RE: Labilization and diversification of pyrogenic dissolved organic matter by microbes (bg-2021-23)

Foreword

In this document, we have placed all referees' comments, and we describe the manuscript edits that were made to address them. In addition to the comments of the two referees, we have also included an "unsolicited revisions" section below. Comments by reviewers are colored in **black**, our responses are in **red**, text from the modified manuscript is in **blue**. The line numbers mentioned below correspond to the numbering in the "tracked changes" documents of the manuscript and the supplemental material.

Commonly used acronyms in this document:

- pyDOM = pyrogenic dissolved organic matter
- ESI = electrospray ionization
- FT-ICR-MS = Fourier transform – ion cyclotron resonance – mass spectrometry
- ROS = reactive oxygen species
- TOC = Total Organic Carbon
- KMD = Kendrick mass defect

Referee 1

In the study, the authors used art-of-the-state technologies to understand the transformation of pyrogenic dissolved organic matter by soil microbes. The authors reveal that the alternation of pyDOM was uniform and that a large portion of the bio-produced compounds is peptide-like. The results of this study can definitely improve the current understanding of the biogeochemical cycle of pyDOM.

We thank the referee for their review of our manuscript and are pleased that they see its value for improving the current understanding of the biogeochemical cycle of pyDOM.

1 Method. FT-MS. Positive or negative ESI? These two modes are suitable for acidic and basic compounds, respectively. If only one mode was used, only partial results could be obtained.

We apologize for omitting this critically important detail. We corrected he manuscript to indicate the ionization mode:

Line 218: Samples were analyzed in negative ionization mode.

We also note the limitations of the employment of negative-mode ESI and provide appropriate citations:

Lines 290-295: It is important to note that the electrospray ionization (ESI) source is prone to biases, and the analytical window of FT-ICR-MS depends most critically on it. Thus, it may not identify compounds that are present if they are not ionizable (Stenson et al., 2002; Patriarca et al., 2020). Therefore, our observations are influenced by the limited analytical window, and it is essential that observations by FT-ICR-MS are always paired with supplementary quantitative techniques (optical analyses, NMR, etc.) in order to determine if the identified trends are real or an artifact of ESI charge competition (D'Andrilli et al., 2020).

Lines 333-339: Bio-degradability trends derived from FT-ICR-MS molecular data match those from the UV-VIS data from chromophoric pyDOM (Figure S1) revealing a similar inability of UV-VIS to detect LMW compounds which do not absorb UV-VIS light. In summary, we observe a degradation of a variety

of different molecular classes as well as a producion of many molecules that appear to be of high biological lability. However, we caution that there are observed discrepancies among carbon loss and molecular/chromophoric data for the Oak 400 pyDOM systems, an observation that highlights the need to clearly understand methodological analytical windows when interpreting molecular and spectroscopic data.

Lines 773-778: It must be noted that the results of our study were acquired using negative-mode ESI which is only effective for electronegative (carboxyl-rich, hydroxyl-rich) compounds (Stenson et al., 2002; Patriarca et al., 2020). Thus, the trends of degradation and labilization are skewed to fit this criterion and do not provide a complete overview of all molecules that are bio-labile or bio-produced. Future studies should employ positive-mode ESI and/or different ionization sources (such as atmospheric pressure photoionization) to better elucidate the molecular degradability of pyDOM.

2 Results 3.1 The pyDOM produced at a higher temperature is more recalcitrant than that produced at a lower temperature. The photo-irradiated pyDOM should be more labile than the fresh one. However, it was not the case in this study (as shown in Figure 1). Why? How about the results using TOC loss and CO_2 respiration?

The differences among the four incubations have been described in greater detail. We also incorporated TOC loss quantities in all figures (Figure 1, Figures S2-S8) and clarified that TOC losses are equivalent to CO_2 respiration:

Lines 314-339: The organic carbon loss was also found to be equivalent to mineralized CO2 (± 4%, Bostick et al., 2021) indicating that microbial respiration had occurred though CO2 mineralization can happen abiotically as well. Using the number of formulas lost as a proxy for bio-lability here, it appears that Oak 400 Fresh (1646 bio-labile formulas, 16% carbon loss) is more bio-labile than Oak 650 Fresh (1364 bio-labile formulas, 15% carbon loss). This was expected because of the richness of Oak 400 Fresh in smaller less-aromatic compounds (Wozniak et al., 2020). Upon photo-irradiation, both Oak 400 Fresh and Oak 650 Fresh experience significant changes in their molecular composition as previously described in detail by Goranov et al. (2020). The photo-transformed pyDOM is much more aliphatic and richer in nitrogen and LMW compounds which render pyDOM to be much more biologically labile (Goranov et al., 2020). Surprisingly, it was found that Oak 400 Fresh (1646 bio-labile formulas) is more bio-labile than its photo-irradiated counterpart (Oak 400 Photo, 1242 bio-labile formulas). However, this observation using molecular data does not agree with quantitative carbon loss results for the 10-day incubation (Oak 400 Fresh: 16% carbon loss, Oak 400 Photo: 25 % carbon loss). The observed discrepancy is because LMW compounds contribute to a large fraction of the degraded carbon in the Oak 400 pyDOM systems and LMW species are not observed following the employed PPL sample preparation and FT-ICR-MS detection. A similar discrepancy is observed when comparing Oak 400 Photo (1242 bio-labile formulas, 25% carbon loss) and Oak 650 Photo (1410 bio-labile formulas, 23% carbon loss). In contrast, Oak 650 Fresh (1364 bio-labile formulas) was observed to be less bio-labile than Oak 650 Photo (1410 bio-labile formulas) via both FT-ICR-MS and the observed quantitative carbon losses (Oak 650 Fresh: 15% carbon loss, Oak 650 Photo: 23 % carbon loss). LMW species are less abundant in the Oak 650 pyDOM systems resulting in consistent trends between the analyses. Bio-degradability trends derived from FT-ICR-MS molecular data match those from the UV-VIS data from chromophoric pyDOM (Figure S1) revealing a similar inability of UV-VIS to detect LMW compounds which do not absorb UV-VIS light. In summary, we observe a degradation of a variety of different molecular classes as well as a producion of many molecules that appear to be of high biological lability. However, we caution that there are observed discrepancies among carbon loss and molecular/chromophoric data for the Oak 400 pyDOM systems, an observation that highlights the need to clearly understand methodological analytical windows when interpreting molecular and spectroscopic data.

3 Result. As stated by the authors, the photo-degradation of pyDOM is also very interesting; why not to compare the structure change before and after photo-irradiation in the supplement.

The photochemical changes to these pyDOM samples have been a focus of two previously published manuscripts: we report the quantitative changes in Bostick et al. (2020) and the qualitative (structural and molecular) changes are described in detail in Goranov et al. (2020). As the current manuscript and its supplement are already of significant length, we prefer to not further focus on the photochemical changes of these samples. The reviewer is referred to those previously published manuscripts, and clarification has been made to the manuscript to likewise refer the readers:

Lines 319-322: Upon photo-irradiation, both Oak 400 Fresh and Oak 650 Fresh experience significant changes in their molecular composition as previously described in detail by Goranov et al. (2020). The photo-transformed pyDOM is much more aliphatic and richer in nitrogen and LMW compounds which render pyDOM to be much more biologically labile (Goranov et al., 2020)

4 Discussion. 4.1.1 It should be very careful to draw a conclusion on the biodegradability of pyDOM with various structures. Only polar compounds can be ionized by the ESI. Therefore, the results obtained by the ESI-FT-MS are biased. Some compounds are with high aromaticity index; they are still polar if the ESI can ionize them. If possible, more ionization modes can be tested using the same samples.

We agree with the referee that the observed trends from negative-mode ESI-FT-ICR-MS must be viewed in the context of the analytical window of this technique. This has been noted in the manuscript:

Lines 290-295: It is important to note that the electrospray ionization (ESI) source is prone to biases, and the analytical window of FT-ICR-MS depends most critically on it. Thus, it may not identify compounds that are present if they are not ionizable (Stenson et al., 2002; Patriarca et al., 2020). Therefore, our observations are influenced by the limited analytical window, and it is essential that observations by FT-ICR-MS are always paired with supplementary quantitative techniques (optical analyses, NMR, etc.) in order to determine if the identified trends are real or an artifact of ESI charge competition (D'Andrilli et al., 2020).

Lines 773-778: It must be noted that the results of our study were acquired using negative-mode ESI which is only effective for electronegative (carboxyl-rich, hydroxyl-rich) compounds (Stenson et al., 2002; Patriarca et al., 2020). Thus, the trends of degradation and labilization are skewed to fit this criterion and do not provide a complete overview of all molecules that are bio-labile or bio-produced. Future studies should employ positive-mode ESI and/or different ionization sources (such as atmospheric pressure photoionization) to better elucidate the molecular degradability of pyDOM.

