Response prepared for Referee #2 comments egusphere-2026-3815:

"Soil horizon development and altitudinal-vegetational zonation decouple microbial biomass from bulk soil nutrient availability across a Himalayan treeline ecotone."

(Note: for the clear distinction, reviewer's comments are in light blue color and corresponding response are in automatic text color.)

This manuscript investigates microbial biomass C, N, and P and microbial stoichiometry across soil horizons and altitudinal–vegetational zones in a Himalayan treeline ecotone. The study addresses an interesting topic and provides a useful dataset across contrasting vegetation zones and genetic soil horizons. However, several methodological and interpretative issues need further clarification. The main concerns relate to the preparation of soil samples before microbial biomass analysis, the calculation and interpretation of relative horizon contributions from mass-based concentrations, and several mechanistic interpretations that extend beyond the variables directly measured in this study. The statistical interpretation and consistency between the results and conclusions should also be carefully checked. My specific comments are provided below.

R: We thank the reviewer for the careful evaluation of our manuscript and for recognizing the value of the dataset across contrasting vegetation zones and soil horizons. We appreciate the constructive comments, which will help us improve the clarity, methodological transparency, and interpretation of our findings. In response, we will clarify the potential effects of soil sample preparation on microbial biomass measurements, revise the calculation and interpretation of horizon contributions, and ensure that our statistical results are described consistently with their significance levels. We will also revise the Discussion and Conclusion to distinguish the observed patterns in microbial biomass, soil nutrient concentrations, and microbial stoichiometry from potential mechanisms that were not directly assessed in this study. The specific comments are addressed individually below.

Abstract

Horizon contributions

The statement that the O-horizon accounted for 55.7%, 62.0%, and 62.8% of MBC, MBN, and MBP should be reconsidered. These values are based on mass-based concentrations rather than stocks. Concentrations from different horizons cannot be directly summed to represent their contribution to the whole soil profile without considering horizon thickness and soil mass or bulk density. Please revise this interpretation throughout the manuscript.

R: Thank you for this comment. We agree that the previously reported values of 55.7%, 62.0%, and 62.8% should not be interpreted as stocks. We will therefore remove these percentage-contribution estimates and the associated interpretation throughout in the revised manuscript.

The results will be presented in terms of microbial biomass concentrations for each soil horizon, and the interpretation will be revised accordingly. For example, we will describe the O-horizon as having the highest microbial biomass concentrations rather than assigning it a percentage contribution to the overall soil profile. This revision will be made consistently in the Abstract, Results, Discussion, and Conclusion to avoid interpreting horizon-level concentrations as profile-level biomass stocks.

Stoichiometric plasticity

The statement that the microbial biomass stoichiometry of 3.4:0.3:1 indicates stoichiometric plasticity is too strong. The observed ratios show differences from globally reported values, but they do not directly demonstrate microbial physiological plasticity. Please revise this interpretation.

R: Thank you for this valuable comment. We agree and therefore will revise the manuscript to distinguish the observed variation in microbial biomass stoichiometry from mechanistic interpretations.

Statements implying that the observed ratio directly demonstrates stoichiometric plasticity or nutrient constraints will be revised. We will make these changes in the Discussion and Conclusion to ensure that our interpretations remain consistent with the variables measured in this study.

Introduction

Environmental stress

Please check the discussion of environmental stress. High pH is mentioned as an environmental stress, whereas the soils at the study site are described as strongly acidic. Please revise this paragraph to better reflect the conditions relevant to the study area.

R: We sincerely appreciate the reviewer's careful observation. and will correct this statement and paragraph to refer to low pH in the revised manuscript to ensure consistency with the soil conditions at our study site.

Microbial CUE

Please use caution when discussing microbial CUE because CUE was not measured in this study. The relationship between the observed biomass stoichiometry and CUE should not be presented as a mechanism directly evaluated here.

