

SOIL-2019-58

Topical Editor Comments and Response

07/07/2020

Dear Prof Sleutel,

We thank you for your supportive review and positive decision. We have addressed your questions and comments below (all highlighted line numbers refer to the revised pdf file), and particularly appreciate your helpful insights about the role of mineral soil N₂O reduction. Together with the revisions in response to the three reviewers, this manuscript is much improved and we are very pleased with your support for publication in SOIL.

Sincerely,

Kate Buckeridge, on behalf of all co-authors

Topical Editor Decision: Reconsider after major revisions (01 Jul 2020) by Steven Sleutel

Comments to the Author:

The present research tried to tackle three relevant questions about global warming and denitrification at once. Not all was answered here in spite of the interesting experimental design but the authors did manage to bring up some relevant new questions. Particularly intriguing to see that mineral soil would be able to reduce N₂O emitted from the organic soil layer, in spite of lower nosZ abundances. The introduction sets the stage well and makes a case for the presented work. I agree with the authors in that the three hypotheses are sufficiently well under-built and no further elaboration is needed. In response to comments raised by referees some further methodological clarifications are now presented as well. The results were already efficiently presented.

The discussion equally reads well, but I have a few comments:

While the methodology is relatively well thought through but it is somewhat regretful that incubations were carried out in a completely O₂-free atmosphere, which really renders the results less representative. This needs to be brought up in the discussion near L434: N₂O reduction was probably strongly promoted in these experiments with nearly no O₂ present. In a field situation it may well be that N₂O-reduction in the quite porous mineral layer is a much lesser likely process and so would then the 'soil horizon interactions' be a lot smaller.

>> We have added the following sentence to the end of the paragraph (prev l. 434, now l. 461-464) to clarify this possible experimental artifact: “A caveat to this soil horizon interaction is that while our O₂-limited experimental environment was necessary to promote denitrification, this design may have exaggerated bulk soil reduction processes that occur naturally in anaerobic microsites.”

I partially do agree with referee 3 that generally observations on N₂O emission and reduction are not very much discussed mechanistically, viz. with respect to drivers of these processes. But it is equally understandable that the authors chose not to do so as these experiments were run in O₂ free air. One occasion where I hope the authors could still complement is L450: just why in your view would N₂O reduction be particularly favoured in the mineral topsoil vs. organic layer? Perhaps because with a smaller porosity and probably also finer pore size distribution vs. the organic layer hot-spots for complete denitrification have always been more common in the mineral soil. This would then have led to a microbial community more fit (efficient) to reduce N₂O? Or perhaps, N₂O reduction is mostly simply uncommon in the organic layer as it is too porous and more directly in contact with the atmosphere so that N₂O residence times are just too short to allow further reduction. I did find the hypothesis on L138-142 plausible but again, at present it is not discussed why the opposite result was found. It is for referees or readers not possible to reflect a bit on this with very little information provided on the sampled soil horizons. To the least also soil texture and SOC concentration should be added to table 1, but perhaps the authors also still see some room to further contemplate on just why mineral soil would harbour a microbial community more fit for N₂O reduction.

>> We have added the following sentence to the end of the paragraph (prev l. 450, now l. 479-484) to clarify that this finding was in contrast with our original hypothesis, and a possible mechanism: “Contrary to our original hypothesis, shallow mineral soils in situ may be better suited than organic soils to N₂O reduction, given that mineral soils experience frequent inputs of leached NO₃⁻ and DOC from the surface organic soils, and represent a sudden change in the soil structure and porosity towards well-packed fines and smaller pores. These conditions may promote leachate pooling, anaerobic microsites, and a microbial community that proves more effective at reduction.”

I recommend publication after final minor revision.

Some smaller comment:

L59 I suggest you omit “the emergent”

>> We have removed this phrase so that this sentence now reads: ‘...the temperature sensitivity of denitrification’ (l. 62)

L70-79 should be shortened a bit. The message is clear but lengthy

>> We have done our best to make this more concise, but carefully, as the clarity of this concept has been a sticking point for several reviewers (l. 73-81)

L131 replace “predicted” by “expect” or “hypothesize”

>> We have replaced 'predicted' with 'expected' (l. 133)

L779 at the end of a 60 h incubation +

>> We are unsure of the meaning of this comment. The incubation was indeed conducted over 60 h.

Figure captions: in my view no need to repeat the three names of the sites once more, "coolest" etc. suffices

>> If at all possible, we would like to keep the site names in the figure captions, given that they are part of a long-standing latitudinal transect supporting many studies; multiple readers will be familiar with the sites.

L794 C instead of (B); In tab A the orange dot at 25°C is invisible, is that correct?

>> The orange dot is behind the red dot – both sets of error bars are evident, and now that we have changed the shapes, the orange square is now visible behind the red triangle.

L400 I wonder if "our predictions" is really the best term here. You are mainly bringing up an expectation/hypothesis, no?

>> We have replaced 'predictions' with 'hypothesis' (l. 421)

L425 'increased net N₂O production to temperature' sounds awkward

>> We have replaced 'to warming' with 'with higher temperatures' (l. 451)

L426 'did not contradict' seems a bit overly careful, should the authors want to then this phrasing could be omitted.

>> We removed 'did not contradict' (l. 452)

L442-444 is not that readable; the phrasing "our horizon interactions" should be reworded. Probably this sentence can do without "although these results do not contradict the possibility of mineral soil reduction"

>> We have rewritten this sentence as follows: 'Large variation in ¹⁵N₂O abundance among forest sites led to no significant difference between soil horizons and did not allow us to confirm the direction of horizon interactions.' (l. 472-474)

SOIL-2019-58

Response to Reviewers

22/05/2020

Dear Reviewers,

We thank you for taking the time to review our manuscript and hope that we have addressed your questions clearly. Our answers follow your questions below and are preceded by '>>'. The line numbers refer to our post-review, revised version of the text.

Anonymous Referee #1

Received and published: 24 April 2020

Overall, this is a very interesting manuscript that is presented in a clear and easy to follow manner. The objectives and hypotheses are presented well and are followed with an exceptional design and conclusions. I thought the use of the combined incubations was a novel approach to provide a link to landscape-scale processes from the experimental setup. The site selections provide a very useful [...]. The manuscript presents very interesting results especially regarding the potential for N₂O reduction in mineral soils, but the impact of this finding is somewhat muddled in the presentation of the data.

In figure 4, the combination effect is presented and presented as a percent of the 'expected N₂O production rate', but the definition of this rate is not clear. The logic behind these calculations is not clearly explained in the manuscript itself.

Is the expected rate actually the rate at the end of the 60h incubation of the soils incubated in isolation?

>> We have added the following text to the methods (l. 251-259):

"To assess the potential for N₂O to be reduced to N₂ by denitrifiers in the other horizon when incubated together, we calculated the combination effect (ng N₂O-N g dw⁻¹ h⁻¹) as the difference between observed net N₂O fluxes when soil horizons shared the incubation headspace (observed) and the expected flux determined as the linear, additive effect of rate for horizons in separate headspaces (((organic + mineral)/2) = expected). The combination effect was also expressed as a percent of the expected flux:

Combination effect (%) = (observed - expected)/expected*100,

where a negative combination effect implies reduction caused by inclusion of one of the horizons."

We have also added a condensed version of this text to the legend of Figure 4, to clarify how we calculated this combination effect.

The expected rate is from the soils incubated in isolation, but it is calculated the same way as net rate, as the average of each time step.

So that the values expressed in Fig 4B are the absolute rates of Fig4A divided by the rates in Fig3 A &C?

>> The values in 4B are expressed as a percent of the expected, so they are calculated as $((\text{observed} - \text{expected})/\text{expected}) * 100$

It would be important for the reader to understand the logic behind figure 4 and clearly explain the calculations within the manuscript.

>> As noted above, we hope these calculations are now clear.

Additionally, the results from the incubations of mineral soil horizons demonstrate low rates of N₂O production, but without confirmation of ¹⁵N-N₂ measurements, how are the authors confident that these low production rates correlate to high N₂O reduction rates?

This can be addressed with the rate of ¹⁵N-NO₃ throughout the experiment, but it is not clear in the text as the manuscript is currently written, please elucidate on this in the discussion.

>> We have elaborated on our explanation of the two methods of deducing that mineral soils are the dominant reducers in the discussion, supported primarily from the lower net N₂O flux in our combination incubation (lower than organic soil alone), and we show that our ¹⁵N₂O method does not confirm or deny the evidence for mineral soil reduction of organic horizon efflux (l. 451-474). We have also added an additional explanation when the isotopic results are first presented (l. 348-354).

Finally, one very minor comment regarding the figures. To help distinguish between the incubated soils, please use symbols additional to the colors.

>> We have remade the figures to include treatment-specific shapes.

Anonymous Referee #2

Received and published: 30 April 2020

The study is important, since emissions from terrestrial systems dominate N₂O fluxes which may be further enhanced by warming climate leading also to warming of the soil. In this study, the key studied

function was denitrification, both producing and consuming N₂O. The N₂O flux potentials were measured from boreal ecosystem soils where mean annual temperature spans from 0 – 5.2 C. Furthermore, incubations by adding 15N-KNO₃ were done in temperature range from 5 to 25 C in order to see the effect of warming to fluxes. Both short-term warming and a long-term warmer climate enhance net N₂O production, and N₂O production was bigger than N₂O reduction during the incubation. There was reduction of N₂O by mineral soil, not by organic soil. Combining horizons of mineral soil and organic soil decreased the combined net N₂O flux by up to 200% of the expected, combined net production rate from separate horizons. There was decoupling between gene abundances and biogeochemical outcomes. Generalization of the results was done and may be enough from the potentials made on anaerobic conditions and with added NO₃⁻. Possibly name would already indicate, that this was a laboratory experiment. MS is well written and figures are clear. Methodology is mostly the same as used earlier by Billings and Tiemann, 2014, except that $\delta^{15}\text{N}$ is measured from N₂O but not from produced N₂ gas.

There is some points needing further clarification.

Results are expressed as ng N₂O-N g⁻¹ h⁻¹. It is unclear to me is this dry weight or fresh weight and same used in all soil weight (gen copies, added 15N etc.) based measurements?

>> All results are presented corrected by the soil oven dry weight. We have now clarified that in the text (l. 222-3, 248, 290).

Soil samples in half-pint jars (240 ml) were 40 g mineral and 50 g of organic soil, and in combined experiment 20 g for mineral and 25 g for organic soil. Their bulk density (supposedly dry BD? >> Yes) is $\rho_{0.1}$ for organic and $\rho_{0.7}$ g cm⁻³ for mineral soil. is there wet bulk density also available in order to compare thinks based on volume of the soil.

>> Bulk density at 80% WHC was not determined, as bulk density was calculated from field conditions, prior to the incubation.

This may explain why actual soil volumes in jars are different, it is not explained further. Or how close volumes are, when in both WHC is adjusted to 80% (which is of course a big difference in water content). Knowing actual volumes is especially important in combined setting, where volumes probably have an effect to ratios of produced and consumed N₂O.