Referee 2

This work investigated the molecular changes of water extracted chars (pyDOM) during microbial degradation using FT-ICR-MS and other methods. The topic is interesting and the manuscript is well written. But I have some major comments about the discussion about the role of ROS in the transformation of pyDOM.

We thank the referee for their review of our manuscript and we are pleased that they find the topic of research interesting.

The author said this is a parallel study of the same samples (Bostick et al., 2020a), and "Over the 96-day incubation, up to 48% of the carbon was respired to CO2 following first-order kinetics," However, this study only incubated 10 days. The DOC loss or mineralization is very important in the biodegradation of DOM, but I did not see any contents about this in results or discussions in this paper.

We recognize that these results have not been clearly discussed in our manuscript and thank the referee for reminding us of their importance. These data have been incorporated in the results of the revised manuscript. We also edited Figure 1 and Figures S2-S8 to provide the carbon loss quantities.

Lines 314-339: The organic carbon loss was also found to be equivalent to mineralized CO2 (± 4%, Bostick et al., 2021) indicating that microbial respiration had occurred though CO2 mineralization can happen abiotically as well. Using the number of formulas lost as a proxy for bio-lability here, it appears that Oak 400 Fresh (1646 bio-labile formulas, 16% carbon loss) is more bio-labile than Oak 650 Fresh (1364 bio-labile formulas, 15% carbon loss). This was expected because of the richness of Oak 400 Fresh in smaller less-aromatic compounds (Wozniak et al., 2020). Upon photo-irradiation, both Oak 400 Fresh and Oak 650 Fresh experience significant changes in their molecular composition as previously described in detail by Goranov et al. (2020). The photo-transformed pyDOM is much more aliphatic and richer in nitrogen and LMW compounds which render pyDOM to be much more biologically labile (Goranov et al., 2020). Surprisingly, it was found that Oak 400 Fresh (1646 bio-labile formulas) is more bio-labile than its photo-irradiated counterpart (Oak 400 Photo, 1242 bio-labile formulas). However, this observation using molecular data does not agree with quantitative carbon loss results for the 10-day incubation (Oak 400 Fresh: 16% carbon loss, Oak 400 Photo: 25 % carbon loss). The observed discrepancy is because LMW compounds contribute to a large fraction of the degraded carbon in the Oak 400 pyDOM systems and LMW species are not observed following the employed PPL sample preparation and FT-ICR-MS detection. A similar discrepancy is observed when comparing Oak 400 Photo (1242 bio-labile formulas, 25% carbon loss) and Oak 650 Photo (1410 bio-labile formulas, 23% carbon loss). In contrast, Oak 650 Fresh (1364 bio-labile formulas) was observed to be less bio-labile than Oak 650 Photo (1410 bio-labile formulas) via both FT-ICR-MS and the observed quantitative carbon losses (Oak 650 Fresh: 15% carbon loss, Oak 650 Photo: 23 % carbon loss). LMW species are less abundant in the Oak 650 pyDOM systems resulting in consistent trends between the analyses. Bio-degradability trends derived from FT-ICR-MS molecular data match those from the UV-VIS data from chromophoric pyDOM (Figure S1) revealing a similar inability of UV-VIS to detect LMW compounds which do not absorb UV-VIS light. In summary, we observe a degradation of a variety of different molecular classes as well as a producion of many molecules that appear to be of high biological lability. However, we caution that there are observed discrepancies among carbon loss and molecular/chromophoric data for the Oak 400 pyDOM systems, an observation that highlights the need to clearly understand methodological analytical windows when interpreting molecular and spectroscopic data.

My biggest concern: The results and discussions about "Radical oxygenation as a potential source of molecular diversity" contained too many over-interpretations. Only the results of FT-ICR MS cannot support the obtained conclusions. (1) no data about the detection of ROS were present in this study. In addition, the control experiment by addition of ROS inhibitors during incubation was lacking. (2) the conclusions like "the bio-produced formulas could be classified as products of oxygenation reactions, likely driven by ROS species such as the hydroxyl radical (•OH)" obtained by the KMD analysis using oxygen (O) series (eg. Figure 4) are severe over-interpretation of the FT-ICR MS data. There is no evidence to support that $C_cH_hO_{o+1}$ is produced from $C_cH_hO_o$ via oxygenation by hydroxyl radical (•OH) attacks. Combined (1) and (2), no evidence support the conclusions about the pathway of radical oxygenation of pyDOM.

