Response prepared for referee #1 comments on 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, the reviewer's comments are in light blue color, and the corresponding responses are in automatic text color)

In this manuscript, Adhikari et al. investigated the spatial distribution and stoichiometric relationships of soil microbial biomass (MBC, MBN, MBP) across an altitudinal–vegetational gradient and four soil horizons in the Rolwaling Himalayan treeline ecotone, Nepal. This manuscript addresses a critical knowledge gap regarding soil microbial dynamics and nutrient stoichiometry in high-elevation ecosystems. The dataset combined both soil horizon differentiation and altitudinal-vegetational zonation in an understudied region (the Himalayas). A particularly interesting and well-presented finding is the empirical demonstration of a clear decoupling between bulk soil nutrient availability (SOC, TN) and microbial biomass (MBC, MBN), as well as the unusually low C:N:P ratio (3.4:0.3:1) indicating microbial stoichiometric plasticity under environmental stress. Overall, the manuscript is well-written and logically structured. However, there are several conceptual, methodological, and interpretive limitations that need to be addressed before publication. Below are my detailed major and minor comments:

R: We sincerely thank the reviewer for the careful and constructive evaluation of our manuscript and for recognizing the relevance of the study. We greatly appreciate the reviewer's detailed comments, which have helped us identify areas requiring clarification, methodological detail, and more cautious interpretation. Specifically, reviewer comments were very helpful to minimize reflective speculations in the discussion. We have carefully considered each point and will revise the manuscript accordingly, with particular attention to strengthening the conceptual framing, clarifying methodological procedures, and ensuring that our interpretations remain appropriately supported by the data. Our point-by-point responses to all comments showing how we will revise the new version of the manuscript are provided below:

line 48, please specify the altitude information (elevation range in meters above sea level, e.g., m a.s.l.) for the treeline in the Rolwaling Himal to provide necessary spatial context for the reader.

R: We agree with the reviewer's suggestion, and we will add the treeline elevation range (approximately 3900–4000 m a.s.l.) in the Study Area section of the manuscript to provide spatial context for the study system.

Line 66: The statement that "In high-altitude treeline regions, low soil temperature and recalcitrant organic inputs slow down the decomposition of organic matter" is somewhat too absolute and generalized. In Section 4.2, you correctly highlight that substrate quality plays an important role in regulating microbial processes. To ensure logical consistency throughout the manuscript, please rephrase this statement and briefly mention the modulating influence of substrate quality and micro-environmental variability.

R: We agree with the reviewer's suggestion and will revise this statement. The revised text will emphasize that decomposition and nutrient mineralization in high-altitude treeline ecosystems are influenced by the combined effects of temperature, substrate quality, vegetation composition, soil properties, and micro-environmental variability with added supporting references.

Lines 101–105: The main objective is not clearly described here. Please consider rephrasing this section.

R: Thank you so much for your comment pointing this out. We will revise the final paragraph of the Introduction in the revised manuscript to clearly state the overall objective of the study and the specific aspects investigated before presenting the hypotheses (e.g. "this study aims to investigate how soil

horizon development and altitudinal–vegetational zonation influence microbial biomass C, N, and P distributions and microbial stoichiometric relationships across the Rolwaling Himalayan treeline ecotone“).

Figure 1: Please add the directional indicators (E for East longitude and N for North latitude) to the geographic coordinate labels on the map to ensure standard cartographic formatting.

R: We agree with the reviewer’s suggestion and will revise Figure 1 by adding directional indicators (N and E) to the geographic coordinate labels to improve cartographic clarity.

Figure 2: Please consider adding explicit elevation tick marks and values to the vertical axis (elevation axis) to make the altitudinal gradient clearer and easier for readers to interpret visually.

R: We agree with the reviewer’s suggestion. We will revise Figure 2 by adding explicit elevation marks and values (m a.s.l.) to the vertical axis to improve visualization and interpretation of the altitudinal gradient.

Figure 2 & Table 1: In the caption of Figure 2, you refer to "NE and NW aspects", whereas in Table 1, you use "NE slope, NW slope". Slope (gradient/inclination) and slope aspect (cardinal direction/exposure) are distinct topographic terms. To maintain consistency, please adopt a uniform terminology, e.g., "aspect" or "slope aspect", throughout the entire manuscript, including figures, tables, and main text.

R. Thank you so much for pointing out this. We agree with the reviewer’s comment. We will revise the terminology throughout the manuscript and now use “slope aspect” when referring to slope exposure direction (e.g., NE and NW slope aspects) to ensure consistency between figures, tables, and text.