>> The organic:mineral soil mass ratio were chosen to represent (approximately) the average ratio of organic:mineral soil mass ratio in the peds that were collected from all the forest sites. The dry mass

and fresh volumes of the two soil horizons were not similar; there was less dry mass and more fresh volume occupied by the less-dense organic soil. However, in spite of this attempt to mimic realistic O and mineral horizon ratios, organic and mineral soil were next to each other, as opposed to incubating the organic layer on top of the mineral soil. Thus, O horizons and mineral soils shared the same headspace, as described in the methods (l. 186-190). Regardless of our attempts to mimic (in part) some relationships between organic and mineral soils in situ, we recognize that our incubation is not representative of ecosystem-level in situ process rates, which is why all data are reported on a mass, rather than an areal, basis.

Also, in methods (r: 252) the 0.25 g added soil (ww, fresh weight, dw) for functional gene analyses is unclear, and was this wet weight of soil straight from incubation flasks, and thus about 80% WHC? And the result based on this added amount of fresh (WHC 80%) or dry weight of added soil. It may also be worth to mention this and possible volume differences in discussion regarding functional gene abundances.

>> Actual mass extracted was approximately 0.25 g fresh (80% WHC) weight, as per extraction kit protocol – we have updated the text to indicate this (l. 269). The precise amount extracted was recorded for each sample and converted to oven dry weight equivalent based on the dry:fresh weight ratio of an oven-dried subsample collected at the same time (post-incubation). All gene abundance data (and all data) are therefore presented as corrected by soil dry weight, as now indicated (l. 289-90).

I have difficulties to understand this tracing method (could be also my lack of knowledge), so maybe you explain it a bit more carefully (please see next answer). Why not adding $^{15}\text{N}-\text{N}_2\text{O}$ in the beginning and measuring $^{15}\text{N}_2$ would not work?

>> Adding $^{15}\text{N}-\text{N}_2\text{O}$ and tracing it to $^{15}\text{N}_2$ would theoretically allow us to detect N_2O consumption, but it would require quantification of the dilution rate of $^{15}\text{N}-\text{N}_2\text{O}$ with $^{14}\text{N}-\text{N}_2\text{O}$ to quantify N_2O production. In theory, this could work, but the applied approach of watching ^{15}N -labeled NO_3^- be transformed into N_2O is also robust, and is the protocol used in our lab, successfully to date (Billings and Tiemann 2014).

When you add $^{15}\text{N}-\text{NO}_3^-$, you assume that it will first produce enough big amount of $^{15}\text{N}-\text{N}_2\text{O}$ in three initial hours. From this concentration increases in $^{15}\text{N}_2\text{O}$ (production > consumption) or decreases (production < consumption) are visible in the incubation time 60h. In this method you also assume, that ^{14}N N_2O released from soil own N stores is not diluting $^{15}\text{N} + ^{14}\text{N} -\text{N}_2\text{O}$.

In equation at row 238, it would be easier to reader to show the real times (initial 3 h and final 60 h).

We do not assume that all $^{15}\text{NO}_3$ will be consumed in the first three hours, rather that the rate of $^{15}\text{N}_2\text{O}$ production will decline as substrate declines, whereas consumption will increase relative to production as we approach 60 h, a reasonable assumption given that NO_3 pools decline over the course of the incubation and the substrate for consumption, N_2O , increases over this same time frame (l. 236-250). We do assume that $^{14}\text{N}_2\text{O}$ will not be an important agent of diluting the isotopically labeled N_2O pool, because the tracer was highly concentrated (l. 234-236).

Also jump from added ^{15}N 3000 ‰ first to ng ^{15}N - N_2O g⁻¹ soil (dw,ww, fresh?) maybe needs to be explained more clearer. It would be nice to see (or have a reference) how you get from headspace N_2O ppm:s and ^{15}N values to ng g⁻¹ ^{15}N - N_2O in headspace, since there is also lot of ^{14}N - N_2O added. In any case recovery of ^{15}N as N_2O is big, almost 75% at the warmest experiment at the time point 60 h.

>> We have hopefully clarified these calculations by including calculation steps from ppm to rates on l. 220-222 and rates to $^{15}\text{N}_2\text{O}$ on l. 241-244.

Would be nice to see also course of N_2O concentration increase with time – and d ^{15}N - N_2O for the time points used. Maybe as supplement.

>> We have added net N_2O concentration over time as Supplementary Figure 1. $^{15}\text{N}_2\text{O}$ was only analysed at 3h and 60h.

Some typos (or not) and just asking:

r, 226 : there is range of ppm, but what was the range of d ^{15}N - N_2O standards, 0.1 ‰ precision looks for me extremely good in highly enriched N_2O .

>> IRMS precision is typically presented as the standard deviation across five natural abundance standards, and 0.1 ‰ precision (standard deviation) is the value that UC Davis SIF provided to us. We have clarified this in the text (l. 230-1). We agree that it is normal and likely that enriched samples will have a lower precision (higher standard deviation), although we did not submit five replicate enriched samples for comparative analysis.

r. 347 and elsewhere. “ g⁻¹ _ ” between standard deviation (or error?) or after that.

>> We agree that g⁻¹ should be after the standard error. We have changed this for all the gene abundances in this section (l. 360-377).

r. 246, r. 252: is 0.25 g also based on fresh weights?

>> Yes, we have clarified this in the text (l. 269).

reference list: many typos in subscripts N₂O, spaces N 2 O and letters, like “Dur??n”

>> Thank you for checking these, we believe we have corrected all the typos in the references.

Good luck with MS!

>> Thank you!

Interactive comment on SOIL Discuss., <https://doi.org/10.5194/soil-2019-58>, 2019.

Anonymous Referee #3

Received and published: 7 May 2020

In this manuscript the authors present a laboratory-based study to address three uncertainties in climate projections of net N₂O fluxes from soil: (1) short v long-term responses to warming, (2) interactions among soil horizons, and (3) temperatures responses of different steps in the denitrification pathway. While the study itself is sound (although see my comment below about clarification of how net N₂O fluxes were estimated), the authors treat denitrifiers superficially and interpret their results without deep consideration of the mechanisms driving the observed patterns that is, they never mention any of the known controls on nitrate reduction and nitrous oxide reduction by denitrifiers and how warming or soil property differences among soil horizons would affect those controls. Below I detail some of my concerns and hope that my suggestions will help the authors improve the manuscript such that their findings can clearly advance our understanding of how warming affects soil denitrification.

The physiological rationale for enhanced rates of complete denitrification under longterm temperature regimes should be explained in order to justify this hypothesis. The following language is currently used to justify and describe this hypothesis: “less effective processing” by denitrifiers (Line 75) leading to more incomplete denitrification; denitrifying communities as “efficient transformers of NO₃⁻ to N₂” (Line 137); “a soil denitrifying community well-adapted to its temperature regime is adept at complete

denitrification”; and “denitrifier performance” (Line 415). But what is meant by “effective, “efficient,” “adept,” and “performance” as it relates to microbial physiology?

>> We agree that these terms were not well-explained. We have changed the wording at these locations to use a controlled vocabulary. Instead of using this large number of terms, we use the term “effective” and define what we mean by it on lines 141-142. There, we state our interpretation of more ‘effective’ denitrification as more complete denitrification (i.e. higher N₂:N₂O ratio) (l. 76-77). This concept is valuable when considering long- and short-term responses of denitrifiers to environmental conditions.

Denitrifiers are mostly facultative anaerobes that can utilize various metabolisms other than nitrate reduction or nitrous oxide reduction depending on environmental conditions. There was no mention of controls on the actual processes of nitrate reduction and nitrous oxide reduction (e.g., nitrate availability, soil redox) anywhere in the manuscript, which severely undercuts the hypothesis and the interpretation of the results.

>> By working with organic matter-rich soils, we attempted to maximize natural C substrate availability and make that point on line 200. While we agree that the absence of O₂ (and associated redox potential of a system) is a dominant driver of denitrification, one of the goals of the study was to investigate N₂O production in upland, fairly well-aerated soils (l. 162-163).

We agree that the availability of C, NO₃ and O₂ dominate the effect of warming on the production of net N₂O and have clarified this important point in the text by modifying some of our description of the study’s aims (l. 58-61). Specifically, our aim is to explore issues related to temperature sensitivity beyond these proximate controls and their indirect effects. We expect that these proximate controls, or changes to them, will have indirect effects that are complexly packaged in our long-term warming treatment, and make this point in section 4.1 of the discussion (l. 406-410), and acknowledged the importance of these controls in the conclusion (l. 566-567).

Lines 138-146: These three hypotheses are actually predictions (i.e., expected results). In the introduction, there is little justification presented for why these results would be expected other than similar patterns have been observed for heterotrophic respiration. As the authors acknowledge, denitrification is a more complicated process because it includes multiple enzymatic steps. But physiologically, the controls on denitrification are also different from heterotrophic respiration, and that needs to be considered.

>> We respectfully disagree that there is little justification for our hypotheses. In the introduction, we have provided one paragraph explaining the rationale behind each of the 3 hypotheses, then summarized these justifications in the final prediction paragraph of the introduction. Each of these hypotheses addresses a knowledge gap, with varying levels of prior evidence. We have provided the

knowledge gap, the evidence, and our evidence-based hypotheses, in addition to one prediction (short-term warming response), which is based on past results.

>> We agree with the reviewer that physiologically, the controls on denitrification and respiration are probably different because they are carried out by different microbes, or the same microbes in different environmental conditions. Nonetheless, we believe we are justified to use an example of a community-level, physiological (i.e., microbial respiration) response to warming and to hypothesize that it may also apply to denitrification. Because we have very little a priori knowledge about long-term denitrifier responses to warming, and because aerobic respiration and denitrification both represent microbial respiratory pathways, using results from studies of heterotrophic respiratory responses to warming to predict one result in this study seems a valid starting point for developing an analogous knowledge base about denitrifiers. Indeed, it tests the idea that warming can prompt respiratory pathways across microbial taxa to respond in similar ways, which represents a way forward for more generalized hypotheses about ecosystem responses to warming.

The calculation of net N₂O fluxes is core to the validity of the results of this study, so this vague statement on lines 202-203 needs to be clarified.

Please explain what is meant by “the robustness of the final 60 h time point measure,” how the multiple time points were used to verify this, and what is meant by “the net results of these samples.”

>> We apologise that this was unclear. We have now removed that sentence since our method of averaging is already described in greater detail elsewhere (l. 222-226).

In addition, I recommend that the authors add a supplementary figure that shows the net N₂O fluxes calculated for each of the time points so that the readers can see what patterns got washed out by averaging the fluxes observed over the 60 hour incubation (Lines 219-222).

>> We have added this figure as Supplementary Figure 1.

Line 227-242: Based on the equation presented in line 238, it is not the change in ¹⁵N enrichment of the N₂O that is used to estimate N₂O reduction rates as stated on line 227 but rather ¹⁵N₂O abundance.

Also, on lines 231, 234, 440, and elsewhere in the manuscript, only “¹⁵N₂O” is referred to but it would be clearer to the reader if “¹⁵N₂O abundance” was specified.

>> Thank you, we have changed this as suggested.

