Effect of mounding, drainage and fertilization on CH₄ fluxes and methane-cycling functional genes in waterlogged forest stands

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Abstract

Site preparation techniques including mounding, drainage and nitrogen (N) fertilization can enhance seedling survival and site productivity, particularly in waterlogged, low-productivity forest stands. However, practices that alter soil conditions and site hydrology can lead to the unintended alteration of biogeochemical process rates, such as CH₄ fluxes. This study sought to measure CH₄ fluxes measured using static closed chambers at a sub-boreal spruce site and a coastal cedar-hemlock site that underwent mounding and drainage, respectively, to manipulate water table depth relative to planted seedlings, as well as fertilization. The abundance of methyl coenzyme M reductase (*mcrA*) gene found in methanogenic archaea and the particulate methane monooxygenase (*pmoA*) gene found in methane-oxidizing bacteria (MOB) were examined. The use of sulphate as a potential method to stimulate sulphate-reducing bacteria (SRB) and reduce methanogen activity was also investigated using the dissimilatory sulfite reductase β -subunit (*dsrB*) gene. qPCR was used to link *mcrA*, *pmoA* and *dsrB* gene abundance to soil factors and GHG fluxes. Mounding created hot-spots of CH₄ emissions at the spruce site. Drainage improved soil aeration at the coastal cedar-hemlock site and reduced CH₄ emission rates. Fertilization did not affect CH₄ emissions from either site. CH₄ rates were influenced by soil water content and *mcrA* abundance. Measurements of microbial functional genes can elucidate the effects of site preparation on GHG fluxes in waterlogged forest stands.

Introduction

Elevated atmospheric concentrations of greenhouse gases (GHGs) are of major concern worldwide. Methane (CH_4) is the second most-important GHG in terms of radiative climate forcing, its concentrations has increased by about 150% in the last two centuries to about 1.8 ppm (Forster et al., 2007). CH_4 fluxes in forest and wetland soil is a major component of the global carbon (C) cycle. 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). Alterations to the soil C cycle can lead to the release of substantial stored carbon, which is exacerbated in wet forests due to the incomplete degradation of plant biomass carbon in anaerobic zones.

Forest site preparation such as drainage, mounding as well as nitrogen (N) fertilization 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 soil C (Laiho and Finér, 1996; Laiho and Laine, 1997; Canary et al., 2000; Johnson and Curtis, 2001; Bond-Lamberty et al., 2002; 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; Mojeremane et al., 2012). This study examines the microbial communities involved in CH_4 fluxes in wet forest soil ecosystems.

Methanogens from phylum *Euryarchaeota* produce nearly all biogenic CH_4 using a variety of metabolic pathways, though methanogenesis in terrestrial ecosystems is primarily hydrogenotrophic ($\text{CO}_2 + \text{H}_2$) or aceticlastic (acetate) (Conrad, 1999, 2005). Molecular characterization of methanogens target the *mcrA* gene, which encodes the methyl coenzyme M reductase enzyme common to all known methanogenesis pathways (Luton et al., 2002). Aceticlastic methanogenesis is responsible for about two-thirds of methane production in soil (Le Mer and Roger, 2001). Studies of methanogen community structure, including *mcrA* and 16S rRNA characterization, suggest that both hydrogenotrophic methanogens and aceticlastic methanogens are common in waterlogged forest soil (Kemnitz et al., 2004; Conrad, 2005; Frey et al., 2011; Kanokratana et al., 2011). Methanogen diversity (measured by *mcrA* and 16S rRNA) and methanogenesis 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). Methanogenesis has very low energy yields ($\Delta G^\circ = -131$ and -136 kJ for hydrogenotrophic and aceticlastic pathways, respectively) and generally occurs in very low redox potential soils, as methanogens are generally out-competed for acetate and protons by

other biological reducers, e.g., sulphate-reducing bacteria (SRB) ($\Delta G^{\circ} = -152.2$ kJ) (Thauer et al., 1989; Muyzer and Stams, 2008). It is, therefore, predicted that SO₄-S fertilization of waterlogged soil can stimulate the SRB (characterized using the dissimilatory sulfite reductase β -subunit (*dsrB*) gene) and suppress aceticlastic CH₄ production.