We agree with the referee that our results here are not definitive and that the presence of oxygenation reactions driven by ROS is speculated. In the current work we cannot provide direct evidence for the presence of ROS reactions. Throughout the manuscript we describe in detail the indirect evidence for ROS reactions. We have addressed this in the manuscript as shown below:

Lines 647-651: However, it must be noted that this KMD analysis does not directly prove the existence of radical processes and the suggested radical processes are speculated based only on indirect observations. Future studies need to directly test the presence of radical reactions by performing biotic incubations of pyDOM with radical quenchers as well as by quantifying radical fluxes in these microbiological systems.

We also provide recommendations for future studies to directly test this hypothesis.

Lines 958-962: Future microbiological studies must aim to investigate these pathways further by designing radical quenching experiments (to test for presence/absence of radical oxygenation pathways) as well as employ bio-analytical techniques (e.g., genetic sequencing; Nalven et al., 2020) for assessing what microbes are responsible for the labilization and diversification of pyDOM.

m was converted to square, eg. line 150, line 469

We thank the referee for spotting this. These have been corrected:

Line 165: 100 µL

Lines 536-537: is from β-hydrogens to a heteroatom

Figure 1: Present bio-resistant formulas in Figure 1?

Unfortunately, when bio-resistant formulas are plotted in Figure 1, the figure becomes cluttered and the trends are very difficult to see. To overcome this, we tried using several different color schemes, marker sizes and shapes. No figure that had the bio-resistant formulas present was visually appealing. Furthermore, the bio-resistant formulas are not important for understanding the major findings in our study. Thus, if bio-resistant formulas are plotted on Figure 1 they will likely distract the readers from the main trends in the data. Therefore, we prefer to keep the bio-resistant formulas shown on Figures S3 and S7.

Unsolicited revisions

Collectively most of the unsolicited edits we made are mainly cosmetic (addition/removal of articles, commas, etc.) and did not alter the meaning of the text. We made edits that provided clarification and streamlined the manuscript. Several redundant sentences were removed as well. We removed unnecessary and added necessary references. We also reorganized section 4.1.2 for a more logical flow. All changes can be viewed in the document that shows the "Tracked Changes" (except when noted below) in order to be identified during the next round of editorial assessment. Several edits of importance are noted below:

Line 8: There was a typo in affiliation #2. Correction done in both manuscript and the supplemental.

Lines 253-254: The numerator and denominator of the KMD series factors (S) were mistakenly written in an inverted form. This was corrected. The KMD analysis calculations were double-checked and no mistake was found indicating that our error here was only in the transcription of the formula rather than the calculation.

Section 5 of Supplemental: While the manuscript was in review, we identified an error in the formatting of the environmental DOM data package. We re-analyzed the data and obtained different results that are reported in the new Supplement (Section 5). We identified many more common formulas among our bio-produced formulas and previously acquired environmental DOM data (DOM across an estuarine transect and oceanic DOM samples). Any common formulas were also evaluated to determine whether they could be attributed to carboxyl-rich alicyclic molecules (Hertkorn et al., 2006). This new information is mentioned in the manuscript as shown below:

Section 9 of the Supplemental: We provided p-values for each correlation.

Lines 39-42: Some of the bio-produced molecules (212 – 308 molecular formulas) were identified in surface and abyssal oceanic samples and 81 – 192 of them were of molecular composition attributed to carboxyl-rich alicyclic molecules (CRAM). These results indicate that some of the pyDOM bio-degradation products have an oceanic fate and can be sequestered into the deep ocean.

Figure 2 – added more descriptive panel titles

Figure 4 – changed DOM radical to pyDOM radical

Figure 6 – we had mistakenly swapped the labels "Olefinic groups" and "Methanol". The figure has been fixed. We also improved the statistical analysis and added p-values.

References used in responses to reviewers

Bostick, K.W., Zimmerman, A.R., Goranov, A.I., Mitra, S., Hatcher, P.G. and Wozniak, A.S. (2020) Photolability of pyrogenic dissolved organic matter from a thermal series of laboratory-prepared chars. Science of The Total Environment 724, 1-9.

Goranov, A.I., Wozniak, A.S., Bostick, K.W., Zimmerman, A.R., Mitra, S. and Hatcher, P.G. (2020) Photochemistry after fire: Structural transformations of pyrogenic dissolved organic matter elucidated by advanced analytical techniques. Geochimica et Cosmochimica Acta 290, 271-292.

Hertkorn, N., Benner, R., Frommberger, M., Schmitt-Kopplin, P., Witt, M., Kaiser, K., Kettrup, A. and Hedges, J.I. (2006) Characterization of a major refractory component of marine dissolved organic matter. Geochimica et Cosmochimica Acta 70, 2990-3010.