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Response to RC1

Dear Reviewer #1,

We thank the reviewer for the insightful comments and suggestions for this manuscript. We have incorporated most of the suggestions made by the reviewer. Please see below for a point-by-point response to the reviewer's comments.

Reviewer's comments to the authors:

Reviewer #1

In "Dissolved organic matter composition regulates microbial degradation and carbon dioxide production in pristine subarctic rivers," Saarela et al. examine differences in DOM composition and CO₂ production between clearwater and brownwater rivers in subarctic Finish Lapland. They find clearwater river DOM more biolabile, with interesting implications for how future increases in terrestrial DOM inputs may not increase CO₂ fluxes. The manuscript covers an understudied region and could be a useful contribution to our processed based understanding of CO₂ production. However, there are a few major issues with the study design and methods that will need addressing and clarifying in the manuscript itself.

Author response: We thank the reviewer for the feedback and for pointing out the issues concerning the study design and methodology of this study.

Incubation design: As laid out in figure S3, the overall incubation design causes me some concern and I do not think its potential implications are addressed in the paper. While I understand the desire to use the incubation (C in the Figure S3) without added inoculum for FT-ICR MS so as to avoid the influence of the inoculum on molecular formulae, I am worried about the direct comparison between CO₂ analyses conducted in

a set of bottles with an inoculum added and ICR metrics for a final time point derived from a completely different set of bottles with no inoculum added. To me it seems very much like comparing apples and oranges. I understand that in lines 120-122, the authors explain that there was no difference in potential CO₂ production between bottles with the inoculum and those without, though I admit I do not understand how this was analyzed since Figure S3 shows that treatment 2 (C, the one with no inoculum) never had its CO₂ measured, so I am not sure where that potential CO₂ production measurement comes from. Please explain this in the manuscript. But CO₂ production is not the only change that the presence of microbes can cause—they can change the composition of DOM through partial consumption, and it is possible the different size classes of microbes present in the filtered (treatment 2) and inoculum (treatment 1) bottles would do different things to DOM composition.

Thus, I do not think it is valid to compare CO₂ production to ICR composition at the end of the incubation period, after the processing both sets of bottles have undergone has diverged. Comparing it to the composition at the beginning of the experiment makes sense, since those were the molecular starting conditions that led to the CO₂ production. It would greatly strengthen the manuscript if you removed reference to the final timepoint for FT-ICR MS (or at least the comparison of that time point to CO₂ production), and focused on the relationship between the starting FT-ICR MS data.

Author response: We thank the reviewer for pointing this out. We understand the reviewer's concerns about the direct comparison between the incubation bottles with and without an inoculum added. In addition, we agree that the study design needed more clarification. For comparison of the CO₂ production in bottles with and without inoculum, the CO₂ production was measured from bottles without inoculum at the beginning of incubation and at the final time point (21 days). We have clarified this in the manuscript (page 5 lines 122-138) and updated Figure S3 concerning different treatments and their measurements (page 5 in the supplementary material).

We also thank the reviewer for the suggestions concerning the direct comparison of CO₂ production to DOM compositional changes during the incubation (i.e., the FT-ICR MS data after the incubation). In the revised manuscript, we have focused on discussing the molecular "starting conditions" (incubation day 0) and their impact on the microbial biodegradability and potential CO₂ production in these rivers. Furthermore, in chapter 3.4 (Relationships between

microbial degradation and molecular composition of DOM), we have excluded the final time point (21 days) of FT-ICR MS results and focused on presenting the correlations between the CO₂ production and the molecular compound groups at the beginning of incubation (page 5 lines 336-343 and Table S4 in the supplementary material).

Contaminant peaks: The extremely high relative abundance peaks in the top left corner of all your van Krevelen diagrams that you attribute to the aliphatics class look to me like classic surfactant peaks that are often added to FT-ICR MS analysis through the SPE process. They are often of the O₃S₁ class, and are several series of homologous peaks (separated by a CH₂ unit). If they are these common contaminants, they should be removed from the analysis. This will change the % relative abundances of your compound classes, since these currently (and inaccurately, I believe) dominate.

