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Short- and long-term temperature responses of soil denitrifier net N_2O efflux rates, inter-profile N_2O dynamics, and microbial genetic potentials

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Abstract

Production and reduction of nitrous oxide (N_2O) by soil denitrifiers influences atmospheric concentrations of this potent greenhouse gas. Accurate climate projections of net N_2O flux have three key uncertainties: 1) short- vs. long-term responses to warming; 2) interactions among soil horizons; and 3) temperature responses of different steps in the denitrification pathway. We addressed these uncertainties by sampling soil from a boreal forest climate transect encompassing a 5.2 ^oC difference in mean annual temperature, and incubating the soil horizons in isolation and together at three ecologically relevant temperatures in conditions that promote denitrification. Both short-term exposure to warmer temperatures and long-term exposure to a warmer climate increased N_2O emissions from organic and mineral soils; an isotopic tracer suggested an increase in N_2O production was more important than a decline in N_2O reduction. Short-term warming promoted reduction of organic horizon-derived N_2O by mineral soil when these horizons were incubated together. The abundance of *nirS* (a precursor gene for N_2O production) was not sensitive to temperature, while that of *nosZ clade I* (a gene for N_2O reduction) decreased with short-term warming in both horizons and was higher from a warmer climate. These results suggest a decoupling of gene abundance and process rates in these soils that differs across horizons and timescales. In spite of these variations, our results suggest a consistent, positive response of denitrifier-mediated, net N_2O efflux rates to temperature across timescales in these boreal forests. Our work also highlights the importance of understanding cross-horizon N_2O fluxes for developing a predictive understanding of net N_2O efflux from soils.

Keywords: nitrous oxide, *nosZ*, *nirS*, boreal forest, ^{15}N, climate change

Manuscript highlights:

- short- and long-term exposure to warmer temperatures increased soil net N_2O flux
- short-term warming promoted reduction of organic horizon derived N_2O by mineral soil
- gene abundance - process rate coupling in these soils differed across horizons and timescales

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1. Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas, with ~300 times the global warming potential of carbon dioxide on a 100-y timescale and uncertain climate feedback effects (Ciais et al., 2013; Portmann et al., 2012). Though increases in atmospheric N_2O are attributed to N-fertilizer use (Mosier et al., 1998), emissions from natural systems dominate terrestrial fluxes (Ciais et al., 2013) and experimental manipulations indicate warming may enhance these fluxes (Benoit et al., 2015; Billings and Tiemann, 2014; Kurganova and Lopes de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014). One of the most important biogeochemical pathways of N_2O formation in natural systems is denitrification, the stepwise reduction of NO_3^- to N_2. In this pathway, soil denitrifiers can both produce and reduce N_2O, and incomplete reduction of N_2O during the final step to N_2 can result in N_2O release to the atmosphere (Baggs, 2011; Firestone and Davidson, 1989). Soil microorganisms play a critical role in climate change (Cavicchioli et al., 2019) yet it remains unclear how sensitive the denitrification pathway is to a warming climate.

Translating empirically-derived knowledge about soil denitrifiers into climate projections is difficult due to the dynamic and variable nature of the many interacting steps and their controls (Butterbach-Bahl et al., 2013). In this study we address three key challenges that are associated with the temperature sensitivity of the emergent process of denitrification. First, we do not know if short-term responses of denitrifying communities to warming (Billings and Tiemann, 2014; Kurganova and Lopes de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014) are maintained across longer timescales, and thus we cannot know if laboratory studies can provide the empirical data needed to project longer-term fluxes. Studies of heterotrophic soil CO_2 efflux suggest that enhanced rates of microbial respiration with warming may be dampened over the long-term, prompted by a combination of microbial acclimation and adaptation (Billings and Ballantyne, 2013; Bradford, 2013), and it is feasible that denitrifying communities may also exhibit only ephemeral responses to warming. Such a response is consistent with inconclusive results of multiple *in situ* warming experiments though such studies necessarily reflect both denitrification and other N_2O-producing processes in soils (Bai et al., 2013; Butler et al., 2012; Dijkstra et al., 2012; McDaniel et al., 2013). Assuming microbial acclimation, soils indigenous to a particular climate regime may harbor denitrifying

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communities that are more effective at NO_3^- reduction and transformation to N_2 in that climate's typical temperature range. In principle, this could result in relatively lower rates of N_2O loss in that particular temperature regime (i.e. more complete denitrification) compared to less effective processing by those microbial communities if the mean temperature were to shift. Though this phenomenon has not been demonstrated for the more complicated soil denitrification with its multiple enzymatic steps, the so-called "home field advantage" has been demonstrated in studies exploring rates of other soil microbial processes (Alster et al., 2013; Wallenstein et al., 2013).

A second knowledge gap limiting our ability to project future soil N_2O climate feedbacks is potential variation with temperature in interactions between microbial production and reduction of N_2O across soil horizons. Implicit in the concept that such cross-horizon interactions may control net profile N_2O efflux is the assumption that soil denitrifiers have different patterns of production and reduction in different horizons. This may arise because the conditions that control N_2O production or reduction differ between horizons, or it may arise because the metabolic potentials of the soil microbial community in different horizons are intrinsically different (Blume et al., 2002; Fierer et al., 2003). Consistent with this idea, Goldberg and Gebauer (2009) illustrated clear variation in patterns of $\delta^{15}N$ of N_2O across soil depth in response to drought, which could have been caused by variations in either N_2O production or reduction (Billings, 2008). The exchange of substrates between soil horizons thus can be an important process dictating whole-soil N_2O efflux, and may contribute to apparent inconsistencies between warming effects in the laboratory and the field (reviewed in Bai et al. 2013). Indeed, profile interactions have been recently demonstrated as important drivers of soil CO_2 efflux: temperature responses of whole soil core respiration can be distinct from the sum of those observed for horizons incubated in isolation from each other, likely due to exchange of substrates and microbes among horizons (Podrebarac et al., 2016). Though evidence suggests that N_2O produced in one soil horizon may be reduced in another (Goldberg and Gebauer 2009), the degree to which this may occur, and why, has not been determined.

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A third feature challenging our ability to project soil N_2O effluxes in a warmer climate regime is the potentially different response to warming of distinct steps in the denitrification pathway (this may be for one or multiple microbes within the community, that carryout the enzymatic steps). For instance, if the activity of *nosZ*, a gene that codes for an enzyme catalyzing N_2O reduction, experiences a different response to temperature than *nirK*, a gene coding for an enzyme catalyzing NO_2^- reduction (and thus N_2O production), the net flux of N_2O may either increase or decrease with temperature depending on the direction and magnitude of both responses. Though gene abundances sometimes exhibit decoupling from function (Peterson et al. 2012), quantifying any changes in these functional gene abundances with temperature can help discern the propensity for temperature responses of relevant microbial communities' structure, and thus the driving mechanisms for net N_2O production responses. Differential responses of these genes' abundances to short-term temperature manipulation have been observed in grassland soils (an increase in *nosZ* with short-term temperature increases; Billings and Tiemann, 2014), but it is unknown whether these observations are relevant for soil microbial communities subjected to long-term exposure to distinct temperature regimes.