Methane-oxidizing bacteria (MOB) in temperate upland forest soil provide a net sink of atmospheric CH₄ (Adamsen and King, 1993; Dutaur and Verchot, 2007; MacDonald et al., 1996; Krause et al., 2013), and CH₄-uptake in soils can account for between 15 and 45 Tg CH₄ uptake yr⁻¹ (Wuebbles and Hayhoe, 2002). The MOB contain either soluble or particulate methane monooxygenase (PMO) enzymes to oxidize CH₄, 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 CH₄ fluxes in forest ecosystems.

Mechanical site preparation (i.e., ditch drainage and excavator mounding) can alter site hydrology, 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 CH₄ emissions (Martikainen et al., 1995; Lundmark-Thelin and Johansson, 1997; Smolander et al., 2000; Minkinen et al., 2002; von Arnold et al., 2005; 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., 2005; 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) respiration from forest soil. Decreases in CH₄ uptake in upland soil or increases in CH₄ emissions from wetland soil following N deposition or fertilization can result from NH₄⁺ saturating the binding sites for CH₄ in PMO, suppressing CH₄ oxidation (Basiliko et al., 2009; Jassel et al., 2011), possibly due to evolutionary similarities between PMO and ammonium monooxygenase enzymes from nitrifying bacteria (Bédard and Knowles, 1989; Holmes et al., 1995; Purkhold et al., 2000; Bodelier and Laanbroek, 2004). However, N fertilization has also been shown to increase CH₄ flux rates as a result of decreased CH₄ uptake (Castro et al., 1994; Liu and Greaver, 2009; Mojeremane et al., 2012), suggesting that the role of

N addition to forest soils in regulating CH₄ 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 CH₄ flux differences following site preparation and N fertilization.

This study seeks to use quantitative PCR (qPCR) of the *mcrA*, *pmoA* and *dsrB* genes to estimate the response of the methanogen, MOB and SRB communities, respectively, in two regenerating waterlogged forest stands subject to mounding, drainage and fertilization. We attempt to link these functional groups to CH₄ fluxes measured using static closed chambers to better understand the importance of the microbial community in determining GHG fluxes from managed forest stands.

Materials and Methods

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 waterlogged sites in British Columbia (B.C.), Canada. Field site descriptions are provided in Levy-Booth et al. (2016). Briefly, mounding treatments were installed at Aleza Lake Research Forest (ALRF), in the wk1 variant of the sub-boreal spruce (SBS) biogeoclimatic zone (Green and Klinka 1994), near Prince George, B.C. 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 February 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. Sampling took place on June 23, 2011, June 28, July 17, August 24, October 18, 2012 and June 13, 2013, corresponding to pre-mounding and 24 hours, 1 month, 2 months, 4 months and 1 year following fertilization. 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). Soil is Humo-Ferric Podzols with mor humus. The original 22 ha western redcedar (*Thuja plicata*) and shore pine (*Pinus contorta* var. *contorta*) stand underwent harvesting and slash-

burning in 1993 and 1994, respectively, and planted with western redcedar (*Thuja plicata*) in 1995. Three 120 m x 45 m treatment plots containing 5 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 on July 25, 2012 at the same formulation used in ALRF. Plots were organized in complete-block design and included the following treatments: drained/fertilized, drained/fertilized, drained/unfertilized and drained/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.

Soil sampling and preparation

At ALRF and SDT mineral soil (Ae and some Bf horizons; ~ 0-5 cm) were sampled from three and two randomly chosen locations in each treatment plot, respectively. Volumetric soil moisture was measured using a TH₂OTM portable moisture probe (Dynamax Inc., Houston, U.S.A.) and gravitational soil moisture was measured by oven drying field moist soil. Results of Total C, Total N, NO₃-N, NH₄-N and pH analysis are described in Levy-Booth et al. (2016). In this study, 10 g dry soil was analyzed for total S and SO₄-S by the British Columbia Ministry of Forests, Lands and Natural Resources Operation Analytical Laboratory (Victoria).

