Reviewer #2

In an effort to identify BHPs that can be associated with specific ecological niches or bacteria, Richter and colleagues have characterized the BHP distributions in suspended particle matter and sediments in the lakes and lagoons of the Azores Archipelago. The authors are particularly interested in characterizing BHP distributions and their potential as taxonomic markers in lacustrine settings, which have not been as well studied as marine settings. This study identifies multiple novel BHPs, including ethenolamine-BHPs and formylated-aminoBHPs, and proposes potential taxonomic markers for methane-oxidizing bacteria and low-oxygen conditions in lacustrine settings. In addition, they identify nucleoside-BHPs that can be produced within the water column, which has implications for the R_{soil} proxy, leading the authors to propose a new $R_{soil\text{-}lake}$ proxy. The results of this study will be useful for future work using BHPs to characterize lacustrine settings and I recommend it be published in *Biogeosciences* with minor revisions. My general recommendation is to consider what additional information may be available, like $\delta^{13}C_{TOC}$ or metagenomic data, that could be used to support the potential of certain BHPs as proxies, especially for MOBs.

We would like to thank the reviewer for taking the time to review our manuscript and their constructive comments.

Specific Comments:

Line 61: Do both type I and type II MOB produce the same diagnostic BHPs or do we know of differences between them? If there are known differences, be more specific when discussing the diagnostic BHPs. Lines 421-422 suggest that they produce different diagnostic BHPs, but this should be consistently clear.

Thank you for pointing this out. As mentioned in lines 421-422, it was previously proposed that type I and type II MOB produce diagnostic BHPs, i.e. aminopentol and aminotetrol, respectively. However, more recent studies (see Rush et al., 2016; Kusch and Rush, 2022), show that this previous distinction is not as clear as previously thought. More work is therefore needed to test whether other proxies can provide us with additional information on being able to distinguish between type I and type II MOB. As suggested by the reviewer we will modify the text to clarify this point.

Line 65: The phrase “minor amounts of these BHPs” is vague. Are all of the BHPs mentioned in the previous sentence (aminotetrol, aminopentol, methylcarbamate-aminoBHPs, and 3β-methylated-BHPs) also produced by sulfur-reducing bacteria and nitrite-oxidizing bacteria or just a subset of them? This should be clarified.

Only aminotetrol and aminopentol are produced by sulfur-reducing bacteria in low amounts (Blumenberg et al., 2006). Methylcarbamate-aminotriol was identified in nitrate-oxidizing bacteria by Elling et al. (2022). We will modify the text in the manuscript to clarify this point.

Lines 113-114: It is mentioned that Lakes Verde and Funda experience large cyanobacterial blooms in the summer months. Were either of these lakes experiencing cyanobacterial blooms during sampling?

Yes, Lakes Verde and Funda were both experiencing cyanobacterial blooms during sampling. We will clarify this point in the text.

Line 129: What do the given percentages mean for this co-culture? Were there other organisms present and this was the percentage of bacteria that could be identified? If so, could other bacteria present be producing BHPs in the culture?

This is an enrichment co-culture, so it does contain other bacteria. However, based on the previous study conducted by van Grinsven et al. (2020), *Methylobacter* sp. and *Methylotenera* sp. are the primary bacteria in this enrichment. It is possible that other bacteria are producing some of these BHPs; however, this is likely a minor contribution. Further, the primary BHP profile (i.e., high proportions of aminotriol and aminopentol) of *Methylobacter* sp. resembles that of pure MOB Type I cultures previously analyzed. Future studies, however, should analyze BHPs from pure cultures to verify the BHP profile that we describe in this study for *Methylobacter*. We will add a few sentences to the text to acknowledge this potential discrepancy.

Line 179: TOC content was measured, but no associated $\delta^{13}C_{TOC}$ measurements are reported. This could be a useful additional piece of information to aid in characterizing the MOB input in this environment.

$\delta^{13}C_{TOC}$ measurements are available, and we will add these to the manuscript to support our preliminary interpretations.

Line 252: Are the acylated versions of aminopentol also commonly associated with methanotrophic activity?

Prior to this study, acylated-aminopentols have only been reported by Hopmans et al. (2021) from soil samples collected near a terrestrial methane seep. Based on this previous study and our current study, we hypothesize that acylated-aminopentols are likely associated with methanotrophic bacteria. However, we recommend that further studies are conducted on both environmental samples and cultures of methanotrophs to test whether acylated-aminopentols are actually associated with methanotrophic activity. We discuss this in detail in lines 472-479.

