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Author responses to:

Interactive comment on “Molecular characterization of organic matter mobilized from Bangladeshi aquifer sediment: tracking carbon compositional change during microbial utilization” by Lara E. Pracht et al.

We the authors were initially requested to submit detailed responses to each reviewer comment, but not to prepare a revised manuscript. After reviewing these initial responses, the editor requested that we revise our manuscript. Below we show each reviewer comment in *blue italic* text, our initial responses to these comments in black un-italicized text, and a description of the corresponding revisions we made to the text to address each reviewer comments in ***bold, black italicized*** text. At the end of this document is a marked up version of our manuscript that tracks all of the changes made to the manuscript during the revision process.

Anonymous Referee #1:

The terms and samples collected are unclear to me.

1. Page 2. The term aquifer sediments is in my opinion a misnomer. A lake can have sediments, or an ocean, but I don't see how an aquifer can have sediments. I presume that this is an unconsolidated matrix aquifer consisting of sand and clay particles they must have been deposited by water. But I don't think it is accurate to call the aquifer matrix sediments any more.

We disagree that the use of “sediment” is incorrect. Basic groundwater textbooks (e.g., Fetter (2001) Applied Hydrogeology, 4th edition, Prentice Hall, Upper Saddle River, New Jersey) use sediment to describe aquifer materials. In the Fetter textbook, sediment is defined as: “assemblages of individual grains that were deposited by water, wind, ice or gravity. There are openings called pore spaces between the sediment grains, so that sediments are not solid.” The book goes on to describe porosity and classification of sediments and permeability of sediments as it relates to groundwater aquifers.

We kept the use of “sediment” in the revised manuscript.

That said, this aquifer needs to be described better, please.

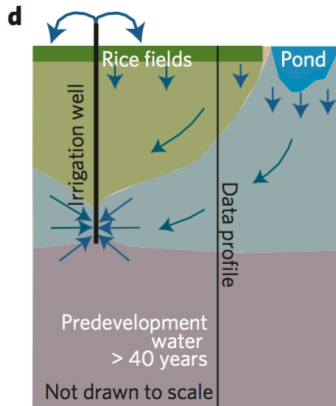
The aquifer is well described in previous publications. We will modify section 2.1 to include a better description of the aquifer and we will point the reader towards these references if they desire additional information about the aquifer.

We added aquifer information to section 2.1

2. Page 2 and 3. What is aquifer-recharge water? How do you collect pond recharge water and rice field recharge water? (page 3). Please define and describe these terms.

3. How does recharge water (pond or rice field) differ from sediment pore water? I am sorry but I find this confusing.

In response to comments 2 and 3 above, we will include a new figure in the manuscript that shows a cross section of the field site and visually indicates what we are calling recharge water and sediment porewater. This figure will build upon Figure 1d in Neumann et al. (2010) *Nature Geoscience*, which is included below for easy reference:



Aquifer recharge water is water that is flowing into the aquifer from surface water sources, recharging the aquifer, but it has not yet entered the aquifer and thus is not yet technically groundwater. As stated in our manuscript (page 3): “Filtered (0.45 μm) aquifer-recharge waters were collected from underneath a rice field and from pond sediments.” We will mark on the figure above where these water samples were collected and we will add information to section 2.1 describing how we collected these water samples.

In contrast to the recharge waters, sediment porewater is water surrounding the collected aquifer sediment, which we vacuum extracted off of the collected sediment back in the laboratory (page 3). We can clarify this point in the added figure.

We added a new figure, Figure 1, which indicates conceptually where incubation materials were collected at the field site. We also added sampling and collection details to section 2.1 and clarified in this section what is meant by sediment porewater and aquifer recharge water.

4. What in reference to these terms above is mobilized SOC?

Mobilized SOC is organic carbon that came off of the aquifer sediment during sampling, homogenization and was dissolved in the sediment porewater. We will add this clarification to section 2.1.

We added this clarification to section 2.1

5. Page 2. How do you know that it's the mobilized SOC that is reacting and not the SOC in place?

As described in the Neumann et al. (2014a) manuscript, the amount of DOC detected in the initial incubation water was fully explained by that detected in sediment porewater before the sediment was used in the incubation. Therefore, the DOC in the incubation water, at least initially, was largely carbon that was in the sediment porewater and thus was carbon that was mobilized off the aquifer matrix. In this previously published experiment, dissolved concentrations of organic carbon then decreased during the incubation and methane was produced. The amount of produced methane was equivalent to the amount of DOC lost during the incubation. These mass balance constraints point to reaction of mobilized SOC.

The mass balance arguments of Neumann et al. (2014) were outlined in section 2.1.

In the current manuscript, we characterized DOC in the sediment porewater (section 3.2)

and then compare the FT-ICR-MS signature of this carbon to that in the initial incubation water (section 3.3). Via this comparison, we similarly conclude that most of the carbon in the initial incubation water was that mobilized from the aquifer sediment. We then go onto to discuss the possibility that between day 1 and day 18 of the incubation, more SOC was abiotically desorbed off the aquifer sediment into the incubation water.

This discussion of abiotic desorption was kept in sections 3.2 and 3.3 and re-iterated in the conclusion section.

We do not deny the possibility that SOC sorbed to or associated with the aquifer sediment could be processed by microbes – but our study is looking at changes in the water, and thus is necessarily tracking microbial processing of SOC mobilized off the aquifer sediment into the water phase.

And if you don't use the terms sediments any more can you call this aquifer matrix organic material?

We intend to keep using the term “sediment.”

We kept the use of “sediment” in the revised manuscript.

6. OC degradation proceeds from large molecules to smaller ones and then to methane and CO₂. page 2. Do you contradict this later saying that larger molecules are more reactive and smaller ones accumulate?

We did not mean to state that small molecules accumulate. Searching our document for the term “accumulate” did not turn up any results. We used the word “persisted” because we are looking at what changes did or did not happen from one time step to the next. The fact that small compounds persisted longer than larger compounds aligns with the pattern of OC degradation proceeding from large molecules to smaller ones and then to methane and CO₂. There is no contradiction. We do clarify our use of the word persistence on page 10: “Our interpretation is that persistent compounds were not necessarily biologically inaccessible, but rather they were more slowly processed or were regenerated during degradation of larger compounds.”

We kept this clarifying sentence in the revised manuscript.

7. page 3 top of page. In addition to what? On the bottom of the last page you didn't describe what you were doing in the field?

We were referring to efforts described previously in the paragraph. In addition to the effort to, “identify the carbon compounds mobilized off aquifer sediment in the Neumann et al. (2014a) experiment and to track chemical compositional changes as SOC was converted into methane,” we also characterized DOC in the aquifer-recharge waters. We will modify this paragraph to make this connection more clear.

We fully revised and re-wrote the introduction. This sentence does not exist in the revised manuscript.

8. page 3, line 5. really? None of the DOC from rice field recharge (what's that) reacted?

Yes. These are peer-reviewed results from Neumann et al. (2010) *Nature Geoscience*. This result was described in the introduction of this manuscript to provide context for the FT-ICR-MS study and results.

We fully revised and re-wrote the introduction. This sentence does not exist in the revised manuscript

9. page 3 line 22. Did these samples come from Neumann's 2014 incubation? How were they preserved all that time? Or was another similar incubation done?

Yes. The samples came from the Neumann et al. (2014) incubation. In section 2.2 we note how the samples were preserved: “Aliquots (100–200 μ L) of vacuum-filtered water samples from destructively sampled incubation bottles were immediately frozen and later packed in dry ice for shipment to the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory in Richland, WA.” We will modify this sentence to clarify that the analyzed samples came from the Neumann et al. (2014) incubation and that they were filtered and kept frozen until analysis by FT-ICR-MS.

We added clarifying information on the samples analyzed by FT-ICR-MS in both section 2.1 and section 2.2.

10. *page 2, line 19. aquifer recharge waters. Define how you collect this?*

See our response to comments #2 and #3. We will clarify how we collected aquifer recharge waters in the method section.

