH_4-N), though negatively with total C and soil water content. These data can be used to better understand how site preparation treatments are reflected in the relationships between functional gene abundance, N_2O flux and soil physico-chemical parameters across large geographic distances, which are explained in the next section.

4.3.6 Effect of soil physico-chemical parameters on N_2O flux and functional gene abundance

4.3.6.1 ALRF

Chapter 2 provided a detailed account of the effects of site preparation and fertilization on soil parameters. Here, constrained multivariate ordination is used to determine soil parameter relationships with functional gene abundances. Soil factors from Chapter 2 significantly explained the variation in the functional gene abundance data following constrained PCA (redundancy analysis (RDA)), where the only the gene abundance variation explained by the soil factors following multivariate regression are ordinated via PCA (Figure 4.10). RDA of ALRF gene abundances (R^2-adjusted: 0.65, $p = 0.005$) did not show distinct treatment effects, although samples clusted by date (Figure 4.10a). N_2O fluxes were greatest in Aug-12, and were most-closely associated with samples from this date following ordination. N_2O fluxes at ALRF showed a positive correlation between soil water content, *nirK*, AOA and AOB *amoA*, *narG* and *nirS* abundance.

Prior to variance partitioning, principal coordinate of a neighbour matrix (PCNM) analysis was used to test the influence of spatial structure on functional gene abundance. Analysis of ALRF sample coordinates resulted in 192 variables between nearest-neighbour sampling locations, four of which showed significant Moran's I statistics. At ALRF, nitrification and denitrification genes were significantly explained by the first PCNM axis. Spatial patterns for nitrification and denitrification genes at ALRF followed patterns of site preparation treatments when averaged by plot (See Appendix K for PCNM axes 1 and 2 values of treatment plots).

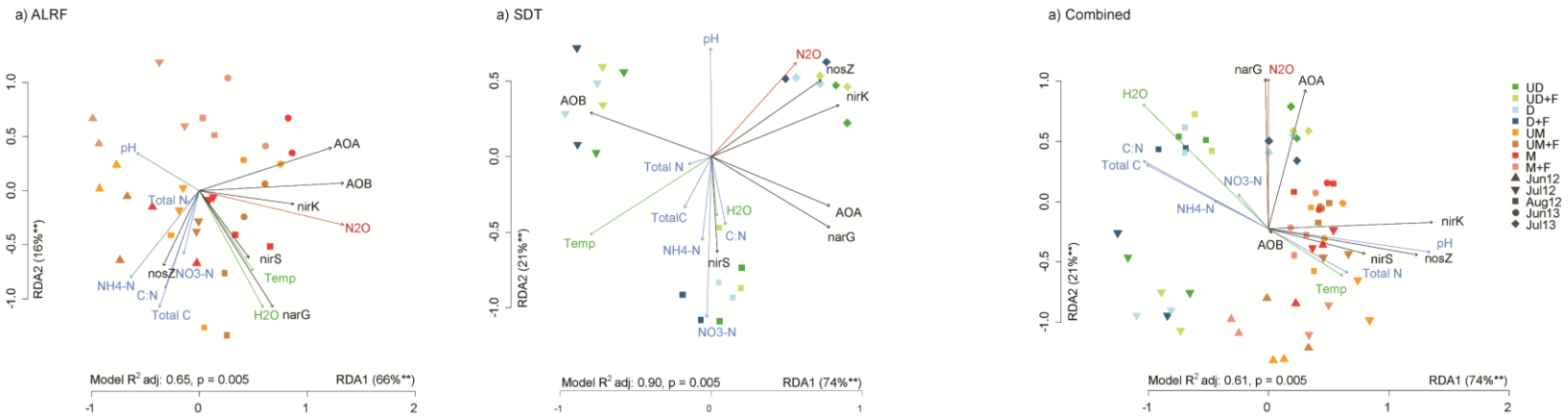

Figure 4.10. Redundancy analysis (RDA) of AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS* and *nosZ* gene abundance (black vectors) constrained by soil physical (green) and chemistry (blue) factors, with N_2O flux rates fit to model (red) for a) Aleza Lake Research Forest (ALRF), b) Suquash Drainage Trial (SDT) and c) combined ALRF and SDT measurements. Model and axis significance determined using Monte-Carlo permutation tests.

Table 4.3a. Canonical variance partitioning of functional gene and greenhouse gas parameters from Aleza Lake Research Forest (ALRF)

Model	df	N	F-Ratio	Total Variance (%)	Space	Physics/ Climate	Chemistry	Genes
Bacterial 16S	9	144	5.78***	18.4	1.5NS	1.0NS	9.5***	NA
Nitrification genes								
AOA *amoA*	4	144	10.84**	17.1		9.4**	15.0**	NA
AOB *amoA*	3	144	13.84***	16.8	10.5***	7.5***	6.0***	NA
Denitrification genes								
narG	7	144	5.64***	12.7	4.0**		0.6NS	2.7**
nirK	9	144	20.63***	48.1	5.1***	14.6***	8.3***	13.1***
nirS	6	144	12.86***	27.1	13.6***	8.3***		2.0*
nosZ	8	144	46.71***	67.3	2.8**	1.6**	0.3NS	43.9***
N_2O	7	144	10.36***	25.5	12.2***	0.8NS		8.8***
Potential Denitrification						Soil Parameters	Transcripts	Genes
PDR	8	18	7.26*	74.7			26.5*	26.8*

Table 4.3b. Explanatory variables in canonical variance partitioning models for Aleza Lake Research Forest (ALRF)

Model	df	Individual Variables			
Bacterial 16S	9	Total C 6.2**	NO_3 3.2**	Total N 2.8**	CN 2.4*
Nitrification genes					
AOA *amoA*	4	H_2O 8.6***	Total C 6.5***	temp 4.8**	
AOB *amoA*	3	H_2O 7.5***	$space_{V2}$ 10.5***	NH_4 6.0***	
Denitrification genes					
narG	7	$Space_{V2}$ 3.7**	AOB 2.7**		
nirK	9	temp 15.7***	NO_3 7.0***	AOB 4.0***	AOA 2.9**
nirS	6	$Space_{lat}$ 13.6***	temp 8.3***	AOA 2.0*	
nosZ	8	*nirS* 42.1***	AOB 2.2***	NO_3 1.6**	$Space_{V4}$ 0.6*
N_2O	7	AOB 11.8***	$Space_{V4}$ 3.7***	$Space_{lat}$ 3.5**	AOA 1.3*
Potential Denitrification					
PDR	8	*NirS*-tr 18.9*	*nirK* 14.6*		

Table 4.4a. Canonical variance partitioning of functional gene and greenhouse gas parameters from Suquash Drainage Trial (SDT)

Model	df	N	F-Ratio	Total Variance (%)	Space	Physics/ Climate	Chemistry	Genes
Bacterial 16S	3	48	12.77***	21.8		22.4***	1.8NS	NA
Nitrification genes								
AOA *amoA*	2	48	9.74***	12.1		9.6**	2.1**	NA
AOB *amoA*	2	48	18.37***	21.5		17.1***	1.3NS	NA
Denitrification genes								
narG	4	48	42.44***	56.6			8.5***	43.4***
nirK	3	48	44.71***	50.6		16.1***		19.3***
nirS	6	48	10.62***	31.2	0.2NS	15.4***	1.7*	11.0***
nosZ	3	48	16.34***	26.6		15.7***	3.5**	10.7***
N_2O	5	48	19.71***	42.4		29.9***		13.3***
Potential Denitrification						Soil Parameters	Transcripts	Genes
PDR	7	12	36.47***	90.6		37.3***		1.2NS

Table 4.4b. Explanatory variables in canonical variance partitioning models from Suquash Drainage Trial (SDT)

Model	df	Individual Variables			
Bacterial 16S	3	temp 22.4***			
Nitrification genes					
AOA *amoA*	2	temp 9.6**	Total C 2.1**		
AOB *amoA*	2	temp 17.1***			
Denitrification genes					
narG	4	AOA 25.7***	NO3 8.5***	AOB 3.8***	
nirK	3	*narG* 19.3***	temp 16.1***		
nirS	6	temp 14.1***	*narG* 11.0***	H_2O 3.4*	NO_3 1.7*
nosZ	3	temp 15.7***	NO3 3.5**	*nirK* 10.7***	
N_2O	5	H_2O 29.9***	aob 7.4***	*nirK* 6.3***	
Potential Denitrification					
PDR	7	N_2O 28.7***	Total N 20.7**		

Table 4.5a. Canonical variance partitioning of functional gene and greenhouse gas parameters from combined Aleza Lake Research Forest (ALRF) and Suquash Drainage Trial (SDT) samples

Model	df	N	F-Ratio	Total Variance (%)	Space	Physics/ Climate	Chemistry	Genes
Bacterial 16S	7	192	16.89***	25.9	10.7***		2.4***	NA
Nitrification genes								
AOA *amoA*	7	192	7.16***	11.9	7.8***	1.6**	7.5***	NA
AOB *amoA*	3	192	3.84**	2.6	1.3*	0.7NS	1.2*	NA
Denitrification genes								
narG	7	192	26.71***	36.1	2.8***		10.5***	7.6***
nirK	8	192	55.49***	57.7	1.8**	1.9**	15.1***	6.2***
nirS	6	192	27.51***	33.3	6.2***	5.6***	1.9**	1.1*
nosZ	10	192	49.21***	60.2		0.2NS	0.2NS	27.51***
N_2O	8	192	26.447***	39	10.2***	4.4***	17.4***	1.7**
Potential Denitrification						Soil Parameters	Transcripts	Genes
PDR	5	30	14.66***	70.2		4.3*	30.3**	6.2*

Table 4.5b. Explanatory variables in canonical variance partitioning models for combined Aleza Lake Research Forest (ALRF) and Suquash Drainage Trial (SDT) samples

Model	df	Individual Variables				
Bacterial 16S	7	ph 6.6***	Total C 4.8***	$Space_{Long}$ 2.4***		
Nitrification genes						
AOA *amoA*	7	space - long 7.8***	Total C 2.5**	H_2O 1.6**	pH 1.2*	
AOB *amoA*	3	space - V2 1.3*	pH 1.2*			
Denitrification genes						
narG	7	AOA 7.6***	pH 5.4***	$Space_{V2}$ 2.8**		
nirK	8	pH 14.9***	AOA 6.9***	NH_4 6.0***	Total C 4.2***	Total N 2.1***
nirS	6	SpaceV1 6.6***	temp 5.5***	AOA 1.1*	NH4 0.8*	Total N 0.7*
nosZ	10	nirk 23.5***	nirs 8.0***			
N_2O	8	pH 14.3***	H_2O 4.8***	NH_4 2.2**	*narG* 1.0*	*nirS* 0.9*
Potential Denitrification						
PDR	5	*nirK*-tr 22.3**	*nirS*-tr 10.2*			

At ALRF, individual gene variation partitioning provided an explanation for 18.4% of bacterial abundance variation with soil chemistry (total C, total N, NH_4-N, NO_3-N, C:N, ratio, pH) significantly explaining 9.5% of variation (Table 4.3a). Broken down into individual factors, total C, total N, NO_3-N and the C:N ratio explained 6.2%, 2.8%, 3.2% and 2.4% of variation, respectively (Table 4.3b). 17.1% of AOA *amoA* variation was explained by variation partitioning, with 15% unique variation explained by soil chemistry (6.5% total C) and 9.4% by soil physico-climactic parameters (8.6% soil water content, 4.8% temperature). 16.8% of AOB *amoA* variation was explained: 10.5% by spatial parameters (PCNM2), 6% by soil chemistry (NH_4-N) and 7.5% by soil physico-climactic parameters (water content). Only 12.7% of *narG* variation was explained: 4% by spatial parameters (3.7% PCNM2) and 2.7% by functional genes preceding *narG* in the coupled nitrification/denitrification pathway (AOB *amoA*). Individual percentages are provided when individual factors significantly explain part of the total group variance, as shown in Table 4.3b, otherwise if a single factor is shown without an associated percentage it explains the entirety of variation allocated to its category. 48.1% of *nirK* variation was explained: 5.1% by spatial parameters (1.2% PCNM4), 8.3% by soil chemistry (7% NO_3-N), 14.6% by soil physico-climactic parameters (15.7% temperature) and 13.1% by preceding genes (4% AOB *amoA*, 2.9% AOA *amoA*). 27.1% of *nirS* variation was explained: 13.6% by spatial parameters (latitude), 8.3% by soil physico-climactic parameters (temperature) and 2% by preceding genes (AOA *amoA*). 67.3% of *nosZ* variation was explained: 2.8% by spatial parameters (0.6% PCNM4), 8.3% by soil chemistry (7% NO_3-N), 14.6% by soil physico-climactic parameters (15.7% temperature) and 13.1% by preceding genes (4% AOB *amoA*, 2.9% AOA *amoA*). For *in situ* N_2O flux rate at ALRF, 25.5% of variation could be explained by spatial structure (12.2%), further divided into PCNM4 (3.7%) and latitude (3.5%), and 8.8% nitrification/denitrification genes, further divided into AOB *amoA* (11.8%) and AOA *amoA* (1.3%) genes. Spatial components were not calculated for PDR; variation of potential denitrification was partitioned into soil abiotic parameters, functional gene abundances and transcript abundances. At ALRF soil parameters did not significantly explain any portion of PDR variation, while functional gene abundance explained 26.8% (14.6% *nirK* gene) and transcripts explained 26.5% (18.9% *nirS* transcript) for a total explained variation of 74.7%.

4.3.6.2 SDT

Soil variabes were able to explain about 90% of the variation of gene abundances measured at SDT following RDA (p = 0.005) (Figure 4.10b). A clear separation of gene abundances due to sampling date was observed. N_2O fluxes were greatest in Jul-13 samples, as were nosZ and nirK abundances, which clustered together with samples from this date and were significantly and positively correlated with pH.