Lines 255-258: Please clarify what is meant by nirS and nosZ clade I as being “the most tractable indicators of N2O production and reduction.” What does “tractable” mean, and how was this assessed?

>> We describe this as ‘based on successful amplification of these genes across all samples’ (l. 277).

Please also specify which other functional gene primers were tested (including citations for the primers used) so that readers can interpret why these genes may have failed to amplify.

>> We have updated this information in the text (l. 273-275) and added the table below to supplementary data.

Gene	Primer	Sequence (5' to 3')	Reference
Nitrite reductase (NO ₂ ⁻ to NO)	nirK-876F	ATY GGC GGV AYG GCG A	Henry et al. 2004
<i>nirK</i>	nirK-1040R	GCC TCG ATC AGR TTR TGG TT	
Nitrite reductase (NO ₂ ⁻ to NO)	nirS-cd3aF	GTS AAC GTS AAG GAR ACS GG	Throbäck et al., 2004
<i>nirS</i>	nirS-R3cd	GAS TTC GGR TGS GTC TTG	
Nitric oxide reductase (NO to N ₂ O)	cnorB2F	GAC AAG NNN TAC TGG TGG T	Braker and Tiedje, 2003
<i>norB</i>	cnorB7R	TGN CCR TGN GCN GCN GT	
Nitric oxide reductase (NO to N ₂ O)	nosZ-F	CGY TGT TCM TCG ACA GCC AG	Röche et al., 2002
<i>nosZ</i>	nosZ-R	CAT GTG CAG NGC RTG GCA GAA	
Nitrous oxide reductase (N ₂ O to N ₂)	nosZ-II-F	CTI GGI CCI YTK CAY AC	Jones et al., 2013
<i>nosZ II</i>	nosZ-II-R	GCI GAR CAR AAI TCB GTR C	

Primers vary in their coverage of the diversity of microbes harboring a given functional gene, so the selected primers may not have been able to detect the relevant organisms present (see Ma et al. 2019, Environmental Microbiology).

For example, only recently was a suite of primers developed that provides better coverage for the many subclades of nosZ clade II (see Chee-Sanford et al. 2020, Journal of Microbiological Methods).

>> We agree that this may have contributed to our lack of success with these primers. We do not delve into this discussion because we did not spend extensive lab time optimizing PCR conditions for all

primers, and because it was always our intent to choose representative (as opposed to exhaustive) denitrifier production and consumption genes.

Lines 411-413: This discussion of differences among the sites representing different long-term climate regimes needs to be expanded on. How would differences in soil organic matter input and nitrogen availability among the sites confound the interpretation of warming effects on N₂O dynamics? The authors should consider how these potential confounding factors influence nitrate reduction and nitrous oxide reduction rates based on our understanding of controls on denitrification.

>> We have clarified in the text that we think these historical differences may be directly important controls for N₂O production in situ, but less so in our incubation where we added a small pulse of NO₃. Therefore, it is more likely that differences in regional microbial responses to short-term warming reflect community-level microbial acclimation to their historical conditions (l. 434-439).

Lines 498-509: I would delete this paragraph about “contrasting efficiencies of N₂O scavenging” which is speculative and not founded in an understanding of microbial physiology. What is meant by “efficiency”? The rationale presented does not consider why microbes would reduce N₂O nor differing conditions in mineral versus organic soils that would cause differences in N₂O reduction rates in these two horizons.

>> We respectfully disagree that contrasting enzyme efficiencies are not based in an understanding of microbial physiology, although we do agree that we know very little about how environmental conditions impact the enzyme efficiencies for the multiple steps in the denitrification pathway. We have added a sentence to illustrate that community structure and resource availability (conditions which differ between soil horizons) have been demonstrated to alter enzyme soil microbial efficiency (l. 533-536). We have also elaborated on the discussion introduced in the previous paragraph about the need to assay more enzyme genes, and suggest that further research into nosZ clade II may help explain the apparent decoupling between rates and gene abundances between organic and mineral soil found in this study (l. 540-543).

1 **Short- and long-term temperature responses of soil denitrifier net N₂O efflux rates, inter-**
2 **profile N₂O dynamics, and microbial genetic potentials**

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14

15 **Abstract**

16 Production and reduction of nitrous oxide (N₂O) by soil denitrifiers influences atmospheric
17 concentrations of this potent greenhouse gas. Accurate projections of net N₂O flux have three
18 key uncertainties: 1) short- vs. long-term responses to warming; 2) interactions among soil
19 horizons; and 3) temperature responses of different steps in the denitrification pathway. We
20 addressed these uncertainties by sampling soil from a boreal forest climate transect
21 encompassing a 5.2 °C difference in mean annual temperature, and incubating the soil horizons
22 in isolation and together at three ecologically relevant temperatures in conditions that promote
23 denitrification. Both short-term exposure to warmer temperatures and long-term exposure to a
24 warmer climate increased N₂O emissions from organic and mineral soils; an isotopic tracer
25 suggested an increase in N₂O production was more important than a decline in N₂O reduction.
26 Short-term warming promoted reduction of organic horizon-derived N₂O by mineral soil when
27 these horizons were incubated together. The abundance of *nirS* (a precursor gene for N₂O
28 production) was not sensitive to temperature, while that of *nosZ clade I* (a gene for N₂O
29 reduction) decreased with short-term warming in both horizons and was higher from a warmer
30 climate. These results suggest a decoupling of gene abundance and process rates in these soils
31 that differs across horizons and timescales. In spite of these variations, our results suggest a
32 consistent, positive response of denitrifier-mediated, net N₂O efflux rates to temperature
33 across timescales in these boreal forests. Our work also highlights the importance of
34 understanding cross-horizon N₂O fluxes for developing a predictive understanding of net N₂O
35 efflux from soils.

36 Keywords: nitrous oxide, *nosZ*, *nirS*, boreal forest, ¹⁵N, climate change

37 Manuscript highlights:

- 38 • short- and long-term exposure to warmer temperatures increased soil net N₂O flux
- 39 • short-term warming promoted reduction of organic horizon derived N₂O by mineral soil
- 40 • gene abundance - process rate coupling in these soils differed across horizons and
41 timescales

42

43 1. Introduction

44 Nitrous oxide (N₂O) is a potent greenhouse gas, with ~300 times the global warming potential
45 of carbon dioxide on a 100-y timescale and uncertain climate feedback effects (Ciais et al.,
46 2013; Portmann et al., 2012). Though increases in atmospheric N₂O are attributed to N-fertilizer
47 use (Mosier et al., 1998), emissions from natural systems dominate terrestrial fluxes (Ciais et
48 al., 2013) and experimental manipulations indicate warming may enhance these fluxes (Benoit
49 et al., 2015; Billings and Tiemann, 2014; Kurganova and Lopes de Gerenyu, 2010; Szukics et al.,
50 2010; Wang et al., 2014). One of the most important biogeochemical pathways of N₂O
51 formation in natural systems is denitrification, the stepwise reduction of NO₃⁻ to N₂. In this
52 pathway, soil denitrifiers can both produce and reduce N₂O, and incomplete reduction of N₂O
53 during the final step to N₂ can result in N₂O release to the atmosphere (Baggs, 2011; Firestone
54 and Davidson, 1989). Soil microorganisms play a critical role in climate change (Cavicchioli et al.,
55 2019) yet it remains unclear how sensitive the denitrification pathway is to a warming climate.

56 Translating empirically-derived knowledge about soil denitrifiers into climate projections is
57 difficult due to the dynamic and variable nature of the many interacting steps and their controls
58 (Butterbach-Bahl et al., 2013). The indirect influences of temperature on strong, proximate
59 controls of denitrification (i.e., availability of C, NO₃⁻, or soil O₂) are likely important features
60 governing soil denitrifier response to climate change (Butterbach-Bahl and Dannenmann, 2011;
61 Wallenstein et al., 2006). Here, we instead address three key challenges that are associated
62 with the temperature sensitivity of denitrification. First, we do not know if short-term
63 responses of denitrifying communities to warming (Billings and Tiemann, 2014; Kurganova and
64 Lopes de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014) are maintained across longer
65 timescales. Therefore, we are uncertain if laboratory studies can provide the empirical data
66 needed to project longer-term fluxes. Studies of heterotrophic soil CO₂ efflux suggest that
67 enhanced rates of microbial respiration with warming may be dampened over the long-term,
68 prompted by a combination of microbial acclimation and adaptation (Billings and Ballantyne,
69 2013; Bradford, 2013), and it is feasible that denitrifying communities may also exhibit only
70 ephemeral responses to warming. Such a response is consistent with inconclusive results of
71 multiple *in situ* warming experiments, though such studies necessarily reflect both

72 denitrification and other N₂O-producing processes in soils (Bai et al., 2013; Butler et al., 2012;
73 Dijkstra et al., 2012; McDaniel et al., 2013). Assuming microbial acclimation, denitrifying
74 communities may be more effective at NO₃⁻ reduction and transformation to N₂ in their
75 acclimated climate's typical temperature range. In principle, this could result in relatively lower
76 rates of N₂O loss in that particular temperature regime (i.e. more complete denitrification)
77 compared to less effective processing by those microbial communities if the mean temperature
78 were to shift. Though this phenomenon has not been demonstrated for the more complicated
79 soil denitrification with its multiple enzymatic steps, the so-called "home field advantage" has
80 been demonstrated in studies exploring rates of other soil microbial processes (Alster et al.,
81 2013; Wallenstein et al., 2013).

82 A second knowledge gap limiting our ability to project future soil N₂O climate feedbacks is
83 potential variation with temperature in interactions between microbial production and
84 reduction of N₂O across soil horizons. Implicit in the concept that such cross-horizon
85 interactions may control net profile N₂O efflux is the assumption that soil denitrifiers have
86 different patterns of production and reduction in different horizons. This may arise because the
87 conditions that control N₂O production or reduction differ between horizons, or it may arise
88 because the metabolic potentials of the soil microbial community in different horizons are
89 intrinsically different (Blume et al., 2002; Fierer et al., 2003). Consistent with this idea, Goldberg
90 and Gebauer (2009) illustrated clear variation in patterns of δ¹⁵N of N₂O across soil depth in
91 response to drought, which could have been caused by variations in either N₂O production or
92 reduction (Billings, 2008). The exchange of substrates between soil horizons thus can be an
93 important process dictating whole-soil N₂O efflux, and may contribute to apparent
94 inconsistencies between warming effects in the laboratory and the field (reviewed in Bai et al.
95 2013). Indeed, profile interactions have been recently demonstrated as important drivers of
96 soil CO₂ efflux: temperature responses of whole soil core respiration can be distinct from the
97 sum of those observed for horizons incubated in isolation from each other, likely due to
98 exchange of substrates and microbes among horizons (Podrebarac et al., 2016). Though
99 evidence suggests that N₂O produced in one soil horizon may be reduced in another (Goldberg
100 and Gebauer 2009), the degree to which this may occur, and why, has not been determined.