We added this clarifying information to section 2.1

11. *page 6. line 30. Concentration and character of OC mobilized off aquifer sediment into sediment porewater differed from DOC in aquifer recharge. Do you mean DOC that comes off the aquifer matrix material relative to that from ponds and lakes flowing down? How old is the aquifer matrix material? How old is this aquifer? Wouldn't you expect this?*

Yes. We are comparing FT-ICR-MS signature of carbon that came off the aquifer sediment to DOC found in the porewater of surface water sources that recharge the aquifer. We agree it is not surprising that the two carbon sources differ. One is presumably a young carbon source (the recharge water) and one is presumably an older carbon source (the aquifer sediment). Therefore, we believe there is value in comparing the two. The sentence at the start of the paragraph sets up this comparison, it is not meant to reflect surprise at this finding.

The aquifer is ~5000 years old. We will add this information to section 2.1 when we describe the aquifer in greater detail (see response to comment #1)

We added this clarifying information to section 2.1

12. *I don't understand how the numbers relate to the pools in line 31 page 6 to line 1 page 7.*

The numbers in the parentheses (line 1 page 7) are comparing DOC concentrations in sediment porewater to DOC concentrations in the two recharge waters. We will modify this sentence to make this comparison more clear.

We modified the text to read: “Sediment porewater, which was shown by Neumann et al. (2014) to have a higher dissolved organic carbon concentration than recharge waters (1059 ± 186 mg/L vs. 17 ± 7 mg/L in rice field recharge water and 30 ± 3 mg/L in pond recharge water), also had a higher total number of assigned formulas (5263 vs. 627 in rice field recharge water and 835 in pond recharge water).”

13. *How do collect mobilized SOC? Is this different from sediment porewater?*

Dissolved organic carbon in sediment porewater (i.e., the water that was vacuum extracted off the sediment) is what we refer to as mobilized SOC. As stated in our response to comments #2 and #3, we will clarify this point in section 2.1 and in the new figure we will add.

We modified the text to read: “Neumann et al. (2014) collected sediment for the incubation experiment from an aquifer depth of 9.1 m (Figure 1), where groundwater arsenic and methane concentrations were relatively low (~ 6 μ g/L and ~ 5 mg/L, respectively). They homogenized the sediment and collected sediment porewater (i.e.,

water surrounding the aquifer sediment) by vacuum filtering (0.2 µm) multiple sediment aliquots. Dissolved organic carbon (DOC) concentrations in sediment porewater ranged between 856 and 1219 mg/L (Neumann et al., 2014) (SI Table S1), which was much higher than ~4 mg/L DOC concentration measured in groundwater at the field site (Swartz et al., 2004). Neumann et al. (2014) concluded that organic carbon was mobilized off the sediment into porewater during sampling and/or homogenization of the sediment. In this manuscript, we refer to organic carbon dissolved in the Neumann et al. (2014) sediment porewater as ‘mobilized SOC.’”

14. *The figures are awfully small and dense. It would help tremendously if the figure captions were on the same page as the figures. Why is this not allowed?*

See reply to comments #15 - #17 – we will modify the figures, increasing their size to facilitate easier axis labeling. As for the figure captions, the issue of them not being on the same page as the figure is isolated to the review process. If accepted, captions will be directly below figures in the published manuscript version.

We increased the size of the figures, including the font in the figures. We added the figure captions to the bottom of the figures.

15. *I am uncertain what the x axis are in Fig. 1 B, C, and E.*

The x-axis values are for the corresponding ratios given at the top of each plot. We will modify the figure and move the names from the top of each plot to under the x-axis to reduce confusion.

We modified the figures as stated.

16. *I am uncertain what the xaxis is in fig3, →0,1,2,3??? 0,1? I thoguth these were day 1 to 18? The figure caption may be accurate but it is too dense.*

17. *fig. 4, see comment 16.*

As was the case for comment #15 – the x-axis values are for the corresponding ratios given at the top of each plot. We will modify the figure and move the names from the top of each plot to under the x-axis to reduce confusion.

We modified the figures as stated.

18. *Conclusions. Lines 15-20, page 11. I don't follow this, seems like a lot of methane is being formed in this aquifer already. What are you warning would happen if this pool of SOC was destabilized in situ? And what does this mean, to destabilize it in situ?*

We were trying to indicate that in the incubation, the organic carbon associated with the aquifer sediment was bioavailable, and if groundwater chemistry changed at the field site such that it facilitated mobilization of organic carbon off the aquifer sediment into groundwater, within the aquifer (i.e., *in situ*), then it could fuel microbial reactions. We were trying to relate the incubation results back to the field site. However, reviewer #2 also had concerns about this approach. Therefore, during revision we will revise our efforts to connect incubation results back to the field site.

We revised this section of the conclusions to read: “As discussed in the Introduction, in the Neumann et al. (2014) experiment, SOC was initially mobilized due to sampling, homogenizing, and/or handling of the sandy aquifer sediment. While such physical perturbations to the subsurface would not occur in situ, geochemical perturbations to aquifers can and do occur, and geochemical perturbations hold potential to mobilize organic carbon off sediment into groundwater. For example, changes to pH or ion concentrations could desorb organic carbon while reductive dissolution of carbon containing oxide minerals could release organic carbon into groundwater (Eusterhues et al., 2003; Fontaine et al., 2007; Gu et al., 1994b; Jardine

et al., 1989; Kaiser and Zech, 1999; Mikutta et al., 2006). FT-ICR-MS and DOC concentration data (Figures 3–4, S2–S3) signify SOC was abiotically released from aquifer sediment during incubation. Release reflected desorption during equilibration of organic carbon between dissolved and sorbed phases. If it were to get mobilized within the anaerobic Bangladeshi aquifer, the characterized pool of SOC would represent a more energetically favorable carbon source than DOC transported into the aquifer with recharge water due to its higher NOSC (Boye et al., 2017; Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011) (Figure 2).”

Overall, pretty confusing paper, confusing terms, with confusing figures.

We are confident that an added overview figure and text in methods section 2.1 will clarify our terms (i.e., mobilized SOC, aquifer recharge water), which was clearly a large contributor to reviewer confusion.

Anonymous Referee #2:

GENERAL COMMENTS

The present study builds on an earlier paper by the group which examined microbial utilization of sedimentary organic carbon upon disturbance (physical homogenization). Here they utilize high resolution mass spectrometry to determine the organic carbon chemistry during microbial processing of the carbon. There are many things to like about the study. Expanding the science of organic carbon in sediments and soils is of importance of understanding the processing (and inversely, storage) of carbon within the subsurface, how it impacts water quality (chemically and biologically), and potential impacts on atmospheric gas exchange. The mass spectral technique utilized within the study offer a means of examining organic carbon with unprecedented resolution, although there are many caveats that the authors should be careful of and be cautious with their conclusions, particularly without secondary supporting techniques. The physical displacement of the organic carbon provide great insight into what is essentially hiding in the sediments, unavailable for microbial decomposition. Following the displacement with incubation studies helps to confirm that these compounds are decomposable, but not in the physically unaltered sediments. This is an important insight that should be the highlight of the manuscript.

We appreciate the positive feedback and the constructive suggestions.

The downside of the manuscript, as it presently stands, is that the primary conclusion, and, in fact, the central theme, is that carbon displaced by physical homogenization will lead to its decomposition by microbial action. There is just no way for me to conceive how sediments 9-m below the ground surface could be homogenized, and making this a central theme of the manuscript is highly problematic. However, by reversing the concept and considering this an examination of the carbon stored in the sediments, the study not only moves to solid ground (no pun intended), but the displacement and subsequent microbial utilization of the sediment organic carbon becomes a highly clever means to illuminate the chemistry of the compounds. I would therefore strongly suggest that the authors consider inverting their view of the central theme and making the paper about the chemistry of organic carbon within the Holocene aquifer of Bangladesh.

We did not intend for our primary conclusion to be that physical homogenization of aquifer sediment 9-m below the ground surface will lead to carbon displacement and microbial decomposition. We agree that there is no conceivable way for sediments at this depth to experience physical homogenization. We intended for our manuscript to be about the chemistry and bioavailability of organic carbon within the studied aquifer in Bangladesh. We were trying to make the argument that the organic carbon studied in the incubation experiment was carbon that was inaccessible in the aquifer, but if subsurface

chemistry changed such that it changed abiotic sorption of carbon to the sediment, or it fueled reductive dissolution of iron oxides that were associated with organic carbon, some portion of this previously inaccessible carbon could get mobilized into the groundwater and become available to microbes. Once available to microbes, the organic carbon could then fuel further reactions. Given the confusion over this point, we will modify the manuscript to make this argument more clear while also noting that homogenization is a large perturbation that likely cannot be fully replicated by the above mentioned *in situ* perturbations, and we will strengthen the theme of organic carbon chemistry in the Holocene aquifer of Bangladesh, as suggested by the reviewer.