PCNM of SDT sampling locations resulted in 128 variables, three of which were positive and had significant Moran`s I statistics for spatial autocorrelation, through forward selection did not produce any PCNM variables that significantly explained variation in functional gene abundance. As a result, variation partitioning at SDT did not include a spatial term. Variation partitioning of SDT samples explained 23.2% of variation of all functional genes (Figure 4.10d). Two variables were significant following forward selection, temperature and NO_3-N, which explained 21% and 3.1% of variation of SDT functional genes, respectively.

Canonical variation partitioning was applied to individual gene measurements at SDT to determine how changes in the distributions of parameters grouped into spatial, chemical and physico-climactic factor categories influence of functional gene abundance. 21.8% of bacterial abundance variation at SDT was explained following variation partitioning, with physico-climactic factors explaining 22.4% of variation (Table 4.4a). The difference between total and single-group explained variation is due to the overlap of factor grouping, which can have negative interactive effects. When RDA is used to determine single-factor contributions to variation, the variation of all other model variables are partialed out. The result for bacterial abundance at SDT was that temperature was shown to explain 22.4% of bacterial abundance variation (Table 4.4b). For nitrification genes, 12.1% of AOA *amoA* variation was explained: 2.1% by soil chemistry (total C) and 9.6% by soil physico-climactic parameters (temperature). 21.5% of AOB *amoA* variation was explained with 17.1% coming from soil physico-climactic parameters (temperature). For denitrification genes, 56.6% of *narG* variation was explained: 8.5% by soil chemistry (NO_3-N) and 43.4% by genes preceding *narG* in the coupled nitrification/denitrification pathway. Of this variation, AOA and AOB *amoA* explained 25.7% and 3.8%, respectively. 50.6% of *nirK* variation was explained with 16.1% by soil physico-climactic parameters (temperature) and 19.3% by preceding genes (*narG*). The remaining 15.2% is overlapping variation shared by the variables included in the partitioning model. 31.2% of *nirS* variation was explained, with 1.7% by soil chemical parameters (1.7% NO_3-N), 15.4% by soil physico-climactic parameters (14.1% temperature, 3.4% water content) and 11% by preceding genes (*narG*). 26.6% of overall *nosZ* variation was explained: 3.5% by soil chemical parameters (NO_3-N), 15.7% by soil physico-climactic parameters (temperature) and 10.7% by preceding genes (*nirK*). 42.4% of N_2O flux rate variation at SDT was explained and partitioned into physico-climactic and gene abundance factors, which accounted for 37.3% and 13.3% of variation, respectively. 29.9% of N_2O flux variation was explained by soil water content, while functional genes AOB *amoA* and *nirK* explained 7.4% and 6.3% of N_2O flux variation, respectively. 90.6% of PDR variation was explained by the canonical model, with 37.3% uniquely explained by abiotic soil parameters, 28.7% as N_2O flux

rate and 20.7% as total N concentration. This is the highest amount of explained variation calculated in this study.

4.3.7 Between-site variation

Samples from ALRF and SDT were combined for constrained ordination with RDA (R^2-adjusted: 0.61, $p = 0.005$), which showed differentiation of ALRF and SDT sites and sampling dates. N_2O fluxes were assocated with samples from undrained plots at SDT, and were positively correlated with AOA *amoA* and *narG* gene abundances, as well as soil moisture and total C. Denitrification genes (*nirK*, *nirS* and *nosZ*) clustered with ALRF samples from Jul-12, where pH, soil temperature and total N were greatest. Unlike ALRF ordination, AOB *amoA* was not significantly correlated with any other factors following combined RDA.

PCNM produced 129 spatial variables, four of which were positive and had significant Moran's I statistics. Of these four, only one significantly explained functional gene abundance in the combined dataset. This spatial parameter (PCNM1) was included in partitioning of variation along with five chemical factors (total C, total N, NH_4-N, NO_3-N and pH) and both physico-climactic factors, which explained 4.4%, 9.5% and 1.5% of variation, respectively, with significant overlap between space and chemistry (9.5%) and all three factor groupings (3.5%) (Figure 4.10f).

Partitioning of functional gene between-site variance using canonical modeling explained 25.9% of bacterial 16S rRNA variation (10.7% space, 2.4% chemistry) (Table 4.5a). Partially variation into unique factors showed that pH, total C and longitude significantly explained 6.6%, 4.8% and 2.4% of bacterial 16S rRNA variation following RDA and model testing with 1000 permutations (Table 4.5b). 11.9% of AOA *amoA* variation was explained: 7.8% by spatial structure (longitude), 7.5% by soil chemical parameters (4.2% total C, 1.2% pH) and 1.6% by soil physico-climactic parameters (soil water content). 2.6% of AOB *amoA* variation was explained: 1.3% by spatial structure (PCNM2) and 1.2% by soil chemical parameters (pH). This was the lowest total variation explained for any factor in this study. 36.1% of *narG* variation was explained: 2.8% by spatial structure (PCNM2), 10.5% by soil chemical parameters (5.4% pH) and 7.6% by nitrification genes (AOA *amoA*). 57.7% of *nirK* variation was explained: 1.8% by spatial structure (PCNM1), 15.1% by soil chemical parameters (14.9% pH, 6% NH_4-N, 4.2% total C, 2.1% total N), 1.9% by soil physico-climactic parameters (temperature and soil water content, neither of which significantly explained *nirK* variation following independent partial regression) and 6.2% by preceding functional genes (AOA *amoA*). 33.3% of *nirS* variation was explained by variance partitioning with RDA. 6.25%, 1.9%, 5.6% and 1.1% of *nirS* variation was allocated to spatial, chemical,

physical and genetic factor categories, respectively, with PCNM1, temperature, AOA *amoA*, NH*4*-N and total N uniquely explaining 6.6%, 5.5%, 1.1%, 0.8% and 0.7% of this variation, respectively. 60.2% of *nosZ* variation was explained by the forward selected model, with 27.51 uniquely explained by preceding functional genes (23.5% by *nirK* and 8% by *nirS*). 39% of N_2O flux variation was explained by the reduced model: 10.2% by spatial parameters (longitude), 17.4% by soil chemistry (14.3% pH, 2.2% NH_4-N), 4.4% by physical factors (4.8% soil water content) and 1.7% by functional gene abundance (1% *narG*, 0.9% *nirS*). When ALRF and SDT PDR are modeled together 70.2% of the total variation explained, with soil parameters accounting for 4.3% (pH of sample, not of incubation), with nitrification/denitrification genes explaining 6.2% and transcripts explaining 30.3%. The only factors that explained significant amounts of variation when partialed against other forward-selected variables were the transcripts of *nirK* and *nirS*, which explained 22.3% and 10.2% of PDR variation, respectively.

4.4 Discussion

4.4.1 Factors influencing N_2O flux

N_2O flux rate was not changed by mounding but elevated by fertilization at both ALRF and SDT for at least one year following fertilization (Figure 4.1). Peak emissions varied between the two sites. N_2O fluxes rates are stimulated by N inputs to soil (Bateman and Baggs, 2005; Jassel et al., 2011; Pielegaard, 2013; Ussiri and Lal, 2013; Wu et al., 2013), though not always (Pang and Cho, 1984; Johnson and Curtis, 2001; Basiliko et al., 2009; Gundersen et al., 2012). For example, fertilization of a 58-year-old coastal Douglas-fir stand in BC with 200 kg N ha^{-1} urea significantly increased N_2O emissions on all sampling dates during the growing season following application (Jassel et al., 2011). Flux rates of up to 30 µmol m^{-2} h^{-1} were recorded in fertilized plots during the growing season, a 650% greater flux rate than the highest rates found in this study at ALRF in Aug-12, when converted to µg m^{-2} h^{-1} (Figure 4.1a). Jassel et al. (2011) found that unfertilized plots did not emit significant amounts of N_2O, nor did treatment plots the year following fertilization. In this study, low N_2O emission rates were measured in soil that were not fertilized. At SDT, the highest flux rates were recorded one year following fertilization (Figure 4.1b). Concentrations of NH_4-N and NO_3-N peaked in soil at SDT towards the end of 2012 (Chapter 2), suggesting that fertilizer entered the soil and was mineralized more slowly at SDT than at ALRF. Vegetation cover, including the presence of a thick salal understory at SDT, likely slowed fertilizer contact with the soil, and plant uptake may have been a major sink for applied N compared to the exposed mineral soils at ALRF. These factors don't entirely explain the large N_2O emissions from 2012 samples, nor does temperature, as it was not the warmest sampling date in this study (13^{o}C mean daily temperature

compared to 14^{o}C in Sep-13). Major rainfall prior to the Jul-13 sampling could have increased fertilizer N infiltration into anaerobic zones in the soil at SDT. Measurement of organic matter decomposition, mineralization and nitrification rates following mid-rotation fertilization could further elucidate the contributions of the various N sources to N_2O emissions in these plots. Soil chemistry did not explain significant amounts of variation in N_2O flux at either ALRF or SDT following variation partitioning (Tables 4.2a, 4.3a), though soil chemistry explained the highest percentage of between-site N_2O flux variation due mainly to the major effect of chemistry on N_2O at SDT (Table 4.4a), with NH_4-N explaining the third-most unique variation of all individual variables. Therefore at ALRF, soil water content, temperature and the abundance of microbial functional groups in the nitrification and denitrification pathways played important roles in N_2O flux regulation.

Soil water content appeared to influence N_2O flux rates at ALRF and SDT, as well as between-site differences in N_2O fluxes. Low-O_2 environments and high NO_3-N availability are required for the denitrification process to result in N_2O production (Smith et al., 1998; Bateman and Baggs, 2005; Dobbie and Smith, 2006). PCA demonstrated that following site preparation and fertilization treatments NO_3-N and soil water content generally correlated positively with N_2O emissions at ALRF and SDT, and at the two sites combined (Figure 4.9). Water table depth likely effected N_2O flux from mound hollows at ALRF. The high water table during early spring sampling dates led to standing water in the hollows. While high soil moisture is associated with N_2O-emitting sites (Weier et al., 1993; Smith et al., 1998; Davidson et al., 2000; Bateman and Baggs, 2005; Pilegaard et al., 2006; Pilegaard, 2013), fully saturated soil can suppress emissions (Hou et al., 2013), and even lead to N_2O uptake (Davidson et al., 2000; Chapuis-Lardy et al., 2007; Goldberg and Gebaur, 2009), as was seen in hollows covered in standing water at ALRF in Jul-12. The greatest efflux rate of N_2O occurred in wet soil (i.e., water content between 40-60%), not in saturated soil (water content between 60-80%) (See chapter 2 for water content), in agreement with field measurements reported in Smith et al. (1998). Water saturation of soil in mound hollows was alleviated as temperature increased and the water table drew down. These factors would be expected to create a flush of N_2O, particularly in hollows that had collected NPK-S fertilizer. By Aug-12 hollows were the largest locational sources of N_2O in mounded plots, the combination of high levels of accumulated mineral N (Chapter 2), high soil moisture and high *nirS* gene abundance (Figure 4.5b) likely contributed to the hot-spots of N_2O fluxes from the hollows in Aug-12 mentioned previously. This study was not designed to test the effect of water table depth on N_2O flux following fertilization; this, therefore, remains an area of necessary future research. In an imperfectly-drained grassland soil, Dobbie and Smith (2006) found a negative exponential relationship between soil water table depth and N_2O emissions following periodic fertilization with 100 kg ha^{-1} NH_4-NO_3-N. In a previous study drainage at SDT

reduced water table depth (Sajedi et al., 2012), and in this study drainage reduced N_2O flux in Jul-12 (Figure 4.1b). However, no effects of drainage on denitrifying genes were observed for this date (Figure 4.6). Nonetheless N_2O flux was correlated with soil water content at SDT (Figure 4.9d) and soil water content accounted for the highest percentage of variation in N_2O flux of any individual variable at SDT (Table 4.5b), and the second-highest for between-site N_2O flux differences (Figure 4.4b). Without the ability of aerobic nitrifiers to supply NO_3^-, denitrifiers require low-energy N_2O-reduction in order to oxidize organic C in soil. The data presented in this study agrees with the conceptual model that N_2O flux in waterlogged forest ecosystems depends not only on the ability of aerobic microorganisms to mineralize and nitrify urea-N following fertilization (and independently produce N_2O in unsaturated soil), but also on the influence of soil water content on the N_2O/N_2 output ratio of O_2-sensitive denitrifying microorganisms (Davidson et al., 2000; Bateman and Baggs, 2005; Chapuis-Lardy et al., 2007).

Other soil factors affected by mounding, drainage and fertilization and that influence N_2O flux include soil C and C:N ratios and pH. Soil pH and C:N ratio have been proposed as the primary landscape-scale drivers of denitrification (Pielegaard, 2013; Gundersen et al., 2012; Klemedtsson et al., 2013). Soil pH has a strong influence on many soil factors that effect N_2O emissions including negative correlations with N transformation rates (Bremner, 1997; Mørkved et al., 2007), ammonia-oxidizing community diversity and activity (de Boer et al., 1996; de Boer and Kowalchuck, 2001; Nicol et al., 2008), nitrification (Dancer et al., 1973; Ste-Marie and Paré, 1999), denitrification (Šimek and Hopkins, 1999; Šimek et al., 2002; Šimek and Cooper, 2002; Čuhel et al., 2010) and N_2O:N_2 ratio (Stevens and Laughlin, 1998; Šimek et al., 2002; Dannenemann et al., 2008; Čuhel et al., 2010). Soil at ALRF had lower concentrations of total C and N as well as available mineral N, relative to soil at SDT, and mounded plots had lower total C, N and mineral N concentrations than unmounded controls (Chapter 2). These data suggest a lower N_2O production potential of ALRF soils and particularly soils in mounded plots at ALRF, though soil at ALRF also had higher mean pH than SDT: 4.7 ± 0.1 compared to 3.6 ± 0.1, which suggests a greater N_2O production potential of ALRF soils. There were no site-related differences in N_2O flux rate ranges between ALRF and SDT, though site differences in N_2O flux variation were allocated primarily to pH. These data seem to support the conclusions of Pielegaard (2013), that N_2O flux over large geographic areas are significantly influenced by soil pH.