101 A third feature challenging our ability to project soil N₂O effluxes in a warmer climate regime is
102 the potentially different response to warming of distinct steps in the denitrification pathway
103 (this may be for one or multiple microbes within the community, that carryout the enzymatic
104 steps). For instance, if the activity of *nosZ*, a gene that codes for an enzyme catalyzing N₂O
105 reduction, experiences a different response to temperature than *nirK*, a gene coding for an
106 enzyme catalyzing NO₂⁻ reduction (and thus N₂O production), the net flux of N₂O may either
107 increase or decrease with temperature depending on the direction and magnitude of both
108 responses. Though gene abundances sometimes exhibit decoupling from function (Peterson et
109 al. 2012), quantifying any changes in these functional gene abundances with temperature can
110 help discern the propensity for temperature responses of relevant microbial communities'
111 structure, and thus the driving mechanisms for net N₂O production responses. Differential
112 responses of these genes' abundances to short-term temperature manipulation have been
113 observed in grassland soils (an increase in *nosZ* with short-term temperature increases; Billings
114 and Tiemann, 2014), but it is unknown whether these observations are relevant for soil
115 microbial communities subjected to long-term exposure to distinct temperature regimes.

116 In this study, we explore these three issues: short- vs. long-term responses of soil denitrifying
117 communities' net production of N₂O to warming, the exchange of denitrification-derived N₂O
118 among horizons as a driver of temperature response of net N₂O efflux, and the potentially
119 different responses of the relative abundances of microbial genes linked to N₂O production vs.
120 reduction to temperature. We invoked a space for time substitution to test our long-term
121 warming hypothesis, using a climate transect along which mean annual temperature (MAT)
122 varies but dominant vegetation, soil type, and soil moisture are similar. To elucidate both short-
123 and long-term temperature responses of soils' denitrifying communities, we incubated soils
124 that came from different latitudes and climate regimes along this transect (long-term warming)
125 for 60 h at 5, 15 and 25 °C (short-term warming), to reflect typical current (5 and 15 °C) and
126 projected future (25 °C) soil temperatures. Specifically, laboratory incubations of mesic organic
127 and mineral boreal forest soil horizons were established in conditions that promote
128 denitrification. To understand the potential for interactions among soil horizons as a driver of
129 temperature response of net N₂O efflux, we incubated organic and mineral soils both

130 individually and in combination. We measured net rates of N₂O efflux and abundances of
131 representative functional genes linked to production and reduction of N₂O, and estimated N₂O
132 reduction using an isotopic tracer.

133 We **expected** that short-term warming would enhance net N₂O production in these boreal soils,
134 as in the majority of past incubation studies (Billings and Tiemann, 2014; Kurganova and Lopes
135 de Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014). As outlined above, we also tested
136 the hypothesis that a warmer temperature regime over a longer timescale would show the
137 opposite effect: a dampened net N₂O efflux from the historically warmer soils, where organic N
138 turnover is faster (Philben et al., 2016), and where denitrifying communities presumably can
139 function **effectively** as transformers of NO₃⁻ to N₂ at warmer temperatures compared to their
140 more northern counterparts. **Here, we define "effective" as a denitrifier community being able**
141 **to transform NO₃⁻ to the end product, N₂.** We also hypothesized that N₂O produced in one
142 horizon would be reduced in the other when incubated together, resulting in lower net N₂O
143 efflux than a simple linear combination of these horizons' individual efflux rates. Specifically, we
144 anticipated that organic soils, relatively rich in microbial abundance and diversity compared to
145 mineral soils, would reduce mineral-produced N₂O, following dominant diffusion gradients.
146 Finally, we hypothesized that soils exhibiting higher rates of net N₂O production would exhibit
147 some combination of increased *nir* abundance and decreased *nos* abundance and associated
148 higher ratios of *nir:nos* gene abundances, reflecting shifts in microbial genetic potentials with
149 temperature regime.

150 **2. Materials and method**

151 *2.1 Study site and soil sampling*

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

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

170 Two large (30 cm²) peds of organic (LFH or O horizon) and mineral (B horizon) soil were
171 collected at each forest stand on a different calendar date but an equivalent ecological date:
172 22-24 October 2013 in Eagle River, 4-5 November 2013 in Salmon River, and 22-23 November
173 2013 in the Grand Codroy. This pre-freeze, post-growing season period typically exhibits
174 relatively large and active microbial biomass in northern latitude organic soils (Buckeridge et al.,
175 2013). The A_h and A_e horizons were not present at all sites so were not included in the
176 incubation at any site. Each collection was shipped to the University of Kansas (4-5 days transit
177 in insulated coolers, on ice) and processed immediately. Because regions were processed as
178 separate experimental blocks we cannot separate the region and block effects. However, we
179 confounded these factors knowingly, because we believed ecological date and rapid processing
180 were more important than minimal differences in laboratory practice between blocks.

181 *2.2 Incubation and headspace gas collection*

182 Aboveground vegetation (i.e. moss, herbaceous plants, tree seedlings) was removed from the
183 peds with scissors. The two peds of organic and mineral soil from each forest site were pooled
184 within horizon and mixed by hand, producing an organic and mineral sample for each forest.
185 This process was repeated nine times, for three forests in each of three regions. Subsamples

186 (fresh mass, organic: 50 g; mineral: 40 g) were placed in half-pint (237 ml) Mason jars. To test
187 the potential for N₂O producers and reducers from one horizon to interact with their
188 counterparts in the other horizon, 'combined' samples were also prepared in which an open
189 container of mineral soil (20 g) was placed within a jar, next to organic soil (25 g) such that they
190 had a shared headspace but were not physically mixed. Each sample was replicated for three
191 temperature incubation scenarios (5, 15 and 25 °C), and three blank jars (no soil) were included
192 for each temperature. To maximize the potential for denitrification we promoted anaerobic
193 conditions and substrate diffusion to by evacuating headspace air and replacing with He, and
194 adjusting water-holding capacity to 80% with a K¹⁵NO₃⁻-N solution (δ¹⁵N 3000 ‰) that added 18
195 and 1.3 μg N g⁻¹ dw soil to the organic and mineral soil samples, respectively (18x background
196 levels at the time of sampling, although within the annual range of soil NO₃⁻ availability based
197 on unpublished field data). Our approach was distinct from a potential denitrification assay,
198 which calls for non-limiting C and NO₃⁻ additions to soils (Pell et al., 1996); instead, we intended
199 to promote conditions conducive to denitrification using natural C pools and as close to natural
200 NO₃⁻ concentrations as was feasible. Therefore, this experiment is not predictive of bulk soil
201 N₂O rates and instead explores controls on N₂O rates in soil zones with low O₂ concentrations.
202 Such 'hot spots' for biogeochemical cycles in soils are well-documented (McClain and others
203 2003).

204 Over 60 h of incubation, we collected headspace gas eight times for determination of N₂O
205 concentration. The first sample was collected immediately after initiating the incubations, the
206 second sample was collected at ~3 hours, and then further samples were collected every ten
207 hours afterwards. At each collection point 14 ml of headspace gas was removed with a needle
208 and gas-tight syringe and injected into pre-evacuated 12 ml borosilicate vials with a silicone
209 septum and aluminum crimp (Teledyne Instruments, Inc., CA, USA); at the second and last
210 collection an additional 14 ml headspace gas was removed and injected into pre-evacuated
211 Exetainers (Labco Ltd., High Wycombe, UK) for isotopic analysis of N₂O in the headspace. After
212 each gas sampling, He of an equivalent volume was injected into the incubation vessels to
213 maintain pressure in the containers. At the end of the incubation all jars were opened and soils
214 were destructively harvested to quantify soil inorganic N, and for DNA extraction.

215 2.3 N₂O concentration and isotope analysis

216 Headspace samples were analyzed for N₂O concentration in an auto-injected 5 ml subsample
217 on a gas chromatograph fitted with an electron capture detector (CP-3800, Varian), and
218 calibrated against a four-point standard curve that encompassed the sample range. Blank
219 corrected headspace concentrations were adjusted for the dilution at each sampling with He
220 replacement, converted to rate of net N₂O-N production (ng g dw⁻¹ h⁻¹) by application of the
221 ideal gas law ($n = PV/RT$), multiplication by the molar mass of N in N₂O, and correction by g dry
222 weight of soil in the sample and change in time since the previous sample. Then rates of net
223 N₂O production were calculated as the average of the 8 sample collections' rates. Net N₂O flux
224 changed throughout the course of the 60 h incubation (Supplementary Figure 1); we focus on
225 the average of these rates to integrate both production and reduction into an aggregate value
226 across the whole incubation. Samples for isotope analysis ($\delta^{15}\text{N}$ of N₂O) were submitted to the
227 University of California, Davis, Stable Isotope Facility, where they were analyzed on a
228 ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a
229 ThermoScientific Delta V Plus isotope ratio mass spectrometer (Bremen, Germany). Analysis
230 was conducted with 4 standards of 0.4-10 ppm N₂O in He, with a precision (standard deviation
231 on five replicate natural abundance standards) of 0.1‰ ¹⁵N.

232 The change in the percent of added ¹⁵N found in the N₂O between incubation sampling times at
233 3 h and 60h was used to quantify gross reduction of N₂O to N₂ (Billings and Tiemann 2014).
234 Because our tracer contained far more ¹⁵N than is present naturally, any natural fractionation
235 during N₂O reduction was negligible compared to the isotopic signature of the tracer in the N₂O
236 pool, and we can use ¹⁵N₂O abundance as a means of assessing N₂O production vs. reduction. If
237 ¹⁵N₂O abundance at 60 h is higher than at 3 h, it suggests the tracer was continuing to flow into
238 the N₂O pool more so than out of it, and thus that N₂O production outpaced N₂O reduction
239 (transformation into N₂) at that time point. In contrast, if ¹⁵N₂O abundance at 60 h is lower than
240 at 3 h, it suggests that the tracer was flowing out of the N₂O pool at a greater pace than it was
241 flowing into it, and thus that N₂O reduction outpaced N₂O production at that time point. We
242 calculated ¹⁵N₂O by multiplying the isotopic ratio of the sample by the concentration of N₂O in
243 that sample. Then we computed the change in percent of the ¹⁵N tracer added that was found

244 in headspace N₂O across incubation time as:

245 *Change in*¹⁵N₂O (%)

$$246 = \left(\left(\frac{{}^{15}\text{N}_2\text{O}}{{}^{15}\text{NO}_3\text{-N added}} \right) * 100 \right)_{\text{final}} - \left(\left(\frac{{}^{15}\text{N}_2\text{O}}{{}^{15}\text{NO}_3\text{-N added}} \right) * 100 \right)_{\text{initial}}$$

247

248 where ¹⁵N₂O is ng of ¹⁵N in headspace N₂O per g of dry weight soil, ¹⁵NO₃⁻-N is ng of ¹⁵N in NO₃⁻
249 per g dw of soil, final refers to the end of the incubation (~60 h), and initial refers to the first
250 time point at which change in ¹⁵N of N₂O was assessed (~3 h).