In this study, we explore these three issues: short- vs. long-term responses of soil denitrifying communities' net production of N_2O to warming, the exchange of denitrification-derived N_2O among horizons as a driver of temperature response of net N_2O efflux, and the potentially different responses of the relative abundances of microbial genes linked to N_2O production vs. reduction to temperature. We invoked a space for time substitution to test our long-term warming hypothesis, using a climate transect along which mean annual temperature (MAT) varies but dominant vegetation, soil type, and soil moisture are similar. To elucidate both short- and long-term temperature responses of soils' denitrifying communities, we incubated soils that came from different latitudes and climate regimes along this transect (long-term warming) for 60 h at 5, 15 and 25 ^oC (short-term warming), to reflect typical current (5 and 15 ^oC) and projected future (25 ^oC) soil temperatures. Specifically, laboratory incubations of mesic organic and mineral boreal forest soil horizons were established in conditions that promote denitrification. To understand the potential for interactions among soil horizons as a driver of temperature response of net N_2O efflux, we incubated organic and mineral soils both

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individually and in combination. We measured net rates of N_2O efflux and abundances of representative functional genes linked to production and reduction of N_2O, and estimated N_2O reduction using an isotopic tracer.

We predicted that short-term warming would enhance net N_2O production in these boreal soils, as in the majority of past incubation studies (Billings and Tiemann, 2014; Kurganova and Lopes de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014). As outlined above, we also tested the hypothesis that a warmer temperature regime over a longer timescale would show the opposite effect: a dampened net N_2O efflux from the historically warmer soils, where organic N turnover is faster (Philben et al., 2016), and where denitrifying communities presumably can function as efficient transformers of NO_3^- to N_2 at warmer temperatures compared to their more northern counterparts. We also hypothesized that N_2O produced in one horizon would be reduced in the other when incubated together, resulting in lower net N_2O efflux than a simple linear combination of these horizons' individual efflux rates. Specifically, we anticipated that organic soils, relatively rich in microbial abundance and diversity compared to mineral soils, would reduce mineral-produced N_2O, following dominant diffusion gradients. Finally, we hypothesized that soils exhibiting higher rates of net N_2O production would exhibit some combination of increased *nir* abundance and decreased *nos* abundance and associated higher ratios of *nir:nos* gene abundances, reflecting shifts in microbial genetic potentials with temperature regime.

2. Materials and method

2.1 Study site and soil sampling

Soil was collected from three mature forest stands at each of three regions along the Newfoundland and Labrador Boreal Ecosystem Latitudinal Transect (NL-BELT), Canada (Table 1, Fig.1; (Ziegler et al., 2017)). NL-BELT spans the north-south extent of the balsam-fir dominated boreal biome in eastern Canada, from southwest Newfoundland to southeast Labrador. This transect has long-term (century-scale) temperature regime differences, but otherwise similar conditions. For instance, the three study regions along this transect (from south to north), the Grand Codroy, Salmon River, and Eagle River watersheds (Fig. 1), have similar Orthic Humo-

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Ferric Podzols (Spodosols; Soil Classification Working Group, 1998) and balsam fir (*Abies balsamea*)-dominated vegetation. The difference in MAT and precipitation is 5.2 °C and 431 mm between Grand Codroy (southern-most) and Eagle River (northern-most) climate stations (Environment and Climate Change Canada 2108). The soils are mesic and the regions have an evaporative demand gradient (Table 1) that considerably reduces the precipitation gradient, making the transect an excellent proxy for investigating soil temperature responses while mitigating confounding features of differing soil moisture. Three replicate forest stands were established in each of the three climate regions, allowing us to assess the influence of long-term differences in MAT (and associated differences in climate) along the transect without concerns about pseudoreplication, a rarity in large-scale space-for-time substitutions (Ziegler et al., 2017)

Two large (30 cm^2) peds of organic (LFH or O horizon) and mineral (B horizon) soil were collected at each forest stand on a different calendar date but an equivalent ecological date: 22-24 October 2013 in Eagle River, 4-5 November 2013 in Salmon River, and 22-23 November 2013 in the Grand Codroy. This pre-freeze, post-growing season period typically exhibits relatively large and active microbial biomass in northern latitude organic soils (Buckeridge et al., 2013). The A_h and A_e horizons were not present at all sites so were not included in the incubation at any site. Each collection was shipped to the University of Kansas (4-5 days transit in insulated coolers, on ice) and processed immediately. Because regions were processed as separate experimental blocks we cannot separate the region and block effects. However, we confounded these factors knowingly, because we believed ecological date and rapid processing were more important than minimal differences in laboratory practice between blocks.

2.2 Incubation and headspace gas collection

Aboveground vegetation (i.e. moss, herbaceous plants, tree seedlings) was removed from the peds with scissors. The two peds of organic and mineral soil from each forest site were pooled within horizon and mixed by hand, producing an organic and mineral sample for each forest. This process was repeated nine times, for three forests in each of three regions. Subsamples (fresh mass, organic: 50 g; mineral: 40 g) were placed in half-pint (237 ml) Mason jars. To test

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the potential for N_2O producers and reducers from one horizon to interact with their counterparts in the other horizon, 'combined' samples were also prepared in which an open container of mineral soil (20 g) was placed within a jar, next to organic soil (25 g) such that they had a shared headspace but were not physically mixed. Each sample was replicated for three temperature incubation scenarios (5, 15 and 25 °C), and three blank jars (no soil) were included for each temperature. To maximize the potential for denitrification we promoted anaerobic conditions and substrate diffusion to by evacuating headspace air and replacing with He, and adjusting water-holding capacity to 80% with a $K^{15}NO_3^-$-N solution ($\delta^{15}N$ 3000 ‰) that added 18 and 1.3 µg N g^{-1} dw soil to the organic and mineral soil samples, respectively (18x background levels at the time of sampling, although within the annual range of soil NO_3^- availability based on unpublished field data). Our approach was distinct from a potential denitrification assay, which calls for non-limiting C and NO_3^- additions to soils (Pell et al., 1996); instead, we intended to promote conditions conducive to denitrification using natural C pools and as close to natural NO_3^- concentrations as was feasible. Therefore, this experiment is not predictive of bulk soil N_2O rates and instead explores controls on N_2O rates in soil zones with low O_2 concentrations. Such 'hot spots' for biogeochemical cycles in soils are well-documented (McClain and others 2003).

