Response to comments by Reviewer #2 (Anonymous Referee)

This article outlines important results, highlighting that bacterial lipid distributions can be impacted by soil water content, likely due to redox changes, in "permafrost soils". Oxygen levels can also influence GDGT distributions, where more methylation and a higher $MI_{ob/br}$ was present in low oxygen conditions. These results are supported by analysis of bacterial genera that may be associated with GDGT production, which has been lacking in the literature and can help constrain additional and more specific sources of these lipids. Discussion of certain taxa being more present in terrestrial or marine sediments helps to outline that differences in GDGT abundances may be associated with differences in microbial communities. These GDGT abundances can also be influenced by environmental conditions that cause physiological adaptation within the bacterial membranes, but this may not be the main driver. The comparison between terrestrial and marine sites also allows for discussion of indices that can be used to estimate redox changes that should be applied in terrestrial soils settings as well as in marine settings. Overall, this paper is appropriate for Biogeosciences but requires some adjustments.

We sincerely thank the reviewer for the insightful comments and positive assessment of the novelty and significance of our research. We have carefully addressed all comments and revised the manuscript accordingly. Our point-by-point responses are provided below in bule, while the corresponding revisions to the manuscript are highlighted in orange.

General comments:

1) Definition of permafrost soils & impact of depth on dataset. The definition of permafrost is ground that has remained at or below 0°C for two or more consecutive years. However, some of the soil you analyze is not at a freezing temperature and, therefore, is not permafrost. How much of each core was active layer (i.e., ground that thaws each summer) vs. permafrost? Defining the size of the active layer could help

clear this up. Tang et al. (2023) refer to the soil samples as from the “active layer of thawing permafrost” and define the approximate depth of the active layer.

We thank the reviewer for this important clarification. All soil samples analyzed in this study were collected from the active layer within the sporadic permafrost zone of the Tibet Plateau. These samples were from the same soil profiles investigated by Tang et al. (2023), where the active-layer thickness was approximately 130 cm at NVI and exceeded 210 cm at LR, confirming that both soil profiles analyzed in this study were within the active layer.

To avoid ambiguity, we have revised the terminology throughout the manuscript, including the Title, Abstract, Introduction, Materials and methods and Conclusions. Specifically, the title of this manuscript has been revised to: Enhanced production and methylation of bacterial branched tetraether lipids responding to water content-mediated redox changes in Tibetan Plateau soils from a permafrost region.
We have also clarified this in the revised manuscript as following:

“Here, we investigated the distributions of sb/br/ob-GDGTs, together with bacterial community composition in two soil profiles with 130-200 cm active-layer thickness from the Tibetan Plateau permafrost region.” (Page 2, Lines 37-39)

“Two soil cores were collected in July and August 2018 from the seasonally thawed active layer in the sporadic permafrost zone of the QTP, specifically at Longren (LR, 30°30'N, 91°13'E, 4257 meters above sea level; hereafter m a.s.l.) and NamCo (NVI, 30°43'N, 91°20'E, 4860 m a.s.l.) (Fig. 1a). These samples were from the same soil profiles investigated by Tang et al. (2023), with detailed site environmental information are shown in Table S1. Samples were collected at 10 cm intervals to maximum depths of 200 cm at LR and 130 cm at NVI, within the active-layer at both sites.” (Page 5, Lines 135-141)

"Based on a comprehensive analysis of sb-, br-, and ob-GDGTs in two active-layer soil profiles from the Tibetan Plateau permafrost region, we found that NVI soils were cooler, more alkaline, and drier, with lower sb-, br-, and ob-GDGT concentrations and methylation indices than LR soils." (Page 26, Lines 612-615)

2) Could depth play a role in the oxygen levels and soil water content percentages? If so, maybe coring deeper in the future could be beneficial and suggesting this as potential future work could help make this point clearer.