Testing the hypothesis that GHG emissions are related not only to soil physico-chemical factors but to functional gene abundances is a current goal in molecular ecology, including in forest ecosystems (Morales et al., 2010; Rasche et al., 2011; Petersen et al., 2012). Morales et al. (2010) found that the difference between *nirS* and *nosZ* gene copies was positively correlated to *in situ* N_2O flux rates in agricultural and deciduous forested sites in Michigan containing Hapludalf (Gray Brown Luvisol) soil.

Rasche et al. (2011) found positive correlations between AOA *amoA*, *nirS* and *nosZ* with N_2O emissions in a 65-year-old Austrian beech forest on Dystric Cambisol (Dystric Brunisol) soil. In this study, *narG*, *nirK*, *nirS* and *nosZ* did not correlate significantly with N_2O (Figure 4.9), nor did linear combinations of these genes, though they did have weak positive relationships following multiple comparisons (Appendices 5a-5c). At spatial and temporal scales relevant to the management of forests for GHG emissions, models of N_2O flux from a lowland tropical forest in Costa Rica using functional gene (*nirK*, *nirS*, *nosZ*) abundances were equivalent to models constructed using NO_3^-, pH and soil moisture (Graham et al., 2013). While the authors were able to improve multiple regression models of soil process rates, including N_2O flux, in individual sampling months by using functional genes, they did not find that the inclusion of these terms improved linear models of temporally-pooled datasets. In this study, AOB *amoA* abundance was significantly correlated to N_2O flux at ALRF, though N_2O flux at SDT and in pooled analysis was correlated with soil physico-chemistry (soil water content and pH) (Figure 4.9). Following variation partitioning of N_2O flux at ALRF and SDT, similar trends emerged, with variation of N_2O flux at ALRF being primarily explained by AOB *amoA* abundance, though also weakly, but significantly, by AOA *amoA* (Table 4.3b), variation of N_2O flux at SDT being explained by AOB *amoA* and *nirK* abundance (Table 4.4b), and between-site N_2O flux variation being weakly influenced by *narG* and *nirS* abundance (Table 3b). In all cases gene abundance explained less variation than other significant sources of variation, including spatial patterns at ALRF (Table 4.3a), soil physico-climactic patterns (SDT, Table 4.4a) or soil chemistry (combined, Table 4.5a). These data suggest that the abundance of functional groups involved in nitrification and denitrification plays an essential, though secondary, role in the regulation of N_2O fluxes in the wet forest ecosystems measured in this study. Of particular interest is the consistent importance of nitrifying organisms, primarily the AOB, in explaining variation of N_2O fluxes in this study. While several studies report increases in AOB *amoA* abundance following N addition to soil (Long et al., 2012; Zhang et al., 2013) or associations between AOA *amoA* abundance and N_2O flux (Rasche et al., 2011), this study is the first to my knowledge to show positive correlation between AOB *amoA* abundance and N_2O flux rates. The importance of high soil moisture levels and NO_3-N concentrations for denitrification are discussed in previous sections. It seems likely that nitrifying communities in high water content, low-N environments (such as ALRF, and to a lesser extent SDT) are stimulated by spatial and temporal windows of low-water content and pulses of mineral N addition. The abundance and activity of nitrifiers (and AOB in particular) appear to be crucial links between moisture levels, N concentrations and the production of N_2O, either directly through nitrifier denitrification (Wrage et al., 2001; Clough et al 2004; Bateman and Baggs, 2005) or denitrification coupled to the availability of NO_3-N. While this study shows that AOB, not AOA, increase in abundance in response to N fertilization (a result also shown by Di et al. (2010), Long et al. (2012) and Wertz et al. (2012)), further research is

required to determine the role of the AOB in N_2O fluxes in wet forest soils subject to fertilization. This relationship could be clarified, for example, by measuring AOA and AOB *amoA* expression in a ^{15}N-labelling study at varying soil moisture and N input (type and concentration) regimes.

4.4.2 Factors influencing potential denitrification

Assays of potential net and gross denitrification using soil from Jun-13 and Jul-13 from ALRF and SDT, respectively, were used to understand the links between treatments, soil conditions, the microbial community and N_2O production under ideal conditions. While site preparation did not affect PDR at SDT, at ALRF, soil from the mounded plots, unexpectedly, had greater PDR than soil from unmounded plots (Figure 4.2a). Soil C:N ratio and pH were higher in these mounded plots than in unmounded plots (Chapter 2). C:N ratio negatively correlated with *in situ* N_2O flux rates over large C:N ranges in forests in Germany (Klemedtsson et al., 2005). However, at ranges measured at ALRF (<20), Klemedtsson et al. (2005) reported that other factors such as climate, pH and groundwater depth are more important than C:N ratios for regulating N_2O flux. While pH and *in situ* N_2O output (i.e., the $N_2O/(N_2O + N_2$ ratio) were negatively correlated in German acidic fen soil (Palmer et al., 2010), Simek and Cooper, 2002) reported that the optimum pH for PDR is unclear. PDR in fertilized treatments were significantly greater than those from unfertilized treatments at both ALRF and SDT (Figure 4.2). Mineral N concentrations were higher in the fertilized plots compared to unfertilized plots, as shown in Chapter 2 and previously discussed in this section, though during PDR the added NO_3-N provides abundant substrate for denitrification in all incubated soils. PDR in soil from SDT showed strong positive correlation to pre-incubation NO_3-N concentrations (Appendix G), indicating that potential denitrification at this site was influenced primarily by soil chemistry. When RDA was used to allocate variance of PDR at SDT, 90.6% of PDR variation was accounted for, with soil parameters being the only significant source of this variation (Table 4.5a). Total N and *in situ* N_2O rate were the primary individual factors explaining unique sources of PDR variation (Table 4.5b).

Other than NO_3-N and organic C limits on N_2O production via denitrification (Bijay-singh et al., 1988; McCarty and Bremner, 1992; Weier et al., 1993; Hill and Cardaci, 2004), the abundance and activity of nitrifying and denitrifying communities can also influence N_2O flux rates and PDR. Fertilization was not shown to influence total C concentrations at ALRF or SDT. However, *in situ* (Figure 4.3b) and post-incubation (Figures 4.7, 4.8) abundance of AOB *amoA* genes were greater in soil from fertilized plots than in unfertilized plots at the dates from which soil was removed for PDR incubation. The abundance of *nirK* genes and transcripts were also greater in fertilized soil after incubation at ALRF

(Figure 4.7). In a study of the relationship between soil physico-chemical parameters, microbial functional genes and PDR in a range of natural ecosystems in Alaska, Petersen et al. (2012) found correlations between AOB *amoA*, *nirK*, *nirS* and *nosZ* gene abundance were with PDR in forest soil and developed a structural equation model to demonstrate how denitrification genes can be used to predict PDR. The extent to which the soil's genetic potential for denitrification influences PDR is yet to be resolved. Attard et al. (2011) found significant positive correlation between *nirK* gene abundance and PDR in agricultural soil, yet they concluded that the role of soil characteristics such as soil organic C, water-filled pore space (WFPS) and NO_3^- explained a greater portion of the PDR variance than functional genes in the denitrification pathway following multiple regression analysis. It remains to be established that organism or gene abundance directly affects activity rates. At ALRF potential rates were positively correlated with abundance of nitrite reductase genes and transcripts (*nirK* and *nirS*) (Appendix G) and *nirK*:*nosZ* and *nirS*:*nosZ* transcript ratios. *NirK* and *nirS* abundance have previously been linked with PDR (Dandie et al., 2008; Miller et al., 2008; Baudion et al., 2009; Djigal et al., 2010; Song et al., 2010; Petersen et al., 2012). Positive correlations have also been measured between *nirS* genes and *in situ* N_2O flux rates (Rasche et al., 2011), but not in all environments (Čuhel et al., 2010; Morales et al., 2010). Liu et al. (2010) showed that *nirS* and *nosZ* transcript abundance was not detectable *in situ* from peat soil in western Norway, but rapidly peaked to about 10^6 copies g^{-1} soil within 1-5 hours following the start of PDR incubation, after which time transcript copy number declined with equal rapidity. The *nirS* and *nosZ* transcript abundance measured by Liu et al. (2010) is up to two orders of magnitude greater than the abundance of the transcripts measured in this study (Figures 4.7, 4.8), although *nirK* transcripts were detected at greater abundances in this study than shown by Liu and colleagues. In this study *nirS* and *nirK* transcript abundances were positively correlated with PDR at ALRF (Appendix G) and the abundance of *nirS* transcripts explained the greatest about of PDR variation at ALRF, with *nirK* genes explaining the second-most (Table 4.4b), though when the variation of PDR from both ALRF and SDT sites was partitioned together, *nirK* and *nirS* transcripts were both found to significantly explain PDR variation. Despite the limitations of transcript detection from field soil likely due to low transcript rates (Liu et al., 2010), the expression of denitrification genes is probably a major influencing factor on PDR in these soils.

The relationship between AOB and PDR is probably due to reported high degrees of correlation between AOB and nitrification rate, which has a strong, positive relationship with PDR (Petersen et al., 2012). There is also evidence that even in O_2 concentrations of 0.5% nitrifier denitrification accounts for about half of N_2O production during PDR incubation (Zhu et al., 2013). It is unlikely that the PDR method used in this study completely removed O_2 from the incubation. No AOB *amoA* transcripts were

detected following the PDR incubation and sources of *nirS* and *nirK* transcripts were not differentiated. Future work should focus on sequencing transcripts of denitrification genes to determine their origin to determine contribution of nitrifying and denitrifying functional groups to N_2O production. There is much debate over the ecological role of AOB and AOA. AOB abundance can be greater (Mertens et al., 2009; Petersen et al., 2012) or significantly less than AOA (Leininger et al., 2006; He et al., 2007; Adar and Schwartz, 2008; Onodera et al., 2010; Bru et al., 2011) in a range of ecosystems, with N-enriched ecosystems generally favouring AOB. In this study AOB were less abundant but more responsive to fertilization relative to AOA. Wertz et al. (2012) also found that AOB *amoA* increased with fertilization (200 kg N ha^{-1}), as did soil NO_3^- concentrations and potential nitrification rates in lodgepole pine (*Pinus contorta* ssp. *latifolia*) and spruce (*Picea glauca*) stands in BC. This study suggests that AOB are more responsive to N fertilization than AOA, and that bacterial nitrification gene abundances may be a strong predictor of PDR and field N_2O emissions across gradients of mineral N availability. The use of both microbial gene and transcript abundance in models of denitrification from soil depends on the ability to measure and link genes and transcripts to denitrification potential, and to demonstrate that they are important measures for the prediction of soil N_2O flux rates.

4.4.3 Factors influencing functional gene abundance

4.4.3.1 Nitrification genes

AOA were three orders of magnitude more abundant than AOB across all sites and treatments (Appendix F). AOA *amoA* was more abundant than AOB *amoA* at both sites, with mean AOA:AOB ratios of 305 at ALRF and 1460 at SDT (Figures 4.2, 4.3). AOA are numerically (Leininger et al., 2006) and transcriptionally (Nicol et al., 2008) important to nitrification in soil. AOA:AOB ratios of 10 to 400 have been reported (Leininger et al., 2006; Onodera et al., 2010; Bru et al., 2011), though ratios < 1 occur at some sites (Bru et al., 2011; Petersen et al., (2012). Mounding disturbed the soil environment to improve planting sites for seedling survival, reducing competition from vegetation, increasing soil temperature, made a sizeable portion of the soil's organic C and N inaccessible through burial below the rooting zone and changed the soil water content within plots (see Chapter 2). These alterations to the soil environment likely removed of competitors for mineralized N for AOA and AOB, increasing substrate availability to slow-growing ammonia-oxidizers.

While AOA abundance was greater in undrained control plots at SDT compared to drained plots, AOB were greater in the drained plots (Figure 4). While no significant relationships were observed between AOA and pH, drainage decreased soil pH (Chapter 2), which can reduce AOA *amoA* copy

numbers, as AOA are also sensitive to soil pH, though it is not clear the exact relationship between these factors. For example, positive correlations between soil pH and AOA have been reported (Hallin et al., 2009; Jia and Conrad, 2009; Bru et al., 2011; Long et al., 2012), yet Lehtovirta et al. (2009) and Nicol et al. (2008) showed that AOA *amoA* abundance and transcription declines rapidly as pH increases from 3.9 to 6.6 and 4.5 to 7.5, respectively, while AOB *amoA* transcription increases gradually over this range. Drainage increases soil aeration, which can stimulate AOB (Szukics et al., 2010; Rasche et al., 2011). AOA have a lower O_2 demand than AOB, allowing the AOA to inhabit anoxic zones in soil (Schleper and Nicol, 2010; Walker et al., 2010). This could explain their abundance in undrained soil. AOB likely outcompete AOA in drained environments, particularly in the relatively oxygen-rich forest floor layers, as shown at SDT.