Field measurement and gas chromatography analysis of CH₄ fluxes

Measurement of *in situ* GHG flux at ALRF and SDT took place as described in Basiliko et al. (2009) and in Levy-Booth et al. (2016). 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. 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 CH₄.

Nucleic acid extraction and quantitative PCR

DNA was extracted from 0.25 g dry soil using the MoBio PowerClean soil DNA isolation kit and quantified using the Quant-iT™ PicoGreen® dsDNA assay (Life Technologies Corp., Carlsbad, U.S.A.). qPCR used Power SYBR® Green PCR Master Mix (Life Technologies Corp., Carlsbad, U.S.A.) in the Applied Biosystems® StepOnePlus™ real-time PCR system. Gene copy numbers were expressed as copy number g⁻¹ soil (dry weight (dw)). *McrA* and *pmoA* quantification are described in Christiansen et al. (2016) using primers from Luton et al. (2002) and Bourne et al. (2001), respectively, and amplification protocols in Frietag et al. (2010). Standard curves for calibration of *mcrA* qPCR were created using triplicate 10-fold dilutions from 10³ to 10⁸ copies of clonal 420-bp *mcrA* segments from *Methanolinea mesophila*, amplified from soil near the SDT site as in Christiansen et al. (2016). Standard curves for *pmoA* were created using triplicate 10-fold dilutions from 10³ to 10⁸ 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°C and 40 cycles of 95°C denaturation for 30 s, 55°C annealing for 45 s, 72°C extension for 45 s, after which fluorescence was measured. Standard curves for *dsrB* were created using triplicate 10-fold dilutions from 10² to 10⁸ copies of *Desulfosporosinus orientis* and *Desulfomicrobium baculatum* genomic DNA.

Statistical analysis

Statistical analysis was performed using in R v. 2.15.3 (R Core Team, 2013). Data were fitted with the linear mixed-effects model and subject to two-factor analysis of variance (ANOVA) using the *lme* and *anova* functions in the *nlme* and *car* packages, respectively, with fertilization and mounding/drainage as fixed effects and blocking as a random effect. qPCR data were analyzed as log₁₀ values and plotted using 25%-75% quartile boxplots with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). GHG data that violated ANOVA assumptions were logarithmically transformed and qPCR data that violated assumptions were analyzed as normal values. Unconstrained, exploratory ordination was carried out using principal component analysis (PCA) on scaled parameters with the *prcomp* function for ordination by singular value decomposition and visualized using the *ggbiplot* package in 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 dependant 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 dependant variable or matrix variance uniquely explained by each soil parameter, or groupings of parameters, by constraining linear partial regression by all other variables. Bonferroni-corrected p-values were used for multiple comparisons.

Results

Site preparation altered forest soil CH₄ flux rates

Site preparation had a significant but inconsistent effect on CH₄ flux rates in both ALRF and SDT sites. At ALRF, the highest rates CH₄ efflux were measured following fertilization on Jul-12 (Figure 1a). At this date, CH₄ efflux in mound hollows rates was 422.4 ± 176.6 and 1171.3 ± 368.8 $\mu\text{g CH}_4 \text{ m}^{-2} \text{ h}^{-1}$ in unfertilized and fertilized plots, respectively. By Aug-12—the warmest, driest date—79% of chambers demonstrated CH₄ uptake. Mounding appeared to reduce CH₄ flux rates after one year, owing primarily to the reduction of efflux in exposed mounds. There was consistent CH₄ uptake measured in the drained plots at SDT, particularly in chambers located in unfertilized plots (Figure 1b). While there was CH₄ uptake in all plots throughout the course of the field measurements, the undrained control plots had significantly greater emissions throughout the experiment (Jul-12, Aug-12 and Jun-13). SDT fluxes did not suggest seasonal or fertilization effects, having less temporal fluctuations than at ARLF.