Yes, we fully agree with the reviewer's comment. With increasing soil depth, concurrent changes in temperature, porosity, permeability, drainage conditions, and other edaphic properties may influence water retention and oxygen availability. In permafrost regions, the underlying frozen layer may further restrict vertical drainage and promote water accumulation within the active layer, potentially favoring oxygen-limited conditions. Therefore, soil depth and SWC are not independent environmental variables. Our RDA analysis (Fig. S3) identified SWC as the environmental variable most strongly associated with variations in GDGT distributions among the variables measured in this study. We therefore interpret SWC as an important hydrological factor potentially influencing GDGT distributions through its effects on soil oxygen availability and redox conditions, rather than as an independently demonstrated causal driver. While deeper sampling may help resolve vertical environmental gradients, extending the profile into underlying permafrost would introduce distinct physicochemical and microbial conditions, shifting the focus from GDGT variability within the active layer to GDGT distributions across the active layer-permafrost boundary.

3) A summary graphic or table in the results section, or a summary paragraph in the conclusion could help outline the main trends in the NVI vs. LR sites to highlight the main differences between the sites. This would help future studies to compare paleoclimate proxy index results.

We thank the reviewer for this helpful suggestion. We have added a summary paragraph to the Conclusions section to highlight the major differences between the NVI and LR soil profiles. Specifically, we summarized the contrasting environmental conditions (soil temperature, pH, and SWC), the abundances and dominant compositions of sb-, br-, and ob-GDGTs, and their methylation indices in the revised manuscript.

"Based on a comprehensive analysis of sb-, br-, and ob-GDGTs in two active-layer soil profiles from the Tibetan Plateau permafrost region, we found that NVI soils were cooler, more alkaline, and drier, with lower sb-, br-, and ob-GDGT concentrations and methylation indices than LR soils. Among br-GDGTs, non-cyclized IIa and IIIa were prominent in NVI, while Ib and IIb were relatively enriched in LR. OB-1064 and OB-1106 dominated ob-GDGTs in NVI and LR, respectively, and SB-966 dominated sb-GDGTs at both sites." (Page 26, Lines 612-618)

4) Would be good to add some points that the IIIa and IIIa' brGDGTs are commonly found in higher concentrations in cold regions (e.g., Zhao et al., 2023; Otiniano et al., 2023; Raberg et al., 2024). This trend was also observed in another brGDGT study from the Tibetan Plateau at an elevation of 3500-4000m and ~580km southeast of this study (Liang et al., 2023).

We have now expanded the Discussion to acknowledge the higher abundances of IIIa and IIIa' reported in cold-region soils and sediments in the revised manuscript.

"The prominence of IIIa in the colder NVI soils is consistent with previous reports of elevated hexamethylated brGDGTs, including IIIa and IIIa′, in cold-region environments (Zhao et al., 2023; Otiniano et al., 2023; Liang et al., 2023; Raberg et al., 2024)." (Page 18, Lines 415-418)

Specific Comments:

5) Title: With the definition of permafrost soils needing to be adjusted, perhaps the ending of the title could be changed to "…Tibetan Plateau soils from a permafrost region".

We have revised the title to "Enhanced production and methylation of bacterial branched tetraether lipids responding to water content-mediated redox changes in Tibetan Plateau soils from a permafrost region".

6) Abstract: Great abstract that summarizes the main findings of your research. Line 38 refers to the cores analyzed as "permafrost peatland soil profiles", but this terminology is not used again in the paper until the summary/conclusion. This links back to the idea of adjusting the paper depending on whether this was permafrost that was analyzed.

We have revised the abstract to clarify that the samples were collected from two active-layer soil profiles in the Tibetan Plateau permafrost region, ensuring consistency with the terminology used throughout the manuscript.

"Here, we investigated the distributions of sb/br/ob-GDGTs, together with bacterial community composition in two active-layer soil profiles from the Tibetan Plateau permafrost region." (Page 2, Lines 37-39)

7) Introduction: Well done introduction that explains the background required to understand the paper. For further strengthen your introduction, it may be beneficial to add a sentence or two about the specific environmental parameters analyzed (i.e., linking in the redox idea here) at or around line 115.

We have expanded the Introduction to link redox condition with elevated SWC in soil in the revised manuscript.

