Egusphere-2024-3813: Long-term nitrogen fertilization alters microbial respiration sensitivity to temperature and moisture, potentially enhancing soil carbon retention in a boreal Scots pine forest

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Reply to editor Kees Jan van Groenigen (on comments of reviewer #1 and #2)

Your revised manuscript has now been seen by two reviewers, one of which was also involved in the first review round. Whereas the comments by reviewer #1 are easy to address, you will notice that reviewer #2 raises several substantial concerns. These need to be addressed before the manuscript can be accepted. I believe that most of these concerns can be addressed by clearly stating the limitations of your study, by better justifying some of the choices you made in your approach, and providing additional clarifications regarding your interpretation, rather than making major changes to the actual analyses. As such, I suggest minor revisions, but please note that I may contact reviewer #2 if I am not sure if the concerns have been adequately addressed.

Dear Editor,

thank you for evaluating the comments of reviewers and favorable decision for minor revision.

We agree that the comments of reviewer #1 are easy to address and we implemented all as suggested.

We also agree that the comments of reviewer #2 could be considered by clarifying raised concerns in the text of our paper instead of changing the analysis. Below we reply in detail to each reviewer #2 comment and indicate corresponding text revision.

This manuscript addresses the impact of long-term nitrogen (N) fertilization on microbial respiration and greenhouse gas (GHG) fluxes in a boreal forest ecosystem. The authors employ empirical measurements and modeling to evaluate Rh/SOC responses to temperature and soil water content (SWC), presenting this as evidence for enhanced carbon (C) retention under N addition. While the study utilizes a valuable long-term experimental site and extensive measurements, the manuscript suffers from several critical scientific, methodological, and interpretative issues that significantly undermine its novelty and reliability. Detailed recommendations for improvement are provided below.

We appreciate reviewer #2 thoughtful comments and addressed these accordingly to substantially improve the clarity of our paper.

Major Comments

1. The experiment lacks true replication. Only three plot pairs (CTR vs. N+) were used, separated by an average of 122 m. This design raises concerns about pseudoreplication and site effects (e.g., topography, soil heterogeneity). As the authors acknowledge (e.g., line 211),

microtopography may have affected results, yet this source of variation is not accounted for in the analysis.

We acknowledge the reviewer's concern regarding replication and the potential influence of site-specific effects such as microtopography and soil heterogeneity. The experiment was conducted on three trenched areas per treatment (N+ and CTR), each containing two replicated 706 cm² measurement plots (i.e., 6 plot pairs in total, as clarified in line 121). These two replicate plots were treated as independent spatial observations to capture within-trench variability, while the three trenched areas per treatment were considered representative of site-level variability under each treatment.

We clarified the structure of the sampling design in the methods to avoid any confusion (revised text in line 121):

"Measurements were taken from 12 plots (six per treatment). Two plot pairs (2 × 706 cm^2) were used to account for local heterogeneity in soil and microtopography at the trench level of each trenched area (1 m^2), while three trenched areas per treatment were used to capture spatial heterogeneity of each treatment at the site level (Fig. 1d)."

However, we also fully recognize the limitations of this spatial design, and as such, we have explicitly framed the study as a case study in the conclusions (line 415), to avoid overgeneralizing the results.

Revised conclusions (line 415): ***Although our experiment design allowed exploratory insights into N fertilization effects, caution is needed in extrapolating beyond this site. While results represent a case study, …***

2. Thinning treatments conducted in 1990 and 2015 differed between the control and N-fertilized plots (line 90), potentially introducing confounding effects on litter input, SOC accumulation, and soil respiration. Since thinning alters stand structure and litter quality, failing to account for these differences undermines the attribution of observed effects solely to nitrogen fertilization. A clearer separation or statistical control of thinning effects is necessary to support the current conclusions.

We thank the reviewer for this important observation. While we acknowledge that thinning can influence soil respiration through effects on stand structure and litter inputs, previous studies suggest that these effects are generally modest (see lines 321–322). The thinning was carried out according to Finnish silvicultural guidelines (Tapio) with the aim of applying consistent intensity across all treatments.

We have now clarified this in the manuscript (line 95): ***"To minimize potential confounding, both CTR and N-fertilized (N+) plots were thinned in 1990 with similar intensity (~20%), and again in 2015 with nearly identical intensity, reducing basal area by***

36.7% (CTR) and 40.1% (N+), following the Finnish silvicultural guidelines (Tapio, www.tapio.fi).”

Although we cannot fully rule out legacy effects of thinning, the consistent application and timing across treatments support our conclusion that N fertilization was the primary driver of the observed SOC and respiration responses.