Over 60 h of incubation, we collected headspace gas eight times for determination of N_2O concentration. The multiple time points verified the robustness of the final 60 h time point measure and the net result of these samples was used to compare across treatments. The first sample was collected immediately after initiating the incubations, the second sample was collected at ~3 hours, and then further samples were collected every ten hours afterwards. At each collection point 14 ml of headspace gas was removed with a needle and gas-tight syringe and injected into pre-evacuated 12 ml borosilicate vials with a silicone septum and aluminum crimp (Teledyne Instruments, Inc., CA, USA); at the second and last collection an additional 14 ml headspace gas was removed and injected into pre-evacuated Exetainers (Labco Ltd., High Wycombe, UK) for isotopic analysis of N_2O in the headspace. After each gas sampling, He of an equivalent volume was injected into the incubation vessels to maintain pressure in the

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containers. At the end of the incubation all jars were opened and soils were destructively harvested to quantify soil inorganic N, and for DNA extraction.

2.3 N_2O concentration and isotope analysis

Headspace samples were analyzed for N_2O concentration in an auto-injected 5 ml subsample on a gas chromatograph fitted with an electron capture detector (CP-3800, Varian), and calibrated against a four-point standard curve that encompassed the sample range. Blank corrected headspace concentrations were adjusted for the dilution at each sampling with He replacement, and rates of net N_2O production were calculated as the average of the 8 sample collections' rates. Net N_2O flux changed throughout the course of the 60 h incubation; we focus on the average of these rates to integrate both production and reduction into and aggregate value across the whole incubation. Samples for isotope analysis ($\delta^{15}N$ of N_2O) were submitted to the University of California, Davis, Stable Isotope Facility, where they were analyzed on a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (Bremen, Germany). Analysis was conducted with 4 standards of 0.4-10 ppm N_2O in He and a precision of 0.1‰ ^{15}N.
The change in ^{15}N enrichment of the N_2O between incubation sampling times at 3 h and 60h was used to quantify gross reduction of N_2O to N_2 (Billings and Tiemann 2014). Because our tracer contained far more ^{15}N than is present naturally, any natural fractionation during N_2O reduction was negligible compared to the isotopic signature of the tracer in the N_2O pool, and we can use $^{15}N_2O$ as a means of assessing N_2O production vs. reduction. If $^{15}N_2O$ at 60 h is higher than at 3 h, it suggests the tracer was continuing to flow into the N_2O pool more so than out of it, and thus that N_2O production outpaced N_2O reduction (transformation into N_2) at that time point. In contrast, if $^{15}N_2O$ at 60 h is lower than at 3 h, it suggests that the tracer was flowing out of the N_2O pool at a greater pace than it was flowing into it, and thus that N_2O reduction outpaced N_2O production at that time point. We computed the change in percent of the ^{15}N tracer added that was found in headspace N_2O across incubation time as:

$$Change\ in^{15}N_2O = \left(\left(\frac{^{15}N_2O}{^{15}NO_3^-\text{-N}\ added}\right) * 100\right)_{final} - \left(\left(\frac{^{15}N_2O}{^{15}NO_3^-\text{-N}\ added}\right) * 100\right)_{initial}$$

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where $^{15}N_2O$ is ng of ^{15}N in headspace N_2O per g of soil, $^{15}NO_3^-$-N is ng of ^{15}N in NO_3^- per g of soil, final refers to the end of the incubation (~60 h), and initial refers to the first time point at which change in ^{15}N of N_2O was assessed (~3 h).

2.4 Soil nutrient analysis

To observe changes in extractable inorganic N during the incubation, we extracted soil subsamples prior to and following the incubation (fresh mass, organic: 12 g; mineral 10 g) by shaking for 1 h with 40 ml 0.5 M K_2SO_4. After shaking all samples were filtered and extracts frozen at -20 °C until further analysis. Soil NO_3^--N and NH_4^+-N in the extracts were analyzed on a Lachat 8500 Autoanalyzer (Hach Co., Loveland, CO, USA) using the cadmium reduction and phenol red methods, respectively.

2.5 Functional gene abundance

Soil DNA was extracted from 0.25 g soil using MoBio Power Soil DNA extraction kit and purified with MoBio PowerClean DNA Clean-up kit (MoBio Laboratories, Carlsbad, CA, USA, now Qiagen). DNA was quantified with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), diluted by a factor of ten and stored at -20 °C until further analysis. We assayed several functional gene primers in the denitrification pathway via PCR, and selected *nirS* (Geets et al., 2007) and *nosZ clade I* (Wallenstein and Vilgalys, 2005) as the most tractable indicators of N_2O production and reduction in these soils using quantitative PCR (qPCR), based on successful amplification of these genes across all samples. Note that we were not able to amplify *nirK* or *nosZ clade II* in these soils. qPCR was accomplished using the ABI StepOnePlus (Applied Biosystems) with Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent/Life Technologies, Carlsbad, CA, USA). Each reaction consisted of 5 µl (~2 ng) genomic DNA, 400 nM each primer, 300 nM reference dye and 1 X Brilliant III in a final volume of 20 µl. The qPCR program consisted of an initial denaturing temperature of 95 °C for 3 min followed by 40 cycles of denaturing at 95 °C for 5 s and a combined annealing and extension step of 10 s at 60 °C for both *nirS* and *nosZ* genes. Melt curves were calculated at the end of each qPCR run to confirm

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product specificity. Each qPCR plate contained one primer pair, three negative controls and a four-point standard curve (ranging from 300 to 300,000 copies). Standard curves were generated using genomic DNA from lab stock of cultured *Pseudomonas fluorescens* and gene copy numbers were calculated assuming a mass of 1.096 x 10^{-21}g per base pair (Wallenstein and Vilgalys, 2005), one gene copy per genome, and a genome size of 7.07 Mb (NCBI).

2.6 Statistical analysis

We used a three-way ANOVA to assess the influence of the fixed effects of soil horizon, 'region' (historical temperature), 'temperature' (short-term, incubation temperature) and their interactions on: inorganic N pools, net N_2O flux averaged across the incubation, change in percent of added ^{15}N tracer found in headspace N_2O, the effects of mixing horizons in the incubation on net N_2O flux, and functional gene abundances. For all analyses, we followed up significant main effects with a Tukey's posthoc analyses and report adjusted *P*-values. For all variables, we assessed whether they met assumptions required for performing these statistical tests, and log-transformed variables before analysis when required. All statistical analyses were performed in R (R Core Team, 2014), using the MASS package (Venables and Ripley, 2003). All significant ($\alpha = 0.05$) results and interactions are reported except significant main effects when significant interactions of their terms are reported instead. Errors reported are one standard error of the mean.

3. Results

3.1 Changes in inorganic N pools after the incubation

Temperature altered the pool sizes of NH_4^+-N differently in each region and horizon (temp x region x horizon: *P*=0.05), increasing relative to pre-incubation pool sizes in the organic soils at some of the incubation temperatures (coolest region, 25 ^oC: *P*=0.04; intermediate region, 25 ^oC: *P*=0.02; warmest region, 15 ^oC: *P*<0.0001, 25 ^oC: *P*=0.0001) (Fig. 2 A and B). Mineral soil NH_4^+-N pool sizes post-incubation did not differ from pre-incubation pool sizes.