251 To assess the potential for N₂O to be reduced to N₂ by denitrifiers in the other horizon when
252 incubated together, we calculated the combination effect (ng N₂O-N g dw⁻¹ h⁻¹) as the
253 difference between observed net N₂O fluxes when soil horizons shared the incubation
254 headspace (observed) and the expected flux determined as the linear, additive effect of rate for
255 horizons in separate headspaces (((organic + mineral)/2) = expected). The combination effect
256 was also expressed as a percent of the expected flux:

$$257 \text{Combination effect (\%)} = \frac{\text{observed} - \text{expected}}{\text{expected}} * 100,$$

258 where a negative combination effect implies reduction caused by inclusion of one of the
259 horizons.

260

261 2.4 Soil nutrient analysis

262 To observe changes in extractable inorganic N during the incubation, we extracted soil
263 subsamples prior to and following the incubation (fresh mass, organic: 12 g; mineral 10 g) by
264 shaking for 1 h with 40 ml 0.5 M K₂SO₄. After shaking all samples were filtered and extracts
265 frozen at -20 °C until further analysis. Soil NO₃⁻-N and NH₄⁺-N in the extracts were analyzed on a
266 Lachat 8500 Autoanalyzer (Hach Co., Loveland, CO, USA) using the cadmium reduction and
267 phenol red methods, respectively.

268 2.5 Functional gene abundance

269 Soil DNA was extracted from approximately 0.25 g fresh weight soil using MoBio Power Soil
270 DNA extraction kit and purified with MoBio PowerClean DNA Clean-up kit (MoBio Laboratories,

271 Carlsbad, CA, USA, now Qiagen). DNA was quantified with a Qubit 2.0 Fluorometer (Invitrogen,
272 Carlsbad, CA, USA), diluted by a factor of ten and stored at -20 °C until further analysis. We
273 assayed several functional gene primers in the denitrification pathway via PCR (*nirK* (Henry et
274 al., 2006), *nirS* (Throbäck et al., 2004), *norB* (Braker and Tiedje, 2003), *nosZ* (Rösch et al., 2002),
275 *nosZ* clade II (Jones et al., 2013); Supplementary Table 1), and selected *nirS* and *nosZ* as the
276 most tractable indicators of N₂O production and reduction in these soils using quantitative PCR
277 (qPCR), based on successful amplification of these genes across all samples. qPCR was
278 accomplished using the ABI StepOnePlus (Applied Biosystems) with Brilliant III Ultra-Fast SYBR®
279 Green QPCR Master Mix (Agilent/Life Technologies, Carlsbad, CA, USA). Each reaction consisted
280 of 5 µl (~2 ng) genomic DNA, 400 nM each primer, 300 nM reference dye and 1 X Brilliant III in a
281 final volume of 20 µl. The qPCR program consisted of an initial denaturing temperature of 95 °C
282 for 3 min followed by 40 cycles of denaturing at 95 °C for 5 s and a combined annealing and
283 extension step of 10 s at 60 °C for both *nirS* and *nosZ* genes. Melt curves were calculated at the
284 end of each qPCR run to confirm product specificity. Each qPCR plate contained one primer
285 pair, three negative controls and a four-point standard curve (ranging from 300 to 300,000
286 copies). Standard curves were generated using genomic DNA from lab stock of cultured
287 *Pseudomonas fluorescens* and gene copy numbers were calculated assuming a mass of 1.096 x
288 10⁻²¹g per base pair (Wallenstein and Vilgalys, 2005), one gene copy per genome, and a genome
289 size of 7.07 Mb (NCBI). All gene abundance data were corrected by soil oven dry mass based on
290 the dry:fresh mass ratio of an oven-dried subsample collected post-incubation.

291 2.6 Statistical analysis

292 We used a three-way ANOVA to assess the influence of the fixed effects of soil horizon, 'region'
293 (historical temperature), 'temperature' (short-term, incubation temperature) and their
294 interactions on: inorganic N pools, net N₂O flux averaged across the incubation, change in
295 percent of added ¹⁵N tracer found in headspace N₂O, the effects of mixing horizons in the
296 incubation on net N₂O flux, and functional gene abundances. For all analyses, we followed up
297 significant main effects with a Tukey's post-hoc analyses and report adjusted *P*-values. For all
298 variables, we assessed whether they met assumptions required for performing these statistical

299 tests, and log-transformed variables before analysis when required. All statistical analyses were
300 performed in R (R Core Team, 2014), using the MASS package (Venables and Ripley, 2003). All
301 significant ($\alpha = 0.05$) results and interactions are reported except significant main effects when
302 significant interactions of their terms are reported instead. Errors reported are one standard
303 error of the mean.

304 **3. Results**

305 *3.1 Changes in inorganic N pools after the incubation*

306 Temperature altered the pool sizes of NH_4^+ -N differently in each region and horizon (temp x
307 region x horizon: $P=0.05$), increasing relative to pre-incubation pool sizes in the organic soils at
308 some of the incubation temperatures (coolest region, 25 °C: $P=0.04$; intermediate region, 25 °C:
309 $P=0.02$; warmest region, 15 °C: $P<0.0001$, 25 °C: $P=0.0001$) (Fig. 2 A and B). Mineral soil NH_4^+ -N
310 pool sizes post-incubation did not differ from pre-incubation pool sizes.

311 Temperature also altered the pools sizes of NO_3^- -N differently for each region and horizon
312 (temp x region x horizon: $P=0.03$), decreasing relative to pre-incubation pool sizes in the organic
313 soils at all temperatures in all regions (coolest, 5 °C: $P=0.001$, 15 °C: $P=0.0007$, 25 °C: $P=0.003$;
314 intermediate, 5 °C: $P=0.04$, 15 °C: $P=0.002$, 25 °C: $P=0.008$; warmest, 5 °C: $P<0.0001$, 15 °C:
315 $P<0.0001$, 25 °C: $P<0.0001$). NO_3^- -N pool sizes also decreased in the mineral soils at all
316 temperatures in the coolest (5 °C: $P=0.0005$, 15 °C: $P=0.0008$, 25 °C: $P=0.002$) and intermediate
317 (5 °C: $P=0.02$, 15 °C: $P=0.002$, 25 °C: $P=0.0004$) regions, although not in the warmest region (Fig.
318 2 C and D). These results imply that the anaerobic conditions we generated by replacing
319 headspace air with He and keeping 80% water holding capacity generally supported
320 denitrification and limited nitrification.

321 *3.2 N_2O net production rates with short- and long-term warming*

322 Net N_2O flux was influenced by regions ($P=0.002$), incubation temperature ($P=0.006$), and soil
323 type ($P<0.0001$) without any significant effect of any interaction among or between these
324 independent variables. When averaged across all incubation temperatures and the two soil
325 horizons, the warmest region ($3.8\pm 0.8 \text{ ng } \text{N}_2\text{O-N g}^{-1} \text{ h}^{-1}$) had a higher rate than the intermediate

326 (1.9±0.6 ng N₂O-N g⁻¹ h⁻¹, *P*=0.008) and coolest region (1.2±0.3 ng N₂O-N g⁻¹ h⁻¹, *P*=0.003),
327 whereas the intermediate latitude and coolest regions' net N₂O production did not differ from
328 each other (Fig. 3). Averaged across all regions and the two soil types, the warmest incubation
329 temperature (3.4±0.8 ng N₂O-N g⁻¹ h⁻¹) exhibited a higher net N₂O flux than the lowest
330 temperature (1.1±0.3 ng N₂O-N g⁻¹ h⁻¹, *P*=0.003). Averaged across all regions and soil
331 temperatures, the organic soil (4.9±0.8 ng N₂O-N g⁻¹ h⁻¹) exhibited a higher rate than the
332 mineral soil (0.6±0.2 ng N₂O-N g⁻¹ h⁻¹, *P*<0.0001) and the combined incubation (1.3±0.3 ng N₂O-
333 N g⁻¹ h⁻¹, *P*<0.0001), which had a higher rate than the mineral soil alone (*P*=0.005).

334

335 We used N₂O emission from organic and mineral soil in isolation (Fig. 3 A & C) to compute
336 expected net N₂O flux for the combined soils (Fig. 4 A & B). Observed rates of net N₂O
337 production in the headspace surrounding combined organic and mineral soils (Fig. 3 B) were
338 less than expected values (Fig. 4 A & B) and often exhibited net N₂O reduction, implying inter-
339 profile interactions and differential temperature responses of the two horizons. The absolute
340 effect of the combined horizons' reduction of N₂O differed by incubation temperature
341 (*P*=0.002), with higher net reduction in the warmest incubation as compared to the coolest (25
342 vs. 5 °C: *P*=0.001) and a trend towards more reduction in the intermediate latitude region as
343 compared to the coolest (*P*=0.098). In proportional terms, the effect of combining horizons
344 decreased the combined net N₂O flux by up to 175% of the expected combined net production
345 rate, and this effect differed by temperature (*P*=0.009). In particular, it was more pronounced
346 at 15 °C relative to 5 °C (*P*=0.004). There was no significant interaction between region and
347 temperature on this combined-horizon rate.

348 We used the change in ¹⁵N in the N₂O (t_{60h}-t_{3h}) as a proxy for estimating how the relative
349 contribution of production and reduction of N₂O varied among regions, across horizons, and
350 with incubation temperature. Specifically, a negative net ¹⁵N abundance in N₂O from t_{60h}-t_{3h}
351 would indicate that consumption outpaced production, given that all the ¹⁵NO₃⁻ was reduced
352 over this period. Instead, the change in ¹⁵N abundance in N₂O across incubation time was
353 consistently positive, suggesting that rates of N₂O production consistently outpaced rates of

354 **N₂O reduction during the 60h incubation**. These values differed by region ($P=0.001$), a feature
355 driven by the warmest region exhibiting the largest change compared to the coolest region
356 ($P=0.0007$), and a similar trend between the warmest and intermediate-latitude regions
357 ($P=0.081$; Fig. 5). There was no significant effect of incubation temperature or soil type or any
358 interaction between temperature, region and soil type on this change in N₂O-¹⁵N.

359 3.3 Functional gene abundance

360 At the end of the 60 h incubation period, the abundance of one functional gene indicative of
361 N₂O production, *nirS*, did not vary significantly by incubation temperature or region but differed
362 strongly by soil horizon ($P<0.0001$). There was a higher abundance of this gene in the organic
363 soil ($0.73 \times 10^6 \pm 0.04 \times 10^6 \text{ g}^{-1}$) vs. the mineral soil ($0.18 \times 10^6 \pm 0.02 \times 10^6 \text{ g}^{-1}$) (Fig. 6). There
364 was no significant effect of any interaction among or between the independent variables on
365 *nirS* abundance. Functional gene abundance for N₂O reduction, *nosZ*, differed by region
366 ($P=0.0002$), incubation temperature ($P=0.04$) and soil ($P<0.0001$). It was higher in soils from the
367 warmest region ($8.4 \times 10^6 \pm 1.9 \times 10^6 \text{ g}^{-1}$) relative to the intermediate latitude region ($4.0 \times 10^6 \pm$
368 $0.8 \times 10^6 \text{ g}^{-1}$, $P=0.0006$) and the coolest region ($4.9 \times 10^6 \pm 1.1 \times 10^6 \text{ g}^{-1}$, $P=0.001$), at the coolest
369 ($6.7 \times 10^6 \pm 1.6 \times 10^6 \text{ g}^{-1}$) relative to the warmest incubation temperature ($5.2 \times 10^6 \pm 1.7 \times 10^6 \text{ g}^{-1}$,
370 $P=0.02$), and in organic ($10.55 \times 10^6 \pm 0.95 \times 10^6 \text{ g}^{-1}$) relative to mineral soils ($0.98 \times 10^6 \pm 0.08$
371 $\times 10^6 \text{ g}^{-1}$). There was no significant effect of any interaction among or between the independent
372 variables on *nosZ* abundance, although there was a near-significant trend for soil type to alter
373 the regional effect ($P=0.052$). The resulting *nirS:nosZ* ratio ranged from 0.03 to 0.55 and
374 displayed an interaction between region and soil horizon ($P=0.04$), driven by lower *nirS:nosZ*
375 ratios in organic soil in the warmest relative to intermediate latitude region ($P<0.0001$) and
376 warmest relative to coolest region ($P=0.003$); these effects were not exhibited in the mineral
377 soil.