At both ALRF and SDT, fertilization increased AOB, but not AOA, *amoA* gene copies. AOB *amoA* was significantly correlated with N_2O flux at ALRF (Figure 4.6; Appendix H). Nitrifying organisms can contribute directly to soil N_2O pools by nitrifier denitrification, or indirectly though linked nitrification-denitrification (Martikainen, 1985; Wrage et al., 2001; Clough et al., 2004; Kool et al., 2011; Rasche et al., 2011; Petersen et al., 2012). In a study of the effect of different N fertilization regimes on functional genes, the abundance of AOA *amoA* was positively correlated with C:N ratio and negatively with total N in an agroecosystem (Hallin et al., 2009). Fierer et al. (2009) and Zeglin et al. (2011) also found links between AOB *amoA* and the soil C:N ratio. Autotrophic AOA and AOB are slow-growing and do not require organic C sources, though mixotrophic strains have been characterized (Prosser and Nicol, 2008). While AOA and AOB are likely out-competed for mineralized N in soils with abundant plant and heterotrophic microbial biomass due to their slow growth rates, mounding plots with reduced vegetation cover and soil C can alleviate competitive constraints on AOA and AOB abundance. AOB *amoA* abundance was correlated to NO_3-N concentrations at SDT, though not at ALRF, suggesting that at least at SDT AOB were partly responsible for mediating the nitrification rate. The addition of N to soil can stimulate AOB without stimulating AOA (Hallin et al., 2009; Petersen et al., 2012). The abundances of AOA and AOB *amoA* copies were weakly explained following variation partitioning, with soil chemical and physico-climactic parameters explaining significant portions of variation of both gene targets at ALRF (Table 4.4a), with soil water content explaining the highest amount of variation for each (Table 4.4b). At SDT, variation of AOA and AOB *amoA* abundance was also weakly explained, mostly by soil temperature (Table 4.5a, b). Total C explained small percentages of variation of AOA *amoA* abundance at both ALRF and SDT. A possible explanation is that AOA have the capacity for mixotrophic or heterotrophic growth in soil with low available mineral N (Hallam et al., 2006; Pratscher et al., 2011; Tourna et al., 2011; Pester et al., 2012). While their role in N_2O emissions at ALRF and SDT are unclear,

the increasing AOB abundance after fertilization can elucidate the effects of fertilization on N_2O emissions.

4.4.3.2 Denitrification genes

Gene quantity decreased stepwise along the denitrification pathway at ALRF and SDT (Appendix F). For example, 0.52% of bacteria, as quantified using total bacterial 16S (described in Chapter 3), contained the *narG* gene. The proportion of bacteria with the ability to reduce nitrate would be greater, as not all known variants of nitrate reduction genes (e.g., *napA*) were quantified in this study. This value is still much lower than the 5-20% reported by Bru et al. (2011). Nitrite reduction and nitrous-oxide reduction potential were characterized using *nirS/K* and *nosZ* genes, which were found in about 0.1% and 0.001% of bacteria, respectively. Despite the vast underestimation of functional gene quantities due to current limitation in primer design (Penton et al., 2013), these data agree that denitrifying organisms represent a fraction of total bacterial abundance, and that a sizeable portion of the denitrifying community lacks the ability to reduce N_2O to N_2 (Jones et al., 2008; Richardson et al., 2009; Bru et al., 2011).

Prior to the initiation of mounding or fertilization treatments, there were no differences in denitrification gene abundance between plots or sampling locations at ALRF. Following site preparation denitrification genes were consistently lower in mounded plots. Mounding at ALRF buried and dispersed forest floor C (Chapter 2). The mechanical removal of forest floor organic C from soil through mounding has been shown previously (Johansson, 1994; Liechty et al., 1997; Lundmark-Thelin and Johansson, 1997). Denitrification is a process that allows facultatively anaerobic heterotrophic organisms to oxidize organic C in low-O_2 environments. The mounding effect can be explained by the suppression of denitrifiers in plots with reduced organic C. Levy-Booth and Winder (2010) found positive correlations between *nirK* and *nirS* gene abundance in mineral soil layers with organic and total C in operationally-thinned coastal Douglas-fir forests in BC, while Hallin et al. (2009) found no correlation between organic C and denitrification gene quantity or composition in a fertilized agroecosystem. Further research is needed to elucidate the connection between disturbance of organic C and denitrification gene quantity in soil.

The soil layer in which genes were located had no consistent effect on the quantity of functional genes (Figures 4.3, 4.4, 4.5, 4.6), suggesting depth within the soil layers analyzed in this study was not an important spatial driver of gene abundance. In contrast, Kandeler et al. (2009) showed denitrification gene abundance varied between soil layers of a 57-year-old Norway spruce plantation on weakly podzolized silt-loam soil, with forest floor horizons (Oe and Oa) having a greater abundance of NO_3-reducing genes

(*narG* and *napA*) and mineral horizons (Ah and Bw) having higher *nosZ* abundance. Abundance of *nirK* was significantly higher in forest floor layers, though the gradient was less pronounced than that of NO_3-reducing genes.

Several sampling dates showed a significantly greater abundance of denitrification genes in fertilized plots than in unfertilized controls at both sites. The *narG* gene was the only denitrification gene that was significantly correlated to NH_4-N and NO_3-N when samples from both sites were combined (Appendix J). The *nosZ* gene was also elevated in fertilization plots, though at ALRF it was elevated within 24-48 hours of fertilization. It is unclear whether *nosZ* gene stimulation resulted from the elevated levels of NH_4-N and NO_3-N 24 hours after following fertilizer application; NH_4-N and NO_3-N concentrations increased between 1.2 and 8 times and 1.4 and 5 times, respectively, within 24-48 hours of fertilization. An alternate explanation is that soil may have undergone compaction during manual fertilizer application, as soil compaction can stimulate denitrification (Ruser et al., 2006). Drainage had little effect on nitrate reduction and denitrification genes, though several sampling dates had fewer *narG*, *nirS* and *nosZ* gene copies following drainage, likely due to the sensitivity of these dissimilatory reduction process to O_2. Drained and undrained plots differed little when all soil factors were ordinated by PCA, while fertilization separated SDT plots following PCA, suggesting a lack of physical as well as biological disturbance due to ditch drainage.

Spatial location on the landscape influenced denitrification gene abundance at ALRF, with a course-scale variable (PCNM2) and latitude explaining significant portions of variation in AOB *amoA*, *narG* and *nirS* abundance (Table 4.4b). Fine-scale spatial structure (PCNM4) explained relatively minor amounts of variation of *nirK* and *nosZ* abundance. Denitrification gene abundance exhibited some spatial autocorrelation. Latitude, indicating the west-to-east layout of the treatment plots at ALRF (See Chapter 2) and mounding treatment (Appendix K) primary influenced spatial structure of denitrification gene abundance at ALRF. Spatial structure was found to occur in the distributions of AOB *amoA*, *narG*, *nirK*, nirS, *nosZ*, bacterial 16S and crenarchaeal 16S rRNA abundance across the landscape scale in Burgundy region (Bru et al., 2011), and in the distribution of *narG*, *napA*, *nirK*, *nirS*, *nosZ* and bacterial 16S rRNA abundance in a pasture grassland in the Czech Republic (Philipott et al., 2009). Philipott et al. (2009) found that the spatial distributions of *narG*, *napA*, *nirK* and *nosZ* were closely related and not correlated with measured soil parameters, though the spatial distribution of *nirS* was unique and was positively correlated to the spatial distribution of NH_4-N, NO_3-N, pH and soil moisture. PDR and $N_2O/(N_2O + N_2)$ were associated with the spatial distribution of the *nosZ*/16S rRNA abundance ratio. In a 44-ha agroecosystem in Sweden, the spatial distributions of AOA *amoA* and AOB *amoA* abundance and community structure showed distinct, uncorrelated patterns that failed to explain potential nitrification

activities, which were positively correlated instead to pH and dissolved organic C (DOC concentrations (Wessén et al., 2011). In the same ecosystem, *nirK* and *nirS* abundance and community structure displayed distinct spatial patterns at the field scale, which only partially explained the spatial distribution of PDR measurements (Enwall et al., 2010). The spatial patterns observed in soil physico-chemical parameters such as pH and DOC were positively correlated to denitrification gene abundance and PDR, and matched the spatial arrangement of two cropping systems. These data suggest that management practices that create spatial patterns of soil physico-chemical parameters can impact the spatial distribution of nitrification and denitrification genes, and ultimately the denitrification potential of the soil. This observation has not been made in forest soil, but could have implications for understanding the spatial component of N_2O emissions from managed forest ecosystems.

Variation partitioning revealed important relationships between denitrification genes and the soil parameters measured in this study. The inclusion of a term encompassing the functional genes preceding each denitrification gene in the nitrification/denitrification pathway significantly improved the amount of variation that was able to be explained. Between 2% (*nirS*) and 43.9% (*nosZ*) of variation was able to be explained in this manner at ALRF (Table 4.4a). For example, *nirS* and AOB *amoA* explained significant portions of *nosZ* variation: 42.1% and 2.2%, respectively. At SDT, preceding denitrification genes also significantly improved variation partitioning models, despite the use of adjusted-R_2 values to account for the benefit of increased model terms. At SDT, *narG* abundance was significantly partitioned into soil chemistry (NO_3-N) and abundance of AOA and AOB *amoA* (Figure 4.4a). *NirK* variation modeling was improved by 19.3% by adding *narG* abundance to the reduced model. *NirS* and *nosZ* models were improved by the inclusion of *narG* and *nirK* terms, respectively.

For *nirK*, *nirS* and *nosZ*, however, soil temperature was also an important component of the variation partitioning model at SDT. Soil was positively correlated to *nosZ* abundance in an Inner Mongolian grassland soil (Zhang et al., 2013) but was negatively correlated to *nirS* and *nosZ* abundance in an Austrian beech forest, despite positive correlation between soil temperature and N_2O flux rate (Rasche et al., 2011). Variation partitioning explained over 50% of variation in several denitrification genes, which were some of the highest amounts of explained variation using this method reported in this study. These values are much less than variation partitioning models for denitrification genes across natural and managed ecosystems throughout the Burgundy region (Bru et al., 2011). In that study between 55% and 83% of variation of denitrification genes was successfully allocated, with soil chemistry (importantly pH and total C) being the primary driver of gene abundance at the landscape scale. The authors had a larger set of soil factors into which variation was able to be partitioned, though they did not allocate variation of denitrification genes to preceding genes in the nitrification/denitrification pathway as

I have here. This study demonstrates the usefulness of using canonical variation partitioning to elucidate the relationships between denitrification genes with both abiotic and biotic soil factors, including the abundance of related functional genes.

4.5 Conclusions

The objectives of this study were met by determining how site preparation techniques including excavator mounding and ditch drainage alter the soil environment and lead to changes to the abundance of microbial functional groups that regulate N-cycling soil process including N_2O flux. The interaction between mounding, water content and N_2O fluxes was complex. Mounding did not significantly alter N_2O flux rates, but created a spatial moisture gradient from mound top to mound hollow that also declined temporally as the water table lowered throughout the growing season. As such, the highest rates of N_2O flux occurred with an optimal gravimetric water content of about 40%, with N_2O uptake measured in hollows when water content reached about 80%. These data appear to support hypothesis i, though revision to our understanding of mounding effects on N_2O fluxes need to take into account temporal fluctuation of the water table depth, which was not directly measured in this study. Drainage did not reduce N_2O fluxes as hypothesized, as the highest N_2O fluxes measured at SDT were in drained plots, again showing optimum water content of 40%, with N_2O flux generally suppressed, or reversed, above this range. N_2O flux was increased by fertilization at both sites, supporting hypothesis ii. Mounding treatments resulted in an increase in *amoA* genes from ammonia-oxidizing microorganisms and a decrease in denitrifying genes, relating to alterations in total soil C, water content and pH, supporting hypothesis iii. Ditch drainage decreased soil water content without disturbing soil structure or stratification. This led to increase in AOB, but not AOA. AOA flourished in undrained control soils as a result of their low-O_2 demands relative to AOB. Therefore hypothesis iii was supported for drainage effects on AOA, but not AOB. Drainage had little effect on denitrification genes, although most genes were lower following drainage at one or more sampling dates, also partially supporting hypothesis iii. Fertilization increased AOB, but not AOA, and increased some denitrification gene abundances (*narG* at ALRF, *narG*, *nirK* and *nirS* at SDT), further supporting hypothesis iii, though not for AOA or *nosZ*. However, there existed mounding/drainage interactions that supported increased denitrification gene abundance following fertilization when soil water content was optimal (see above). Few layer effects were observed for nitrifier and denitrifier abundance, though what differences existed mostly confirmed current knowledge of niche optima for these functional groups. AOB and AOA were higher and lower, respectively, in aerated forest floors at one date at SDT, suggesting that the lower O_2 demands of AOA (see Chapter 1) allow these organisms to colonize the oxic/anoxic threshold in soil. Nitrite reducers (*nirK*/*S*) were higher in forest

floor soil relative to mineral in waterlogged undrained plots, an effect largely diminished in drained plots, suggesting an optima of moisture and organic C for these organisms, while nitrous oxide reducers (*nosZ*) were greater in mineral soil on one date at SDT, and therefore likely have a higher moisture content optima due to activity in lower redox potential soils. While hypothesis iv for potential denitrification was largely supported, the lower PDR in mineral soil of unmounded plots compared to mounded plots indicates the presence of N_2O-producing microorganisms in these locations that are not active *in situ* but are were rapidly activated by the PDR incubation process. The apparent importance of AOB abundance (though not activity) in potential denitrification was also not predicted and is a rich area for future study. N_2O flux and PDR were positively correlated to AOB *amoA* abundance at the mounding installation, and PDR was positively correlated to *nirS* and *nirK* genes and transcript abundance, demonstrating that at the site level, measurement of functional genes and transcripts may improve predictive models of GHG emissions; these data generally support hypothesis v. This study was the first to my knowledge to a) use quantification of nitrification and denitrification genes to determine the underlying reasons for site preparation and fertilization-induced differences in N_2O fluxes in waterlogged forest ecosystems, b) highlight the importance of the abundance of AOB *amoA* gene copies for both *in situ* and incubation-based measurements of net N_2O flux and c) use canonical variation partitioning to allocate important amounts of explained variation of N_2O flux, PDR and functional gene abundances to functional genes in the nitrification and denitrification pathways.