We fully revised the re-wrote the introduction as well as the conclusion of the manuscript. We believe these revised sections more clearly present our ideas and arguments surrounding aquifer perturbations and they strengthen the theme of SOC chemistry in the Bangladeshi aquifer.

One final general comment is that there seems to be a number of locations in the manuscript where microbial processing of organic carbon is not correctly portrayed. I would therefore also suggest the authors seek to ensure microbial and biochemical accuracy (possibly consulting with a microbiologist).

In the manuscript we used “fermentation of organic carbon into methane,” to encompass the entire process of anaerobic methane production. We recognize that methane production is a multi-step process and that fermentation is the first step and that the microbes that actually produce methane from acetate or hydrogen are not the same set that are fermenting organic carbon. We will fix this phrasing to be more technically correct. In addition, we have reached out to a microbiologist.

We fixed the phrasing throughout the manuscript.

DETAILS

Page 1

Lines 17-18: Organic carbon is not fermented into methane. It's at two (to three) step process where first the organic carbon (that less than 600 Da) is bacterially fermented (sometimes with a secondary fermentation step), then the metabolites are respired by archaea to methane (methanogenesis).

See our response above. We are well aware of the multiple steps involved with anaerobic methane production. We were using this phrasing to encompass the entire process. We will modify this phrasing in the manuscript to be more technically correct.

We fixed the phrasing to read: “mobilized SOC was converted into methane”.

Lines 19-20: Stick with abiotic sulfidization; as noted below, the methanogen pathway is not warranted here.

After consulting with a microbiologist, we have decided to broaden the possible pathways for the formation of organo-sulfur compounds to include anabolism in general (i.e., microbial formation of organo-sulfur compounds for any purpose, not just methanogenesis) and sulfate reduction leading to abiotic sulfidization.

The text now reads: “We reason that these detected compounds formed abiotically following microbial reduction of sulfate to sulfide, which could have occurred during incubation but was not directly measured, or they were microbially synthesized.”

Line 22: Here and elsewhere, the term or inference of “recalcitrance” needs to be clarified. As

noted in citation later in the manuscript (Schmidt et al, 2011; Lehmann and Kleber, 2015), “molecular recalcitrance” has become an antiquated notion and should be placed in the correct context here. In short, the new realization is that recalcitrance is an ecosystem specific feature. As summarized in Schmidt et al. (2011), lignin and other complex aromatic structures degrade at rates not dissimilar to many starches.

We are aware of the ecosystem specific nature of recalcitrance and reference Schmidt et al. (2011) in the manuscript. However, based on responses at conferences and other meetings, there are still many researchers that hold onto the idea of molecular recalcitrance. We were trying to highlight that molecular recalcitrance did not control carbon use in the experiment, which aligns with this emerging understanding that all carbon types are molecularly available. We will add a sentence to the abstract to help make this connection to and alignment with current understanding more clear (i.e., clarify and contextualize our use of molecular recalcitrance as requested by the reviewer).

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Page 2

Lines 10-11: Again, the inference to recalcitrance needs to be clarified (or avoided).

Similar to our response above, we will modify the manuscript to clarify and contextualize our use of molecular recalcitrance in the manuscript, focusing on how this is an outdated but still used concept and highlighting how our results align with emerging understanding of carbon processing in the environment.

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Lines 22-23: same comment as for lines 17-18 on page 1.

See reply to same comment above.

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Line 30: Exoenzymes are indeed needed for depolymerization but not necessarily to monomers. The size cutoff is the critical factor and is largely considered in the 600 Da range (as noted in the manuscript).

We will remove monomers from this sentence and replace it with the critical ~600Da-size cutoff.

The introduction was re-written and this section/sentence was cut.

Page 3

Line 7: The analysis provides insight into the chemistry of the DOC; it does not, however, translate to the bioavailability, which is a far more complex pathway that varies between specific organisms. Even bioaccessibility is not really tracked here – possibly the lack of bioaccessibility is obtained.

We agree that bioavailability is complex and varies between specific organisms. However, the rate of carbon usage during the incubation does give insight into anaerobic

microbial processing of organic matter for the studied system (i.e., the captured microbial community under the conditions of the incubation). We will modify the manuscript to reflect this more nuanced perspective of “bioavailability” and move away from broadly connecting bioavailability with chemical compositional changes tracked in the incubation.

In the revised manuscript we clearly state that “accessibility and microbial ecology control carbon bioavailability”

Lines 16-17: I think this statement of “aquifer sediments” is splitting hairs. There is an abundance of work done on marine sediments that should be noted, and which could help guide the authors to stronger conclusions, and an extensive body of literature on soils is also available. For marine sediment analogies, Hedges and co-workers, for example, have done extensive work that has been, in my opinion, groundbreaking and could help with the interpretation here. Also, two recent papers using FT-ICR- MS (Bailey et al., Soil Biol Biochem. 2017, 107: 133-; Boye et al., Nature Geosci. 2017, 10, 415-), in which one of the authors was involved, could also be helpful and describe organic carbon physically isolated in soils and thermodynamically protected in terrestrial sediments.

We appreciate the references and will include them in our revised manuscript.

We fully integrated Boye et al.(2017) and Bailey et al (2017) into the revised manuscript.

Lines 22-32: How long were the samples stored? And at what temperature?

After collection, water samples were filtered in an anaerobic glove box and transferred to vials that were kept frozen at -18 C for a period two to three months before shipment to EMSL for FT-ICR-MS analysis. We will add this information to the methods section.

This information was added to section 2.2.

Line 24: Homogenization of the sediments alters that physical accessibility massively. As noted in Bailey et al. (Soil Biol Biochem. 2017, 107: 133-), the chemistry of organic carbon changes with pore-size. Adding in the displacement of physically occluded organic carbon, and the system has been changed massively. This can be used to the authors advantage, but a realization that no disturbance can release this proportion of the OC needs to be made. Rather, it provides an ability to access what is not being processed by the microbiota.

We appreciate the reference and agree with this comment. See our reply to this similar comment in the General Comment section above.

We have revised our introduction section to include this point: “While mobilization was an artifact of the experiment, it allowed us to study a pool of sedimentary organic carbon that was highly bioavailable after mobilization and would have otherwise been inaccessible.”

Page 5

Lines 17-: What proportion of the mass spec data was successfully assigned? Or, in other words, what proportion of the mass spectral data were left unassigned. This is a really important aspect of the analysis when (semi-) quantification is being attempted.

43 to 47% of the mass spectra data were successfully assigned. This percentage assignment was consistent for all of the samples and was at a level that we considered good given the samples were not concentrated or otherwise treated prior to analysis. We will add this information to the methods section of the manuscript.

We added this information to section 2.2.

Line 25: This is already an outdated concept. The present manuscript notes this later in the references to Schmidt et al. (2011) and Lehmann and Kleber (2015), and the authors should hold to the updated theory of sediment/soil organic carbon.

As stated above, we will modify the manuscript to clarify and contextualize our use of molecular recalcitrance in the manuscript, focusing on how this is an outdated but still used concept, and highlighting how our results align with emerging understanding of carbon processing in the environment.

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Line 22-23: The process described here for microbial metabolism is not correct. Catabolism is the transfer of compounds into useful energy. The authors are correct that for the organisms to utilize carbon compounds for catabolism or anabolism they must be less than ca. 600 Da. However, to decrease polymer size, extracellular enzymes are (typically) used, and this is independent of catabolism. In fact, catabolic energy is needed to synthesize the molecules, and thus would be an endergonic process.

We appreciate the correction. We have consulted with a microbiologist and will modify the language accordingly.

We fixed this language. The section now simply reads: “the dimensions of bacterial porin structures can lead to the exclusion of large organic solutes (e.g. >600Da), requiring extracellular enzymes (exoenzymes) to breakdown these larger molecules before they can be assimilated by microbial cells (Benz and Bauer, 1988; Nikaido and Vaara, 1985; Weiss et al., 1991)”

Line 27-29: As noted in the M-M section, FT-ICR-MS is not quantitative, and thus providing the % differences should be done with caution.