3. The authors use Rh/SOC to infer decomposition intensity, but Rh is inherently influenced by SOC content (i.e., autocorrelation risk). This may mask real changes in decomposition activity. The justification for SOC normalization as a proxy for microbial efficiency is weak and not supported by microbial process data (e.g., enzyme activities, community structure).

We appreciate the reviewer’s thoughtful comment regarding the potential autocorrelation between Rh and SOC, and the limitations of using $R_{h/SOC}$ *as a proxy for microbial decomposition efficiency.*

We agree that normalizing Rh by SOC does not eliminate the autocorrelation issue. However, this ratio is widely used in soil carbon studies to express decomposition intensity relative to substrate availability, especially when comparing soils with differing SOC content and is commonly applied in incubation studies to represent relative microbial activity.

We added a cautionary note in the Discussion to acknowledge the interpretive limitations of this approach (lines 317-324).

“Normalizing respiration by SOC provides a meaningful way to interpret respiration rates relative to carbon availability, especially when comparing treatments with differing SOC stocks. Although, this normalization does not fully resolve the issue of R_h ***dependence on the amount of SOC, it has been widely adopted in field and incubation studies, as well as in soil carbon modeling frameworks (e.g., Tuomi et al., 2008; Davidson et al., 2012; Curiel Yuste et al., 2007; García-Angulo et al., 2020). Nonetheless,*** $R_{h/SOC}$ ***should be interpreted with caution, as it does not capture underlying microbial mechanisms such as enzyme kinetics or community structure, which introduce nonlinearities in the decomposition kinetics. Whereas normalizing*** R_h ***by SOC as a proxy of the decomposition rate constant assumes a linear relation between decomposition rate and SOC. In the absence of microbial process data, it serves as a useful, though imperfect, indicator of decomposition rates.”***

4. Although N fertilization increased Rh in absolute terms (Fig. 3a), it did not change Rh/SOC (Fig. 3b). Nevertheless, the authors claim that N enhances carbon retention (Fig. 6, lines 275–280), which lacks a consistent mechanistic link and may simply reflect dilution effects on SOC stocks.

We appreciate the reviewer’s concern regarding the interpretation of carbon retention under nitrogen (N) fertilization. While absolute Rh increased under N fertilization (Fig. 3a), Rh *normalized by SOC ($R_{h/SOC}$) did not increase (Fig. 3b), suggesting that the rate of CO_2 emission per unit of SOC remained unchanged or slightly decreased.*

As discussed in response to Comment 3, $R_{h/SOC}$ is a widely used proxy for SOC-specific decomposition intensity. In the N-fertilized plots, increased litter input resulting from enhanced tree growth contributed to higher SOC stocks. The unchanged (or slightly lower) $R_{h/SOC}$ ratio, despite increased litter input, suggests that a smaller fraction of incoming carbon is lost as CO_2 - indicating more efficient carbon retention.

We agree that this interpretation is indirect and may partly reflect dilution effects. However, the combination of increased litter inputs and stable $R_{h/SOC}$ supports the hypothesis of enhanced SOC accumulation. We have revised lines 270–280 to more clearly present this reasoning while also noting the potential limitations and need for microbial process measurements to confirm the mechanisms involved.

"Annual $R_{h/SOC}$ rates (expressed as % SOC respired per year) based on daily model estimates ranged from 9.85 (±0.14 SE) to 12.95 (±0.23 SE) % and increased over 2021–2023 (Fig. 6). On average, $R_{h/SOC}$(T, SWC) values were 1.90 (±0.41 SE) % higher in control (CTR) plots compared to N-fertilized (N+) plots. This suggests that, despite increased litter inputs in N+ plots due to enhanced tree growth, the relative decomposition rate per unit SOC remained unchanged or declined, potentially favoring greater SOC retention.

The difference in modeled R_h (calculated as $R_{h/SOC}$(T, SWC) × SOC) between CTR and N+ plots corresponds to a reduction of –345.4 (±73.6 SE) g CO_2 m^{-2} yr^{-1} in heterotrophic CO_2 emissions (Table 3). This potential reduction in CO_2 emissions outweighed the global warming potential (GWP) associated with increased N_2O emissions (1.1 ± 0.1 g CO_2 m^{-2} yr^{-1} reduced CH_4 uptake (10.1 ± 0.5 g CO_2 m^{-2} yr^{-1}) and fertilizer production emissions (6.5 g CO_2 m^{-2} yr^{-1}). Overall, the net GWP balance suggests an annual reduction of -327.6 (±73.6 SE) g CO_2 m^{-2} yr^{-1} attributable to N fertilization."