Author response: We thank the reviewer for this comment. We carefully checked each molecular formula with extremely high relative peak intensity. Since the molecular formula assignments in this study only included C, H, O and N, thus excluding S containing formulas, these peaks cannot be O₃S₁ peaks. However, we cannot exclude that these peaks were other contaminants, and as reviewer pointed out, these peaks inaccurately dominated in the former FT-ICR MS data version. After careful consideration, we decided to remove the unusually high relative peak intensities (> 0.10) from the FT-ICR MS analysis. We added an explanation of the peak removal to Methods (page 6 line 181).

At this point it isn't clear where your study system is situated compared to the systems you say are highly and less studied; the difference between "boreal catchments" (line 44) and "northern high-latitude streams" (line 46) is not clear—boreal catchments certainly are one type of northern high-latitude stream. In line 71 you say "subarctic rivers," which might be a good description to use in line 46 to contrast with boreal catchments. Further, right now you cite five sources for boreal catchments and five for high-latitude streams, completely contradicting the point you are making in the text that one is more studied than the other. Perhaps you don't need to set it up as an either/or scenario, but simply explain why it is good to study subarctic rivers.

Author response: We agree with the reviewer that the former sentence was misleading, as the term “high-latitude streams” can refer to both boreal and subarctic streams. We have now edited this sentence and used the term “subarctic” (page 2 line 44).

Section 2.6: I have a few concerns about the FT-ICR MS methods you describe, and it would be helpful if you could clarify these points in this methods section. Why was only m/z range 150-500 analyzed? Normally DOM masses extend well into m/z 800-1000. Please add a short (even half a line long) explanation. Further, it sounds as if formulae were assigned one by one rather than in homologous series (the standard and far more powerful way of assigning formulae). If that is true, please justify it, or add some acknowledgement of how this may impact the results. If anyone is undertaking ultrahigh resolution mass spectrometry, they should work with the best software possible to ensure that their assignments are correct, and homologous series assignments are far more accurate than single formulae assignments. If, however, for some reason that is not what was done in this study, it at least needs to be acknowledged explicitly in the paper with a small explanation.

Author response: We thank the reviewer for these important comments concerning FT-ICR MS analysis. We acknowledge that several studies in northern freshwater ecosystems have used wider m/z range in their analyses (e.g., up to m/z 800-1000). On the other hand, previous studies have found that intensity maxima of natural organic matter (NOM) typically occurs in the range of m/z 350–500 (Reemtsma 2009), which was the case also in our study (see Fig. 1 presenting an example of the sample spectra in our FT-ICR MS analysis). Based on these factors, we decided to use the mass range of m/z 150-500 in this analysis. We have added an explanation of the m/z range selection to the revised manuscript (page 6 lines 175-177).

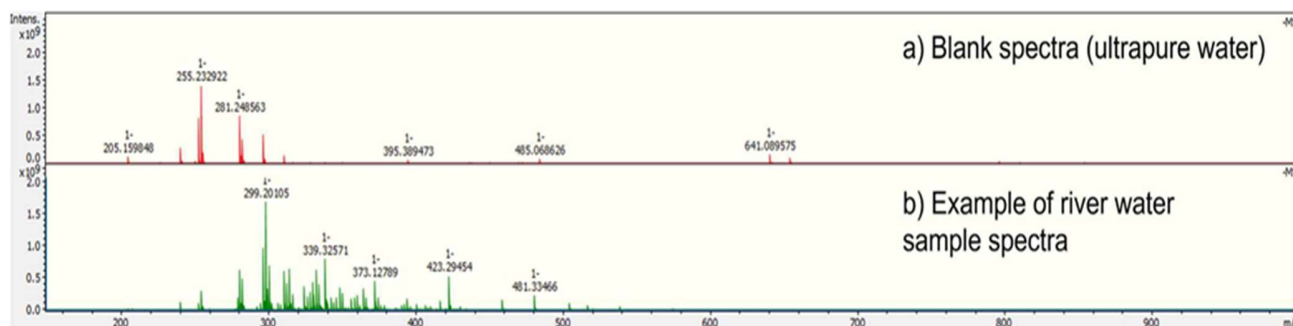


Figure 1. An example of the mass spectra in a) blanks and b) river water samples in this study.