Temperature also altered the pools sizes of NO_3^--N differently for each region and horizon (temp x region x horizon: *P*=0.03), decreasing relative to pre-incubation pool sizes in the organic

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soils at all temperatures in all regions (coolest, 5 °C: P=0.001, 15 °C: P=0.0007, 25 °C: P=0.003; intermediate, 5 °C: P=0.04, 15 °C: P=0.002, 25 °C: P=0.008; warmest, 5 °C: P<0.0001, 15 °C: P<0.0001, 25 °C: P<0.0001). NO_3^--N pool sizes also decreased in the mineral soils at all temperatures in the coolest (5 °C: P=0.0005, 15 °C: P=0.0008, 25 °C: P=0.002) and intermediate (5 °C: P=0.02, 15 °C: P=0.002, 25 °C: P=0.0004) regions, although not in the warmest region (Fig. 2 C and D). These results imply that the anaerobic conditions we generated by replacing headspace air with He and keeping 80% water holding capacity generally supported denitrification and limited nitrification.

3.2 N_2O net production rates with short- and long-term warming

Net N_2O flux was influenced by regions (P=0.002), incubation temperature (P=0.006), and soil type (P<0.0001) without any significant effect of any interaction among or between these independent variables. When averaged across all incubation temperatures and the two soil horizons, the warmest region (3.8±0.8 ng N_2O-N g^{-1} h^{-1}) had a higher rate than the intermediate (1.9±0.6 ng N_2O-N g^{-1} h^{-1}, P=0.008) and coolest region (1.2±0.3 ng N_2O-N g^{-1} h^{-1}, P=0.003), whereas the intermediate latitude and coolest regions' net N_2O production did not differ from each other (Fig. 3). Averaged across all regions and the two soil types, the warmest incubation temperature (3.4±0.8 ng N_2O-N g^{-1} h^{-1}) exhibited a higher net N_2O flux than the lowest temperature (1.1±0.3 ng N_2O-N g^{-1} h^{-1}, P=0.003). Averaged across all regions and soil temperatures, the organic soil (4.9±0.8 ng N_2O-N g^{-1} h^{-1}) exhibited a higher rate than the mineral soil (0.6±0.2 ng N_2O-N g^{-1} h^{-1}, P<0.0001) and the combined incubation (1.3±0.3 ng N_2O-N g^{-1} h^{-1}, P<0.0001), which had a higher rate than the mineral soil alone (P=0.005).

We used N_2O emission from organic and mineral soil in isolation (Fig. 3 A & C) to compute expected net N_2O flux for the combined soils (Fig. 4 A & B). Observed rates of net N_2O production in the headspace surrounding combined organic and mineral soils (Fig. 3 B) were less than expected values (Fig. 4 A & B) and often exhibited net N_2O reduction, implying inter-profile interactions and differential temperature responses of the two horizons. The absolute effect of the combined horizons' reduction of N_2O differed by incubation temperature

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(*P*=0.002), with higher net reduction in the warmest incubation as compared to the coolest (25 vs. 5 °C: *P*=0.001) and a trend towards more reduction in the intermediate latitude region as compared to the coolest (*P*=0.098). In proportional terms, the effect of combining horizons decreased the combined net N_2O flux by up to 200% of the expected combined net production rate, and this effect differed by temperature (*P*=0.009). In particular, it was more pronounced at 15 °C relative to 5 °C (*P*=0.004). There was no significant interaction between region and temperature on this combined-horizon rate.

We used the change in ^{15}N in the N_2O (t_{60h}-t_{3h}) as a proxy for estimating how the relative contribution of production and reduction of N_2O varied among regions, across horizons, and with incubation temperature. The change in $^{15}N_2O$ across incubation time was consistently positive, suggesting that rates of N_2O production consistently outpaced rates of N_2O reduction during the incubation. These values differed by region (*P*=0.001), a feature driven by the warmest region exhibiting the largest change compared to the coolest region (*P*=0.0007), and a similar trend between the warmest and intermediate-latitude regions (*P*=0.081; Fig. 5). There was no significant effect of incubation temperature or soil type or any interaction between temperature, region and soil type on this change in N_2O-^{15}N.

3.3 Functional gene abundance

At the end of the 60 h incubation period, the abundance of one functional gene indicative of N_2O production, *nirS*, did not vary significantly by incubation temperature or region but differed strongly by soil horizon (*P*<0.0001). There was a higher abundance of this gene in the organic soil (0.73×10^6 $g^{-1} \pm 0.04 \times 10^6$) vs. the mineral soil ($0.18 \times 10^6$ $g^{-1} \pm 0.02 \times 10^6$) (Fig. 6). There was no significant effect of any interaction among or between the independent variables on *nirS* abundance. Functional gene abundance for N_2O reduction, *nosZ*, differed by region (*P*=0.0002), incubation temperature (*P*=0.04) and soil (*P*<0.0001). It was higher in soils from the warmest region (8.4×10^6 $g^{-1} \pm 1.9 \times 10^6$) relative to the intermediate latitude region (4.0×10^6 $g^{-1} \pm 0.8 \times 10^6$, *P*=0.0006) and the coolest region (4.9×10^6 $g^{-1} \pm 1.1 \times 10^6$, *P*=0.001), at the coolest (6.7×10^6 $g^{-1} \pm 1.6 \times 10^6$) relative to the warmest incubation temperature ($5.2 \times 10^6 \pm 1.7 \times 10^6$, *P*=0.02), and in organic ($10.55 \times 10^6 \pm 0.95 \times 10^6$) relative to mineral soils ($0.98 \times 10^6 \pm 0.08 \times$

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10^6). There was no significant effect of any interaction among or between the independent variables on *nosZ* abundance, although there was a near-significant trend for soil type to alter the regional effect (*P*=0.052). The resulting *nirS:nosZ* ratio ranged from 0.03 to 0.55 and displayed an interaction between region and soil horizon (*P*=0.04), driven by lower *nirS:nosZ* ratios in organic soil in the warmest relative to intermediate latitude region (*P*<0.0001) and warmest relative to coolest region (*P*=0.003); these effects were not exhibited in the mineral soil.