Table 1: under the "Zone characteristics" column for the UD zone, the descriptive text is set in italics. Only the Latin botanical names should be italicized. Please ensure that all Latin vegetation/species names throughout the manuscript are consistently formatted in italics, and that non-taxonomic text remains in standard font (e.g., check Table 1, line 155, line 432, line 480, among others).

R: We agree with the reviewer’s comment and will carefully check the manuscript for botanical nomenclature formatting. We will correct the italicization so that only Latin taxonomic names are italicized, while descriptive ecological terms and non-taxonomic text remain in regular font.

Section 2.3 Field Sampling: Please provide the explicit sampling depth information (depth ranges in centimeters, e.g., 0–10 cm, 10–20 cm) for each genetic soil horizon/site sampled in Section 2.3. Additionally, please consider summarizing these depth ranges clearly in Table 1 or Table 2 to give readers a complete vertical reference for the soil horizon layers.

R: Thank you so much for pointing this out. We will add the depth information of the sampled soil horizons in the Field Sampling section and will summarize the corresponding depth ranges of each horizon in Table 1 to provide a clearer vertical reference for the soil profile.

Section 2.4 Laboratory Analysis. (i)Line 170: Please briefly explain the rationale for choosing 20 °C as the incubation temperature (e.g., standard laboratory incubation protocol vs. field temperature relevance) and provide the corresponding reference. (ii)Lines 172–173: The authors state that "a total of 7.5 g of moist soil" was used. Please explain why a 7.5 g sample mass was selected instead of a more standard weight (e.g., 5 g or 10 g). (iii)Lines 188–189: You mention that "carbonates were absent from the soil." Please state how this was confirmed. Did you perform a field/laboratory test (such as dilute HCl effervescence or total inorganic carbon analysis)? If so, please specify the analytical method used here.

R: We appreciate the reviewer’s request for clarification. We will revise the Methods section to clarify that incubation at 20 °C was applied as a standard laboratory procedure for microbial biomass

estimation under controlled conditions, rather than to reproduce the actual soil temperature in the field. We will also clarify that the 15 g samples were weighed. The volume of organic layer soil was very large to adjust our lab equipment and revised as per the local adjustment for the lab facility (7.5 g for fumigated and 7.5 g for non-fumigated). Moisture correction was applied later in the calculation. In addition, we will specify that the absence of carbonates was confirmed using the dilute HCl effervescence test in the field.

Section 2.5 Statistical Analysis. Line 206: (i)Please assign a formal equation number (e.g., Eq. 1) to the model formula for easy reference. (ii) If I understand correctly, in the formula log10(Y) ~ (zone + aspect + soil horizon)2 + (1|plot id), the notation "2" uses R formula syntax to represent all main effects and two-way (second-order) interactions, right? To avoid confusion with quadratic or polynomial transformations (e.g., squaring a variable), please explicitly clarify in the text that "2" denotes the inclusion of all two-way interaction terms among the three fixed factors.

R: We agree with the reviewer's suggestion. We will assign an equation number to the model formula and will clarify that the "^2" notation in the R formula represents the inclusion of all main effects and two-way interactions among the fixed factors (zone, aspect, and soil horizon), rather than a quadratic transformation.

Figure 3: The abbreviations LC, UC, LD, and UD are referred to as "four topographic zones" in the caption of Figure 3, but as "four altitudinal–vegetational zones" in other parts of the manuscript. Please confirm whether both terms refer to the exact same zones. If so, please adopt a single, uniform naming convention throughout the manuscript (e.g., "altitudinal–vegetational zones"). Consider briefly describing their key topographic features (e.g., convex, depression) in the main text or summarize them in Table 1 to clarify the distinction between topographic and vegetational drivers.

R: We agree with the suggestion. The terms referred to the same four zones; however, "topographic zones" was not the most appropriate terminology because the zones were defined based on elevation and vegetation characteristics. We will revise the manuscript to consistently use "altitudinal–vegetational zones" throughout Figure captions, tables, and text.

Use of Abbreviations.The abbreviation for Total Nitrogen (TN) is defined multiple times in the main text (e.g., Line 169, Line 238). An abbreviation should be defined at its first mention and used consistently thereafter. In addition, for terms like Soil Organic Carbon (SOC), the full name is repeated in later sections even after the abbreviation was already established. This undermines the purpose of using abbreviations. Please carefully proofread the entire manuscript to ensure that all technical abbreviations (SOC, TN, MBC, MBN, MBP) are defined once upon first occurrence and used consistently throughout all subsequent text (caption of tables and figures, abstract are not included).

R: Thank you so much for pointing this out. We will carefully check the manuscript and will ensure that abbreviations such as SOC, TN, MBC, MBN, and MBP are defined at their first occurrence and used consistently thereafter. Repeated definitions and unnecessary use of full terms will be removed.