We also thank the reviewer for the comment concerning the use of different molecular formula assignment strategies. We agree with the reviewer that molecular assignment using homologous series is certainly a powerful method and widely used in recent studies. However, one-by-one molecular formula assignment is still a commonly used method, and no major problems have been reported. In addition, since we aimed to evaluate the molecular composition of natural water in this study, we wanted to assign not only the molecules that could form a series, but also other molecules under the same conditions. Therefore, we used the one-by-one method in this study.

While we acknowledge the weaknesses of this approach (e.g., Koch et al. 2007), we have conducted other measures that significantly decrease the possibility of assignment errors in our FT-ICR MS data analysis. For example, since high errors are associated with the assignments containing S and P (Mostovaya et al. 2017), these formulas were excluded from further analysis. In this study, the analyses were restricted to molecular formulas with C, H, O, and max. 2 N, which can be considered to significantly decrease the possibility of false assignments (Koch et al. 2007). Furthermore, considering that the number of possible molecular formulas as well as the number of assignment errors increase with increasing m/z (Stenson et al. 2003; Sleighter et al. 2008), the use of m/z range below 500 Da also decreases the possibility of assignment errors.

Section 2.7: I believe this is not the normal way of counting bacterial abundance, and flow cytometry would have been far more accurate. Please add some explanation of why this method was chosen to assess abundance.

Author response:

We thank the reviewer for this comment concerning different bacterial quantification approaches and their accuracy. In previous studies, methods using fluorescent dyes to stain the bacterial cells and qPCR have been compared, and e.g. Chen and Li (2005) conclude that concentrations measured by real-time qPCR were highly associated with the total number concentrations measured by epifluorescence microscopy (EFM) and flow cytometry (FCM).

Zhang et al. (2017) suggest that the differences in FCM and qPCR observed in some studies could be due to the combination of the inherent biases of all experimental steps, such as the efficiency of cell/DNA extraction, PCR inhibition due to the amplification efficiency and primer specificity, or underestimated counts of bacterial cells with FCM. We acknowledge the high variability and other weaknesses that qPCR might include. However, in this study, the bacterial abundance was quantified to use as an estimation to compare the relative differences between the studied rivers, and therefore, we consider the accuracy of this method sufficient for these purposes. In addition, considering the remote location of the sampling area, the use of 16S qPCR with frozen glass microfiber filters was a practical approach to quantify the bacterial abundance.

Lines 155-157: Please include whether the CO₂/DOC ratio is using the DOC concentration at the beginning or end of the measurement period.

Author response: We thank the reviewer for pointing this out. The CO₂/DOC ratio (i.e., the cumulative CO₂ production per DOC) was calculated based on the CO₂ production rates ($\mu\text{mol l}^{-1} \text{d}^{-1}$) measured during the four 24 h gas samplings divided by the DOC concentration in each bottle at each timepoint. We have clarified this in the text (page 5 line 156).

Lines 244-251: Please specify whether you are talking about the mean or mean weighted average m/z, O/C, and H/C in this section. In Table 1, you say mean, but it's not clear how that is calculated. Did you add up all the m/zs for each sample and divide by the number of samples? That's what mean implies. If you mean mean weighted average, as in the mean m/z weighted by the relative abundances, please specify that. Same for O/C and H/C. At this point it's unclear what this metric is.

Author response: Thank you for this comment. Indeed, the values presented in Table 1 are the mean values of m/z, O/C, H/C, and AI_{mod} , i.e. the sum of the values divided by the number of samples ($n = 6$ in the brown-water river and $n = 5$ in the clearwater river).

Lines 252-253: Do you mean the percent of formulae based on number of formulae, or based on percent relative abundance? They can mean very different things. Please clarify.