"Soil hydrology here is regulated by pH, temperature, precipitation, seasonal snowmelt, freeze-thaw processes, and the presence and thickness of the active layer, resulting in pronounced spatial variation in soil water content (SWC). Elevated SWC restricts oxygen diffusion through soil pores, promoting reducing conditions that can restructure microbial communities and associated biogeochemical processes (Marschner, 2021; Pett-Ridge and Firestone, 2005). Such changes may alter the sources and distributions of br-GDGTs, thereby complicating their application as paleoclimate proxies in water-saturated soils (Liang et al., 2023; Raberg et al., 2024; Tang et al., 2024)." (Page 4, Lines 111-119)

Materials & Methods:

8) At Line 130 soil pH measurements were mentioned. Were any pH measurements taken in the field?

Soil pH was measured in the laboratory using soil-water suspensions, rather than directly in the field.

9) It may also be good to include a figure (or a subplot to one of the existing figures) of the sb/br/ob-GDGTs to show the methylation of each lipid.

Thank you for this suggestion. We have revised Fig. 2 to more clearly illustrate the distributions of individual sb-, br-, and ob-GDGTs alongside their methylation indices ($MI_{ob/br}$ and $M_{Iob/br/sb}$).

10) The relative abundances for cren are not listed in the supplemental as well as the values used to calculate Methanobacteria/(Methanobacteria+Thaumarchaeota).

We have added the relative abundances of crenarchaeol and the relevant data used to calculate Methanobacteria/(Methanobacteria + Thaumarchaeota) to the Supplementary Tables.

Results:

11) On line 341, you mention that eight genera had positive correlations, but only list six. Only six of these genera are listed in Table S5, I was just unsure on why two were left out of the explanation. Looking at Fig. 3c, it appears that *Ralstonia* and *Acinetobacter* were the ones left out, which are aerobic. Could these genera be more specific to brGDGTs, but not specifically linked with high SWC or low oxygen levels?

Eight bacterial genera exhibited positive correlations with sb-, br- and ob-GDGT groups, of which six were discussed because of their reported anaerobic physiological traits. The remaining two, *Ralstonia* and *Acinetobacter*, were not highlighted because they have been described as aerobic in the literature. We would like to clarify that their oxygen requirements were classified based on previous studies, whereas their associations with GDGTs were identified through statistical correlations. Nevertheless, the predominance of anaerobic taxa among GDGT-associated bacterial genera supports our hypothesis that oxygen availability may influence GDGT distributions in the soil. We have revised the Results section to clarify this distinction.

"Eight bacterial genera exhibited positive correlations with all three GDGT groups (sb-, br-, and ob-GDGTs), of which six were classified as anaerobic: *Syntrophus*, *LCP-26*, *LCP-6*, *Treponema*, *SJA-88*, and *C1_B004*." (Page 16, Lines 365-367)

Discussion:

12) Missing source for information on lines 458 to 459.

We have clarified this in the revised manuscript.

"For example, *SAR202*, *Rhodospirillales* and *DG-56* are strongly correlated with branched GDGT abundance in marine sediments (Zeng et al., 2023), whereas *LCP-26*, *Syntrophus*, *LCP-6*, *Treponema*, and *C1_B004* are associated with branched GDGTs in peat soils (this study; Fig. 3c)." (Page 22, Lines 502-506)

13) A large portion of the discussion involves comparison between terrestrial and marine GDGTs. The points discussed are very interesting and allow you to compare past findings to your terrestrial results, but it may be good to introduce this comparison earlier in the paper (i.e., marine environments are briefly mentioned in line 91, but not brought up again until line 416).

We thank the reviewer for this suggestion. We have revised the Introduction to highlight previous observations of oxygen-dependent sb- and ob-GDGT distributions in marine environments (Liu et al., 2014; Zeng et al., 2023).

"At the same time, previous observations that the distributions of sb- and ob-GDGTs vary with oxygen availability in marine environments (Liu et al., 2014; Zeng et al., 2023), raising the possibility that these lipids may also respond to hydrologically mediated redox gradients in terrestrial soils." (Page 5, Lines 119-122)

14) Lines 483-485: it may be good to also mention depth here and mention how your results had SWC that increased with depth for LR, which was the site that also had higher GDGT concentrations.

We thank the reviewer for this suggestion. We have expanded the Discussion to link the depth in soil profiles in the revised manuscript.