Fertilization transiently increased SO₄-S concentrations

An important component of this study was to determine how fertilization with S affected the SRB and the methane-cycling community. Trace concentrations of total S were only measured at ALRF in the forest floor F and H layers (data not shown). However, SO₄ was measured in almost all of the mineral samples taken from this location (Figure 2a). Treatment effects were measured at ARLF throughout the study, with a significant increase after 24 hours of fertilization. This effect was only observed in the mounded plots, which lacked an organic layer. As a result there was a significant interaction between mounding and fertilization at this date. The fertilization effect continued in Jul-12, although the SO₄-S

concentrations in the fertilization plots had decreased. SDT had trace amounts of total S in 52% of forest floor samples (data not shown) from both fertilized and unfertilized plots. In contrast to ALRF there were no treatment or locational effects of mounding and fertilization on SO₄-S at SDT, with the exception of Jul-13, where fertilized drained soil samples had significantly greater SO₄-S than control and drained unfertilized samples (Figure 2b). Soil SO₄-S was greater at SDT than at ARLF, with concentrations reaching a peak of 90.1 ± 35.2 and 16.5 ± 4.2 $\mu\text{g g soil}$ at these sites, respectively.

Microbial gene abundance altered by site preparation

mcrA abundance was affected by mounding, with significantly lower abundance in mounded plots in Oct-12 (Figure 3a). In Jun-13, as the mounded plots once again became waterlogged, fertilized mound hollows and unmounded plots had significantly greater *mcrA* abundance than mound tops. A consistent drainage effect on *mcrA* abundance was observed at SDT (Figure 4b). Undrained control plots had significantly greater *mcrA* abundance than drained plots in Aug-12 and Jul-13 and Sep-13. A significant effect of fertilization also occurred in Jul-13, one year following the treatment.

The abundance of *pmoA* was significantly greater in mounded plots than in unmounded plots in Oct-12 (Figure 3b). *PmoA* abundance in ALRF and SDT soil appeared to follow a seasonal trend. The lowest measured abundance (5.3 ± 0.2 *pmoA* copies g⁻¹ soil (dw)) was measured in undrained SDT fertilized plots in Oct-12, which had a soil temperature of 8.1°C. Mirroring *mcrA* abundance, control plots had significantly greater *pmoA* abundance than drained plots in Aug-12 as well as Jul-13 and Sep-13. There was significant interaction between drainage and fertilization in Aug-12, as fertilization counteracted the effect of drainage on *pmoA* abundance.

DsrB abundance at the ALRF site was significantly lower in mounded plots in Jun-13 (Figure 3c), mirroring to *mcrA* abundance at this date. At SDT, *dsrB* was significantly lower in drained soil in Aug-12 and Jul-13. There was no increase of *dsrB* genes related to application of SO₄-S fertilizer, nor were interactions between fertilization and drainage observed.

Influence of site preparation and fertilization on soil parameters, gene abundance and GHG flux

Redundancy analysis (RDA) was used to determine the relationships between soil parameters and microbial gene abundance. In addition to the variables reported in this study, soil chemical variables, bacterial 16S rRNA gene abundance and CO₂ flux rates from Levy-Booth et al. (2016) were included in this analysis. Forward-selected soil variables explained 77% of variation in ALRF gene abundances (Figure 5a). *McrA* abundance was negatively correlated with *pmoA* abundance, CO₂ fluxes and soil temperature, but positively correlated with CH₄ flux rates (Supplementary Table 1). *PmoA* abundance was

strongly and positively correlated with CO₂ fluxes and soil temperature. Canonical variation partitioning using partial regression was used to test variables that explain variation in gene abundances and GHG fluxes. Note that the sum of variation explained by individual variables will not equal the total model variation, as additive or subtractive effects can occur. Bacterial 16S rRNA gene abundance was explained most strongly by total soil carbon and nitrate concentration. Soil temperature explained the most variation in *McrA*, *pmoA* and *dsrB* abundance, with soil water content and NO₃-N concentrations explaining additional variation. *PmoA* was also significantly explained by *mcrA* abundance. In turn, CO₂ fluxes were significantly explained only by soil temperature and NO₃-N concentrations, while *mcrA* abundance, bacterial 16S rRNA gene abundance and NO₃-N explained CH₄ fluxes, albeit only 8.6% of variation in CH₄ fluxes could be explained at ALRF. However, these data indicate that the abundance of microorganisms containing *mcrA*, *pmoA* and *dsrB* genes can fluctuate with temperature, and are influenced by nitrate availability. Further, CH₄ fluxes, while poorly explained by soil variables were most-strongly linked to the population size of *mcrA* containing organisms at ALRF. While fertilization with NO₃-N and SO₄-S was thought to suppress methanogenesis in forest soil, fertilization may lead to a stimulation of these populations through the alleviation of nitrogen limitation.