Figure 4. (i)Letter sequence: Please ensure that the lowercase letters indicating significant differences follow standard scientific convention, starting with 'a' for the horizon with the greatest mean/median value and descending progressively (a > b > c). (ii)Label placement: Please reposition all letters slightly above the upper bound of the error bars to prevent overlap and improve visual clarity. (iii)Missing annotations: In the MBC plot, the letters for the Bh horizon are missing. Please check whether this is a plotting oversight or due to data constraint, provide the missing statistical annotations, and clarify the cause if data for this horizon were excluded from the analysis.

R: We thank the reviewer for pointing this out. We will check the statistical annotations and will revise the placement of the lowercase letters to improve their visibility. Just to make it clear, the absence of the Bh-horizon annotation in the MBC panel is not a plotting omission: the Bh horizon was not present

in the UD zone where soil with shallow depths occur. We will clarify this in the figure caption of the revised manuscript.

Table 2. Please correct the unit for SOC. The current notation "mgg-1 soil" lacks proper spacing. Please update it to standard format: mg C g-1 soil". Please add a space before all "±" symbols in Table 2 (e.g., replace 12.4± 0.5 with 12.4 ± 0.5) to ensure symmetric formatting throughout the table.

R: Thank you so much for pointing this out, we will correct and make the systematic formatting throughout the revised manuscript for unit and symbols. Thank you so much for pointing out in detail, it is very helpful.

Figure 5. (i)The unit for bulk soil organic carbon is given as "g C kg-1 soil" in the horizontal axis and caption of Figure 5, whereas "mg C g-1 soil" is used in Table 2 and Figure 3. Using different unit expressions across figures and tables creates unnecessary confusion. Please adopt a single, uniform unit notation across all figures, tables, and text. (ii)Statistical symbols such as R2 (coefficient of determination) and p (probability value) in the caption of Figure 5 should be italicized. Please check and ensure that statistical abbreviations and variables are consistently italicized throughout the entire manuscript.

R: We thank the reviewer for noting these formatting inconsistencies. We will standardize the SOC unit in Figure 5 and its caption to mg C g^{-1} soil, consistent with Table 2 and Figure 3. We have also checked the manuscript and will revise the formatting of statistical symbols, including R^2 and p, to ensure consistent italicization throughout the revised manuscript.

Please consider increasing font size in figure 6 and figure 4.

R: Thank you so much for the comment, Figures 6 and 4 will be revised with increased font size in revised manuscript.

In Section 3.2.4, you present results from a Principal Component Analysis (PCA). However, there is no corresponding description of this statistical method in the Methodology section. Please add a dedicated subsection or paragraph detailing the PCA methodology.

R: Thank you for pointing this out. We will add a dedicated description of the PCA procedure to the Methods section. The revised manuscript text will specify the variables included in the PCA, treatment of missing observations, standardization of variables prior to analysis (we have done this), the R package and function used for the PCA, and the procedure used to visualize the resulting ordination by vegetation zone.

Line 425-426, The authors attribute the decline in microbial biomass with soil depth primarily to "increasing energetic and chemical constraints in mineral soil horizons." However, as noted in Line 423 regarding organic matter occluded within soil aggregates, physical protection mechanisms significantly limit microbial accessibility to substrate. Besides, oxygen availability decreases substantially with increasing soil depth, imposing severe aerobic metabolic constraints on subsoil microbial communities. Please broaden this paragraph to explicitly incorporate physical protection and depth-dependent oxygen limitation alongside energetic and chemical constraints when explaining subsoil microbial biomass dynamics.

R: Thank you so much for these very crucial comments on the discussion. We will broaden the discussion of the decline in microbial biomass with soil depth to include not only reduced substrate and energetic availability but also potential effects of physical protection of organic matter and reduced oxygen availability with increasing depth (e.g. including the reference from (Wilpiszeski et al., 2019; Tecon & Or, 2017). These mechanisms will be presented as potential explanations rather than directly measured processes.

Line 438, check the format of the unit “1300mm yr-1”.

R: Many thanks for pointing this out, checked and corrected as 1300 mm yr^{-1}.

Lines 465-466, you stated that “soil nutrient concentrations (SOC and TN)”. SOC represents an organic substrate and energy source, rather than a nutrient. While TN, TP, and inorganic nitrogen forms are nutrients, carbon provides the structural/energetic framework for microbial heterotrophy. Please rephrase this phrasing and check the entire manuscript to ensure SOC is not incorrectly classified as a soil nutrient.

R: We thank reviewer for this correction. We will revise the wording to distinguish SOC from nutrients and now describe SOC primarily as an organic carbon substrate and potential energy source for microorganisms. We have also checked the manuscript for similar terminology and will revise the relevant statements for consistency throughout the revised manuscript.