"In both our soil profiles, sb/br/ob-GDGT concentrations exhibited subsurface maxima that broadly coincided with elevated SWC, occurring at approximately 60-100 cm in NVI and 120-150 cm in LR (Fig.2), rather than increasing monotonically with depth." (Page 23, Lines 537-540)

15) Line 489: Missing another transition sentence that could help with flow into the next section.

We have added a transition sentence in the revised manuscript to link the marine-terrestrial comparison with the evaluation of $MI_{ob/br}$ as a potential indicator in terrestrial soils.

"These environmental contrasts highlight the need to evaluate whether $MI_{ob/br}$ can reliably reflect moisture-mediated redox variations in terrestrial soils." (Page 23, Lines 544-546)

16) Line 515: Could also cite Raberg et al. (2025), where this concept is studied in a lake sediment setting; overall the paper could benefit if there was a little more consideration for lake sediment-derived GDGTs.

We have cited Raberg et al. (2025) to the revised Discussion to highlight evidence from lacustrine environments that oxygen availability influence lipids distribution and MBT'_{5ME}.

"Evidence from lacustrine environments further supports that oxygen depletion alters brGDGT distribution and MBT'_{5ME} (Raberg et al., 2025)." (Page 25, Lines 579-581)

Summary & Conclusions:

17) Line 557: Could list all the genera.

Added.

"Co-occurrence network analysis further demonstrates that the distributions of these lipids are strongly associated with specific anaerobic bacterial communities, including *Syntrophus*, *LCP-26*, *Treponema*, *LCP-6*, *SJA-88*, and *C1_B004*." (Page 27, Lines 626-628)

18) Could be beneficial to add a summary here of the main findings of the two sites and how they differed (e.g., NVI was more alkaline than LR; NVI had lower SWC than LR; NVI had cooler temperatures *in situ* than LR; NVI had less GDGTs than LR; the GDGTs that had the highest and lowest concentrations for each site and each type of GDGT; NVR had lower index values than LR).

We have added a summary in the revised manuscript.

"Based on a comprehensive analysis of sb-, br-, and ob-GDGTs in two active-layer soil profiles from the Tibetan Plateau permafrost region, we found that NVI soils were cooler, more alkaline, and drier, with lower sb-, br-, and ob-GDGT concentrations and methylation indices than LR soils. Among br-GDGTs, non-cyclized IIa and IIIa were prominent in NVI, while Ib and IIb were relatively enriched in LR. OB-1064 and OB-1106 dominated ob-GDGTs in NVI and LR, respectively, and SB-966 dominated sb-GDGTs at both sites." (Page 26, Lines 612-618)

Figures:

19) Fig. 1. Could the direction that the slope is facing impact the thawing as well? E.g., could one side of the slope be getting more solar radiation?

The slope aspect may influence active-layer thawing by getting more solar radiation, with sun-facing slopes generally experiencing greater surface warming and deeper seasonal thawing than shaded slopes. These differences may further affect soil moisture distribution and ground thermal conditions, as demonstrated in previous studies on the Tibetan Plateau (Fan et al., 2023). However, slope aspect and its potential contribution were not assessed in our study.

20) Fig. 2. Very useful figure to outline the main trend differences between the two sites, however this figure could be further strengthened if the following are addressed:

20-1) Would be good to add which site is which on the left side of the figure rather than having it only in the figure caption.

20-2) The colors on the left side of the figure that show the compound concentrations make it a little hard to tell which structure is which. This could be strengthened by making the figure larger or maybe moving this section of the figure to supplement and organizing the brGDGTs according to methylation (i.e., tetramethylated brGDGTs are one color gradient, then pentamethylated brGDGTs are another color gradient and hexamethylated are a third color gradient) to show the pattern more clearly. Another option could include having the brGDGTs in greyscale from black to dark grey to light grey according to degree of methylation, then including solid bars, a crosshatched pattern, and a diagonal pattern to organize according to degree of cyclization?

20-3) Please add a note of the differences in the x-axes scale between the sites in the figure caption.

20-4) Label the axes more clearly on the right side by adding text to the x-axes. Having the indices lines be similar colors to the colors used for the compounds makes it appear like some compounds and their concentration values are directly linked to the indices.