5. Equation 1 used to model Rh/SOC responses to temperature and moisture contains five parameters, which is excessive given the limited data and moderate model fit ($R^2 \approx 0.4$). In particular, key parameters for the N-fertilized treatment (e.g., parameter c) are statistically non-significant and have large standard errors (Table 1), suggesting weak parameter identifiability and potential overfitting. Therefore, the reliability of model-based annual extrapolations is questionable, and the results should be interpreted with greater caution.

We acknowledge the reviewer's concern about model complexity and the potential risk of overfitting due to the limited dataset and moderate model fit. The five-parameter formulation was chosen to allow flexibility in capturing nonlinear and asymmetric responses of $R_{h/SOC}$ to temperature and soil moisture. Using a simpler model would have biased the fit, particularly in the control (CTR) plots where $R_{h/SOC}$ showed a clear peak response to soil water content (SWC).

In contrast, for the N-fertilized (N+) plots, the estimated value of the moisture response shape parameter c was close to 1 and statistically non-significant, indicating a more linear or flat response of $R_{h/SOC}$ *to SWC. This aligns with the biological observation that moisture limitation was less apparent in N+ plots, possibly due to structural or microbial changes. Although the c parameter was not significant in N+, we retained the same model structure across both treatments to ensure comparability of model parameters and to enable treatment-based interpretation of response functions.*

To reflect this limitation in interpretation, we added the following clarification to Results (lines 253-256)

"While not statistically significant in N+ plots, the c value near 1 suggests a relatively flat response of $R_{h/SOC}$ ***to SWC. In contrast, a significant c value (≈ 8,*** $p < 0.001$***) in CTR plots indicates a peaked moisture response, supporting the role of moisture limitation in decomposition under ambient conditions (Table 1).***

and in Discussion (lines 396-399):

"However, the lack of significance of the moisture shape parameter c in the N-fertilized treatment reflected both variability in the data and the absence of a distinct moisture optimum. The contrast between the flat response to moisture in N+ and clear peaked moisture response in CTR highlights potential treatment-related shifts in environmental sensitivity, but also underscores the need to interpret model-based extrapolations with caution."

6. Despite a modest R^2 (~0.4; Table 2), the authors extrapolate hourly Rh/SOC fluxes across three years. This modeling approach is risky and not supported by comprehensive seasonal data (limited to May–October), especially given that winter fluxes are modeled without measurements.

We agree that extrapolating $R_{h/SOC}$ *fluxes year-round, particularly in the absence of winter measurements, introduces uncertainty. To address this, we have added a clarification in the Discussion about the potential limitations of annual estimates.*

Although direct winter flux measurements were not available, the model extrapolations are based on measured temperature responses, and both treatments (CTR and N+) showed a similarly strong temperature limitation at low soil temperatures (Fig. 4). Specifically, when soil temperature fell below 5 °C, modeled $R_{h/SOC}$ *values approached zero for both treatments (Fig. 5). Because these wintertime fluxes were minimal and similar between treatments, their contribution to total annual* $R_{h/SOC}$ *was negligible, and the impact on annual treatment differences was minor.*

We have reformulated and added the following statement to the Discussion:

" Annually, N-fertilized plots respired 10.3% of SOC (± 0.3 SE), compared to 12.2% (± 0.5 SE) in CTR plots. Although the difference was derived from the modeled values, the lower respiration rate in fertilized plots suggests a potential increase in microbial carbon use efficiency, which may contribute to long-term SOC accumulation. Despite the winter fluxes not being directly measured, modeled values under low soil temperatures (<5 °C) were close to zero for both treatments due to strong temperature limitation observed in measured data. As a result, differences in winter $R_{h/SOC}$ contributed minimally to annual estimates and are unlikely to have significantly biased treatment comparisons. "

7. The claim of net GHG mitigation (–327.6 g CO_2 m^{-2} yr^{-1}; line 280) is based on a weakly supported model and fails to account for the full life-cycle impacts of fertilization (e.g., leaching, offsite emissions, biodiversity loss).

We agree that the estimated net GHG mitigation of -327.6 g CO_2 m^{-2} yr^{-1} should be interpreted with caution. This value is based on model-derived $R_{h/SOC}$ data from a three-year period and does not fully account for all potential life-cycle impacts of fertilization, including nitrogen leaching, offsite emissions, and changes in biodiversity.