Lines 274-275: Is there any evidence apart from the putative oxazinone-aminotriol for nitrite-oxidizing bacteria in the sediments from Lake Verde? While Elling et al. (2022) identified the same compound in nitrite-oxidizing bacteria, the source of that BHP here is not necessarily the same. Further comment on the potential source of oxazinone-aminotriol in this environment would be useful.

As far as we know, Elling et al. (2022) was the first study to report oxazinone-aminotriol in cultures and our study is the to report oxazinone-aminotriol in environmental samples. At this time, we have not done any additional molecular work on these samples and therefore it is difficult to speculate on other potential sources of oxazinone-aminotriol. We hope to explore this in future studies in more detail.

Line 314: The fieldwork was done at the beginning of the stratification season for deep lakes – is there evidence that the lakes had fully stratified at that point? If not, this could potentially explain the finding that there is no significant difference between the BHP distributions in the surface, chemocline, and bottom water.

The deep lakes (Azul, Funda, and Negra) were stratified at the time of sampling as demonstrated by the water column profiles shown in Fig. 6 and in Table A2 (we will include the full water column profile data as part of the supplementary material). However, as mentioned by the reviewer, it was early in the season and the bottom waters of both Funda and Azul, for instance were not anoxic. Our ANOSIM test is likely also not significant due to the limited number of samples analyzed in our study. Further work is needed to test whether the BHP distributions are significantly different between the surface water, chemocline, and bottom water in other lakes.

Figure 4: It would be easier to distinguish between deep lakes, shallow lakes, and coastal lagoons if the sample site markers for type of site had a different shape.

Thank you for the suggestion, we will modify the figure as recommended by the reviewer.

Line 353: Is it clear why methoxylated BHPs are only present in the lagoons?

At this point no, but it is interesting! Hopefully future studies in coastal settings and marine environments will help us understand if this is a feature unique to marine settings and/or if we can link this to a specific producer.

Lines 444, 452: It is noted that sulfur-reducing bacteria and nitrite-oxidizing bacteria could potentially contribute to the aminotriol, aminotetrol, and aminopentol in the water column, although it is unlikely. Is there any additional data available, like $\delta^{13}C_{TOC}$ measurements or metagenomic data, that could support the MOB origin for these BHPs, especially for the potential origin from nitrite-oxidizing bacteria? Confirmation that nitrite-oxidizing bacteria are not making a significant contribution of aminopentol would also support the interpretation that that there is a higher abundance of Type I than Type II MOB.

$\delta^{13}C_{TOC}$ measurements are available, and we will add these to the manuscript to support our preliminary interpretations as mentioned previously.

Metagenomic work is currently beyond the scope of this study and molecular data is currently not available for these lakes. This is, however, a direction we are interested in pursuing as part of future work.

Line 524: A reference is made to the chironomid community shifting as a result of lake eutrophication. However, in the context of this paper, this line seems unrelated and would require more information on why chironomids would be relevant for understanding MOB contributions to the ecosystem. I would suggest either removing this line or further explaining the connection between chironomids and MOB.

Thank you for this suggestion, we will modify this in the text.

Technical Corrections:

Figure 1C: Sao Jorge Lake is not labeled or obvious in this panel, despite being included in the figure caption. Additionally, in the enlarged map, it would be useful to center and zoom in further on the lagoons of interest because they are currently difficult to see.

Thank you for the suggestion, we will modify this figure.

Figure 1D: Is the enlarged map different from the map of Flores island? The island map has more lakes than the enlarged panel, which makes it challenging to identify the lakes of interest in the larger geographic context.

Thank you for pointing this out, we will modify this figure.

Table 1: Formatting issues make this confusing, especially for the pH, NH_4, and NO_2 columns.

We will modify this to make it easier to read.

Lines 364-366: The caption for Fig. 6 says that dissolved oxygen in plots D and I and pH in plots E and J are pink when they are in black.

Thank you for noticing this mistake. We will correct this in the figure caption.

Appendix B1, page 31 (no line numbers): General typing errors – the appendix could use a proof-reading. Examples: "is similar to **(the)** retention time difference" and "we attribute this **to potential a potential** co-eluting peak."

Thank you for catching these mistakes. We will correct the abovementioned mistakes, and will proofread the Appendix for typing errors.

Figure B2A: The bottom chromatogram (m/z 718.563) does not have any labeled peaks. Based on retention time, I would assume it to be peak o, as labeled in the same chromatogram in panel B. If so, this should be labeled.

We will correct this in the manuscript.

References

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