Chapter 5. Conclusions

The results of the research presented in this thesis demonstrate that the effects of site preparation (mounding and drainage) and fertilization on greenhouse gas (GHG) (CO_2, CH_4, N_2O) flux dynamics from wet, low-productivity forest ecosystems can be clarified by quantifying the size of the communities involved in key biogeochemical transformations. There are conflicting results from studies attempting to resolve the magnitude of GHG emissions following site preparation and fertilization. Key areas of ambiguity include: does mixing of forest floor layers by mounding decrease soil carbon (C) in organic matter (OM) and increase CO_2 emissions (Lunmark-Thelin and Johansson, 1997; Piirainen et al., 2007; Smolander et al., 2000)? Does drainage enhance CO_2 emissions and reduce soil C (Silvola, 1986; Glen et al., 1993; Laiho, 2006; Mojeremane et al., 2012)? Can mounding create anaerobic hotspots of CH_4 and N_2O emissions in areas of standing water (e.g., hollows) (Wachinger et al., 2000; Groffman et al., 2009; van den Heuvel et al., 2009; Christiansen et al., 2012)? Can drainage turn a wet CH_4-emitting forest in to a CH_4 sink (Nykanen et al., 1995; Augustin et al., 1998)? Does nitrogen (N) addition reduce CO_2 emissions in N-limited forest stands (Burton et al., 2003; Bowden et al., 2004; Olsson et al., 2005; Metcalfe et al., 2012)? Does fertilization increase CH_4 emissions due to suppression of CH_4 oxidation (Bodelier and Laanbroek, 2004; Mohanty et al., 2006; Basiliko et al., 2009; Bodelier, 2011)? Can fertilization with SO_4-S stimulate sulphate-reducing bacteria (SRB) to outcompete methanogenic archaea thereby reducing CH_4 emissions (Muyzer and Stams, 2008)? Do nitrifying and denitrifying organisms modulate the soil-surface N_2O flux response to N fertilization (Davison et al., 2000; Johnson and Curtis, 2001)? Furthermore, little data exist regarding the quantitative dynamics of functional genes involved in biogeochemical cycling in forest soil, the impacts of site preparation and fertilization on their abundance or their relationships with soil physico-chemical parameters (Bru et al., 2011; Hartmann et al., 2012; Shrestha et al., 2012). Improving knowledge of the ecology of microbial functional groups can improve our ability to accurately model and predict the effects of management on GHG fluxes from forest ecosystems.

This thesis began with a review of the state of knowledge of the effects of site preparation and fertilization on forest C and N cycles, greenhouse gas fluxes and the microbial communities responsible for GHG fluxes in soils and in particular the molecular biology of N-cycling microbial communities, with specific focus on N-fixing bacteria, nitrifying bacteria and archaea and denitrifying microorganisms in forest soil (Chapter 1). Key areas of knowledge gained by this review included a compressive analysis of the limitation of current quantitative methodologies including primer sets used for amplification of N-cycling functional genes, a synthesis of niche-partitioning models of ammonia-oxidizing archaea (AOA) and bacteria (AOB) and demonstrated that nitrifying (AOA and AOB *amoA*) and denitrifying (*narG*,

nirK, *nirS*, *nosZ*) functional gene abundance were positively related to soil O_2 availability (nitrifiers) and moisture (denitrifiers), mineral N substrate availability and form, pH and in the case of denitrifying genes, organic C, and could be linked to emissions of N_2O and N_2. It was, therefore, determined that population dynamics of nitrifying and denitrifying microorganisms would be investigated in an attempt to determine the effects of site preparation and fertilization on their abundance using parametric and multivariate statistical methods.

The bulk of thesis material describes a series of field studies that took place in the Aleza Lake Research Forest (ALRF), an interior hybrid white spruce stand subject to mounding and fertilization (Figure 2.2) and Suquash Drainage Trial (SDT), a coastal western redcedar-western hemlock-yellow cedar stand subject to drainage and fertilization (Figure 2.3) to investigate the effects of these practices on GHG fluxes (measured using gas chromatography of mixing ratios in static closed chamber headspace) and associated microbial communities (estimated using quantitative real-time PCR of microbial functional genes). Chapter 2 provides a detailed description of soil physico-chemical factors in all treatment plots to determine the effects of site preparation on the soil environment, and provide a point of reference for subsequent chapters. This chapter was designed to answer key management-related questions regarding response of soil chemistry, soil physico-climactic characteristics, soil microbial community structure and GHG potential related to the investigated management practices. Objective 1 was to quantify the physico-chemical response of forest soil to mounding, drainage and fertilization, including soil water content and soil physico-chemical characteristics. The aim of mounding and drainage is to reduce water saturation in soil to prepare sites for planting (Örlander et al,. 1990; Sutton, 1993; Löf et al,. 2012). Gravimetric soil moisture was reduced 15.2% in the mounded planting sites and 20% in the drained plots (Figure 2.4), supporting Hypotheses 1.1, that site preparation would reduce soil moisture content. However, water content of mound hollows increased 40% following mounding, with standing water covering these locations throughout much of the growing season. Mounding reduced total C, N and S at the planting sites (mound tops) in mounded plots instead of mixing it into mineral soil as hypothesized. The removal or reduction of forest floor layers led to C and N concentrations of 282 to 471 g kg^{-1} and 9.7 to 16.8 g kg^{-1}, respectively in mounded plots (Tables 2.1, 2.2). While mounding has been shown to mix forest floor OM into the upper 10 cm of planting sites (Liechty et al., 1997; Piirainen et al,. 2007), the mixed mounding technique used in this study did not. It is, therefore, recommended that mounding techniques that better mix the forest floor into the mound be used for site preparation when enhancing OM in the rooting zone is desired. Desiccation of mound tops and waterlogging of mound hollows was also identified in this study, demonstrating that careful adjustment of mound height for the local climate is needed to produce conditions appropriate to maximize seedling survival and minimize

GHG emission. Forest floor or mineral soil C or N concentrations were increased about 25% 15 years following drainage at SDT (Tables 2.3, 2.4). These data suggest that enhanced post-drainage tree growth results in deposition of OM in soil that is protected from microbial oxidation (Minkkinen and Laine, 1998; Minkkinen et al., 1999; Domisch et al., 2000). These data do not support Hypothesis 1.2, that soil characteristics in drained and mounded plots are equivalent to control plots as there are clear differences in soil chemistry following site preparation. Fertilization transiently increased NH_4-N, NO_3-N and SO_4-S concentrations at both sites (Tables 2.1, 2.3), supporting Hypothesis 1.3 that fertilization would add measurable amounts of mineral N and S to the soil. Net soil respiration rates were reported to meet Objective 2, which was the measurement of GHG flux rates following mounding, drainage and fertilization of wet forests, as CO_2 accounted for between 91.0% and 100.4% of total GHG emissions when CH_4 and N_2O were converted to CO_2-equivalants based on their 100-year global warming potentials (GWP), with few exceptions in water-covered fertilized locations (e.g., hollows) (Appendices 2.2, 2.3). Determining the total GWP of site preparatory and fertilization practices can help researchers, forest managers and regulatory bodies better understand the contribution of forest management to global climate change. CO_2 fluxes were reduced by mounding and increased by drainage at ALRF and SDT, respectively, at a single sampling date each (Figure 2.2), and measurements of locational CO_2 fluxes supporting hypotheses under Objective 2, which were that i) locations with reduced soil water content following site preparation (drained sites, mound tops) will have reduced CH_4 and N_2O emissions and increased CO_2 emissions, and that locations with increased water content (mound-associated hollows) will see elevated CH_4 and N_2O emissions and decreased CO_2 emissions; iii) fertilization will have no effect on CO_2 emissions, decrease CH_4 emissions due to the presence of SO_4-S and increase N_2O emissions in locations of elevated soil moisture. Mounding, drainage and fertilization led to community shifts in bacteria and fungi (Figure 2.6, Table 2.5), supporting Hypothesis 3.1, that site preparation would shift bacterial and fungal community structure, though effects of fertilization on microbial communities were not consistent at both sites. Mounding also reduced diversity of both bacteria and fungi, due to the removal or reduction of litter-associated OTUs. The drainage plots also showed distinct OTU separation based on soil layer but neither bacterial nor fungal diversity were reduced following drainage, suggesting shifts from anerboic to aerobic microorganisms following drainage. It is clear that drainage does not disturb the soil environment to the same extent as mounding and should, therefore, be considered for regeneration of tree species capable of growth on organic soils with intact forest floors (e.g., western redcedar, western hemlock) and appropriate soil texture (low to moderate clay content). Excavator mounding, meanwhile, was substantially less expensive in this study (Jull, M., personal communication) and was appropriate for the high clay content and poor drainage class of ALRF soils and for regeneration of hybrid white spruce, which establishes on mineral soil.

CO_2 was the most important GHG for GWP calculation, though hot spots of other GHGs can transiently impact GWP of management practices. CH_4, for example, is the second-most important GHG by atmospheric mixing ratio and GWP (Forster et al., 2007). Drainage can reduce CH_4 emissions (Glen et al., 1993; Mojeremane et al., 2012) and can even transform CH_4-emitting forests into CH_4 sinks by stimulating methane oxidation (Keller et al., 1990; Keller and Reiners, 1994; Castro et al., 1995; Czepiel et al., 1995; Wang and Bettany, 1995; Le Mer and Roger, 2001). The effects of fertilization on CH_4 fluxes are less clear, though NO_3^- and SO_4^{2-} can lead to nitrate- and sulphate-reducers outcompeting methanogens for low-molecular weight C (Bodelier, 2011) and NH_4^+ can increase CH_4 fluxes by competitive inhibition of methane oxidation (Crill et al., 1994; Maljanen et al., 2006). Chapter 3 was designed to address Objective 2 and 3 for CH_4 fluxes, which were the quantification of CH_4 fluxes and the abundance of associated microbial functional genes (*mcrA*, *pmoA* and *dsrB*). CH_4 fluxes at ALRF remained around zero for the majority of the study, though mounding transiently increased CH_4 emissions in hollows in association with fertilization (Figure 3.1a). Given that mounding reduced total soil C concentrations (Table 2.1), the substrate for methanogenesis in these plots is unresolved, but the measurement of CH_4 fluxes from hollows and sequencing of *mcrA* from the hydrogenotrophic methanogen *Methanolinea mesophila*, (data not shown), suggest that methanogens are present and actively converting oxidized soil C into CH_4. Hydrocarbon formation and eutrophication of hollows was observed following fertilization of mounded plots. Eutrophication can increase methane emissions from sediments by reducing dissolved oxygen, increasing dissolved organic C, increasing methanogen abundance and diversity and supressing MOB (Weaver and Dugan, 1972; Liikanen and Martikainen, 2003; Castro et al., 2004). It is, therefore, likely that eutrophication in the mound water, which drew down over the growing season depositing algal and phytoplankton biomass on the soil surface, affected GHG emissions in mounded plots, which is an area for further research. Drainage consistently reduced CH_4 fluxes (Figure 3.1b) without the hypothesized increase in CO_2 emissions (Figure 2.2b). Therefore, Hypothesis 2.1, that drainage would reduce CH_4 emissions and increase in CO_2 emissions, was supported. SO_4-S fertilization did not reduce CH_4 as hypothesized. Functional gene quantification can elucidate the impact of site preparation and fertilization on the microbial communities responsible for GHG fluxes, specifically MOB *pmoA* and methanogen *mcrA* abundance and transcription, which can regulate CH_4 flux dynamics (Frietag et al., 2010; Shrestha et al., 2012; Nazaries et al., 2013; Tong et al., 2013). Total bacteria were reduced on one sampling date at ALRF due to mounding, as were *mcrA*, *pmoA* and *dsrB* copies (Figure 3.3), likely as a result of the removal of forest floor and soil OM. The abundance of *mcrA* peaked in the hollows at the same date that CH_4 emissions spiked (Figures 3.2, 3.4). Furthermore, drainage increased bacterial abundance while decreasing *mcrA*, *pmoA* and *dsrB* copy abundance at SDT (Figure 3.4), though dates at which functional genes were lower were not always indicative of lower CH_4

fluxes (Figure 3.2b). These findings supported Hypothesis 3.2 , that methanogens would be decreased by all site preparation methods, and partially supported 3.4, that SRB would be decreased by mounding and drainage and elevated by NPK-S fertilization, as *dsrB* abundance was lowered by site preparation and increased by fertilization on at least one date at both ALRF and SDT. Furthermore, aerobic methanotrophs were significantly greater in forest floors while anaerobic SRB were greater in mineral soil. Methenogens were generally higher in waterlogged soil including mineral soil, but were also assocated with higher concentrations of organic matter in the forest floor.