20-5) Specifying that the data are specifically from the UPLC-ESI-qTOF method explained in section 2.2.1 would also help explain why the brGDGT isomers are not included in the figure. You could also add a supplement figure (or two) that has the relative abundances for each of the methods used to measuring the brGDGT relative abundances.

We thank the reviewer for these constructive suggestions. We have substantially improved Fig.2 as well as its caption as below to enhance the clarity of the lipid distributions and environmental profiles.

Specifically, 1) the NVI and LR sites are now clearly labeled; 2) sb-, br- and ob-GDGTs are displayed separately using distinct color gradients to facilitate the compounds identification and their increasing methylation patterns; 3) the x-axes and concentration units have been clarified, with site-specific concentration scales been noted in the caption; and 4) the right line plots are presented using distinct colors and symbols to

avoid confusion; 5) We have clarified the analytical methods underlying the different lipid measurements. Normal-phase HPLC-APCI-MS enables the separation of concentional br-GDGT positional isomers but does not provide the MS^2 fragmentation information required for reliable identification of sb- and ob-GDGTs. Therefore, we combined both method to achieve a more comprehensive characterization of branched GDGTs and their methylation patterns. Notably, the br-GDGT concentrations obtained using the two method were of the same order of magnitude, indicating broad consistency between the datasets. However, as the two approaches involve different analytical conditions and were employed for complementary purposes rather than direct method comparison, a detailed comparison of their relative abundances would provide limited additional insight into the scientific questions addressed here. We have therefore clarified the respective applications of the two methods in the revised manuscript and Fig. 2 caption, rather than introducing an additional supplementary figure.