We agree that FT-ICR-MS is not quantitative and we note this in our methods section, however, multiple previous readers of the manuscript requested such comparisons of the generated data. We will clarify in the manuscript when we compare relative compositions that what we are comparing is the composition detected by the analysis method and not necessarily the full composition of the samples. That said, all samples were treated the same and normalized by the total number of peaks in each sample, making such comparisons more robust.

We added the following text to the start of the results/discussion section: “As previously mentioned, ESI FT-ICR-MS is not quantitative because the ionization efficiency of the different organic compounds vary widely during ESI. Thus, comparisons involve only those compounds detected by the instrument, and not the full composition of each sample. However, all samples were treated the same and each sample was normalized by the total number of detected peaks, making such comparisons more robust.”

We also added a similar clarification directly to Table 1: “percentages reflect only those compounds detected by the FT-ICR-MS analysis method.”

Line 30: Once again, the concept of chemical recalcitrance is now antiquated and should be

placed within the ecological context of the environment (see Schmidt et al., 2011, for example).

As stated above, we will modify the manuscript to clarify and contextualize our use of molecular recalcitrance in the manuscript, focusing on how this is an outdated but still used concept and highlighting how our results align with emerging understanding of carbon processing in the environment.

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Page 8

Line 6: “thermodynamically accessible” is not an appropriate description. A reaction is either thermodynamically viable or it is not. There is no middle ground. And accessible is not a term that would equate to viability. Accessible leads one to think of physical access rather than biochemical viability.

We agree with the reviewer that a reaction is either thermodynamically viable or not. We were using “thermodynamically accessible” to point to the energetic favorability of a reaction. We will remove this phrasing from the manuscript and instead use “energetically favorable.”

The term thermodynamic accessibility was removed from the revised manuscript and replaced with thermodynamic favorability.

Line 25: There is more than fermentation and methanogenesis happening here. Dissimilatory sulfate reduction would be taking place and other respiration may as well (DIRB, for example).

Our sentence was trying to indicate the possibility of microbial sulfate reduction with by our statement “microbes ... generated sulfide.” We will clarify this point by specifically calling out the reaction by name (dissimilatory sulfate reduction) in the sentence and including to the possibility of other reactions.

The text was revised to: “In the later incubation period, new organosulfur compounds were detected in biotic incubations but not in abiotic incubations, indicating that microbes either generated sulfide (via dissimilatory sulfate reduction) that abiotically reacted with DOM during this time period or they directly synthesized organosulfur compounds (see section 3.4)”

Lines 32-33: Are the number of compounds identified meaningful given the noted limitation of the FT-ICR-MS approach?

The number of detected compounds is traditionally reported in FT-ICR-MS studies. Even though the method is not quantitative, the number of detected compounds provides insight into the complexity of the samples and is sometime correlated with DOC concentration. Because of these relationships, the number of detected compounds facilitates comparisons within and between different studies using FT-ICR-MS.

The following sentence was added to section 3.1: “The number of detected compounds provides insight into the complexity of the samples (Sleighter and Hatcher, 2008). These numbers indicate that mobilized SOC was more complex than DOC in surface recharge water.”

Page 9

Line 15-16: I don’t understand this statement. If I look at Figure 2 and compare DOC levels for pond water (b) to rice (d) for the biotic incubation, they look exactly the same; for the abiotic incubations, the rice paddy water created great DOC than the pond water, which is the opposite

of the text.

We are referring to concentration changes over time in the different incubations. At the start of the paragraph we state that DOC concentrations did not change during the incubation for bottles with rice-field recharge water (both biotic and abiotic treatments), but there was an increase in DOC for bottles with pond recharge water between day 1 and day 18 in both the biotic and abiotic treatments. We will modify the text to make it clear that we are talking about a change over time and that we are not comparing the treatments to each other.

The text was simplified to: “If this interpretation is correct, then increases in DOC concentrations between day 1 and day 18 of the incubation can be attributed to desorption of SOC.”

Page 10

Line 4: This is (at least) a two-step process: Fermentation and then methanogenesis.

We acknowledge this point and will modify the manuscript to be more technically correct.

We fixed this phrasing throughout the manuscript.

Line 13: Consider the concepts in these referenced (Lehmann and Kleber, 2015; Schmidt et al., 2011) earlier in the manuscript, and place your findings within the context of current organic matter processing paradigms.

As stated above, we will modify the manuscript to clarify and contextualize our use of molecular recalcitrance in the manuscript.

Throughout the revised manuscript we clarify that the idea of molecular recalcitrance is outdated, and that our results align with current understanding that microbes can process all carbon types.

Lines 17-27: This is an excellent paragraph.

We appreciate the positive feedback.

Page 11

Lines 9-10: I would recommend removing the implication of methanogenesis in sulfidization of organic matter. The sulfur enzymes would not be sufficient to provide this signal.

As noted in our response to this same comment made for page 1, after consulting with a microbiologist, we have decided to broaden the possible pathways for the formation of organo-sulfur compounds to include anabolism in general (i.e., microbial formation of organo-sulfur compounds for any purpose, not just methanogenesis) and sulfate reduction leading to abiotic sulfidization.

The section now reads: “We reason that these detected compounds either formed due to microbial sulfate reduction and sulfide generation during this period, which was not directly measured, followed by abiotic sulfurization of organic matter, or they were microbally synthesized. Both pathways are plausible. Sulfurization of organic matter is well documented (Brown, 1986; Heitmann and Blodau, 2006; Kohnen et al., 1991; Urban et al., 1999), and microbes can synthesize a wide array of organic sulfur compounds (Brosnan and Brosnan, 2006; Madigan et al., 2003; Thauer, 1998).”

Line 18: Again, remove “fermented into methane”. The organic carbon compounds are fermented, and then methanogenesis transpires.

We acknowledge this point and will modify the manuscript to be more technically correct.
We fixed this phrasing throughout the manuscript.

Line 18: The posed “If this pool of SOC were destabilized in situ” is an important point. How would the organic matter be destabilized? In the Neumann et al. (2014) study, the sediments are homogenized, leading to the release of organic matter that is then subject to microbial degradation. It is interesting that the organic matter is rapidly consumed but not surprising. Presumably the OM is physically isolated, or partially mineral protected, and not available for microbial utilization. Residing 9 m below the surface, it is hard to imagine a process that would lead to destabilization. As such, I would recommend moving away from this position and instead focus on the interesting aspect of its chemistry— the metabolism of the OM advances our understanding of their composition and that a protection mechanism must be in play.

See our response to this comment in the “General Comments” section at the start of the review.

We clarified our ideas about in situ destabilization in the introduction section: *“In the Neumann et al. (2014) incubation experiment, SOC was mobilized during sampling and/or homogenization of the aquifer sediment. While this type of physical disturbance to the aquifer matrix could not occur in situ (i.e., within the aquifer), it is plausible that more realistic aquifer perturbations, such as geochemical changes brought about by large-scale groundwater pumping, could mobilize SOC in situ. For example, mineral-associated organic carbon can get mobilized into groundwater if solution pH increases (Gu et al., 1994a; Jardine et al., 1989; Kaiser and Zech, 1999), if concentrations of ions that compete with organic carbon for sorption sites increase (Jardine et al., 1989; Kaiser and Zech, 1999), or if an influx of dissolved organic carbon fuels microbial reactions that target the mineral phase (e.g., reductive dissolution of iron oxide minerals) (Eusterhues et al., 2003; Fontaine et al., 2007; Mikutta et al., 2006).”*

Lines 20-26: I don’t see the present study supporting the conclusions drawn in this paragraph. The summary provided in lines 27-31 are reasonable and should remain the emphasis. Albeit that I support reasonable speculation, the extension of a homogenized sediment release well-protected organic carbon as an inference into field setting is not warranted. As noted in the comment above, the power of this study is in characterizing the OM that is protected, not in biogeochemical processes that follow unrealistic release upon physical homogenization.