378 4. Discussion

379 By promoting the denitrification pathway we aimed to: 1) distinguish short- (via laboratory
380 manipulations) and long-term (via a natural climate gradient) responses of denitrification-
381 derived net N₂O flux to temperature; 2) assess the degree to which net N₂O fluxes in these soils

382 are sensitive to interactions between soil horizons; and 3) leverage the abundance of genes
383 responsible for denitrifier production and reduction of N₂O as a means of assessing differences
384 in these processes' responses to short- and long-term temperature responses. Our first
385 hypothesis was not supported: though short-term warming enhanced net N₂O effluxes from
386 these soils, soils from a historically warmer environment exhibited greater net N₂O efflux than
387 those from cooler environments, suggesting a positive response of net N₂O fluxes to both short-
388 and long-term warming (Fig. 3). Indeed, an isotopic proxy for N₂O reduction derived from use of
389 a stable isotope tracer suggests that enhancement of net N₂O production with long-term
390 warming can be greater than any enhancement in N₂O reduction (Fig. 5). Our second
391 hypothesis was supported in that the combined incubation of mineral and organic soils
392 exhibited net N₂O efflux rates that did not match the linear sum of separate incubation flux
393 rates. However, we observed reduction of N₂O by mineral soil, not by organic soil as we
394 predicted. Specifically, net N₂O production was tempered by more mineral soil N₂O reduction
395 at warmer incubation temperatures (Fig. 4 & 5), indicating that soil horizon interactions may be
396 critical to rates of net N₂O efflux to the aboveground atmosphere. Finally, our third hypothesis
397 that linked gene abundance to process rates was only partially supported. *NosZ* decreased at
398 the warmest incubation temperature (i.e. lower N₂O reduction gene abundance with warming,
399 Fig. 6), consistent with rates. However, in the organic soils, *nosZ* was higher under higher
400 historical temperature (i.e. higher N₂O reduction gene abundance with warming, Fig. 6),
401 inconsistent with rates that increase with warming. There was no response to either short- or
402 long-term warming in *nirS* abundance in either soil horizon, or to long-term warming in *nosZ*
403 abundance in the mineral soil. Combined, these data suggest complex microbial responses to
404 short- and long-term exposure to distinct temperature regimes, which we expand upon below.

405 *4.1 Warming-induced enhancement of N₂O production exceeds that of N₂O reduction*

406 Long-term climate gradients substitute space for time and encompass variation in multiple
407 ecosystem phenomena driven by centuries of exposure to distinct climate regimes. For
408 instance, we know that *in situ* soil N cycling is more rapid (Philben et al., 2016) and likely
409 supports greater forest productivity in the relatively warm, southern-most boreal forests of this

410 transect (Ziegler et al., 2017). The net N₂O efflux rate data from this set of lab incubations
411 suggests that, especially in the organic soil horizons, both short-term warming and a long-term
412 warmer climate enhance net N₂O production, a result consistent with the stable isotope tracer
413 data (Fig. 5). These data correspond with the enhanced, short-term warming-induced N₂O
414 fluxes observed in several systems (Billings and Tiemann, 2014; Kurganova and Lopes de
415 Gerenyu, 2010; Szukics et al., 2010; Wang et al., 2014). The apparent lack of long-term,
416 denitrifier adaptation to rising temperatures (i.e. continued enhancement of N₂O production
417 with long-term exposure to warmer temperatures that outstrips enhancement of N₂O
418 reduction) is consistent with recent work in soils from these same sites demonstrating no
419 change in the responses of microbial biomass-specific decay or CO₂ efflux rates to warmer
420 temperatures over decadal timescales (Min et al., 2019). However, results from the current
421 study contrast with our **hypothesis** of microbial adaptations to a warmer climate over the long
422 term, which assume that a soil denitrifying community well-adapted to its temperature regime
423 is **effective** at complete denitrification with relatively little N₂O byproduct. Such predictions
424 arise from more conceptual studies presenting ideas about microbial metabolic responses to
425 warming (Billings and Ballantyne, 2013; Bradford, 2013) and not collective longer-term warming
426 effects, such as substrate or microbial community compositional changes, that may further
427 control microbial responses.

428 The similar difference in net N₂O rates between the northern region and southern region (2.6
429 ng N₂O-N g⁻¹ h⁻¹) and between the coolest and warmest incubation temperature (2.3 ng N₂O-N
430 g⁻¹ h⁻¹, both 68% of the average range across treatments) indicates that net rates were
431 enhanced to a similar degree by both short-term warming of 20 °C and a long-term MAT
432 difference of 5 °C. Temperature sensitivity (i.e. change per °C) of net N₂O flux increased at
433 lower latitudes, and the isotopic tracer experiment indicated that N₂O production increases
434 outpaced N₂O reduction increases in warmer regions. **Enhanced soil organic matter inputs and
435 nitrogen availability and cycling rates in the warmer climate forests (Philben et al., 2016; Ziegler
436 et al., 2017) may contribute to greater net N₂O production in the incubations, and *in situ*. In this
437 short-term incubation, the pulse of NO₃⁻ added minimized any differences in NO₃⁻ availability
438 for denitrifiers, likely leaving varying abilities of soil denitrifier community to respond to**

439 warming as a key difference across the incubated soils. Therefore, the additive, positive result
440 from both historically warmer soils and warmer incubation temperatures suggests that
441 community-level denitrifier effectiveness declines (i.e. more incomplete denitrification) in
442 warmer temperatures if they are from soils with historically warmer temperatures. This pattern
443 contradicts a “home-field” advantage (Wallenstein et al., 2013) for denitrifiers. More N₂O
444 production in warmer climates may arise from multiple changes that overcome adaptive home-
445 field advantages, such as shifts in the community composition (Delgado-Baquerizo et al., 2016)
446 and an increased number of inefficient N₂O producers, increases in the number of microbial
447 cells and transfer points involved in the denitrification pathway (i.e. nitrifier-denitrification in a
448 single organism vs. coupled nitrification-denitrification in distinct organisms (Butterbach-Bahl et
449 al., 2013), or a changed contribution of alternate, possibly less-efficient electron donors (i.e. co-
450 denitrification (Spott et al., 2011)).

451 Despite increased net N₂O production with higher temperatures, soil horizon interactions
452 temper the response to warming. Two of our methods supported the potential for mineral soil
453 N₂O reduction: (1) calculated differences in flux values between shared headspace N₂O flux
454 values and the isolated headspace N₂O flux values of the two isolated horizons, and (2) the
455 change in isotopic enrichment of the shared and isolated headspace N₂O. The first method
456 demonstrated that short-term warming enhanced the degree of interprofile interaction that
457 increased N₂O reduction during the incubation, while long-term warming did not significantly
458 influence interprofile N₂O dynamics (Fig. 4 A & B). The similarities in net N₂O flux between the
459 combined and mineral soil incubations (Fig. 3 B & C), and the fact that both of these incubations
460 have lower flux than the organic soil alone, indicate that the mineral soil served as a net N₂O
461 reducer, especially in response to short-term temperature increases. A caveat to this soil
462 horizon interaction is that while our O₂-limited experimental environment was necessary to
463 promote denitrification, this design may have exaggerated total soil reduction processes that
464 occur naturally in anaerobic microsites.

465 Our second method of detecting horizon interactions driving net N₂O efflux used ¹⁵N₂O
466 headspace differences from the start to the end of the incubation as an indicator of reduction.

467 We expected an increase in the ^{15}N in the headspace N_2O as $^{15}\text{NO}_3^-$ is reduced, followed by a
468 decline in ^{15}N in the headspace N_2O as the tracer flows into the N_2 pool, with balance of these
469 processes over the 60 h incubation indicating net production or reduction (Billings and
470 Tiemann, 2014). NO_3^- pools declined and the change in our $^{15}\text{N}_2\text{O}$ abundance was positive,
471 suggesting that N_2O production still outweighed reduction at the end of the 60 h for both the
472 individual horizons and the combination incubation (Fig. 5 A). Large variation in $^{15}\text{N}_2\text{O}$
473 abundance among forest sites led to no significant difference between soil horizons and did not
474 allow us to confirm the direction of horizon interactions. Horizon interactions drove net profile
475 N_2O fluxes in a field drought manipulation in a Norwegian spruce forest, during which soils
476 exhibited a net N_2O sink via upper mineral soil reduction of deep mineral soil N_2O production
477 (Goldberg and Gebauer, 2009). It remains unknown if the relatively shallow mineral soils we
478 sampled are analogous reducers of deeper mineral soil N_2O produced in this system, or if they
479 could continue to reduce large portions of organic soil N_2O efflux (Fig. 4) in situ. Contrary to our
480 original hypothesis, shallow mineral soils in situ may be better suited than organic soils to N_2O
481 reduction, as mineral soils experience frequent inputs of leached NO_3^- and DOC from the
482 surface organic soils, and represent a sudden change in the soil structure and porosity towards
483 well-packed fines and smaller pores. These conditions may promote leachate pooling,
484 anaerobic microsites, and a microbial community that proves more effective at reduction.

485 Mineral soil reduction of organic soil-generated N_2O becomes most relevant when diffusion of
486 N_2O from the upper soil profile to the atmosphere is restricted, and N_2O produced in those
487 surface layers diffuses downwards according to Fick's Law as has been discussed in the
488 literature for soil CO_2 dynamics (Oh et al., 2005; Richter et al., 2015). Such a situation is likely to
489 occur in 'hot spots' (McClain et al., 2003) such as frozen surface soil patches during winter.
490 Similarly, 'hot moments' may occur in the spring snow melt or in winter, despite cold
491 temperatures reducing N cycling rates: subnival N_2O production can be an important
492 contribution to annual N budgets in pastures (reviewed in Uchida and Clough 2015), and winter
493 N dynamics also appear to be important in northern temperate forest systems. For example,
494 winter N_2O production equaled ~30% of the summer N_2O production in a SE Canadian forest
495 (Enanga et al., 2016) and ~60% of the annual atmospheric N inputs in a NE U.S. forest (Morse et

496 al., 2015). Mineral soil reduction of winter organic soil-generated N₂O may temper net fluxes
497 and may be an important feature of N cycling in these forests that likely varies with snowpack
498 dynamics.