Lines 475-485. (i)The authors interpret elevated qMIC as evidence for “more efficient microbial utilization” and “faster nutrient cycling.” However, qMIC is a stock ratio (MBC/SOC) and does not directly measure carbon use efficiency (a flux ratio) or nutrient turnover rate. High qMIC indicates greater immobilization of carbon in microbial biomass, which may reflect lower turnover, shifts in community composition, or differences in substrate quality, rather than faster nutrient cycling. (ii) The inference from “reduced surface litter retention and increased exposure to wind and radiation” to “greater incorporation of organic substrates into mineral soil” lacks an explicit mechanism. Exposure to wind and radiation more commonly implies loss (erosion, photomineralization, leaching), not downward incorporation. If the authors wish to argue for enhanced incorporation into mineral soil, they should articulate the intermediate pathway, e.g., photodegradation and physical fragmentation of surface litter into finer, more soluble fragments that are subsequently leached into mineral horizons and ideally support it with relevant measurements (DOC flux, litter fragment size, or isotopic source tracing). Without this, the causal link remains speculative.

R: We appreciate this correction regarding the interpretation. We will revise the interpretation of qMIC to reflect that it is a stock-based MBC:SOC ratio rather than a direct measure of carbon-use efficiency, microbial efficiency, or nutrient turnover in our revised manuscript. We will also revise the discussion of litter exposure and carbon incorporation to avoid implying a causal mechanism that was not directly measured in this study. We will describe photodegradation, physical fragmentation, and subsequent production and leaching of soluble organic compounds into mineral horizons only as potential pathways, supported by previous studies (e.g. (Brandt et al., 2009) rather than as processes demonstrated by our data.

Section4.3. The authors state “microbial stoichiometric ratios differed substantially from globally reported averages, supporting our third hypothesis that microbial communities exhibit localized stoichiometric responses to environmental conditions”. Deviation from a global average only demonstrates that the system differs from the global mean; it does not demonstrate a response to local environmental conditions. The third hypothesis concerns a response, which requires evidence that stoichiometric ratios covary systematically with local environmental drivers. A single deviation from a global constant cannot provide such evidence, because the deviation may arise from any number of causes, e.g., methodological differences, distinct community composition, site history, rather than from local environmental control. Notably, the authors later report "substantial variation in soil stoichiometric ratios both along the soil profile and across altitudinal and vegetation zones," which is precisely the kind of gradient-based evidence that could support the third hypothesis. However, this evidence is not used to support the claim here; instead, the global comparison is presented as the supporting evidence. This is a mismatch between evidence and argument. Consider reframing the comparison with the global average as a descriptive benchmark rather than hypothesis support.

R: Thank you for this important input. We agree that deviation from a global average provides a descriptive comparison but does not, by itself, demonstrate a localized response to environmental conditions. Moreover, we have this result from only one study area, we have therefore revised section 4.3 so that the comparison with globally reported microbial stoichiometric ratios is presented as a descriptive benchmark. We will discuss the observed variation in microbial stoichiometric ratios along the soil profile and across altitudinal-vegetational zones as relevant evidence while avoiding causal interpretations.

Line 515–516. The sentence starting with "Hence, in alpine ecosystems where..." is a sentence fragment lacking a main predicate. Please revise or merge it with the subsequent sentence.

R: We sincerely thank the reviewer for pointing out this sentence structural error. We will revise this paragraph as: „Similar departures from global stoichiometric expectations have been reported from cold alpine ecosystems of the Tibetan Plateau and Gongga Mountain, where microbial communities display elevated stoichiometric plasticity and strong spatial variability in nutrient allocation (Fan et al., 2021; Sun et al., 2013). Hence, in alpine ecosystems, where low temperatures, nutrient limitations, and strong seasonal fluctuations impose physiological constraints on microbial growth, microbial biomass stoichiometry may reflect local environmental conditions rather than a fixed global elemental composition. “

References:

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Sun, H., Wu, Y., Yu, D., & Zhou, J. (2013). Altitudinal Gradient of Microbial Biomass Phosphorus and Its Relationship with Microbial Biomass Carbon, Nitrogen, and Rhizosphere Soil Phosphorus on the Eastern Slope of Gongga Mountain, SW China. *PLoS ONE*, *8*(9), e72952. https://doi.org/10.1371/journal.pone.0072952

Tecon, R., & Or, D. (2017). Biophysical processes supporting the diversity of microbial life in soil. *FEMS Microbiology Reviews*, *41*(5), 599–623. https://doi.org/10.1093/femsre/fux039

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Scales. *Applied and Environmental Microbiology*, *85*(14), e00324-19.

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