We agree that aquifer sediment will not experience homogenization, and thus the carbon probed in our incubation study may remain protected indefinitely. However, we do think it is reasonable that some of the SOC studied in the experiment could get mobilized into the aquifer due to desorption or reductive dissolution reactions. This possibility is one motivation for probing the protected OM. We will clarify our conceptual mechanisms for possible *in situ* carbon mobilization, while noting that these mechanisms will not access all the carbon made available during homogenization. Similarly, we will de-emphasize this point/conclusion in the manuscript and strength the focus on the chemistry of protected OM.

We revised the conclusion section to reference in situ destabilization ideas put forward in the Introduction. The section now reads: *“As discussed in the Introduction, in the Neumann et al. (2014) experiment, SOC was initially mobilized due to sampling, homogenizing, and/or handling of the sandy aquifer sediment. While such physical perturbations to the subsurface would not occur in situ, geochemical perturbations to aquifers can and do occur, and geochemical perturbations hold*

potential to mobilize organic carbon off sediment into groundwater. For example, changes to pH or ion concentrations could desorb organic carbon while reductive dissolution of carbon containing oxide minerals could release organic carbon into groundwater (Eusterhues et al., 2003; Fontaine et al., 2007; Gu et al., 1994b; Jardine et al., 1989; Kaiser and Zech, 1999; Mikutta et al., 2006). FT-ICR-MS and DOC concentration data (Figures 3–4, S2–S3) signify SOC was abiotically released from aquifer sediment during incubation. Release reflected desorption during equilibration of organic carbon between dissolved and sorbed phases. If it were to get mobilized within the anaerobic Bangladeshi aquifer, the characterized pool of SOC would represent a more energetically favorable carbon source than DOC transported into the aquifer with recharge water due to its higher NOSC (Boye et al., 2017; Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011) (Figure 2).”

Page 12

Line 17: The authors are correct to compare their finding to the soil science literature. It's important to note that there is really a fine line between subsurface soils and sediments. In fact, when soil scientist describe a C horizon (or horizons), they have effectively crossed the boundary into sediments. Top soils that undergo greater input and turnover are a different story, but the subsurface across depths is more similar.

We appreciate the positive comment and insight.

Molecular characterization of organic matter mobilized from Bangladeshi aquifer sediment: tracking carbon compositional change during microbial utilization

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10 **Abstract.** Bioavailable organic carbon in aquifer-recharge waters and sediments can fuel microbial reactions with implications for groundwater quality. A previous incubation experiment showed that sedimentary organic carbon (SOC) mobilized off sandy sediment collected from an arsenic-contaminated and methanogenic aquifer in Bangladesh was bioavailable; it was **transformed** into methane. We used high-resolution mass spectrometry to molecularly characterize this mobilized SOC, reference its composition against dissolved organic carbon (DOC) in aquifer recharge water, track compositional changes during incubation, and advance understanding of **microbial processing of organic carbon** in anaerobic environments. Organic carbon mobilized off aquifer sediment (i.e., mobilized SOC) was more diverse, proportionately larger, more aromatic and more oxidized than DOC in surface recharge. **It was predominately composed of terrestrially derived organic matter and had characteristics signifying it evaded microbial processing within the aquifer.** Approximately 50% of identified compounds **in mobilized SOC and in DOC from surface recharge water** contained sulfur. **During incubation, after mobilized** SOC was **converted** into methane, new organosulfur compounds with high S-to-C ratios and high nominal oxidation state of carbon (NOSC) were detected. We **reason that** these detected compounds **formed** abiotically following microbial **reduction of sulfate to sulfide**, which could have occurred during incubation but was not directly measured, **or they were microbially synthesized.** Most notably, microbes transformed all carbon types during incubation, including those **currently considered thermodynamically unviable for microbes to degrade in anaerobic conditions (i.e., those with low NOSC).** **In anaerobic environments, energy yields from redox reactions are small and the amount of energy required to remove electrons from highly reduced carbon substrates during oxidation decreases the thermodynamic favourability of degrading compounds with low NOSC.** While all compound types were eventually degraded, **during incubation,** NOSC and compound size controlled the rates of carbon transformation. Large **more thermodynamically favourable** compounds (e.g., aromatics with high NOSC) were targeted first while small **less thermodynamically favourable** compounds (e.g., alkanes and olefinics with low NOSC) **were used last.** These results indicate that in anaerobic conditions, **microbial communities are capable of degrading and mineralizing all forms of organic matter, converting larger energy-rich compounds into smaller energy-poor compounds.** However, in an open system, where fresh carbon is continually supplied,

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the slower degradation rate of reduced carbon compounds would enable this portion of the organic carbon pool to build up, explaining the apparent persistence of compounds with low NOSC in anaerobic environments.

1 Introduction

Organic carbon can impact groundwater quality both positively and negatively by fueling microbial reactions that remove and contribute dissolved contaminants to groundwater (e.g., natural attenuation of nitrate, mobilization of geogenic arsenic, methane production). The organic carbon fueling these microbial reactions can come from the near-surface environment, transported into the aquifer with recharging water (LeBlanc, 1984; Mailloux et al., 2013; Neumann et al., 2010), or can reside in the aquifer sediment, co-deposited when the aquifer formed (Korom, 1992; Parkin and Simpkins, 1995; Postma et al., 2007). Ultimately, the extent to which these sources of organic carbon can fuel subsurface reactions depends on their availability to the resident microbial community.

Historically, it was thought that molecular structure dictated carbon bioavailability (Lehmann and Kleber, 2015; Lutzow et al., 2006; Schmidt et al., 2011). Simple monomers were considered bioavailable, while more complex compounds, particularly those with aromatic rings, were considered molecularly recalcitrant. However, it is now understood that given the correct conditions, microbes can process all carbon types, continually transforming large, energy rich organic compounds into smaller, energy poor organic compounds (Lehmann and Kleber, 2015). In fact, compounds previously considered recalcitrant often have rapid turnover rates (Amelung et al., 2008).

The current view is that accessibility and microbial ecology control carbon bioavailability (Lehmann and Kleber, 2015; Schmidt et al., 2011). Physical protection mechanisms, such as organic carbon sorption to mineral surfaces, can limit microbial access to carbon (Baldock and Skjemstad, 2000; Petridis et al., 2014), and energetic constraints, nutrient limitations, and metabolic capacities of microbial communities can affect the ability of microbes to process different chemical forms of organic carbon (Fontaine et al., 2007; LaRowe and Van Cappellen, 2011; Trulleyova and Rulik, 2004). Recent work has demonstrated that in anaerobic settings, the nominal oxidation state of carbon (NOSC) influences bioavailability. In a sulfate reducing aquifer, compounds with high NOSC were missing while those with low NOSC persisted (Boye et al., 2017). This outcome reflects the fact that organic carbon oxidation (i.e., removing electrons from carbon) requires energy (LaRowe and Van Cappellen, 2011), which must be offset by energy released from the reduction half-reaction. The amount of energy required to fully oxidize a carbon compound increases with the number of electrons. Thus, in anaerobic conditions, where the amount of energy released from reduction half-reactions is lower than that in oxic conditions, it is, paradoxically, more thermodynamically favorable to oxidize compounds with high NOSC than compounds with low NOSC (Keiluweit et al., 2016). It is these types of physical, energetic and nutritional constraints on carbon bioavailability that help explain the presence of old organic carbon (i.e., thousands of years old) within soil and sediments, and the contemporary processing of this organic carbon, particularly after perturbation events (Fontaine et al., 2007; Gurwick et al., 2008; Parkin and Simpkins, 1995).