4. Discussion

By promoting the denitrification pathway we aimed to: 1) distinguish short- (via laboratory manipulations) and long-term (via a natural climate gradient) responses of denitrification-derived net N_2O flux to temperature; 2) assess the degree to which net N_2O fluxes in these soils are sensitive to interactions between soil horizons; and 3) leverage the abundance of genes responsible for denitrifier production and reduction of N_2O as a means of assessing differences in these processes' responses to short- and long-term temperature responses. Our first hypothesis was not supported: though short-term warming enhanced net N_2O effluxes from these soils, soils from a historically warmer environment exhibited greater net N_2O efflux than those from cooler environments, suggesting a positive response of net N_2O fluxes to both short- and long-term warming (Fig. 3). Indeed, an isotopic proxy for N_2O reduction derived from use of a stable isotope tracer suggests that enhancement of net N_2O production with long-term warming is greater than any enhancement in N_2O reduction (Fig. 5). Our second hypothesis was supported in that the combined incubation of mineral and organic soils exhibited net N_2O efflux rates that did not match the linear sum of separate incubation flux rates. However, we observed reduction of N_2O by mineral soil, not by organic soil as we predicted. Specifically, net N_2O production was tempered by more mineral soil N_2O reduction at warmer incubation temperatures (Fig. 4 & 5), indicating that soil horizon interactions may be critical to rates of net N_2O efflux to the aboveground atmosphere. Finally, our third hypothesis that linked gene abundance to process rates was only partially supported. *NosZ* decreased at the warmest incubation temperature (i.e. lower N_2O reduction gene abundance with warming, Fig. 6),

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consistent with rates. However, in the organic soils, *nosZ* was higher under higher historical temperature (i.e. higher N_2O reduction gene abundance with warming, Fig. 6), inconsistent with rates that increase with warming. There was no response to either short- or long-term warming in *nirS* abundance in either soil horizon, or to long-term warming in *nosZ* abundance in the mineral soil. Combined, these data suggest complex microbial responses to short- and long-term exposure to distinct temperature regimes, which we expand upon below.

4.1 Warming-induced enhancement of N_2O production exceeds that of N_2O reduction

Long-term climate gradients substitute space for time and encompass variation in multiple ecosystem phenomena driven by centuries of exposure to distinct climate regimes. For instance, we know that *in situ* soil N cycling is more rapid (Philben et al., 2016) and likely supports greater forest productivity in the relatively warm, southern-most boreal forests of this transect (Ziegler et al., 2017). The net N_2O efflux rate data from this set of lab incubations suggests that, especially in the organic soil horizons, both short-term warming and a long-term warmer climate enhance net N_2O production, a result consistent with the stable isotope tracer data (Fig. 5). These data correspond with the enhanced, short-term warming-induced N_2O fluxes observed in several systems (Billings and Tiemann, 2014; Kurganova and Lopes de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014). The apparent lack of long-term, denitrifier adaptation to rising temperatures (i.e. continued enhancement of N_2O production with long-term exposure to warmer temperatures that outstrips enhancement of N_2O reduction) is consistent with recent work in soils from these same sites demonstrating no change in the responses of microbial biomass-specific decay or CO_2 efflux rates to warmer temperatures over decadal timescales (Min et al., 2019). However, results from the current study contrast with our predictions of microbial adaptations to a warmer climate over the long term, which assume that a soil denitrifying community well-adapted to its temperature regime is adept at complete denitrification with relatively little N_2O byproduct. Such predictions arise from more conceptual studies presenting ideas about microbial metabolic responses to warming (Billings and Ballantyne, 2013; Bradford, 2013).

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405 The similar difference in net N_2O rates between the northern region and southern region (2.6
406 ng N_2O-N g^{-1} h^{-1}) and between the coolest and warmest incubation temperature (2.3 ng N_2O-N
407 g^{-1} h^{-1}, both 68% of the average range across treatments) indicates that net rates were
408 enhanced to a similar degree by both short-term warming of 20 °C and a long-term MAT
409 difference of 5 °C. Temperature sensitivity (i.e. change per °C) of net N_2O flux increased at
410 lower latitudes, and the isotopic tracer experiment indicated that N_2O production increases
411 outpaced N_2O reduction increases in warmer regions. Enhanced soil organic matter inputs and
412 nitrogen availability and cycling rates in the warmer climate forests (Philben et al., 2016; Ziegler
413 et al., 2017) may contribute to greater net N_2O production. The additive, positive result from
414 both historically warmer soils and warmer incubation temperatures suggests that community-
415 level denitrifier performance declines (i.e. more incomplete denitrification) in warmer
416 temperatures if they are from soils with historically warmer temperatures. This pattern
417 contradicts a "home-field" advantage (Wallenstein et al., 2013) for denitrifiers. More N_2O
418 production in warmer climates may arise from multiple changes that overcome adaptive home-
419 field advantages, such as shifts in the community composition (Delgado-Baquerizo et al., 2016)
420 and an increased number of inefficient N_2O producers, increases in the number of microbial
421 cells and transfer points involved in the denitrification pathway (i.e. nitrifier-denitrification in a
422 single organsism vs. coupled nitrification-denitrification in distinct organisms (Butterbach-Bahl
423 et al., 2013), or a changed contribution of alternate, possibly less-efficient electron donors (i.e.
424 co-denitrification (Spott et al., 2011)).

425 Despite increased net N_2O production to temperature, soil horizon interactions temper the
426 response to warming. Two of our methods either supported or did not contradict the potential
427 for mineral soil N_2O reduction: (1) calculated differences in flux values between shared
428 headspace N_2O flux values and the isolated headspace N_2O flux values of the two isolated
429 horizons, and (2) the change in isotopic enrichment of the shared and isolated headspace N_2O.
430 The first method demonstrated that short-term warming enhanced the degree of interprofile
431 interaction that increased N_2O reduction during the incubation, while long-term warming did
432 not significantly influence interprofile N_2O dynamics (Fig. 4 A & B). The similarities in net N_2O

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flux between the combined and mineral soil incubations (Fig. 3 B & C) indicate that the mineral soil served as a net N_2O reducer, especially in response to short-term temperature increases.

Our second method of detecting horizon interactions driving net N_2O efflux used $^{15}N_2O$ headspace differences from the start to the end of the incubation as an indicator of reduction. We expected an increase in the ^{15}N in the headspace N_2O as $^{15}NO_3^-$ is reduced, followed by a decline in ^{15}N in the headspace N_2O as the tracer flows into the N_2 pool, with balance of these processes indicating net production or reduction (Billings and Tiemann, 2014). NO_3^- pools declined and the change in our $^{15}N_2O$ was positive, suggesting that N_2O production still outweighed reduction at the end of the 60 h for both the individual horizons and the combination incubation (Fig. 5 A). Large variation in $^{15}N_2O$ changes among forest sites led to no significant difference between soil horizons and did not allow us to confirm our horizon interactions, although these results do not contradict the possibility of mineral soil reduction. Horizon interactions drove net profile N_2O fluxes in a field drought manipulation in a Norwegian spruce forest, during which soils exhibited a net N_2O sink via upper mineral soil reduction of deep mineral soil N_2O production (Goldberg and Gebauer, 2009). It remains unknown if the relatively shallow mineral soils we sampled are analogous reducers of deeper mineral soil N_2O produced in this system, or if they could continue to reduce large portions of organic soil N_2O efflux (Fig. 4) in situ.