Multiple comparisons and unconstrained and constrained multivariate ordination were used to determine correlations between genes, GHG fluxes and soil physico-chemical parameters. CH_4 rates were influenced by soil water content and methanogen gene abundance. Unconstrained ordination using principal component analysis (PCA) showed shifts, but not separation, of soil biotic and abiotic parameters following site preparation, with differences in mounded and unmounded plots being related to soil moisture, and pH and differences between drained and undrained plots driven by changes in aerobic organisms and processes (Figure 3.5). Constrained ordination using redundancy analysis (RDA) was used to test correlations between abiotic and biotic parameters. RDA showed that at ALRF, *mcrA* and *dsrB* abundance were positively correlated with NO_3-N concentration and negatively correlated with CO_2 and *pmoA* abundance, while at SDT *mcrA, dsrB* and bacterial abundance were positively correlated with CH_4 flux rate, and *pmoA*, temperature and CO_2 fluxes were positively correlated at both sites (Figure 3.5), consistent with relationships between these genes and GHG fluxes observed in a variety of terrestrial and aquatic ecosystems (Levine et al., 2011; Ma et al.,, 2012; Tong et al., 2013). RDA-based variation partitioning models of individual genes at ALRF, showed that they were explained mostly by soil physico-climate and primarily by soil temperature (Table 3.4), while genes and GHGs at SDT were influenced by chemistry, physics and other genes, with temperature, *dsrB* and *mcrA* being the main sources of variation (Table 3.5). Together, these data support Hypothesis 4.1, that soil moisture directly increases and decreases methanogen and methanotroph population size, respectively, and partially support 4.2, that increases in the methanogen population increase CH_4 flux rates while increases in the methanotroph population decrease CH_4 flux rates, at SDT. This chapter is, to my knowledge, the first study to use of RDA to partition variance of functional genes and GHGs into categories including related functional communities. This cannot be taken to mean that casual relationships exist, though given the current paradigm of biogenic CH_4 cycling, it is likely that they do. This chapter improves the understanding of the impact of site preparation on the abiotic and biotic drivers of CO_2 and CH_4 fluxes from wet forests.

It is well established that N-fertilization increases N_2O emissions from soil (e.g., Neff et al., 1994; Pilegaard et al., 2006; Jassal et al., 2008; Aronson and Allison, 2012; Mojeremane et al., 2012; Pilegaard, 2013; Ussiri and Lal, 2013; Wu et al., 2013), though the magnitude of N_2O emissions in response to N-addition, potential soil physico-chemical limitations of N_2O emissions and the organisms responsible remain unresolved in managed forest ecosystems (Gundersen et al., 2012; Butterbach-Bahl et al., 2013; Saari et al., 2013). Chapter 4 presents the effect of site preparation and fertilization on N_2O flux and associated communities of nitrifiers and denitrifiers in order to complete Objectives 2, 3 and 4: quantification of N_2O fluxes, nitrification and denitrification genes and relationships between them, respectively. Mounding had no effect on N_2O emissions, though N_2O emissions were significantly but transiently increased by fertilization. N_2O flux peaks at ALRF shifted from mound tops in July of 2012 to hollows in August 2012, culminating in a maximum efflux of 203 ± 147 $\mu g\ m^{-2}\ h^{-1}$ in fertilized hollows (Figure 4.1a) as the mounds became desiccated and the water table in the hollows dropped below the soil surface (Figure 2.3). The minor uptake of N_2O observed in July when standing water covered the hollows indicated that in fully saturated soil denitrifiers relied on low-energy N_2O-reduction for oxidation of soil C (Chapuis-Lardy et al., 2007). Drainage decreased N_2O emissions at one date, though high N_2O fluxes were observed in drained plots throughout the study, with the highest rates observed in fertilized drained plots in July 2013 (Figure 4.1b). Fertilization significantly increased N_2O fluxes for at least 14 months at SDT following application, in contrast to ALRF, as well as studies in Douglas-fir stands that showed fertilization with urea-N transiently increased N_2O fluxes over one growing season, resulting in minute N_2O uptake in fertilized stands the following year (Jassal et al., 2008, 2010). Therefore, the aspects of Hypotheses 2.1 and 2.2 regarding water-content regulation of locational N_2O fluxes require clarification, as water table changes over the growing season alter the N_2O flux direction and capacity irrespective of mounding or drainage, and N_2O fluxes occurred even in locations hypothesized to suppress denitrification, i.e., mound tops and drained plots. The cause of this discrepancy is the temporal variability in the water table height and resulting soil moistures. The highest rates of N_2O flux occurred with an optimal gravimetric water content of about 40%, with N_2O uptake measured in hollows when water content reached about 80%. The highest N_2O fluxes measured at SDT were in drained plots, again showing optimum water content of 40%, with N_2O flux generally supressed or reversed above this range. Further analysis of the effect of water table depth on N_2O flux and related microbial functional groups is required. Hypothesis 2.2, that N_2O emissions would be reduced by mounding and drainage, was only partially supported, as moisture did not fully determine the magnitude of N_2O fluxes following fertilization. Mounding at ALRF generally increased nitrification genes (AOA *amoA*, AOB *amoA*) (Figure 4.3) and decreased denitrification genes (*narG*, *nirK*, *nirS*, *nosZ*) (Figure 4.5). Drainage at SDT generally decreased AOA *amoA* (Figure 4.4) and denitrifying genes (Figure 4.6). Fertilization

consistently increased AOB *amoA* and *narG* genes at both sites, with nitrite reduction genes (*nirK/S*) elevated by fertilization at SDT. AOB *amoA* copies were highest in aerated forest floor soil while *nirK/S* copies were higher in wet forest floor material. Additionally, AOA *amoA* were higher in aerated mineral soil and *nosZ* copies were highest in wet mineral soil, suggesting that AOB have a higher aerobic requirement than AOA and that nitrous oxide reduction takes place in lower redox environments relative to nitrite reduction. These results overall support Hypotheses 3.5 and 3.6 that site preparation and fertilization would reduce and increase microbial functional genes involved in nitrification and denitrification, respectively, with the exception that AOA *amoA* and *nosZ* genes were not affected by fertilization.

In an attempt to resolve ambiguity regarding the effects of site preparation and fertilization on the denitrification capability of the microbial community in soil at ALRF and SDT, a potential denitrification assay was conducted. Nitrifying and denitrifying genes and RNA transcripts were quantified using soil removed from ALRF and SDT one year after fertilization of field plots. Potential denitrification rate (PDR) was increased significantly by mounding and fertilization. PDR of fertilized mounded plots at ALRF were about three times greater than unfertilized mounded plots, leading to significant interactions between mounding and fertilization (Figure 4.2a). Fertilization significantly increased PDR at SDT (Figure 4.2b). Similar patterns were observed between PDR and *in situ* N_2O fluxes at SDT, though unfertilized *in situ* N_2O fluxes were almost zero due to lack of substrate. Similarly at ALRF, the *in situ* N_2O fluxes from fertilized mounds was negligible, while PDR was about 50 ng N_2O-N kg^{-1} h^{-1}, similar to that of fertilized mound hollows. These results show that the denitrifying community (including nitrifying microorganisms) in low-N_2O emitting soils have the potential for rapid emission of N_2O when provided with organic C and NO_3^-. No significant changes in gene copy number for AOA *amoA*, *narG*, *nirS* or *nosZ* were found at ALRF, but AOB *amoA* and *nirK* genes, as well as *nirK* transcripts were significantly greater in fertilized soil following potential denitrification assays compared to unfertilized soil (Figure 4.7). At SDT, AOB *amoA* was also significantly greater in fertilized soil compared to unfertilized soil (Figure 4.8). Significant positive correlation was found between PDR and bacterial 16S, *nirK* genes, AOB *amoA*, *nirK* transcripts and *nirS* transcripts at ALRF (Appendix H), though only *nirS* transcripts were significantly correlated with PDR following Bonferroni correction, a highly conservative correction factor following multiple comparisons that has been applied to studies of the ecology of function genes (e.g., Bru et al., 2011), though not always (Hallin et al., 2009; Basiliko et al., 2009; Wertz et al., 2009). At SDT, PDR was positively correlated with *nirK* and *nosZ* gene abundance and *nosZ* transcript abundance following Pearson correlation and *in situ* N_2O flux rate, NO_3-N concentration, NH_4-N and AOB *amoA* abundance following Bonferonni correction. Therefore, AOB and *nirS/K*-containing organisms are

implicated in determining PDR, with confirmation that *nirS/K* transcription is likely involved in potential denitrification activity. AOB *amoA* transcripts were not detected in any post-incubation extracts, indicating that nitrification was unlikely to be the source of N_2O production. This means either that the nitrification potential of a soil influences denitrification potential due to control of N-cycling rates by ammonia oxidation (Long et al., 2012; Petersen et al., 2012), or that AOB are denitrifying directly (Wrage et al., 2001; Zhu et al., 2013). Sequencing of *nirK* and *nirS* transcripts could resolve the organisms responsible for N_2O production following incubations. Regardless, these data help elucidate how site preparation and fertilization can lead to changes in the nitrifying and denitrifying community including reproduction and transcription, and can be useful for interpreting results from field studies.

This study also sought to determine if nitrifier and denitrifier gene abundance would positively correlate with field N_2O fluxes as they did with PDR, and to determine the associated physico-chemical parameters. The review presented in Chapter 1 highlighted pH and NH_4^+ as factors that would likely positively correlate with AOA and AOB abundance in field studies, while soil C, NO_3^-, pH and moisture would likely positively correlate with denitrifier gene abundance. Unconstrained exploratory ordination was used to determine how mounding, drainage and fertilization affected biotic and abiotic soil parameters. Site differences were caused by greater soil C concentrations and water content at SDT and higher nitrifier and denitrifier gene abundance at ALRF driven by higher soil pH (Figure 4.9). Constrained ordination using RDA showed that at ALRF, N_2O fluxes were most-closely associated with nitrification and denitrification functional gene abundance (AOA *amoA*, AOB *amoA*, *nirK*, *narG*) as well as soil water content and soil temperature (Figure 4.10a), showing that moisture level and soil temperature can be important to the regulation of the size of the denitrification community and its function, and, therefore, suggesting that understanding microsite water content heterogeneity, not simply mean plot or subplot water content, is a key component of predicting N_2O fluxes following site preparation. While AOB *amoA* genes, *nirK* genes, total N and NO_3-N were also positively correlated with N_2O fluxes at ALRF following multiple comparison testing, only AOB was significantly and positively correlated following Bonferonni correction (Appendix H). N_2O fluxes at SDT were positively correlated with AOA *amoA*, narG, *nosZ* and *nirK* abundance following RDA (Figure 4.10b). Between-site differences in N_2O fluxes were positively correlated with soil water content, AOA *amoA* and *narG* abundance, following RDA, and with pH and soil water following Bonferonni-corrected multiple comparisons (Appendix J). The partitioning of variance of individual genes and measures of denitrification using canonical RDA revealed a spatial component to the distribution of AOB, denitrification genes and N_2O fluxes at ALRF using principal component of nearest neighbour matrix (PCNM) analysis, which explained between 4% and 13.6% of variation of these factors (Table 4.2a).

Models were forward-selected using R^2-adjusted values to ensure that the minimum number of variables accounted for the maximum amount of variation. Total variation explained for bacterial 16S, AOA *amoA*, AOB *amoA* and narG was low (< 20%), while variation of 25.5% to 74.7% was explained for other factors at ALRF. Soil physico-climate was an important source of variance for most variables as was variation in nitrification and denitrification genes that precede denitrification genes and N_2O fluxes in the denitrification pathway. For example, N_2O flux variation at ALRF was significantly explained by variation of AOB *amoA*, AOA *amoA* and two spatial variables, which were in turn explained by variation in soil water content. PDR variation was equally explained by nitrification and denitrification gene and transcript abundance, showing that while functional gene abundance is an important factor for explaining N_2O production capacity in a soil, measures of gene transcription increases the power of explanatory models. 42.4% of N_2O flux variation at SDT could be allocated to abiotic and biotic soil parameters soil moisture accounting for 29.9% and functional genes (AOA *amoA* and *nirK*) accounting for 13.3%. Soil pH, moisture, NH_4-N concentration, *narG* abundance and *nirS* abundance all significantly contributed to between-site variation of N_2O fluxes. Variation partitioning clarified the relationships between abiotic and biotic soil parameters and the *in situ* N_2O flux rate following mounding, drainage and fertilization. Objective 4 was therefore achieved and Hypothesis 4.3 further clarified, in that pH was significantly associated with nitrification genes but was not a primary site-specific driver. NH_4-N was a significant source of variation for AOB *amoA* at ALRF, though soil water and temperature had a greater impact on nitrifier gene abundance. Total C consistently influenced AOA *amoA* abundance, suggesting possible mixotrophic or heterotrophic growth (Prosser and Nicol, 2008), and AOB *amoA* exhibited spatial structure that was related to site and management practices. Variance partitioning supported Hypothesis 4.4, as soil C, soil N, pH and water content all significantly accounted for denitrification gene abundance, though soil temperature, spatial structure and genes proceeding our target denitrification genes in the nitrification and denitrification pathways also accounted for significant portions of functional gene variation.

Laboratory-based research contributes important knowledge regarding the basic ways that soil factors, organisms and processes interact, for example the denitrification potential experiment described above, as well as the elucidation of AOA and AOB community structure and function (Nicol et al., 2008; Tourna et al., 2008; Verhamme et al., 2011), contribution of nitrifier and denitrifiers to N_2O fluxes in response to soil moisture (Zhu et al., 2013) and relationships between soil pH, denitrification gene abundance, transcription and N_2O production (Liu et al., 2010). However, soil environments are physically, chemically and biologically complex with many competing and interacting factors, that exhibit many levels of temporal and spatial structure and variation, so understanding the how soil

physico-chemical factors and microbial functional groups affect each other and soil process rates in field studies is an ongoing objective of forest ecology. Chapter 1 is a comprehensive review of the current theoretical and technical framework for understanding key N-cycling processes from a microbial perspective in forests. Chapter 2 provides data regarding the changes in the soil environment, total microbial community and GHG-emitting potential of site preparation and fertilization of waterlogged forest stands to improve management decisions by forest managers and improve understanding of these practices for researchers and stakeholders. Chapter 3 elucidates the response of CH_4 fluxes and CH_4-cycling microbial functional groups to site preparation and fertilization and builds a model of variance partitioning to better understand how microbial functional genes are altered by management-related changes in soil physico-chemical parameters and contribute to CH_4 fluxes. Chapter 4 clarifies the relationships between soil physical chemical parameters, nitrifier and denitrifier community abundance and N_2O fluxes in waterlogged low-productivity forest stands subject to mounding drainage and fertilization. As a whole, this study contributes to a wider understanding of the importance of microbial functional group characterization for understanding their role in GHG flux rates in forest ecosystems.