RDA of gene abundances in SDT soil revealed a much stronger correlation between *mcrA* abundance and CH₄ fluxes at this site (Figure 5b). *McrA* abundance was negatively correlated with SO₄-S concentrations at this site, yet positively correlated to *dsrB* abundance (Supplementary Table 2). PCNM of SDT sampling locations resulted in 128 PCNM variables between nearest-neighbor sampling locations. Of these, three PCNMs had significant Moran's I statistics, representing increasingly fine levels of spatial structure. The first PCNM variable at SDT positively correlated with *dsrB*, *mcrA* and CH₄ fluxes, these variables exhibiting spatial organization. As with ALRF variation partitioning models, SDT variables were strongly explained by soil temperature (Table 1). 84.4% of CO₂ flux variation could be explained, with temperature explaining 42.7% on its own. More CH₄ flux variation was explained at SDT than at ALRF, with *mcrA* abundance again acting as the strongest predictive variable.

Discussion

CH₄ flux varies widely in forests associated with different climatic, vegetation and management parameters. In a meta-analysis of CH₄ flux from UK soils, Levy et al. (2012) report that soil depth, C content, volumetric water content and vegetation cover were the primary site-specific determinants of CH₄ flux. Site preparation alters many of these influencing factors. For example, Mojeremane et al. (2012) found that drainage of peatland reduced CH₄ emissions by 57-76%, owing to increased soil

temperature and decreased soil water content. Our data generally agree with these observations, and suggest that soil turnover following mounding, and soil water table reduction following drainage, decreases methanogens and dissimilatory SRB.

While it was hypothesized that $\text{SO}_4\text{-S}$ may reduce CH_4 emissions by stimulating SRB to outcompete methanogens (Muyzer and Stams, 2008), there were no correlations between $\text{SO}_4\text{-S}$ and CH_4 (Figure 5). Therefore, there was no evidence from this study that $\text{SO}_4\text{-S}$ fertilization at a concentration of 50 kg ha^{-1} can be used to reduce CH_4 emissions in waterlogged soils. Alternatively, increased CH_4 efflux is not uncommon in waterlogged forest soils subject to fertilization (Augustin et al., 1998; von Arnold et al., 2005). In a meta-analysis of wetland soil, low N application rates tended to stimulate CH_4 uptake while larger amounts tended to inhibit uptake by the soil (Aronson and Helliker, 2010). 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 (Krause et al., 2013; Zhuang et al., 2013).

McrA and *pmoA* abundance can serve as indicators of the potential for a soil community to emit methane under favourable conditions (Freitag et al., 2010). The abundance of *mcrA* genes unexpectedly responded positively to mineral nitrogen concentrations, suggesting that in nitrogen limited environments methane emissions can be stimulated rather than reduced following fertilization. This result is not without precedent: 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. Rather than competition between SRB and methanogens for acetate and other substrates, the correlation between *dsrB* and *mcrA* suggests another relationship. Hydrogenotrophic methanogens can syntrophically reduce CO_2 to CH_4 using H^+ from SRB, allowing both processes to occur in nutrient poor soil (Pester et al., 2012). The recovery of hydrogenotrophic *M. mesophila* *mcrA* amplicons from soil adjacent to SDT suggests this may be the case, although this hypothesis requires experimental validation. Alternatively, the use of N fertilization levels $>100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ or continuous N deposition can increase CH_4 fluxes by overwhelming the CH_4 binding sites in the PMO enzymes in MOB (Basiliko et al., 2009; Jassal et al., 2011; Bodelier, 2011; Gundersen et al., 2012; Zhuang et al., 2013).