R: Thank you for this important comment. Yes, it's true that microbial carbon-use efficiency was not directly measured in this study. We will therefore revise this statement in the discussion section to avoid implying that the observed microbial biomass stoichiometry directly demonstrates changes in CUE. The discussion will now distinguish measured microbial biomass C:N ratios from "potential" mechanisms involving microbial carbon allocation and utilization.

Hypothesis 3

Please revise H3. Differences in microbial biomass stoichiometry can be tested directly, but "nutrient constraints" and "microbial adaptation" are mechanistic interpretations that were not directly measured in this study.

R: Thank you for this helpful suggestion. We agree and therefore will revise H3 to focus on measurable differences in microbial biomass C:N ratios among soil horizons and altitudinal–vegetational zones. This revision aligns the hypothesis with our measured variables and statistical analyses while avoiding assumptions about underlying mechanisms that were not directly evaluated. And we also consider the similar suggestion of Referee #1 to revise the H3 accordingly.

Materials and Methods

Soil sampling and laboratory analysis

Soil drying before microbial biomass analysis — major concern

Please justify the use of soil dried at 40°C for microbial biomass analysis. Drying followed by rewetting and three weeks of incubation may alter the microbial biomass compared with the original field condition. Please discuss how this sample preparation may affect the interpretation of MBC, MBN, and MBP.

R: Thank you for raising this important methodological concern. Soils were dried at 40°C, rewetted and pre-incubated for three weeks prior to analysis, MBC, MBN and MBP represent the microbial biomass re-established under standardized laboratory conditions rather than the in-situ field stock and should not be compared directly with absolute values measured on field moist soils. Drying rewetting is known to reduce microbial biomass, particularly MBP, and to alter community composition and elemental stoichiometry (Khan et al., 2019; Sawada et al., 2019; Sparling & Ross, 1988). Since all samples were

treated identically, relative differences among sites are consistent and interpretable. They suit comparisons between treatments or sites that were all handled the same way.

Expression of soil properties

Relative contribution of soil horizons — major concern

Please reconsider the calculation and interpretation of the relative contribution of each horizon. Concentrations expressed per unit soil mass cannot be directly summed across horizons to represent their contribution to the whole soil profile without considering horizon thickness and soil mass or bulk density.

R: Thank you for highlighting this important calculation and interpretation of the result. As also addressed in our response to the reviewer's earlier comment on horizon contributions in the Abstract, we agree that mass-based microbial biomass concentrations from different soil horizons should not be summed to estimate their relative contributions to the whole soil profile. The previously reported percentages were derived from horizon-specific concentration values and therefore should not have been interpreted as contributions to total soil-profile microbial biomass. We will take this into account throughout the text.

Statistical analysis

MBP model simplification

Please provide more details on the simplification of the MBP model. Specify which interaction terms were removed and how the final model structure was selected after the singularity problem was identified.

R: Thank you for this important comment. We agree that the statistical approach used for MBP requires further clarification. In our analysis, the mixed-effects model for MBP encountered a singularity issue. We therefore fitted an ordinary linear model, retaining Zone, Aspect, and Soil Horizon as fixed effects, together with all two-way interactions among these factors, but without the random intercept for Plot ID, which was used for MBC and MBN. Thus, the model structure was changed by excluding the random effect rather than by removing individual interaction terms. We will revise the Statistical Analysis section to clarify the model specification and distinguish the MBP analysis from the mixed-effects models used for MBC and MBN in our revised manuscript.

Zero or negative CFE flushes

Please provide further justification for treating all zero or negative CFE flushes as experimental artifacts and assigning them as missing values. Please clarify whether these observations were checked for analytical or procedural problems before exclusion.

R: Thank you for this important comment. We have checked the analytical records and calculations for the observations with zero or negative CFE flushes to assess potential analytical or procedural problems. As these non-positive net flushes do not provide valid positive estimates of microbial biomass using our calculation procedure, the affected observations were excluded from the corresponding biomass analyses and treated as missing values. We will revise the Methods section to clarify the checks performed, the rationale for exclusion, and the number of affected observations for MBC, MBN, and MBP. We will also acknowledge that these values may reflect very low microbial biomass or measurement variability and should not automatically be interpreted as confirmed experimental artifacts.