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Deleted: Organic carbon (OC) in aquifer sediments can impact groundwater quality, both positively and negatively, by fueling microbial reactions that remove and contribute dissolved contaminants to groundwater (e.g., natural attenuation of nitrate (Korom, 1992), mobilization of geogenic arsenic (Postma et al., 2007), methane production (Parkin and Simpkins, 1995)). Ultimately, the bioavailability of sedimentary OC (SOC) will dictate the extent to which it can fuel subsurface reactions. In aquifers, OC is often considered to have low reactivity relative to that in soils and marine sediments (Christensen et al., 2000; Hartog et al., 2004; Jakobsen and Postma, 1994), with increasing age frequently corresponding with decreased reactivity (Chapelle et al., 2009; Jakobsen and Postma, 1994; Postma et al., 2012). Over time, more bioavailable OC is thought to be preferentially degraded, leaving more molecularly recalcitrant and/or inaccessible forms behind. ... [2]

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5 Within aquifers, field studies have demonstrated that the reactivity of sedimentary organic carbon (SOC) decreases with increasing age (Chapelle et al., 2009; Jakobsen and Postma, 1994; Postma et al., 2012). With contemporary understanding of the factors that control carbon bioavailability, this diminishment in reactivity can be attributed to the development of constraints limiting microbial processing of sedimentary organic carbon rather than to a complete absence of SOC. Results from an incubation experiment conducted with sediments collected from a methanogenic, alluvial aquifer in Munshiganj, Bangladesh align with such an interpretation (Neumann et al., 2014). Sampling and homogenization of the aquifer sediments mobilized 0.33 ± 0.06 mg of organic carbon per gram of sediment or $8.8 \pm 0.7\%$ of total SOC (SI section 1). During incubation, the mobilized SOC was rapidly converted into methane by the native microbial community (Neumann et al., 2014). The experiment demonstrated that the ~5000 year-old, sandy aquifer sediments contain a notable amount of organic carbon that, if mobilized within the aquifer, could fuel microbial reactions.

15 In the Neumann et al. (2014) incubation experiment, SOC was mobilized during sampling and/or homogenization of the aquifer sediment. While this type of physical disturbance to the aquifer matrix could not occur *in situ* (i.e., within the aquifer), it is plausible that more realistic aquifer perturbations, such as geochemical changes brought about by large-scale groundwater pumping, could mobilize SOC *in situ*. For example, mineral-associated organic carbon can get mobilized into groundwater if solution pH increases (Gu et al., 1994a; Jardine et al., 1989; Kaiser and Zech, 1999), if concentrations of ions that compete with organic carbon for sorption sites increase (Jardine et al., 1989; Kaiser and Zech, 1999), or if an influx of dissolved organic carbon fuels microbial reactions that target the mineral phase (e.g., reductive dissolution of iron oxide minerals) (Eusterhues et al., 2003; Fontaine et al., 2007; Mikutta et al., 2006).

20 At two different sites in Asia, there is published evidence that large-scale groundwater pumping has facilitated mobilization of sedimentary organic carbon. At the site in Bangladesh where materials for the Neumann et al. (2014) incubation were collected, dry-season groundwater irrigation has pulled surface recharge water down to a 30-m depth where irrigation wells are screened (Harvey et al., 2006). Field measurements showed that dissolved organic carbon (DOC) concentrations in groundwater remained constant down to this depth, but that DOC age increased (Harvey et al., 2002). This situation is only possible if SOC was released along the groundwater flow path as younger DOC was mineralized or sequestered into sediment (Neumann et al., 2010). In Vietnam, urban groundwater pumping for the city of Hanoi has pulled organic-rich river water into a Pleistocene-aged aquifer (van Geen et al., 2013). Along the recharge pathway, groundwater DOC concentrations peaked where microbial reduction reactions were occurring (i.e., iron reduction and arsenic mobilization), and mass balance calculations demonstrated that the river could not have supplied enough carbon to fuel these reactions. The data indicated that DOC in river water fuelled microbial reactions that mobilized SOC and that the mobilized SOC then fuelled further reduction reactions.

30 Given the potential importance of SOC for fueling subsurface reactions, we sought to chemically characterize the carbon compounds mobilized off the aquifer sediment in the Neumann et al. (2014) incubation. While mobilization was an artifact of the experiment, it allowed us to study a pool of sedimentary organic carbon that was highly bioavailable after mobilization and would have otherwise been inaccessible. For characterization, we used Fourier transform ion cyclotron

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Deleted: Neumann et al. (2014a) previously conducted an incubation experiment using sediment and waters from Munshiganj, Bangladesh, designed to evaluate the ability of OC in aquifer-recharge waters and aquifer sediment to fuel microbial reduction reactions. Groundwater at the site is contaminated with arsenic and supersaturated with methane (Harvey et al., 2002). The incubation uncovered a pool of bioavailable OC in aquifer sediments, equating to 0.33 ± 0.06 mg OC g⁻¹ sediment, or $8.8 \pm 0.7\%$ of total SOC (SI Section 1). The native microbial community fermented the mobilized SOC into methane. The tested sediment was a fine-grained sand and lacked peat, (Chapelle, 2000a) silt, and clay components often implicated as sources of SOC fueling subsurface reactions (Aravena and Wassenaar, 1993; Chapelle, 2000b; Desbarats et al., 2014; McMahon and Chapelle, 1991). The experiment demonstrated that old (~5000 years old), sandy aquifer sediments can contain notably large amounts of microbially-available OC. ... [3]

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Deleted: (2014a) experiment and to track chemical compositional changes as SOC was converted into methane. In anaerobic conditions, OC degradation occurs via multiple steps involving a consortium of microbes (Megonigal et al., 2003). Hydrolytic exoenzymes depolymerize OC into monomers. Monomers are then fermented into low molecular weight alcohols and volatile fatty acids, which are eventually mineralized into CO₂ and CH₄. Our effort not only characterizes a previously undocumented pool of bioavailable OC in sandy aquifer sediments but also advances understanding of how OC chemical composition relates to bioavailability in anaerobic environments. ... [4]

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Deleted: conducted in aerobic conditions indicated that ~30% of DOC in pond recharge and ~0% of DOC in rice field recharge was degradable (Neumann et al., 2010). Molecular characterization of DOC in these two waters provides additional insight into relationships between chemical composition and bioavailability, and enables a comparison of carbon compounds found in aquifer sediment to those found in surface recharge. ... [5]

resonance mass spectrometry (FT-ICR-MS) coupled with electrospray ionization (ESI) in negative ion-mode. FT-ICR-MS is capable of characterizing the thousands of different compounds contained in natural organic carbon; though the technique is not quantitative, because the ionization efficiency of compounds vary, making some compounds more difficult or impossible to identify (Sleighter and Hatcher, 2007). The technique has been applied to natural organic matter in a variety of environments (Chipman et al., 2010; Evert, 2015; Kim et al., 2006; Longnecker and Kujawinski, 2011; Spencer et al., 2014; Tfaily et al., 2013).

Through FT-ICR-MS analysis of the Neumann et al. (2014) experimental samples, we were not only able to chemically characterize SOC mobilized from the Bangladeshi aquifer sediments, but were also able to compare the chemical composition of mobilized SOC to that of DOC in surface-water sources that recharge the aquifer and to track compositional changes in mobilized SOC as it was converted into methane by the native microbial community during incubation. These efforts generate knowledge about the chemistry of organic carbon within surface recharge waters and sediments of the Bangladeshi alluvial aquifer — the two possible sources of organic carbon for fueling subsurface microbial reactions — and advance understanding of microbial processing of organic carbon within anaerobic environments.

2 Material and Methods

2.1 Incubation

FT-ICR-MS samples were collected from the Neumann et al. (2014) incubation experiment, which used aquifer sediment and aquifer recharge waters collected from a field site in Munshiganj, Bangladesh. The field site has been well characterized hydrologically and geochemically (Harvey et al., 2002, 2006; Swartz et al., 2004). The site is overlain by 3.5 m thick clay layer that, at deeper depths, remains saturated year round. This clay layer hosts rice fields and man-made ponds that serve as the two primary recharge sources for the upper aquifer (Harvey et al., 2006). The upper aquifer, in turn, supplies irrigation water for dry-season rice production (Figure 1). This aquifer is ~5,000 years old and contains Holocene deposits composed of gray colored fine and silty sands between 3.5 m and 12 m depths, and grayish-green colored fine to medium sands between 12 m and 110 m depths (Swartz et al., 2004). The entire aquifer is anaerobic. Groundwater at the site is contaminated with arsenic and supersaturated with methane (Harvey et al., 2002).