Mounding and drainage significantly reduced CH_4 fluxes after one year. While fertilization significantly increased $\text{SO}_4\text{-S}$ concentrations at ALRF, there was no effects on methane rates or methanogen abundance at the conclusion of this study. This work demonstrates that drainage led to a lasting reduction of methanogen populations and CH_4 emissions, while results were less certain for the mounded site. Therefore, ditch drainage techniques may be a useful method of site preparation to reduce overall GHG emissions from waterlogged, CH_4 -emitting soil. The use of microbial genes abundance can

help resolve how the complex changes to the soil community following site preparation can result in alterations to GHG fluxes in waterlogged forest stands.

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Figure Captions

Figure 1. a) CH₄ fluxes emissions from a) undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at ARLF and b) undrained control (C) and drained (D) subject to fertilization at 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 (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Figure 2. SO₄-S concentration (mg kg⁻¹) in soil from a) from undisturbed control (C) and mounded plots (M, mounds; H, hollows) subject to fertilization at ARLF, and b) control (C) and drained plots (D) subject to fertilization at SDT. Shaded arrow shows time of fertilization. $\pm$ 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 (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Figure 3. 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 ARLF. 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. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Figure 4. 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 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. Treatment effects and interactions following two-way ANOVA are provided if significant (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Figure 5. Redundancy analysis (RDA) of microbial gene abundance (black) by soil variables (blue) and spatial structure (pink) following fertilization. A) Ordination of functional genes constrained by significant soil factors for ARLF samples; b) ordination of SDT functional genes constrained by significant soil factors. Abbreviations: UD, undrained; D, drained; UM, unmounded; M, mounded; F, fertilized (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

530 Figure 1

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Table 1. Canonical variance partitioning of gene abundance and greenhouse gas flux rates from ALRF and SDT sites.

Site:	ALRF Mounding and Fertilization Trials				
	Variation				
Model	(%)	Variation by Individual Variables (%)			
<u>Gene Abundance</u>					
Bacterial			NO3		
16S	18.4***	TC 6.2**	3.2**	TN 2.8**	CN 2.4*
		Temp			
mcrA	26.5***	8.1***	H2O 7.0**	NO3 3.6**	
		Temp			
pmoA	34.8***	29.9***	mcrA 1.5*		
		Temp			
dsrB	23.6***	16.2***	NO3 7.6**		
<u>Greenhouse Gases</u>					
		Temp			
CO2	27.6***	13.1***	NO3 2.9*		
CH4	8.6**	mcrA 5.5**	16S 4.6**	NO3 4.2*	

Site:	SDT Drainage and Fertilization Trials				
	Variation	Variation Explained by Individual			
Model	(%)	Variables (%)			
<u>Gene Abundance</u>					
Bacterial		Temp			
16S	21.8***	22.4***			
		dsrB			
mcrA	33.9***	15.1***	NH4 3.5**		
		Temp	dsrB		
pmoA	58.4***	39.1***	6.2***	pH 2.6*	CN 1.5*
		mcrA	pmoA		
dsrB	42.8***	25.9***	5.2**		
<u>Greenhouse Gases</u>					
		Temp	pmoA		
CO2	84.4***	42.7***	2.4***	TC 0.7*	
		mcrA			
CH4	24***	5.7***	H2O 3.9**	TN 2.2*	pmoA 1.6*

Supplementary Material

Supplementary Table 1. Pearson correlation coefficients between measured variables at ALRF.

	Microbial genes				GHG emissions		Soil characteristics			Climate
	<i>mcrA</i>	<i>pmoA</i>	<i>dsrB</i>	Bact.16S	CO ₂	CH ₄	SO ₄ -S	pH	H ₂ O	Temp.
<i>mcrA</i>		*			*	*	***		***	***
<i>pmoA</i>	-0.26				***					
<i>dsrB</i>	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 ₂ O	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)

Supplementary Table 2. Pearson correlations between measured variables at SDT.

	Microbial genes				GHG emissions		Soil characteristics			Climate
	<i>mcrA</i>	<i>pmoA</i>	<i>dsrB</i>	Bact.16S	CO ₂	CH ₄	SO ₄ -S	pH	H ₂ O	Temp.
<i>mcrA</i>			***			***	**	***		
<i>pmoA</i>	0.03		**	***		**		**		***
<i>dsrB</i>	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 ₂ O	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)