499 *4.2 Linking biogeochemical process rates to genetic potential*

500 The functional gene associated with N₂O reduction that we could quantify in these soils was
501 sensitive to both short-term and historical temperature, though it was not consistently
502 associated with process rates. Although we did not detect the atypical *nosZ clade II* in these
503 soils, other, yet unknown genes that we did not measure may be responsible for N₂O reduction.
504 Beyond this possibility, our results suggest a decoupling of process rates and denitrifier genetic
505 controls, or that the long-term temperature-related increase in genetic potential for N₂O
506 reduction did not translate to rates as effectively as the short-term temperature-related
507 decrease in genetic potential for N₂O reduction.

508 Consistent with enhanced net N₂O production in these soils at warmer incubation
509 temperatures, the *nosZ* abundances were reduced after 60 h exposure to 25°C relative to
510 cooler incubations. Although functional gene abundances are assumed to integrate longer-term
511 changes in the microbial community and thus have a reduced dynamism relative to
512 instantaneous rates (Petersen et al., 2012), our results appear to reflect a capacity of
513 denitrifiers to respond rapidly to temperature, as indicated in other laboratory incubations that
514 assayed temperature responses of denitrification functional gene abundances (Billings and
515 Tiemann, 2014; Cui et al., 2016; Keil et al., 2015). However, inconsistent with enhanced net N₂O
516 production in the soils from warmer historical temperatures, we found a reduced *nirS:nosZ*
517 ratio in the southern forest soils. A possible explanation of this apparent decoupling between
518 gene abundances and biogeochemical outcomes may be an interference between potential and
519 transcription (i.e. better detected with mRNA), or inadequate measurement of all genes
520 relevant to N₂O dynamics in these soils. Although our experimental set up promoted
521 denitrification, our incubation may have also supported dissimilatory nitrate reduction to
522 ammonium (DNRA (Schmidt et al., 2011)). This pathway is poorly characterized, but has been
523 detected in both aerobic and anaerobic environments of many soil types; it may account for a

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

532
533 Contrasting efficiencies of N_2O scavenging is another possible explanation for the decoupling
534 between gene abundances and biogeochemical fluxes in these soils, as the catalytic efficiency
535 of enzymes can vary with community structure and resource availability (Tischer et al., 2015),
536 conditions which vary between boreal soil horizons. The observation that mineral soil has the
537 capacity to reduce a substantial amount of organic soil-derived N_2O even as *nosZ* abundances
538 are reduced in mineral compared to organic soil provides a strong indication that *nosZ* in
539 mineral soil is more efficient at scavenging N_2O from the headspace than *nosZ* in the organic
540 horizon. Alternatively, it would be beneficial to increase efforts to detect the *nosZ* clade II in
541 boreal forest soil organic and mineral horizons, as this clade is not detected by the *nosZ* primer
542 and has a higher N_2O consumption capacity than *nosZ* in European mineral soils (Jones et al.,
543 2014). Consistent with our combination samples in the current study, there is increasing
544 evidence that soils can serve as sinks for atmospheric N_2O (Chapuis-Lardy et al. 2007), and
545 interestingly, that this phenomenon can be particularly evident when soil water is limited
546 (Goldberg and Gebauer, 2009). Therefore, given the varying gene abundance and enzyme
547 efficiency with depth implied in this study, a likely fruitful area of research would be to explore
548 mineral soil N_2O sink capacity and mineral soil genetic response as moisture availability varies,
549 as happens particularly during snowmelt periods and in fall within these boreal soils.

550

551 **5. Conclusions**

552 The sensitivity of soil N₂O efflux to global change factors such as rising temperature can be high,
553 as supported by this study, but the mechanisms driving N₂O sources and sinks remain
554 challenging to elucidate. Indeed, variation of net soil denitrifier N₂O efflux within climate
555 region in this study, though less than variation across regions, warrants further consideration of
556 within-region controls on N₂O efflux. The meaningful differences in responses to temperature
557 that we observed across regions, though, permitted us to address the three critical issues
558 framed at the outset of this study; we conclude with three observations and questions for
559 future research. To improve Earth system models of greenhouse gas emissions we need to
560 address the importance of varying N₂O dynamics with soil depth. Indeed, this research
561 highlights potentially different effectiveness of organisms possessing N₂O-relevant functional
562 genes as we move across depth. Is it ubiquitous that organisms possessing *nosZ* are more
563 effective at reducing N₂O to N₂ in sub-surface soils? We have taken the first step towards this
564 characterization, but similar studies should address this question in diverse ecosystems. Our
565 results also illustrate that both denitrifier-mediated rates of N₂O production and reduction can
566 increase with warming, over both short- and long-term timescales, in boreal forest soils. *In situ*
567 variables would undoubtedly alter the *ex situ* fluxes observed in this study, but we demonstrate
568 that when conditions promote denitrification, the net response to warming in these boreal
569 forest soils is dominated by N₂O production. Finally, we remain uncertain of the relative
570 importance of the denitrification pathway in N₂O emissions in boreal forest soils (i.e. as
571 compared to nitrification, co-denitrification, DNRA and others) and suggest similar approaches
572 to explore the importance of historic climate regime, shorter-term temperature variation, and
573 interactive responses among soil horizons in other biochemical pathways of soil N₂O emission.

574

575 **Acknowledgements**

576 We gratefully acknowledge field assistance from Andrea Skinner, and laboratory assistance
577 from Carl Heroneme, Samantha Elledge, Yanjun Chen and Mitch Sellers. Research funding was
578 provided by the National Science Foundation (NSF-DEB 0950095) to SAB, Natural Sciences and

579 Engineering Research Council of Canada (RGPIN#341863) to SZ, an Association for Women
580 Geoscientists Graduate Research Scholarship and the University of Kansas, and the Kansas
581 Biological Survey Graduate Summer Research Fund to KM. The Canadian Forest Service of
582 Natural Resources Canada provided valuable logistical support.

583

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766

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

768

Region	Coolest			Intermediate			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

769

770 **Figure legends**

771 Figure 1. a) Map and b) pictures of the three forests in each region along the Newfoundland
772 and Labrador Boreal Ecosystem Latitude Transect in Canada.

773 Figure 2. Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ pools in the organic (A and C) and mineral soil (B and D), pre-
774 incubation ('Pre-inc.') and at the end of the incubations at 5, 15, and 25°C of soils from along a
775 boreal forest latitudinal transect. Pre-incubation values for nitrate are calculated as ambient
776 concentrations plus added $\text{NO}_3^-\text{-N}$. Note different y-axis values. 'MAT' = mean annual
777 temperature; the 'coolest' region is the Eagle River watershed (northern boreal), the
778 'intermediate' region is the Salmon River watershed (mid-boreal), and the 'warmest' region is
779 the Grand Codroy watershed (southern boreal). See text for description of sites. Values
780 provided as the mean \pm one standard error (n=3 forests per latitudinal region).