Mineral soil reduction of organic soil-generated N_2O becomes most relevant when diffusion of N_2O from the upper soil profile to the atmosphere is restricted, and N_2O produced in those surface layers diffuses downwards according to Fick's Law as has been discussed in the literature for soil CO_2 dynamics (Oh et al., 2005; Richter et al., 2015). Such a situation is likely to occur in 'hot spots' (McClain et al., 2003) such as frozen surface soil patches during winter. Similarly, 'hot moments' may occur in the spring snow melt or in winter, despite cold temperatures reducing N cycling rates: subnivial N_2O production can be an important contribution to annual N budgets in pastures (reviewed in Uchida and Clough 2015), and winter N dynamics also appear to be important in northern temperate forest systems. For example, winter N_2O production equaled ~30% of the summer N_2O production in a SE Canadian forest

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(Enanga et al., 2016) and ~60% of the annual atmospheric N inputs in a NE U.S. forest (Morse et al., 2015). Mineral soil reduction of winter organic soil-generated N_2O may temper net fluxes and may be an important feature in forest N cycling.

4.2 Linking biogeochemical process rates to genetic potential

The functional gene that we could quantify in these soils that is associated with N_2O reduction was sensitive to both short-term and historical temperature, though it was not consistently associated with process rates. Although we did not detect the atypical *nosZ clade II* in these soils, other, yet unknown genes that we did not measure may be responsible for N_2O reduction. Beyond this possibility, our results suggest a decoupling of process rates and denitrifier genetic controls, or that the long-term temperature-related increase in genetic potential for N_2O reduction did not translate to rates as effectively as the short-term temperature-related decrease in genetic potential for N_2O reduction.

Consistent with enhanced net N_2O production in these soils at warmer incubation temperatures, the *nosZ* abundances were reduced after 60 h exposure to 25°C relative to cooler incubations. Although functional gene abundances are assumed to integrate longer-term changes in the microbial community and thus have a reduced dynamism relative to instantaneous rates (Petersen et al., 2012), our results appear to reflect a capacity of denitrifiers to respond rapidly to temperature, as indicated in other laboratory incubations that assayed temperature responses of denitrification functional gene abundances (Billings and Tiemann, 2014; Cui et al., 2016; Keil et al., 2015). However, inconsistent with enhanced net N_2O production in the soils from warmer historical temperatures, we found a reduced *nirS:nosZ Clade I* ratio in the southern forest soils. A possible explanation of this apparent decoupling between gene abundances and biogeochemical outcomes may be an interference between potential and transcription (i.e. better detected with mRNA), or inadequate measurement of all genes relevant to N_2O dynamics in these soils. Although our experimental set up promoted denitrification, our incubation may have also supported dissimilatory nitrate reduction to ammonium (DNRA,(Schmidt et al., 2011)). This pathway is poorly characterized, but has been detected in both aerobic and anaerobic environments of many soil types; it may account for a

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large proportion of NO_3^--N reduction in forest soils (Bengtsson and Bergwall 2000). DNRA represents a process that can reduce NO_3^- via a different nitrite reduction enzyme (*nrf*) than denitrification (*nir*) and can result in an accumulation of NH_4-N, as we observed during our incubation. The process also produces and reduces N_2O (Luckmann et al., 2014). The potential existence of this alternate pathway of NO_3^- reduction and N_2O production and reduction does not negate the observed N_2O efflux or *nosZ* response to short-term and historical temperature shifts; however, it does imply that a deeper understanding of the complex genetic N-cycle is required to link soil process rates to genetic potential.

Contrasting efficiencies of N_2O scavenging is another possible explanation for the decoupling between gene abundances and biogeochemical fluxes in these soils. The observation that mineral soil has the capacity to reduce a substantial amount of organic soil-derived N_2O even as *nosZ* abundances are reduced in mineral compared to organic soil provides a strong indication that *nosZ* in mineral soil is more efficient at scavenging N_2O from the headspace than *nosZ* in the organic horizon. Consistent with our combination samples in the current study, there is increasing evidence that soils can serve as sinks for atmospheric N_2O (Chapuis-Lardy et al. 2007), and interestingly, that this phenomenon can be particularly evident when soil water is limited (Goldberg and Gebauer, 2009). Therefore, given the varying gene abundance and enzyme efficiency with depth implied in this study, a likely fruitful area of research would be to explore mineral soil N_2O sink capacity and mineral soil genetic response as moisture availability varies, as happens particularly during snowmelt periods and in fall within these boreal soils.

5. Conclusions

The sensitivity of soil N_2O efflux to global change factors such as temperature can be high, as supported by this study, but the mechanisms driving N_2O sources and sinks remain challenging to elucidate. Indeed, variation of net soil denitrifier N_2O efflux within climate region in this study, though less than variation across regions, warrants further consideration of within-region controls on N_2O efflux. The meaningful across-climate region responses we observed, though, permitted us to address the three critical issues framed at the outset of this study; we

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conclude with three observations and questions for future research. To improve Earth system models of greenhouse gas emissions we need to address the importance of varying N_2O dynamics with soil depth. Indeed, this research highlights potentially different efficiencies of N_2O-relevant functional genes as we move across depth. Is it ubiquitous that *nosZ* is more efficient in sub-surface soils? We have taken the first step towards this characterization, but similar studies should address this question in diverse ecosystems. Our results also illustrate that both denitrifier-mediated rates of N_2O production and reduction can increase with warming, over both short- and long-term timescales, in boreal forest soils. *In situ* variables would undoubtedly alter the *ex situ* fluxes observed in this study, but we demonstrate that when conditions promote denitrification, the net response to warming in these boreal forest soils is dominated by N_2O production. Finally, we remain uncertain of the relative importance of the denitrification pathway in N_2O emissions in boreal forest soils (i.e. as compared to nitrification, co-denitrification, DNRA and others) and suggest similar approaches to explore the importance of historic climate regime and interactive responses among soil horizons in other biochemical pathways of soil N_2O emission.

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700 **Table 1.** Characteristics of the nine forests in the three study regions in NL-BELT.

701

Region	**Coolest**			**Mid**			**Warmest**		
Forest ID	Muddy Pond	Sheppard's Ridge	Harry's Pond	Hare Bay	Tuckamore	Catch-A-Feeder	O'Regans	Maple Ridge	Slug Hill
Latitude	53°33'N	53°33'N	53°35'N	51°15'N	51° 9'N	51° 5'N	47°53'N	48° 0'N	48° 0'N
Longitude	56°59'W	56°56'W	56°53'W	56° 8'W	56° 0'W	56°12'W	59°10'W	58°55'W	58°54'W
Watershed	Eagle River			Salmon River			Grand Codroy		
Closest weather station [∞]	Cartwright (53°42'N, 57°02'W)			Main Brook (51° 11'N, 56° 01'W)			Doyles (47° 51'N, 59° 15'W)		
Mean annual precipitation (mm)	1073.5			1223.9			1504.6		
MA PET (mm) [¶]	432.9			489.1			608.1		
Mean annual temperature (°C)	0.0			2.0			5.2		
Organic horizon depth (cm)	6.5	4.6	6.1	9.4	7.4	6.6	7.9	8.8	4.3
Bulk density (organic) (g cm^{-3})	0.09	0.07	0.10	0.09	0.09	0.12	0.09	0.14	0.10
Bulk density (mineral) (g cm^{-3})	0.80	0.72	0.76	0.59	0.59	1.20	0.68	0.68	0.66
Soil pH (organic)	5.3	5.3	5.4	4.4	4.4	5.7	4.3	3.7	4.6
Soil pH (mineral)	5.0	5.0	5.0	4.8	4.8	5.9	4.5	4.7	4.9