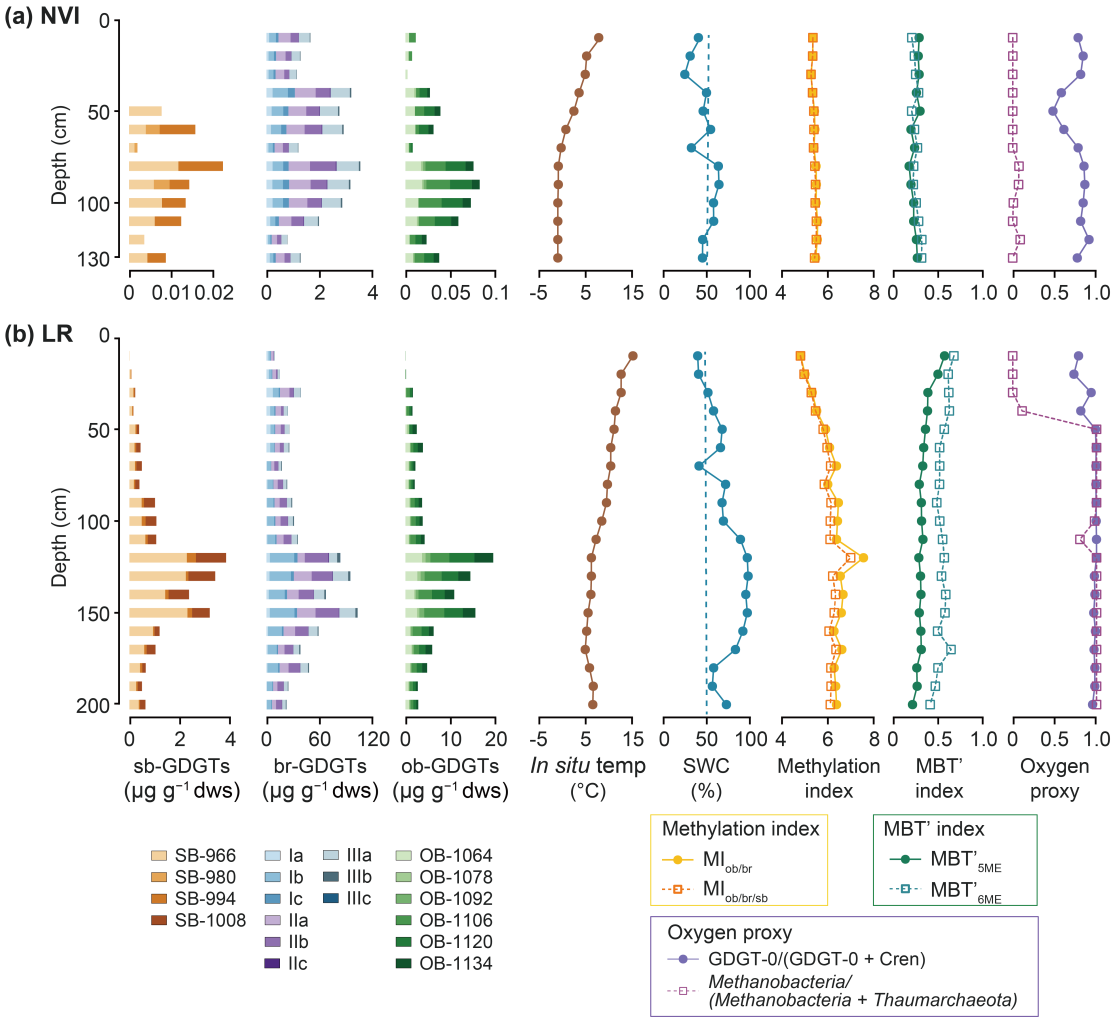


Fig 2. Vertical profiles of lipid concentrations, environmental parameters and relevant proxies with soil depth at (a) NVI site and (b) LR site. Stacked bar charts show concentrations (μg g^{-1} dws) of sb-, br- and ob-GDGTs. Line plots illustrate depth-dependent variations in *in situ* temperature (°C), SWC (%), proxies and ratios derived from lipid and sequencing data, including $MI_{ob/br}$ and $MI_{ob/br/sb}$, MBT'_{5ME} and MBT'_{6ME}, GDGT-0/(GDGT-0 + Cren) and *Methanobacteria*/(*Methanobacteria* + *Thaumarchaeota*). Lipids concentrations and methylation indices were derived from reversed-phase UPLC-ESI-qTOF measurements, whereas MBT′ indices were calculated from normal-phase HPLC-APCI-MS measurements resolving conventional brGDGT positional isomers. Please note that concentration axes use different scales between sites to accommodate differences in lipid abundances.

21) Fig. 4. Includes reference data from Lü et al. (2019). Explaining why these data are included in the figure for comparison could help strengthen this section. Is it included

because the wetland sediments from Lü et al. (2019) are similar to your "permafrost soils"?

We included the data from Lü et al. (2019) because it represents one of the limited terrestrial datasets currently available that simultaneously report ob- and sb-GDGT distributions together with sediment water content. Although the mangrove setting differs hydrologically from our permafrost soils, the dataset provides a useful terrestrial reference for comparing how $MI_{ob/br}$ varies with water content under contrasting hydrological regimes.

22) Fig. 5. Is there opportunity to add an example of lake sediments to this section too?

We thank the reviewer for this helpful suggestion. Fig. 5 aims to compare the distributions of sb-, br-, and ob-GDGTs and their associated $MI_{ob/br}$ indices across global environments. However, comparable quantitative datasets that simultaneously include both sb- and ob-GDGTs in lacustrine sediments are currently not available, despite the extensive study of conventional brGDGTs in lake systems. We therefore did not include lacustrine environments in this comparison. Future work we will detect the occurrence and distribution of sb- and ob-GDGTs in lacustrine settings to evaluate whether the relationships observed here are also applicable to lake sediments.

Technical corrections:

23) Line 72: "paleoclimate proxies" should be changed to "paleoclimate proxy indices".

Changed.

Their distributions are strongly correlated with environmental factors such as temperature and soil pH, forming the basis of widely used paleoclimate proxy indices. (Page 3, Lines 71-72)

24) Table in section 2.3 has a typo for equation 5. The tetramethylated brGDGTs do not have isomers and thus should not have the prime symbols.

Corrected.

References

Fan, X., Li, W., Wu, X. et al. Heterogeneity of Surface Heat Exchange of Slopes and Potential Drivers of the Initiation of Thaw Slump, Qinghai-Tibet Plateau. Int J Disaster Risk Sci 14, 549–565 (2023). https://doi.org/10.1007/s13753-023-00508-8