

Reviewer #1

General comments

The study by Richter et al. describes distributions of BHPs in lacustrine and coastal environments of the Azores. In addition to the environmental samples, an enrichment culture enriched for methanotrophs was also studied. Using LCMS, the authors identified several novel BHPs and report detailed information of their mass spectral identification. The BHP distributions are then discussed in combination with geochemical parameters and the potential as taxonomic markers for different ecological niches is evaluated.

I like the manuscript and think it is well written and structured. The type of figures and the selected statistical methods are chosen well. The detailed identification of the new compounds seems sound and the reported mass spectra in the appendix will be very useful for further research. I am very much looking forward to the final version where the raw data are also accessible. Besides the analytical part, the discussion of the findings in context of previous studies is well balanced and adds important conclusions. Overall, not much should be changed before final publication.

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

Specific comments

It would be great if the authors could also make the inclusion lists for the analytical method available (see also minor comment). This would help “jump-start” implementation of the LCMS method in other labs.

The appendix is great in explaining in detail how the fragment spectra of the BHPs are used for identification. The main text then only contains a brief mentioning (according to line 212) – this is true for all novel compounds except the formylated-aminoBHPs where lines 262-278 are very similar to section B6 in the appendix. I suggest to shorten the main text version so that it is similar to the summary of findings of the other compounds and keep the details in the appendix B6.

Thank you for the recommendation, we will modify the main text and the appendix. We expanded on the formylated-aminoBHPs in the main text because this is the first time this class of compounds is described. Versions of the other novel compounds discussed in this manuscript were previously described by Hopmans et al. (2021) and Elling et al. (2022). Therefore, we would like to keep some of the more expanded description in the main text of the manuscript, but we will shorten it to make it easier to follow. We will also make the inclusion list available as part of the supplementary materials for this manuscript.

Since there are so many compounds in the NMDS plot of Fig. 4, I wonder if it is possible to use colored font for the compounds clustering together in Fig. 4. For example color A for cluster of amino-containing compounds (referenced in line 323), color B for the nucleoside compounds (line 338) and so on (e.g. in line 466). This should then be indicated in the text (section lines 314-349) and in the caption of Fig. 4.

Thank you for the suggestion, we will modify the figure as recommended by the reviewer. We agree that this will make the figure easier to interpret.

Section 3.5 (implications for the Rsoil proxy) is not reflected in the abstract and conclusions and should be added.

Thank you for the suggestion. We will add a sentence about the Rsoil proxy to the abstract and conclusions.

Minor comments and technical corrections

Thank you for the minor comments and technical corrections, we will address these in the revised manuscript.

L67: replace “nor “ with “or”

We will correct this.

L154: specify MS model?

We will add this.

L159: resolution does not have unit “ppm”

The unit “ppm” is present, but was moved to the following line.

L159-160: why three separate inclusion lists? Can these lists be provided as supplemental material, other research would benefit a lot from this

The surface sediment samples were analyzed initially with the inclusion list we had available at the time. After the identification of several novel compounds, we analyzed the water column samples with an updated inclusion list. Finally, we re-analyzed a select number of samples to obtain a better MS2 and verify our tentative identification of these novel compounds. We have modified this in the text to clarify this point. We will include the final inclusion list in the supplementary material.

L169: how was the normalization between sample runs done? Please explain.

We will expand on this in the text. Briefly, we used the same internal standard for all of our sample runs. We used the internal standard to normalize the measured peak areas of our BHPs.

L256: Fig 3e? I might have missed it, but Fig. 3e is not referenced?

Thank you for noticing this mistake, we will correct this in the manuscript.

L303-305: “Using ANOSIM we find a significant difference...”

We will correct this.

Fig. 4: colors are not easy to distinguish (two shades of green, two of blue), choose additional other colors

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

L311: delete “there is”: “we find no significant difference”

Will correct.

L327: “was previously described”

Will correct.

Fig. 6 caption: RU should be “response units”, not “relative units”

Will correct.

Fig. 9 caption: indicate that structures are tentative structures

Will correct.

L478: instead of “an NMDS” use “the NMDS analysis”. This also reads a bit odd, the NMDS analysis shows many compounds. Do you mean that these compounds, the amino BHP cluster, is close to the sites mentioned in line 482?

Yes, what we meant to say is that ethenolamine-BHpentol, ethenolamine-BHhexol, acylated-ethenolamine BHhexols (C_{15:0}, C_{16:0}, C_{17:0}), formylated-aminotetrol, formylated-aminopentol, MC-aminopentol, aminopentol, aminotetrol and the acylated-aminopentols all cluster near the surface sediments from Azul, Verde, Funda, and Negra and the bottom water from Funda and Negra. We will rephrase this in the text to make it clearer.

L493: suggestion – “observe ... in all sediment samples except Cubres East and West (Fig. 2)...”, move outside of parentheses

Will correct.

L538: “BHP distribution”

Will correct.

L603: perhaps “... appear to be produced in the water column...”

Will correct.

Appendix B1 second paragraph last sentence: typo – “...attribute this to a potential co-eluting...are unable to distinguish.”

Will correct.

Appendix B114: m/z 191 was searched in fragment spectra: "...in fragment spectra revealed two..."

Will correct.

Appendix B120: "...is not located in the ring system."

Will correct.

References

Elling, F. J., Evans, T. W., Nathan, V., Hemingway, J. D., Kharbush, J. J., Bayer, B., Spieck, E., Husain, F., Summons, R. E., and Pearson, A.: Marine and terrestrial nitrifying bacteria are sources of diverse bacteriohopanepolyols, *Geobiology*, 20, 399–420, <https://doi.org/10.1111/gbi.12484>, 2022.

Hopmans, E. C., Smit, N. T., Schwartz-Narbonne, R., Sinninghe Damsté, J. S., and Rush, D.: Analysis of non-derivatized bacteriohopanepolyols using UHPLC-HRMS reveals great structural diversity in environmental lipid assemblages, *Organic Geochemistry*, 160, 104285, <https://doi.org/10.1016/j.orggeochem.2021.104285>, 2021.