[∞] Climate normal data (1981 - 2000) (http://climate.weather.gc.ca/climate_normals/index_e.html)
[¶] MA PET, mean annual potential evapotranspiration

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703 **Figure legends**

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707 incubation ('Pre-inc.') and at the end of the incubations at 5, 15, and 25°C of soils from along a
708 boreal forest latitudinal transect. Pre-incubation values for nitrate are calculated as ambient
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710 temperature; the 'coolest' region is the Eagle River watershed (northern boreal), the
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714 Figure 3. Net N_2O flux ('production rate') averaged for 60 h of incubation at 5, 15, and 25°C
715 from organic soil alone (A), combined organic and mineral soil (B) and mineral soil alone (C)
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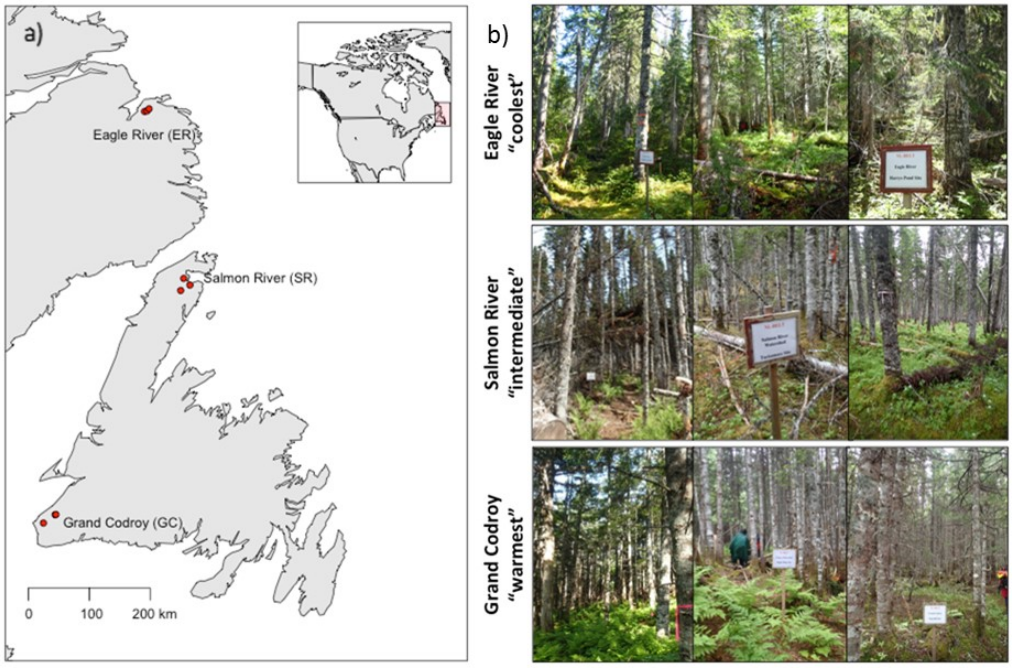

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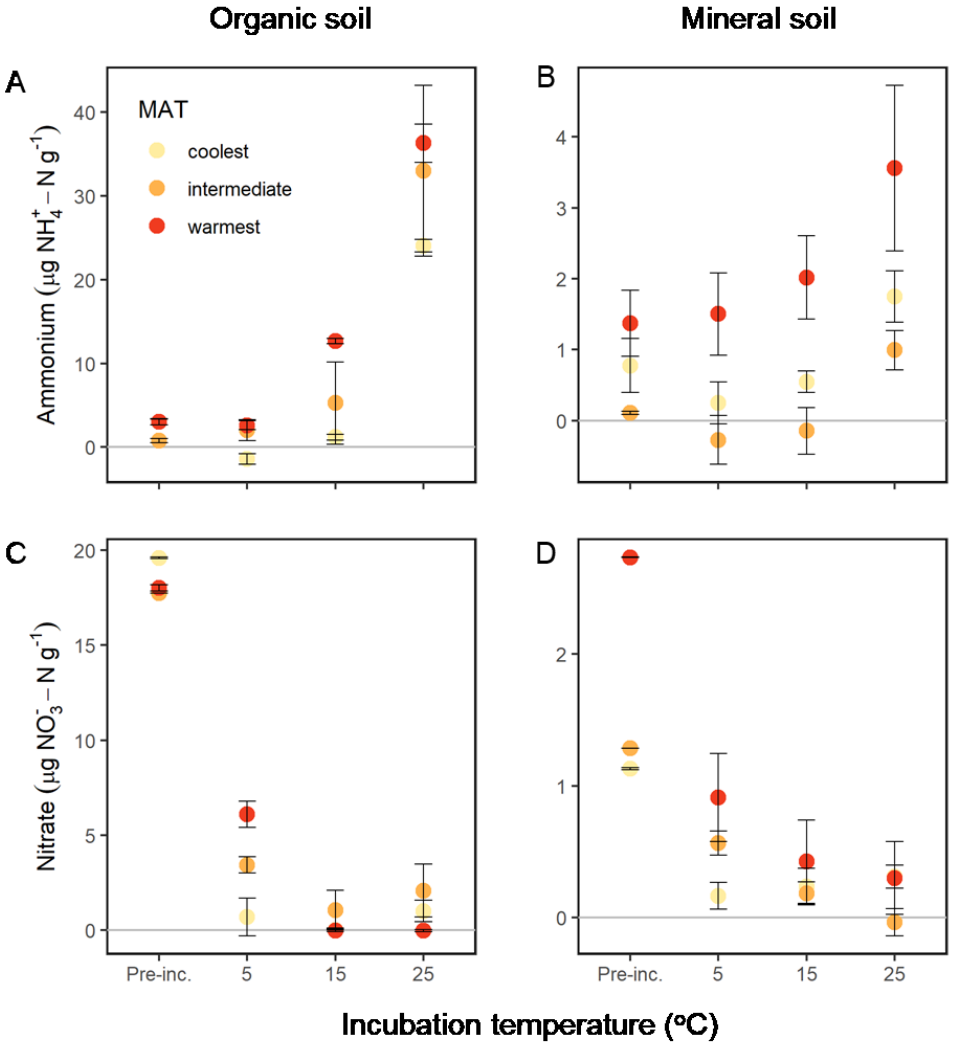