To reflect this, we have revised the Conclusions to include the following statement (lines 435-439):

"The estimated net GHG mitigation of -327.6 g CO_2 m^{-2} yr^{-1} based on $R_{h/SOC}$ model outputs from a three-year period provides a first-order approximation. However, this estimate likely underrepresents the full climate impact of fertilization, as it does not account for longer-term dynamics or potential offsite carbon and nitrogen losses, such as leaching, indirect emissions, or biodiversity-related feedback. Therefore, broader system-level assessments over longer time scales are needed to confirm these findings."

8. Claims such as "enhanced microbial carbon use efficiency" (e.g., line 385) are not empirically validated. No microbial data (e.g., biomass, CUE assays, extracellular enzyme activity) are presented.

We agree with the reviewer that the claim of "enhanced microbial carbon use efficiency" was not directly supported by microbial process data (e.g., biomass, CUE assays, enzyme activity). To avoid overinterpretation, we have added following sentence to discussion (line 405):

"However, in our study CUE could not be directly inferred from our data, as microbial process measurements were not conducted."

9. The inference that N-induced changes in Rh/SOC response curves reflect "microbial adaptation" (line 360) is speculative without supporting microbial community or functional data.

We removed the only claim on "microbial adaptation" (line 336).

10. CH_4 and N_2O fluxes are near detection limits, and treatment differences are marginal (e.g., line 200). Nevertheless, they are used to calculate CO_2-equivalent changes with unrealistic precision (±0.5 g CO_2-eq m^{-2}; Table 3), which exaggerates their ecological relevance.

Thank you for the comment. As noted on line 204, the standard errors (SE) of the mean CH_4 (0.002 mg CH_4 m^{-2} h^{-1}) and N_2O fluxes (0.09 μg N_2O m^{-2} h^{-1}) used in the calculation of annual CO_2-equivalent SE (Table 3) are indeed smaller than the calculated detection limits for CH_4 (0.0238 μg m^{-2} h^{-1}) and N_2O (0.0524 μg m^{-2} h^{-1}). This is because the SE reflects variability among replicate measurements rather than the detection limit of individual measurements. Therefore, although individual flux measurements are near detection limits, the precision of the mean flux estimates and their propagated uncertainty in CO_2-equivalents remains reliable and justifies the reported SE values.

Specific Comments

1. Line 25: "Enhancing annual Rh/SOC" is contradictory if Rh/SOC is reduced annually under N+ (Fig. 6).

*To avoid any contradiction, we revised the sentence "**contrasting with a distinct humped SWC response enhancing annual $R_{h/SOC}$ in control plots**"*

2. Figure 3e–f: Soil temperature and moisture differ only slightly between plots, yet these small differences are used to explain large GHG flux differences. This is overstated.

We agree that the overall differences in soil temperature and moisture between plots are small. However, it is not the magnitude of these differences alone that explains the GHG flux variation, but rather the differing sensitivities and dependencies of soil respiration to soil moisture and temperature under different treatments.

We clarified this point in the manuscript to avoid overstating the role of small absolute differences in environmental variables.

3. Figures 5–6: These figures show model outputs rather than direct observations and should not be interpreted as empirical findings without caution.

We revised the manuscript (e.g., revised chapter 4.3) to clearly distinguish between model outputs and empirical measurements, emphasizing the model-based nature of simulated lines in Figures 5a and annual sums in Figure 5b and the associated uncertainties.

4. Line 300: "Reduced microbial respiration" contradicts earlier statements that Rh increased in fertilized plots.

Thank you for pointing this out. To clarify, the phrase "reduced microbial respiration" in line 300 was intended to refer specifically to a reduction in the $R_{h/SOC}$ ratio, rather than an absolute *decrease in R_h. We corrected this in added text (line 324).* ***"Although, results from our study suggested a reduction in the $R_{h/SOC}$ ratio, rather than an absolute decrease in R_h."***

5. Stating that the models showed "relatively large residuals" but "mean bias errors were close to zero" is not a valid justification for model adequacy.

We agree that simply stating "mean bias errors close to zero" is insufficient to justify model adequacy. Although our empirical models used to evaluate the sensitivity of soil respiration to soil temperature and moisture showed a moderate coefficient of determination ($R^2 \approx 0.4$), other performance metrics - such as root mean square error (RMSE), mean absolute error (MAE), and mean bias error (MBE) - were relatively low, suggesting that the models captured overall trends with acceptable accuracy.

However, ***we removed these details in the conclusions to focus on the actual results***

6. All tables should be formatted using the standard three-line table format.

Thank you for this formatting reminder which we now follow accordingly.