Neumann et al. (2014) collected sediment for the incubation experiment from an aquifer depth of 9.1 m (Figure 1), where groundwater arsenic and methane concentrations were relatively low (~6 µg/L and ~5 mg/L, respectively). They homogenized the sediment and collected sediment porewater (i.e., water surrounding the aquifer sediment) by vacuum filtering (0.2 µm) multiple sediment aliquots. Dissolved organic carbon (DOC) concentrations in sediment porewater ranged between 856 and 1219 mg/L (Neumann et al., 2014) (SI Table S1), which was much higher than ~4 mg/L DOC concentration measured in groundwater at the field site (Swartz et al., 2004). Neumann et al. (2014) concluded that organic carbon was mobilized off the sediment into porewater during sampling and/or homogenization of the sediment. In this manuscript, we refer to organic carbon dissolved in the Neumann et al. (2014) sediment porewater as ‘mobilized SOC.’

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Deleted: (Chipman et al., 2010; Kim et al., 2006; Spencer et al., 2014; Tfaily et al., 2013), including DOC in groundwater (Evert, 2015; Longnecker and Kujawinski, 2011). But no studies that we could find have assessed the molecular composition of OC associated with aquifer sediment (i.e., SOC). Further, most studies have focused on characterizing OC; only a handful have directly tracked changes in elemental composition as OC was processed by microbes and protozoa (Chipman et al., 2010; Kim et al., 2006; Kujawinski et al., 2004; Longnecker and Kujawinski, 2011; Lu et al., 2015; Osborne et al., 2013).

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After homogenization, Neumann et al. (2014) dried and sterilized by gamma irradiation half of the sediment. They independently incubated both native and sterilized sediment for 200+ days with filtered (0.45 µm) aquifer recharge water that they had collected from a rice field and pond at the Munshiganj field site (8.2 g dry sediment to 10 mL of water). They collected the rice field recharge water from a shallow well installed within a rice field bund (i.e., the raised boundary surrounding a rice field), screened just below the surficial clay layer hosting the rice field (Figure 1); rice field recharge is focused through bunds. They collected pond recharge water by driving a Push Point Harpoon (M.H.E. products) 44 cm into the bottom sediments of a pond (Figure 1). In this manuscript, we refer to DOC in the rice field and pond recharge waters as 'recharge DOC.'

Neumann et al. (2014) destructively sampled incubation bottles 1, 17–20, 80–81, 91, 184 and 273 days after initiation of the experiment (Neumann et al., 2014). They vacuum filtered (0.2 µm) incubation water and analyzed it for dissolved organic carbon. They also analyzed headspaces from a few incubation bottles for methane. Previously published (Neumann et al., 2014) DOC and methane concentrations for all incubation replicates and time points are presented in SI Tables S2–S3. During processing of the incubation bottles, aliquots (100–200 µL) of filtered water were saved and immediately frozen at –18 °C. These frozen subsamples were later analyzed by FT-ICR-MS (see next section). Results from the FT-ICR-MS analysis are the focus of this manuscript.

Neumann et al. (2014) kept all water and sediment samples anoxic during collection, handling and processing steps through the use of an anaerobic chamber, gas-impermeable bags, oxygen scavenging packets with oxygen indicator tabs, glass BOD bottles, and glass serum vials sealed with butyl rubber stoppers. Further sampling details are available in the Neumann et al. (2014) manuscript.

2.2 FT-ICR-MS analysis

Two to three months after collection, frozen aliquots of water from the Neumann et al. (2014) incubation experiment were packed in dry ice for shipment to the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory in Richland, WA. At EMSL samples were diluted 1:2 (v/v) with LC-MS grade methanol (MeOH) less than 30 minutes before analysis to minimize esterification (McIntyre and McRae, 2005), and were injected directly into the instrument. Solid phase extraction was not performed due to sample volume constraints. No salts were observed in the spectra. To avoid biases in ionization efficiency due to differences in DOC concentrations, the ion accumulation time (IAT) was optimized for each sample individually based on previously measured DOC concentrations (Tables S1 and S2). The IAT ranged between 1–1.5s. All samples were run with instrument settings optimized by tuning on the Suwannee river fulvic acid (SRFA) standard.

A standard Bruker ESI source was used to generate negatively charged molecular ions. Negative ion mode was chosen due to its extensive use in characterizing a broad range of environmental DOC samples. Additionally, previous work has shown that organic matter is rich in carboxylic groups, as these groups ionize best in negative mode (Stenson et al.,

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Deleted: Neumann et al. (2014a) thoroughly describe the incubation experiment from which FT-ICR-MS samples were collected. Briefly, incubation materials were collected from Munshiganj, Bangladesh. Aquifer sediment was from 9.1 m depth, where conditions were anoxic. Sediment was homogenized and half of it was dried and sterilized by gamma irradiation. Prior to incubation, aliquots of aquifer sediment taken from the half that was not dried and sterilized were vacuum-filtered (0.2 µm) to collect sediment porewater. Filtered (0.45 µm) aquifer-recharge waters were collected from underneath a rice field and from pond sediments. Aquifer sediment (8.2 g, dry weight) was incubated with both recharge waters (10 mL) in clean sterile serum vials that were destructively sampled 1, 17–20, 80–81, 91, 184 and 273 days after initiation (Neumann et al., 2014a). Samples were kept anoxic during all collection, handling and processing steps through the use of an anaerobic chamber, gas-impermeable bags, oxygen scavenging packets with oxygen indicator tabs, glass BOD bottles, and glass serum vials sealed with butyl rubber stoppers (Neumann et al., 2014a). Dissolved organic carbon (0.2 µm filter) was analyzed in all waters (Sievers TOC analyzer). Headspace from a few incubation bottles were analyzed for methane (GC-FID). Previously published (Neumann et al., 2014a) DOC and methane concentrations for all replicates and time points are presented in SI Tables S1–S3. ... [6]

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2003). Recently, Ohno et al. (2016) have shown that for samples rich in condense aromatic and aromatic molecules, the use of (+)ESI improves ionization of aliphatic and carbohydrate like SOM components. Because we did not compliment (-)ESI with (+)ESI, our study may underestimate the presence of aliphatic and carbohydrate molecules.

5 Samples were introduced to the ESI source equipped with a fused silica tube (200 μm i.d) through a syringe pump at a flow rate of 3.0 $\mu\text{L}/\text{min}$. Experimental conditions were as follows: needle volt-age, +4.4 kV; Q1 set to 150 m/z; and the heated resistively coated glass capillary operated at 180 $^{\circ}\text{C}$. Blanks (HPLC grade MeOH) were run at the beginning and end of the analysis to monitor potential carry over from one sample to another. A series of three free fatty acids with masses around 255, 283 and 325 Da were observed in the solvent blanks. These peaks did not show up when running the samples; analytes in the samples picked up the charges and had higher intensity than the three fatty acids present in the MeOH. The instrument was flushed between samples with a mixture of water and methanol. The instrument was externally calibrated weekly with a tuning solution from Agilent, which calibrates to a mass accuracy of <0.1. ppm and contains the following compounds: $\text{C}_2\text{F}_3\text{O}_2$, $\text{C}_6\text{HF}_9\text{N}_3\text{O}$, $\text{C}_{12}\text{HF}_{21}\text{N}_3\text{O}$, $\text{C}_{20}\text{H}_{18}\text{F}_{27}\text{N}_3\text{O}_8\text{P}_3$, and $\text{C}_{26}\text{H}_{18}\text{F}_{39}\text{N}_3\text{O}_8\text{P}_3$ with an m/z ranging between 112 to 1333 Da.

15 One hundred forty four individual scans were averaged for each sample and internally calibrated using OM homologous series separated by 14 Da ($-\text{CH}_2$ groups). The mass measurement accuracy was 0.4 ppm on average and no larger than 1 ppm for singly charged ions across a broad m/z range (i.e. 200 <m/z <1200). Data analysis software (BrukerDaltonik version 4.2) was used to convert raw spectra to a list of m/z values applying FTMS peak picker with S/N threshold set to 7 and absolute intensity threshold set to the default value of 100. Elemental formulas were assigned using in-house built software following the Compound Identification Algorithm (CIA), described by Kujawinski and Behn (2006) and modified by Minor et al. (2012). Elemental formulas were assigned based on the following criteria: S/N >7, and mass measurement error <1 ppm, taking into consideration the presence of C, H, O, N, S and P and excluding other elements. Formulas were allowed to have the following number of elements: C (1–100 atoms), H (1–200 atoms), O (1–30 atoms), N (0–20 atoms), S (0–10 atoms), and P (0–6 atoms). Additionally, each phosphorus atom required at least four oxygen atoms. CH_2 , H_2 , NH and O were used for propagation. Since molecules containing both phosphorus and sulfur are rare, the elemental formula with the lowest error and the lowest number of heteroatoms was consistently chosen. All elemental formulas with errors above 1ppm were rejected.