Results

Figures 3–6: Please improve the overall quality and readability of these figures. Some labels, symbols, and text are difficult to read at the current size. Please increase the figure resolution and ensure that all graphical elements remain clearly legible at publication size.

R: Thank you for this helpful suggestion. We will revise Figures 3–6 to improve their overall presentation and readability. We will increase the figure export resolution and adjusted text sizes, labels, symbols, and graphical layouts where necessary to ensure that the figures remain legible at publication size.

Soil organic carbon and total nitrogen

Profile contributions

Please revise this interpretation according to the comment in the Methods regarding the calculation of horizon contributions.

R: Thank you for this comment. As explained in our response to the earlier comments regarding horizon contributions, we have agreed that concentration-based measurements cannot be interpreted as contributions to the total soil profile. We will remove the profile contribution percentages and revise the corresponding interpretations of soil organic carbon (SOC) and total nitrogen (TN) throughout the manuscript, presenting the results in terms of horizon-specific concentrations instead.

Microbial biomass

Non-significant zone effect

The effect of altitudinal–vegetational zone was not significant ($p = 0.259$). Please avoid describing these differences as a distinct pattern and present them as descriptive trends only.

R: Thank you so much for pointing this out. We will revise the Results section to present the differences among zones as descriptive trends rather than statistically supported differences and ensure that the interpretation remains consistent with the statistical results.

$p = 0.058$

The interaction was not statistically significant at the stated $\alpha = 0.05$ ($p = 0.058$). Please avoid referring to it as “marginally significant.”

R: Thank you so much for pointing out this. We will remove the term “marginally significant” and revise the Results section to report the interaction as statistically non-significant, avoiding interpretations that imply a statistically supported interaction.

Microbial stoichiometry

Overall, 3.4:0.3:1 ratio

Please clarify how the overall microbial biomass stoichiometry of 3.4:0.3:1 was calculated, particularly because MBC, MBN, and MBP contained different numbers of missing observations.

R: Thank you so much for this comment on how we calculated the tri-ratio. We have derived this tri-ratio by dividing the mean MBC and mean MBN setting MBP to 1.

Very high variability

The MBC:MBN ratio in the E-horizon shows extremely high variability (CV = 396.9%). Please discuss this variability when interpreting the approximately 17.8-fold difference from the O-horizon.

R: Thank you so much for this comment. We will revise the Results and Discussion to explicitly acknowledge the high within-horizon variability and clarify that the fold difference reflects a contrast in mean values rather than a uniform difference across samples.

Discussion

4.1 Vertical pedogenic control

"Biological activity"

Please revise "biological activity" because microbial biomass was measured, but microbial activity was not directly assessed.

R: Thank you so much for correctly pointing out this. Yes, we will revise the text and wording to refer to microbial biomass rather than activities so that the interpretation remains consistent with the measured variables.

4.2 Decoupling between microbial biomass and bulk soil nutrients

qMIC and microbial efficiency

This interpretation is too strong. Higher qMIC indicates a greater proportion of SOC present as microbial biomass, but it does not directly demonstrate more efficient substrate utilization or faster nutrient cycling.

Biologically available nutrient fractions

This statement is not directly supported by the measured variables. Biologically available nutrient fractions and microbial activity were not measured in this study. Please revise this interpretation.

R: Thank you for these comments regarding qMIC and biologically available nutrient fractions. We agreed and will revise the Discussion to ensure that our interpretations remain consistent. Specifically, we will clarify that qMIC indicates the proportion of organic carbon soil present as microbial biomass, rather than directly demonstrating more efficient substrate utilization. We will also rephrase the discussion of biologically available nutrient fractions to distinguish the observed patterns in microbial biomass and soil nutrient concentrations from interpretations involving nutrient availability. Thank you so much for this crucial comment.

4.3 Microbial stoichiometry and nutrient constraints

Stoichiometric plasticity

Please revise this interpretation according to the earlier comment regarding microbial stoichiometric plasticity.