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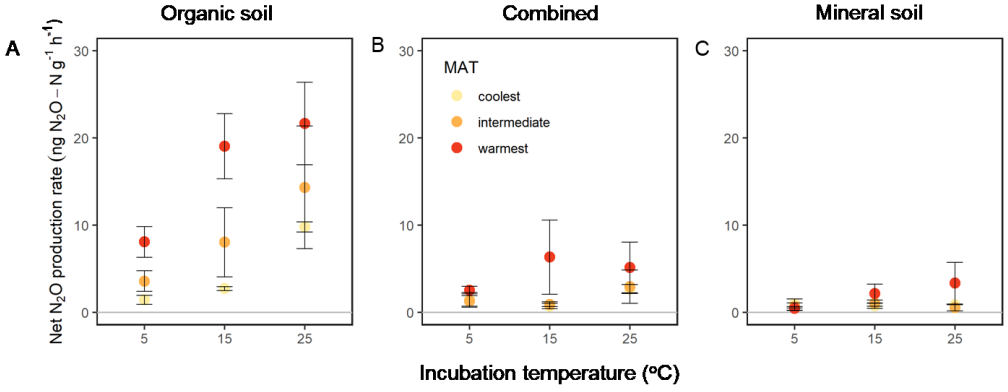

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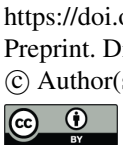

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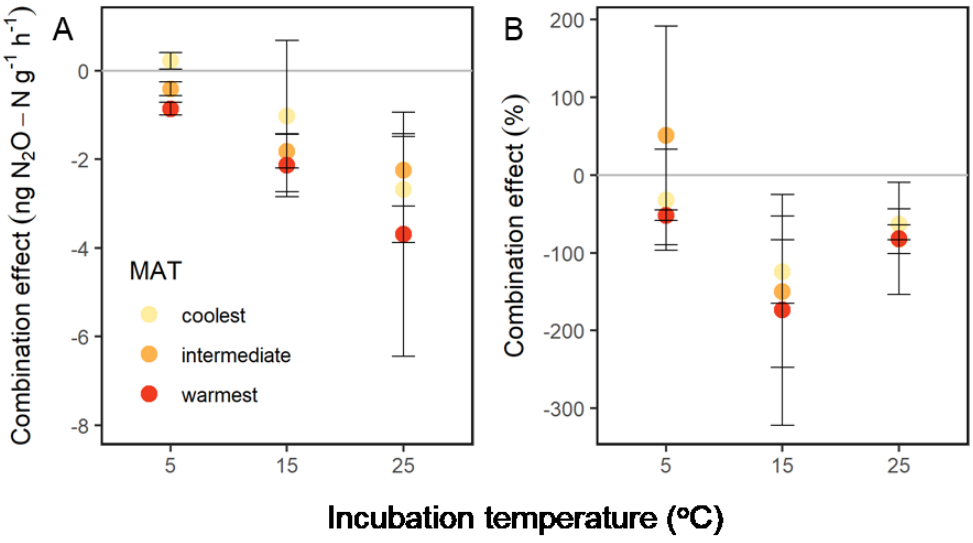

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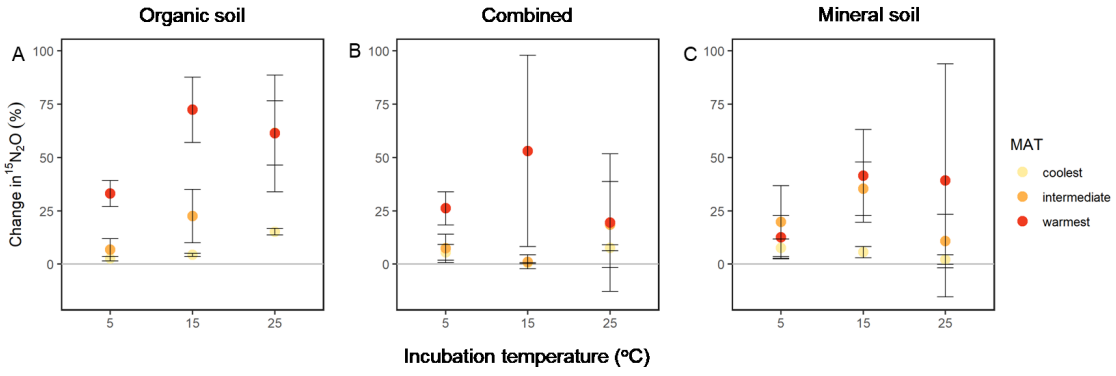

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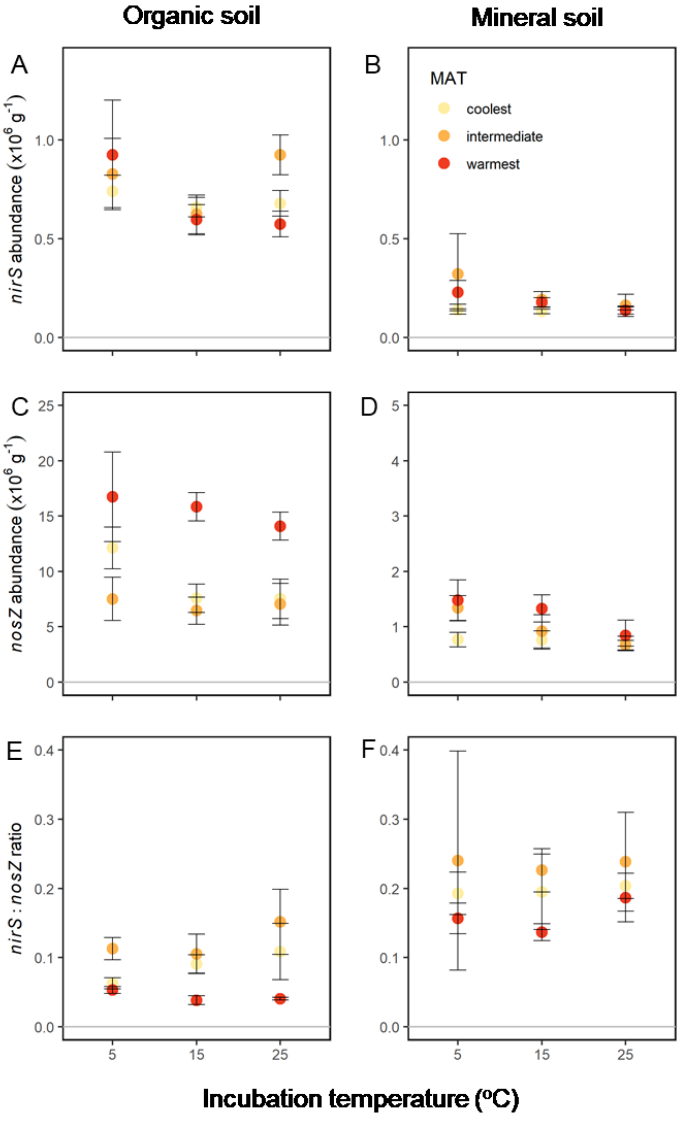

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