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Appendix A. Fertilizer formulation and composition.

Fertilizer	Percentage	kg/ha to get desired elemental percentage	kg/ha of elements	Cumulative kg/ha
Step 1 - determine amount of Thiogro to get 50 kg/ha S				
Thiogro	to get 50 kg/ha S			
N	13.73%	380.23	52.21	52.21
P	15.00%		57.03	57.03
K	14.90%		56.65	56.65
S	13.15%		50.00	50.00
Step 2 - determine amount of NPK to get 100 kg/ha K&P after Thiogro				
NPK	to get 100 kg/ha P			
N	19.00%	429.66	81.63	133.84
P	10.00%		42.97	100.00
K	10.00%		42.97	99.62
S	0.00%		0.00	50.00
Step 3 - determine amount of Urea to get 200 kg/ha N after Thiogro + NPK				
Urea	to get 200 kg/ha N			
N	46.00%	143.83	66.16	200.00
P	0.00%		0.00	100.00
K	0.00%		0.00	99.62
S	0.00%		0.00	50.00
Formulations				
Thiogro	15N-15P-15K-15S - Proprietary, contains $(NH_4)_2SO_4$ and urea			
NPK	20N-10P-10K			
	20% Total N: 1% NO_3-N, 2.8% NH_4-N, 16.2% urea-N			
	10% Total P: P_2O_5			
	10% Total K: K_2O			
Urea	46N-0P-0K - 46% Total N: urea-N			

Appendix B. Greenhouse gas flux rates as 100-year CO_2 equivalents at ALRF and SDT. n = 6; ± standard deviation. Different letters denote statistical differences at $p = 0.05$. Results of linear mixed model ANOVA in Appendix C.

		June 2012				July 2012			
Location		CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.	CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.
ALRF	C	690.8±271.2^{ab}	0.5±0.7	2.0±1.4	693.3±271.2^{ab}	1300.9±240.9^{a}	2.1±3.5^{a}	3.4±6.5^{a}	1306.4±241.0
	C+F	852.5±244.4^{a}	1.9±0.8	-0.6±0.4	853.8±244.4^{b}	962.6±77.5^{ab}	3.7±5.6^{a}	8.7±19.7^{a}	975.0±80.2
	M	423.6±188.0^{bc}	-0.5±0.2	0.4±0.2	423.6±188.0^{a}	1044.4±339.3^{ab}	0.6±1.3^{a}	18.5±20.2^{a}	1063.6±339.9
	M+F	447.8±138.7^{bc}	0.9±1.0	0.3±0.1	449.0±138.7^{ab}	814.0±191.9^{b}	-0.1±0.6^{a}	48.9±15.0^{b}	862.8±192.5
	H	215.6±117.8^{c}	2.5±2.1	-0.4±0.5	217.7±117.8^{a}	658.0±23.9^{b}	9.7±8.1^{ab}	1.1±1.0^{a}	668.9±25.3
	H+F	415.5±183.6^{bc}	0.4±0.8	1.7±1.3	417.5±183.6^{a}	739.5 117.0^{b}	26.9±17.0^{b}	-1.9±3.8^{a}	764.6±118.3
SDT	C					309.7±79.1	2.8±3.7	6.4±11.1	318.8±80.0
	C+F					398.6±111.3	2.0±2.3	9.0±7.6	409.5±111.6
	D					656.1±261.0	-1.2±1.0	-1.1±1.1	653.7±261.0
	D+F					499.7±160.3	-1.2±2.2	1.0±1.4	499.5±160.3
		Aug 2012				June 2013			
		CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.	CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.
ALRF	C	426.9±107.9^{a}	-3.1±0.8^{ab}	13.7±11.8	437.5±108.5	434.2±78.5	0.9±0.7^{ab}	0.3±0.2	435.5±78.5
	C+F	344.4±90.1^{ab}	-2.0±1.2^{ab}	36.1±39.1	378.4±98.2	296.7±105.3	1.5±0.8^{a}	0.4±0.2	298.6±105.3
	M	442.2±119.6^{a}	-5.0±1.2^{a}	5.2±2.1	442.4±119.6	271.5±80.9	-0.1±0.9^{b}	0.0±0.3	271.3±81.0
	M+F	306.2±76.3^{ab}	-3.4±0.9^{ab}	5.9±5.9	308.7±76.5	429.6±209.5	-0.2±0.3^{b}	0.0±0.1	429.4±209.5
	H	203.1±86.1^{ab}	-0.1±1.2^{b}	8.2±17.8	211.2±88.0	188.8±27.2	1.0±0.8^{a}	0.3±0.2	190.1±27.2
	H+F	191.2±108.3^{b}	0.3±0.7^{b}	60.6±88.1	252.1±139.6	351.9±168.4	1.2±0.5^{a}	0.3±0.2	353.4±168.4
SDT	C	260.0±150.8^{a}	3.3±5.2	0.5±1.1	263.8±150.9				
	C+F	382.4±74.6^{ab}	7.9±8.1	3.0±3.1	393.4±75.1				
	D	513.5±64.7^{b}	-1.3±0.9	0.3±1.0	512.6±64.7				
	D+F	394.2±90.2^{ab}	2.3±3.3	11.6±10.2	408.1±90.8				

Appendix B cont. Greenhouse gas flux rates as 100-year CO_2 equivalents at ALRF and SDT. n = 6; ± standard deviation. Different letters denote statistical differences at $p = 0.05$.

		July 2013				Sept 2013			
		CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.	CO_2 (mg m^{-2} h^{-1})	CH_4-CO_2 eq.	N_2O-CO_2 eq.	Total CO_2 eq.
SDT	C	532.5±165.7	7.8±12.5	0.1±0.9	540.4±166.2	575.9±55.4	4.3±9.1	-2.7±2.5	577.5±56.1
	C+F	440.9±181.7	14.2±7.7	19.8±11.6	474.9±182.2	386.5±76.5	4.3±9.8	2.7±0.0	393.4±77.2
	D	703.3±135.0	-1.3±1.9	0.8±0.2	702.9±135.0	450.5±107.6	-1.2±1.7	-0.2±0.8	449.1±107.6
	D+F	535.1±171.3	4.7±3.2	40.3±22.3	580.1±172.8	447.8±164.3	4.6±5.0	21.8±19.9	474.2±165.6

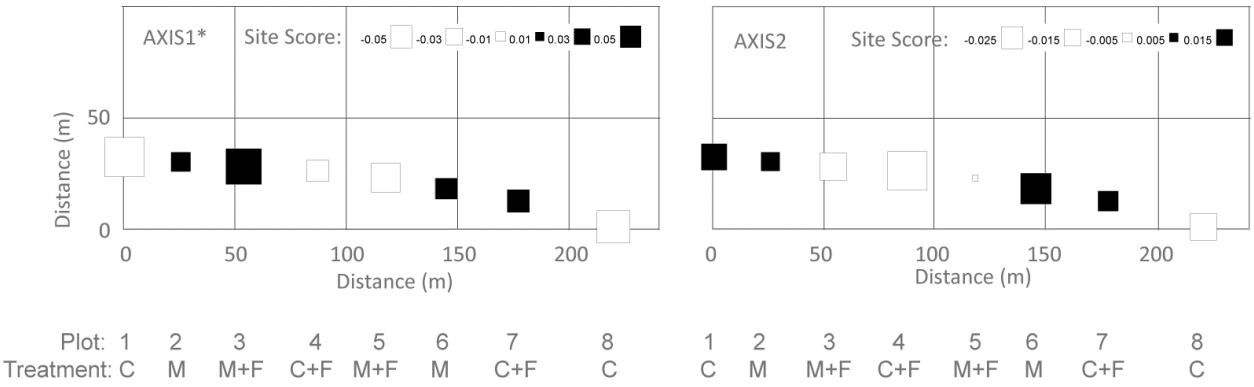
Appendix C. *F* and *p* statistics following two-way ANOVA of drainage and fertilization effects on total 100-year CO_2 equivalent greenhouse gas flux from CO_2, CH_4 and N_2O from ALRF and SDT. Values that are significant at $\alpha = 0.05$ are shown in bold.

		Jun-12		Jul-12		Aug-12		Jun-13		Jul-13		Sep-13	
Factor	Treatment	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
ALRF CO_2-eq.	M	**35.2**	**<0.001**	1.6	0.214	3.1	0.094	2.3	0.144				
	F	**4.8**	**0.038**	0.0	0.915	0.3	0.576	2.2	0.153				
	M × F	0.4	0.527	2.7	0.117	0.0	0.919	**10.2**	**0.004**				
										0.1	0.756	0.0	0.974
SDT CO_2-eq.	D			1.9	0.185	0.2	0.689			4.1	0.055	4.2	0.051
	F			0.4	0.551	3.9	0.061			0.1	0.693	1.3	0.263
	D × F			0.9	0.343	1.6	0.224			0.1	0.756	0.0	0.974

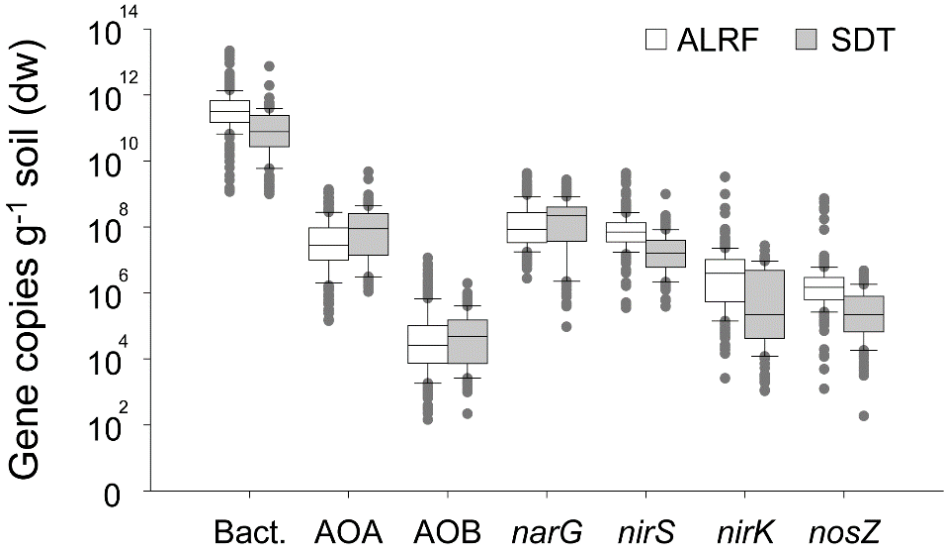
D, Drainage; F, Fertilization; M, Mounding

Appendix D. Sampling dates for soil chemistry, microbial community and greenhouse gas flux at ALRF and SDT.

Site	Sample Type	Sampling Dates					
ALRF	Soil chemistry		Jun-12	Jul-12	Aug-12	Oct-12	Jun-13
	Microbial community	Jun-11	Jun-12	Jul-12	Aug-12	Oct-12	Jun-13
	Greenhouse gases		Jun-12	Jul-12	Aug-12		Jun-13
SDT	Soil chemistry	Jul-12	Aug-12	Oct-12	Jul-13		
	Microbial community	Jul-12	Aug-12	Oct-12	Jul-13	Sept-13	
	Greenhouse gases	Jul-12	Aug-12		Jul-13	Sept-13	



Appendix E. Spatial structure of bacterial 16S, *pmoA*, *mcrA* and *dsrB* genes at ALRF following principal component of neighbour matrices (PCNM) analysis. Mean values of PCNM axis 1 and 2 are plotted on Cartesian coordinates of the eight treatment plots by box size and shade. White boxes represent positive ordination coordinates along the axis while negative coordinates are shown in black. Larger boxes are more positive or negative, while smaller boxes have coordinates closer to zero. PCNM axis 1 significantly explained the spatial structure of the functional genes ($p<0.05$).



Appendix F. Total nitrification and denitrification gene abundances across all treatments at ALRF and SDT. Boxplots show median, 25% quartile and 75% intervals. Whiskers show 5% and 95% intervals. Outliers from these distributions are displayed as individual points.

Appendix G. Correlation matrix showing Pearson coefficients of PDR, field N_2O emissions, soil mineral N availability and gene abundances from ALRF (Jun-13; n = 18; shaded) and SDT (Jul-13; n = 12; unshaded) following potential denitrification incubations. Italicized coefficients are significant at $p < 0.05$ without correction for multiple comparisons and bolded coefficients are significant at $p < 0.05$ following Bonferonni correction.