781 Figure 3. Net N_2O flux ('production rate') averaged for 60 h of incubation at 5, 15, and 25°C
782 from organic soil alone (A), combined organic and mineral soil (B) and mineral soil alone (C)
783 from three regions along a boreal forest latitudinal transect. 'Combined' refers to incubations
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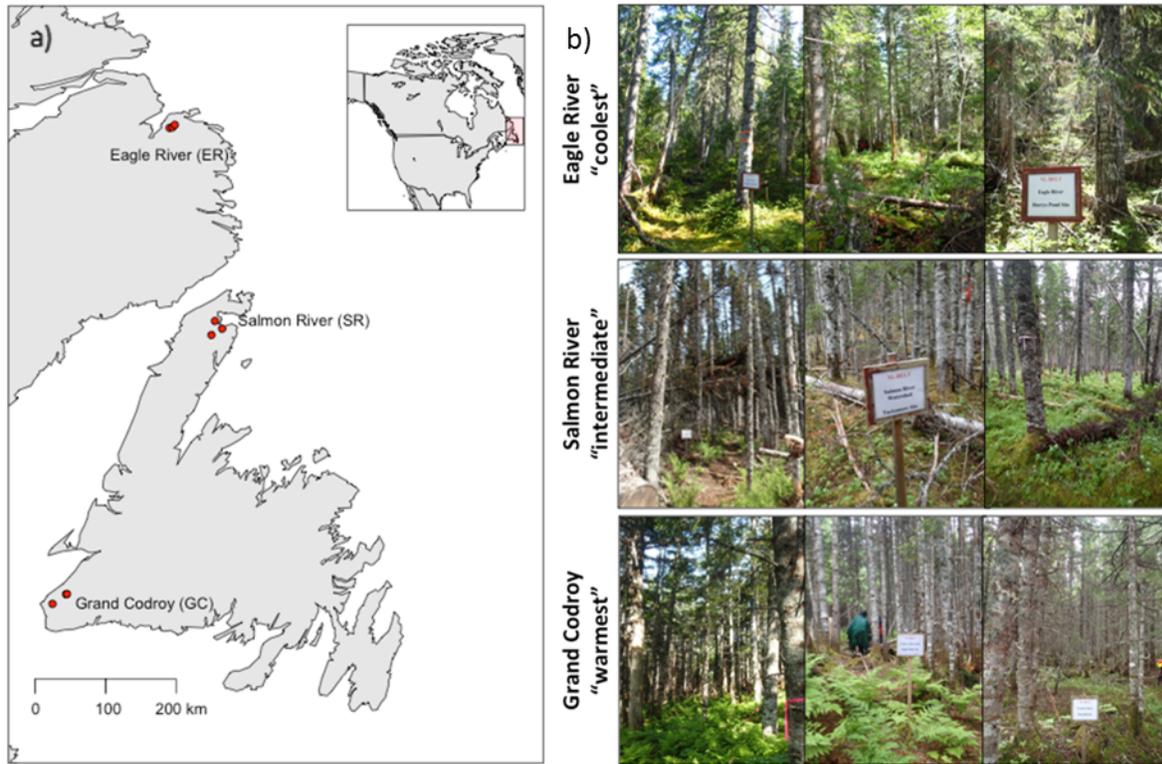
789 Figure 4. The combination effect of shared headspace surrounding physically separated organic
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791 production rate (B), at the end a 60 h incubation at 5, 15, and 25°C, for soils from three regions
792 along a boreal forest latitudinal transect. The combination effect (negative = reduction) is
793 calculated as the difference between observed net N_2O fluxes when soil horizons shared the
794 incubation headspace (observed) and the linear, additive effect of rate differences between
795 horizons in separate headspaces (((organic + mineral)/2) = expected). The percent combination
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806 headspace. 'MAT' = mean annual temperature; the 'coolest' region is the Eagle River watershed
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813 and mineral (F) soil. Note y-axis scales differ for each row, and between (C) and (D). 'MAT' =
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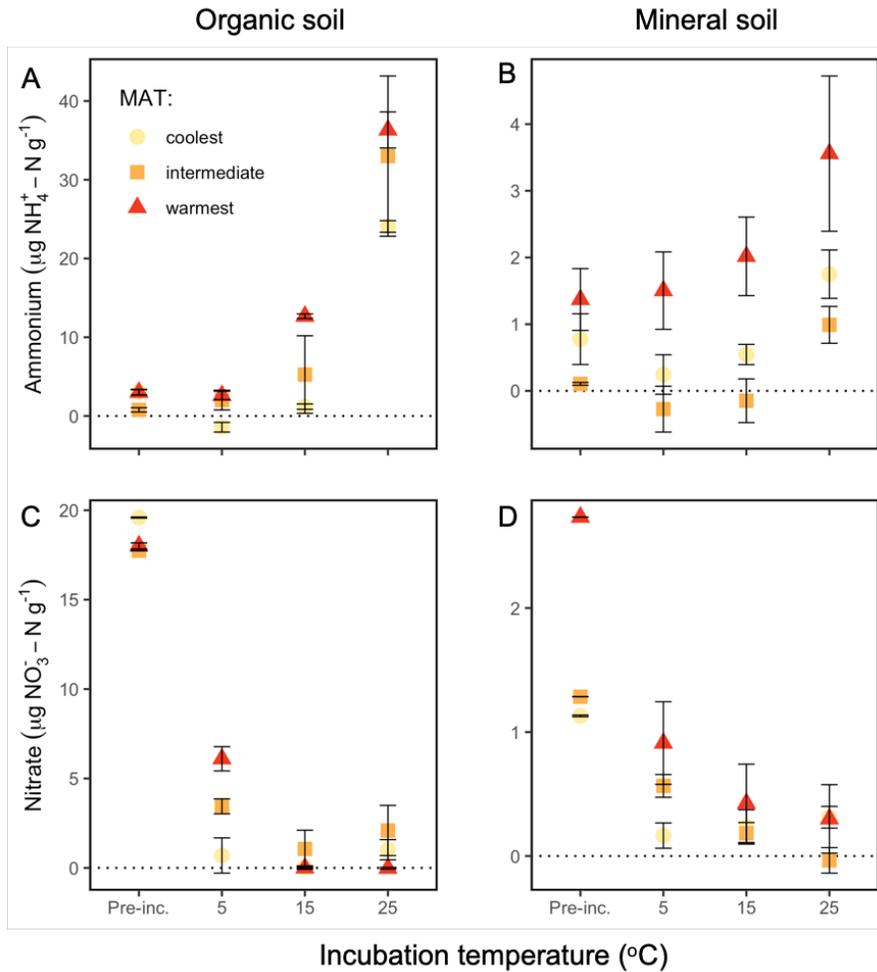
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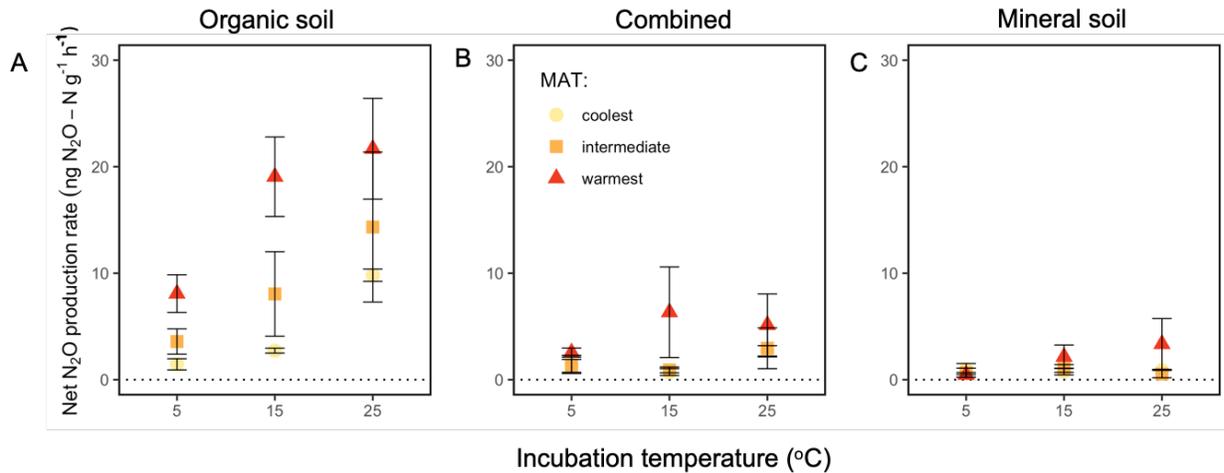
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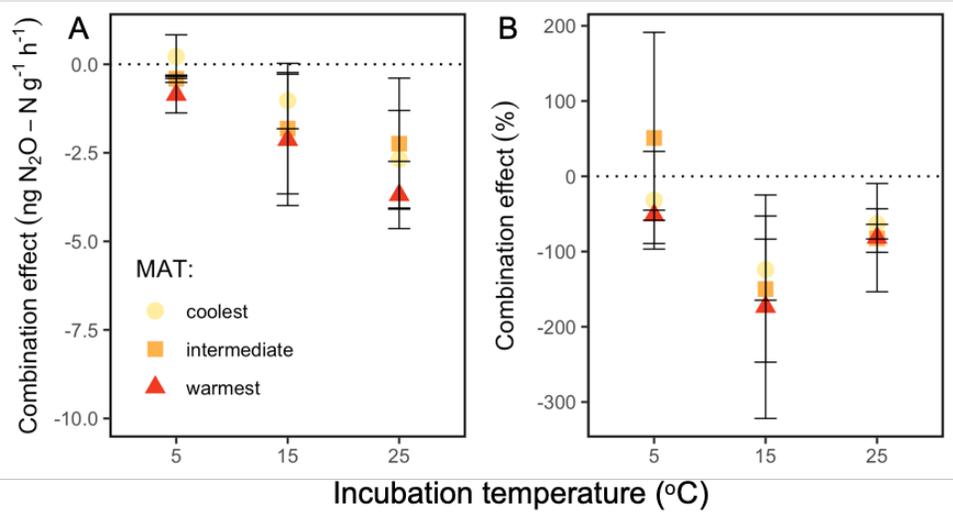


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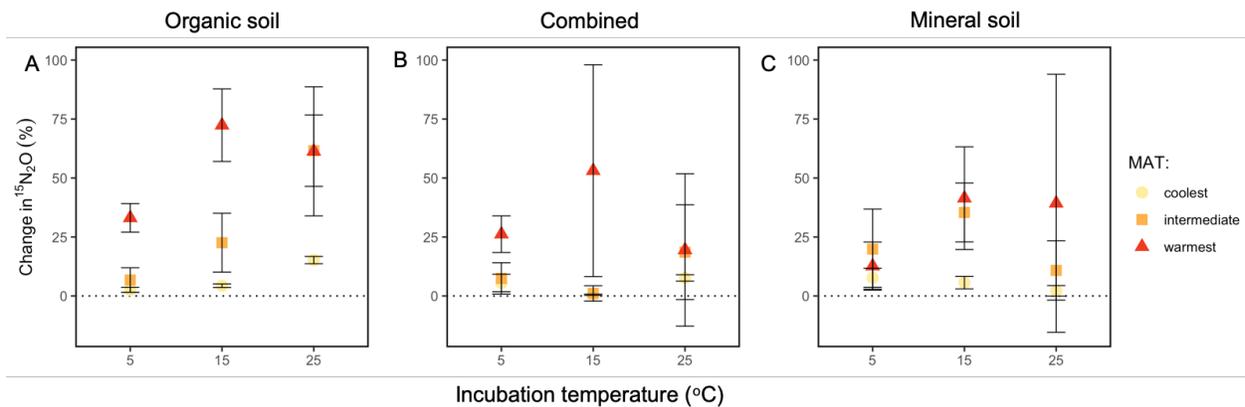


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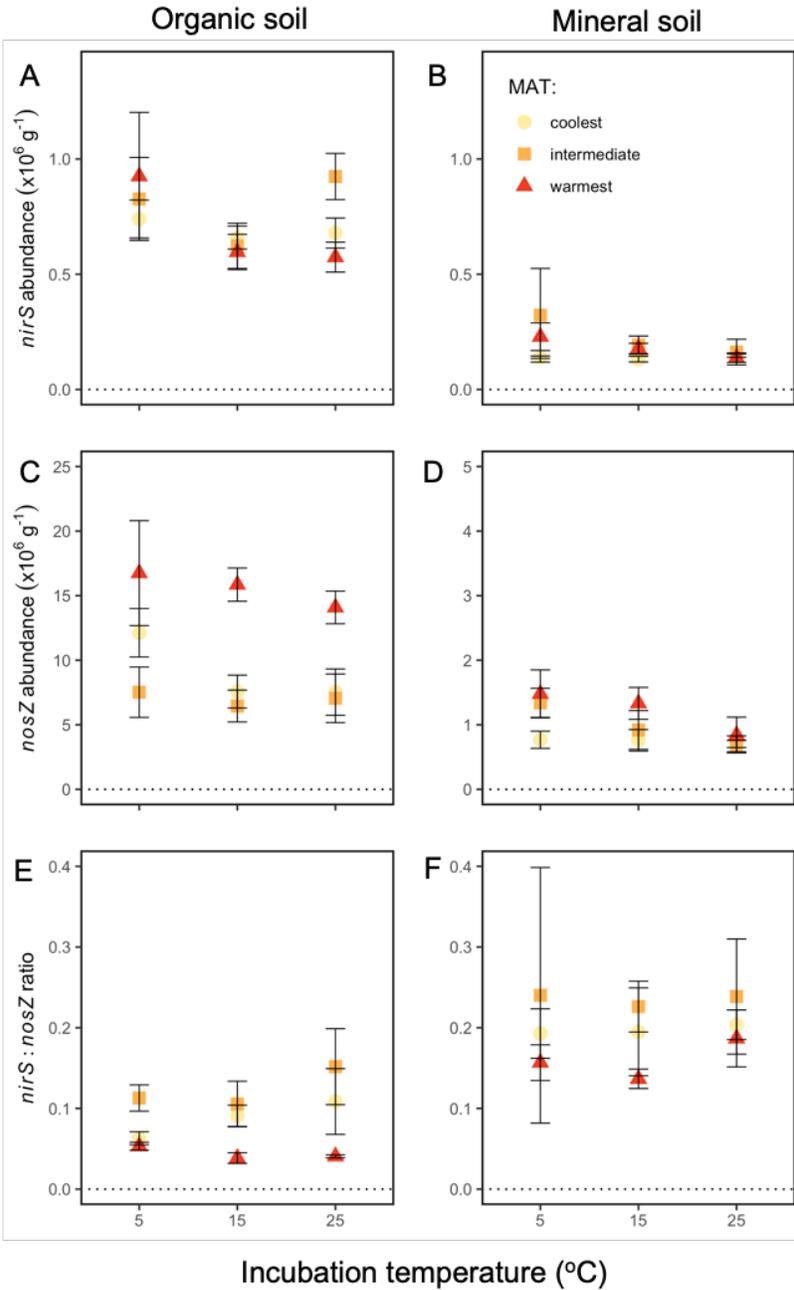
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