R: Thank you for this comment. As addressed in our response to the earlier comment regarding stoichiometric plasticity, we will revise the Discussion to describe the observed differences in microbial biomass stoichiometry without interpreting deviations from globally reported ratios as direct evidence of microbial stoichiometric plasticity or physiological adaptation. We will not claim for direct evidence of microbial plasticity rather discuss deviation from global reported ratios.

Severe N limitation

Please use caution when interpreting high MBC:MBN ratios as evidence of severe N limitation. Nitrogen limitation and the proposed shifts toward maintenance and nutrient scavenging were not directly measured in this study.

R: We agreed and hence will revise the Discussion to present these mechanisms as possible explanations rather than demonstrated processes (e.g. MBC/MBN ratio as a relative amount of carbon and nitrogen in microbial biomass rather quantifying nitrogen availability) and to ensure that our interpretations remain consistent with the variables measured in this study.

4.4 Ecological implications

Nutrient availability and microbial utilization efficiency

Please use more cautious wording here because nutrient availability and microbial utilization efficiency were not directly measured.

R: Thank you so much for this crucial comment. We agreed and we will revise discussion section to distinguish our observed patterns in microbial biomass and soil nutrient concentrations from "potential" ecological explanations, avoiding conclusions about nutrient availability or microbial utilization efficiency.

Length of Discussion

The Discussion is quite lengthy and includes several mechanistic interpretations that were not directly evaluated in this study. Please consider condensing the Discussion and clearly distinguishing the observed results from proposed mechanisms.

R: Thank you for this helpful suggestion. We will condense the Discussion, particularly Sections 4.2–4.4, by removing repetitive content and reducing speculative mechanistic interpretations highlighted in our responses to the previous comments (e.g., explanations involving microbial utilizations efficiency, nutrient availability, and microbial stoichiometric plasticity). We will retain the key findings and focus on the measured variables, while revising or removing passages that are repetitive or extend beyond the evidence provided by our results.

Conclusion

Biologically available substrates

Please use more cautious wording because nutrient accessibility was not directly measured in this study.

Nutrient accessibility

Please use more cautious wording because nutrient accessibility was not directly measured in this study.

Nutrient constraints and stoichiometric flexibility

This conclusion is too strong. Differences from global microbial stoichiometric ratios do not directly demonstrate nutrient constraints or microbial stoichiometric flexibility. Please revise this statement.

R: Thank you for these comments. We will revise the Conclusion to use more cautious wording regarding nutrient accessibility, focused on directly measured variables in this study. Specifically, we will replace statements that imply differences in nutrient accessibility or substrate availability with descriptions of the observed patterns in microbial biomass and soil nutrient concentrations, presenting potential explanations as interpretations rather than direct findings. We will also revise statements concerning nutrient constraints and microbial stoichiometric flexibility. For example, rather than interpreting deviations from globally reported microbial stoichiometric ratios as direct evidence of nutrient constraints or microbial adaptation, we will describe the observed differences in microbial biomass C:N ratios and acknowledge that their underlying mechanisms cannot be established from our measurements alone. These revisions will ensure that the conclusions remain closely aligned with the results and the scope of the study.

References

Khan, S. U., Hooda, P. S., Blackwell, M. S. A., & Busquets, R. (2019). Microbial Biomass Responses to Soil Drying-Rewetting and Phosphorus Leaching. *Frontiers in Environmental Science*, *7*. https://doi.org/10.3389/fenvs.2019.00133

Sawada, K., Funakawa, S., & Kosaki, T. (2019). Immediate and subsequent effects of drying and rewetting on microbial biomass in a paddy soil. *Soil Science and Plant Nutrition*, *65*(1), 28–35. https://doi.org/10.1080/00380768.2018.1534217

Sparling, G. P., & Ross, D. J. (1988). Microbial contributions to the increased nitrogen mineralization after air-drying of soils. *Plant and Soil*, *105*(2), 163–167. https://doi.org/10.1007/BF02376779