	PDR[a]	N_2O[b]	NO_3	NH_4	Bact.[c]	narG[d]	narG[tr e]	nirK	nirK[tr]	nirS	nirS[tr]	nosZ	nosZ[tr]	AOB	AOA	
PDR		0.00	-0.24	-0.16	*0.56*	0.15	-0.24	*0.59*	*0.49*	0.21	**0.64**	-0.08	-0.22	*0.55*	-0.04	ALRF
N_2O	**0.81**		0.16	0.38	0.00	0.08	*0.47*	0.08	0.08	-0.42	-0.17	0.00	0.25	-0.06	-0.03	
NO_3	**0.82**	**0.72**		**0.88**	-0.22	0.00	0.31	-0.21	-0.29	-0.2	-0.17	-0.30	*0.40*	-0.36	-0.24	
NH_4	**0.81**	*0.66*	**0.99**		-0.11	0.00	0.35	-0.12	-0.22	-0.25	-0.29	-0.35	0.36	-0.25	-0.31	
Bact.	-0.47	-0.10	-0.32	-0.35		*0.59*	*-0.43*	0.20	**0.71**	0.33	*0.42*	-0.31	-0.04	**0.71**	-0.22	
narG	0.01	0.09	-0.08	-0.09	0.33		-0.14	0.20	**0.71**	0.37	0.18	-0.05	0.19	*0.48*	0.00	
narG[tr]	0.19	0.45	0.29	0.21	-0.05	0.07		0.12	-0.18	-0.31	-0.17	0.22	-0.26	-0.39	0.18	
nirK	*0.56*	0.24	0.43	0.48	*-0.64*	-0.10	-0.17		0.29	-0.14	0.20	0.04	-0.21	0.15	-0.03	
nirK[tr]	0.48	0.26	0.29	0.29	**-0.80**	-0.32	-0.04	*0.55*		0.37	0.22	0.02	-0.14	**0.87**	0.10	
nirS	0.31	0.33	0.04	0.04	-0.08	0.43	0.15	*0.53*	-0.05		0.5	0.06	-0.01	*0.46*	0.14	
nirS[tr]	0.49	*0.52*	0.26	0.24	-0.10	0.27	0.31	0.48	-0.01	**0.92**		0.12	0.04	0.31	0.14	
nosZ	*0.51*	0.12	0.11	0.14	*-0.56*	0.05	-0.19	*0.71*	*0.50*	*0.60*	*0.59*		-0.03	0.16	**0.97**	
nosZ[tr]	*0.57*	*0.58*	*0.66*	*0.67*	0.07	0.25	-0.04	0.48	0.07	0.27	0.27	0.08		-0.12	-0.01	
AOB	**0.76**	*0.65*	*0.60*	*0.58*	*-0.52*	0.35	0.49	0.29	0.43	0.34	0.49	0.42	0.22		0.25	
AOA	-0.13	-0.35	-0.09	-0.05	0.09	0.11	-0.45	-0.08	-0.33	0.06	0.11	0.19	-0.33	-0.05		

SDT

[a] Potential denitrification rate (ng N kg $soil^{-1}$ h^{-1})
[b] Field N_2O flux (µmol N_2O m^{-2} h^{-1})
[c] Bacterial 16S rRNA
[d] Gene copies g^{-1} soil (dw)
[e] Transcript copies g^{-1} soil (dw)

Appendix H. Pearson correlation coefficients for all soil and gene factors from ALRF

	N_2O	bact	AOA	AOB	*narG*	*nirK*	*nirS*	*nosZ*	H_2O	total C	total N	NO_3	NH_4	pH
N_2O		NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
bact	-0.01		NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS	NS
AOA	0.00	-0.08		*	NS	*	NS	NS	NS	NS	NS	NS	*	NS
AOB	0.33	0.03	0.44		NS	*	NS	NS	NS	NS	NS	NS	NS	NS
narG	0.12	0.24	0.10	0.24		NS	NS	NS	NS	NS	NS	NS	NS	NS
nirK	0.17	0.17	0.39	0.35	0.08		*	*	NS	NS	NS	NS	NS	NS
nirS	0.06	0.46	0.17	0.12	0.04	0.49		*	NS	NS	NS	NS	NS	NS
nosZ	-0.03	0.45	-0.02	-0.07	-0.03	0.34	0.76		NS	NS	NS	NS	NS	NS
H_2O	0.11	0.12	0.04	0.18	0.18	0.10	0.10	0.12		*	NS	NS	NS	NS
total C	0.03	0.24	-0.24	-0.05	0.19	-0.07	0.05	0.15	0.63		*	NS	*	NS
total N	-0.08	0.16	0.08	0.02	0.01	0.14	0.15	0.09	-0.04	-0.3		NS	NS	*
NO_3	0.04	0.12	-0.04	-0.02	0.16	0.23	0.02	0.14	-0.15	-0.21	0.12		NS	*
NH_4	-0.02	0.16	-0.26	-0.15	0.16	-0.17	-0.06	0.08	0.25	0.47	-0.11	0.11		*
pH	-0.07	-0.15	-0.11	-0.11	0.00	-0.13	-0.19	-0.03	-0.02	0.01	-0.29	0.26	0.39	

*, $p < 0.05$ following Bonferroni correction for multiple comparisons; NS, not significant.

Appendix I. Pearson correlation coefficients for all soil and gene factors from SDT

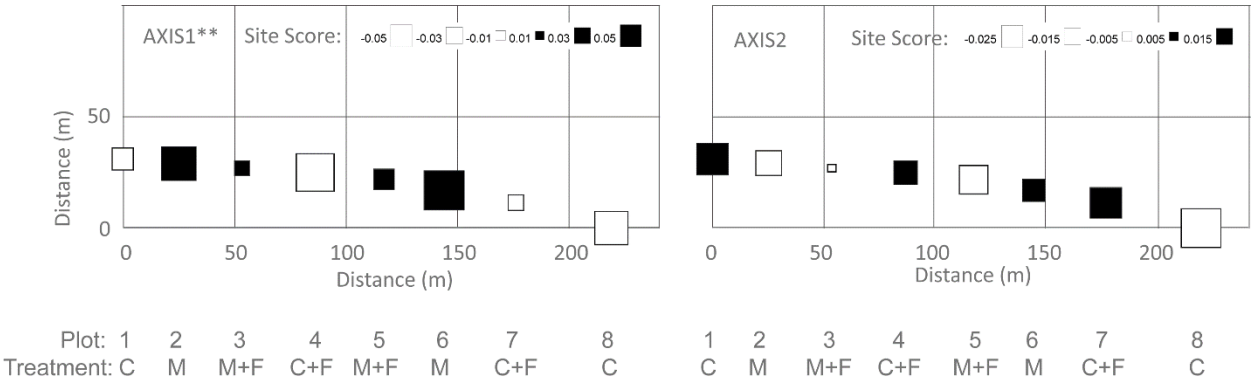
	N_2O	bact	AOA	AOB	*narG*	*nirK*	*nirS*	*nosZ*	H_2O	total C	total N	NO_3	NH_4	pH
N_2O		NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
bact	0.30		*	*	*	*	NS	*	NS	NS	NS	NS	NS	NS
AOA	0.20	0.5		*	*	*	NS	NS	NS	NS	NS	NS	NS	NS
AOB	0.14	-0.33	-0.45		*	NS	NS	NS	NS	NS	NS	NS	NS	NS
narG	0.14	0.6	0.69	-0.43		*	*	*	NS	NS	NS	NS	NS	NS
nirK	0.28	0.64	0.42	-0.22	0.59		NS	*	NS	NS	NS	NS	NS	NS
nirS	-0.08	0.03	0.14	-0.05	0.33	0.15		NS	NS	NS	NS	*	NS	NS
nosZ	0.15	0.41	0.3	-0.24	0.35	0.73	0.09		NS	NS	NS	NS	NS	NS
H_2O	0.55	0.12	0.18	-0.07	0.1	-0.01	0.16	0.03		NS	NS	NS	NS	NS
total C	-0.03	0.08	-0.18	-0.01	-0.02	0.01	0.02	-0.09	-0.14		*	NS	NS	*
total N	0.09	0.11	-0.18	0.07	0	0.03	0.03	-0.1	-0.07	0.79		*	*	NS
NO_3	0.04	0.07	0.01	0.23	0.25	0.05	0.33	-0.13	-0.02	0.29	0.47		*	NS
NH_4	0.12	0.13	0.04	0.2	0.17	0.06	0.26	-0.13	0.05	0.3	0.61	0.83		*
pH	0.06	-0.01	-0.06	0.18	-0.06	0.04	0.06	0	0.11	-0.45	-0.06	0.26	0.43	

*, $p < 0.05$ following Bonferroni correction for multiple comparisons; NS, not significant.

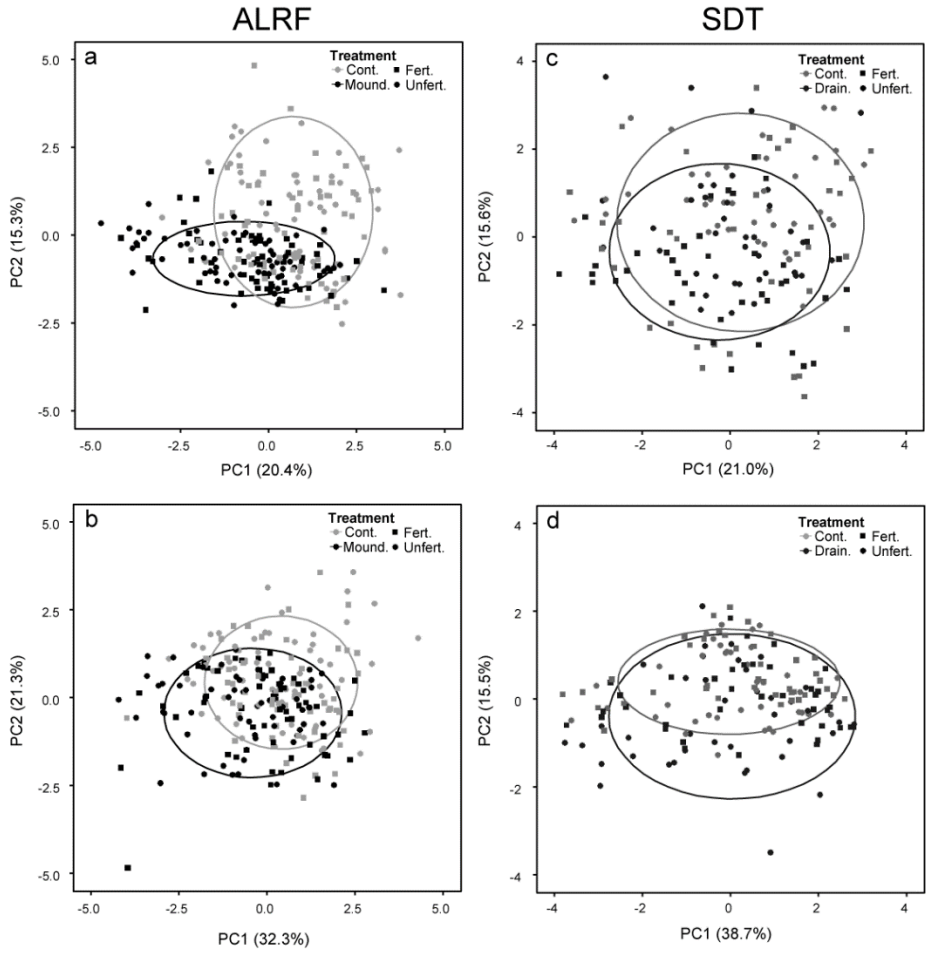
Appendix J. Pearson correlation coefficients for all soil and gene factors from combined ALRF and SDT samples

	N_2O	bact	AOA	AOB	*narG*	*nirK*	*nirS*	*nosZ*	H_2O	total C	total N	NO_3	NH_4	pH
N_2O		NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	*
bact	0.09		NS	NS	NS	*	*	*	NS	NS	*	NS	NS	*
AOA	0.16	0.07		NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
AOB	0.08	-0.08	0.15		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
narG	0.19	0.17	0.37	-0.01		NS	NS	NS	*	*	NS	*	*	NS
nirK	0.04	0.56	0.19	0.1	0.02		*	*	*	*	*	NS	NS	*
nirS	-0.17	0.39	0.03	0.08	-0.07	0.49		*	NS	*	*	NS	NS	*
nosZ	-0.05	0.55	-0.02	-0.08	-0.12	0.69	0.59		*	*	*	NS	NS	*
H_2O	0.39	-0.1	0.17	0.06	0.32	-0.25	-0.15	-0.21		*	*	NS	*	NS
total C	0.17	-0.18	0	-0.07	0.37	-0.43	-0.33	-0.36	0.6		*	NS	*	*
total N	-0.08	0.24	-0.03	0.04	-0.14	0.26	0.26	0.23	-0.2	-0.39		NS	NS	NS
NO_3	0.08	0	0.03	0.08	0.26	-0.02	0.08	-0.12	0.03	0.16	0.03		*	*
NH_4	0.17	0	-0.02	0	0.26	-0.17	-0.04	-0.2	0.25	0.44	-0.1	0.65		*
pH	0.23	0.41	-0.02	0.11	0.02	0.6	0.36	0.43	-0.16	-0.46	0.14	0.25	0.24	

*, $p < 0.05$ following Bonferroni correction for multiple comparisons; NS, not significant.



Appendix K. Spatial structure of nitrification (AOA *amoA*, AOB *amoA*) and denitrification genes (*narG*, *nirK*, *nirS*, *nosZ*) at ALRF following principal component of neighbour matrices (PCNM) analysis. Mean values of PCNM axis 1 and 2 are plotted on Cartesian coordinates of the center of the eight treatment plots by box size and shade. White boxes represent positive ordination coordinates along the axis while negative coordinates are shown in black. Larger boxes are more positive or negative, while smaller boxes have coordinates closer to zero. PCNM axis 1 significantly explained the spatial structure of the functional genes ($p<0.05$).



Appendix L. Principal component analysis (PCA) of microbial gene abundance, N_2O flux and soil characteristics at ALRF and SDT showing ordination coordinates of individual samples and their associated standard deviation ellipses. a) PCA of all ALRF factors, b) PCA of ALRF functional gene factors, c) PCA of all SDT factors and d) PCA of SDT functional gene factors. To match the PCA coordinates calculated using the singular value decomposition (SVD) of the raw data matrix with the *prcomp* function in R (shown here) with coordinates calculated using Eigen decomposition of the correlation matrix with the *FactoMineR pca* function (shown in Figure 4.9), coordinates should be adjusted by using *n* instead of *n*-1 as denominator degrees of freedom normalization factor and transposed by a factor of -1.