Author response: We thank the reviewer for this comment. The percentages of molecular formulas are based on the number of assigned molecular formulas. This has been clarified to manuscript (page 12 line 262). We also included an explanation of this to the caption of Figure 4.

Lines 254-257: Relative peak intensities by definition add up to 100% for a single spectrum, so I do not understand how they could be higher in one river type than another. Do you mean the average relative peak intensity?

Author response: We agree with the reviewer that the comparison of the average relative intensities was not clearly presented. After recalculating the statistical analyses due to the removal of unusually high peaks, we decided to exclude the peak intensity comparison from the chapter 3.2 and focus on the comparison of the parameters presented in Table 1.

Lines 257-259: same as above—do you mean weighted average Al_{mod} ? You need to specify. There is not just one Al_{mod} value for each sample, which is what this sounds like.

Author response: Thank you for this comment. We have used the mean values of Al_{mod} for each sample. We have now explained this in the revised manuscript (page 12 line 264). In addition, we have edited the whole paragraph concerning the significant differences in Al_{mod} between the rivers due to the recalculation of statistical analyses.

Lines 264-779 (and Figure 4): The percentages that are in this section appear to be percent of total molecular formulae in each compound class. Normally in this field folks refer to percent total relative abundances. These two concepts convey different things, and which you want to use depends on what you're looking at (number of specific/rare

formulae versus the contribution that compound class makes to the overall DOM signal). You might want to think about switching to percent relative abundance if you want other papers to be able to cite this for comparison, since most folks work in %relative abundance. A caveat to this is in my next comment—the massive signal peaks you are assigning as aliphatics that are probably surfactant contaminants (O3S1 class, etc) found in most FT-ICR MS spectra that use SPE. These peaks should be removed before calculating %relative abundance.

Author response: We thank the reviewer for this comment. In this section, we present the percentages of different compound groups based on the number of molecular formulas, similarly to e.g. O'Donnel et al. (2016). We agree with the reviewer that % relative abundance is a typical way of presenting these results in this field. The solution to present the relative differences of compound groups in a current way (% of different compound groups) was based on the suggestions and critiques that %RA approach has previously received from the reviewers. Recently, there has been discussion about the use and misuse of peak intensities in organic matter chemistry (e.g., Kew et al. 2022). Therefore, while we acknowledge the benefits of using %RA (i.e., the comparability to other studies), we think that the current way of presenting the data in Figure 4 is reliable and gives a good overview of the quality and composition of DOM in these rivers.

Lines 346-347: Unlike what you are stating in this line, Figure 5 looks to me like the two rivers do not have significant differences in bacterial abundance (just very different ranges). Could you add in some statistic earlier to show they are significantly different, if they are?

Author response: We thank the reviewer for pointing this out. We have edited this statement and removed the part of the sentence concerning the comparison of bacterial abundances due to the lack of significance (page 17 line 354).

Figure 3: First, the font is too small to read, and the dots of color indicating compound category in the legend are too small to see. Please make the figure and legend legible. Of more concern are the high relative abundance peaks at high H/C low O/C ratio (top left

corner)—these look a lot like the surfactant contaminants that are common in FT-ICR MS analysis, and belong to the O₃S₁ or O₄S₁ classes of homologous series. Please see if these peaks are O₃S₁, and remove the contamination series before analysis.

Author response: We thank the reviewer for suggesting these improvements to Figure 3. In the revised figure, we have increased the font size, as well as the size of the dots indicating compound classes in the legend.

As discussed above, we checked all the peaks with unusually high relative peak intensity, and these peaks were not known contaminant peaks from O₃S₁ or O₄S₁ classes. However, since we cannot completely exclude some other possible contamination from sample preparation or other steps of the FT-ICR MS analysis, and the high peaks can distort the results and their interpretation, we decided to remove the peaks with unusually high relative peak intensity (> 0.10). Similar removal of unusually high peaks has been done also in previous studies, e.g., Mostovaya et al. (2017). We have updated all the figures, tables and statistics excluding these peaks from further analysis.

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