25 To further reduce cumulative errors, all sample peak lists within the entire dataset were aligned to each other prior to elemental formula assignment to eliminate possible mass shifts that would impact assignment. Peaks with large mass ratios (m/z values >500 Da) often have multiple possible elemental formulas. These peaks were assigned formulas through the detection of homologous series (CH_2 , O, H_2 , NH). Specifically, when the m/z of a homologous series group and the m/z of an already confidently assigned compound were summed to an m/z that was observed >500 Da, the elemental formula assigned to the smaller compound was appended by the atoms of the homologous series group and the new elemental formula was assigned to the larger compound. If no elemental formula matched an m/z value within the allowed error, the peak was not included in the list of elemental formulas. 43 to 47% of mass spectra data were successfully assigned. This

percentage assignment was consistent for all of the samples and was considered at a good level given the samples were not concentrated or otherwise treated prior to analysis.

2.3 Data processing

5 Due to variation in ionization efficiency of different compounds, peak presence was used for analysis of FT-ICR-MS data rather than peak intensity. Since low DOC concentrations can reduce ionization effectiveness, experimental replicates were combined during data processing, as were incubation samples with similar DOC concentrations collected in close temporal proximity to each other. Tables S1 and S2 indicate the number and DOC concentrations of samples combined together. Peaks present in any individual sample were considered present for the combined sample. Analysis focused only on combined samples, which included the rice field and pond recharge water, sediment porewater, and incubation water from 10 day 1, days 17–20 (called day 18 throughout the manuscript), and days 80–91 (called day 85 throughout the manuscript). Combining replicates and samples with similar DOC concentrations improved robustness of sample characterization and minimized artifactual variability when looking at compositional changes across incubation treatments and across incubation time.

Elemental formulas in combined samples were used to divide organic carbon into 4 distinct heteroatom groups: 15 CHO, CHON, CHO plus P (any compound with P and without S), and CHO plus S (any compound with S). Elemental formulas were also used to calculate double bond equivalent (DBE), nominal oxidation state of carbon (NOSC), aromaticity index (AI), and compound classification. For an organic compound $C_cH_hN_nO_oP_pS_s$, DBE was calculated as (Koch and Dittmar, 2006, 2016):

$$DBE = 1 + \frac{1}{2}(2c - h + n + p) . \quad (1)$$

20 NOSC was calculated as (LaRowe and Van Cappellen, 2011):

$$NOSC = 4 - (4c + h - 3n - 2o + 5p - 2s)/c . \quad (2)$$

AI was calculated as (Koch and Dittmar, 2006, 2016):

$$AI = (1 + c - 0.5 * o - s - 0.5 * (n + p + h))/(c - 0.5 * o - s - n - p), \quad (3)$$

which identifies samples as alkanes (AI=0), olefinics ($0 < AI \leq 0.5$), aromatics ($AI > 0.5$), or condensed aromatics ($AI \geq 0.67$) 25 (Koch and Dittmar, 2006, 2016; Willoughby et al., 2014). Compound class was assigned based on oxygen-to-carbon and hydrogen-to-carbon ratios (i.e., location within van Krevelen plots) (D'Andrilli et al., 2015; Kim et al., 2003). Table S4 includes the ratio ranges used to identify compounds that were lipid-, protein-, carbohydrate-, amino sugar-, lignin-, tannin-, and condensed hydrocarbon-like. Compounds falling outside of these ranges were assigned as 'other.'

Compound changes were tracked between day 1 and day 18 and between day 18 and day 85 of the incubation. 30 Compounds identified at both time points were classified as 'common,' those identified at the first time point but not at the second were classified as 'lost,' and those identified at the second time point but not at the first were classified as 'newly detected.'

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3 Results and Discussion

3.1 Composition of DOC in aquifer recharge waters versus mobilized SOC

Microbial reactions within aquifers are fueled either by sedimentary organic carbon or by dissolved organic carbon transported from the surface into the aquifer with recharging water. Here we compare the chemical composition of these two sources of organic carbon for the Bangladeshi aquifer. As previously mentioned, ESI FT-ICR-MS is not quantitative because the ionization efficiency of the different organic compounds vary widely during ESI. Thus, comparisons involve only those compounds detected by the instrument, and not the full composition of each sample. However, all samples were treated the same and each sample was normalized by the total number of detected peaks, making such comparisons more robust.

The chemical composition of DOC in the pond and rice field recharge waters collected for the Neumann et al. (2014) incubation experiment was highly similar (Figure 2, Table 1). Notable differences between DOC in the two recharge waters only existed in the H-to-C ratio distribution. Pond recharge contained proportionately fewer compounds with H-to-C ratios <0.75 and proportionately more compounds with H-to-C ratios >1.25 compared to rice field recharge (Figure 2b). However, these ratio differences did not result in markedly different compound classifications (Figure 2h; Table 1).

In contrast, the character of organic carbon mobilized off aquifer sediment into sediment porewater during sampling and homogenization of the sediment was distinctly different than DOC in the two recharge waters. Sediment porewater, which was shown by Neumann et al. (2014) to have a higher dissolved organic carbon concentration than recharge waters (1059 ± 186 mg/L vs. 17 ± 7 mg/L in rice field recharge water and 30 ± 3 mg/L in pond recharge water), also had a higher total number of assigned formulas (5263 vs. 627 in rice field recharge water and 835 in pond recharge water). The number of detected compounds provides insight into the complexity of the samples (Sleighter and Hatcher, 2008). These numbers indicate that mobilized SOC was more complex than DOC in surface recharge water. A recent study using FT-ICR-MS to probe the character of organic carbon in intact soil cores found that soluble carbon in the smaller, less physically accessible soil pores was more complex than soluble carbon in the larger, more physically accessible soil pores (Bailey et al., 2017). In this study and in our study, the more complex carbon pool was the one that, due to *in situ* constraints (physical, nutritional and/or energetic), evaded microbial decomposition.

The chemical character of the mobilized SOC and its compositional difference from DOC in recharge water also signify that it avoided microbial processing within the aquifer. The mass distribution for mobilized SOC was wider and skewed toward larger masses relative to DOC in recharge (Figure 2d). The wider distribution aligns with the larger number, and thus, greater diversity of compounds detected in mobilized SOC relative to DOC in recharge. The skew toward larger masses supports the idea that mobilized SOC was protected from *in situ* decomposition. Microbial communities continually transform large compounds into smaller compounds, and the breakdown of large macromolecules is the first step. During the Neumann et al. (2014) incubation, large compounds were lost first while small compounds persisted (see section 3.3 and 3.4). Additionally, the dimensions of bacterial porin structures can lead to the exclusion of large organic solutes (e.g. >600Da), requiring extracellular enzymes (exoenzymes) to breakdown these larger molecules before they can be assimilated

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by microbial cells (Benz and Bauer, 1988; Nikaido and Vaara, 1985; Weiss et al., 1991). Relative to DOC in recharge water, mobilized SOC contained proportionately more compounds that were larger than 600 Da (Table 1), implying that these compounds had not yet been attacked by exoenzymes.

Compositionally, mobilized SOC was more aromatic than recharge DOC. Similar to the mass distribution, the DBE distribution for mobilized SOC was wider and skewed toward higher numbers relative to DOC in recharge (Figure 2e), signifying SOC was composed of a wider diversity of compounds, including those with more double bonds and rings. Based on aromaticity index (AI), aromatic compounds accounted for the larger DBE. Mobilized SOC had proportionately fewer olefinic compounds (i.e., those with double bonds) but more aromatic compounds than DOC in recharge water (Figure 2g; Table 1). According to compound classifications, these aromatic compounds were tannin- and condensed hydrocarbon-like (Figure 2h; Table 1). Mobilized SOC also had a smaller proportion of compounds classified as protein- and lipid-like (Figure 2h; Table 1). These differences in compound classification imply that relative to DOC in recharge, mobilized SOC was composed of proportionately more terrestrially derived organic matter (i.e., cellulose, lignin and tannin-like compounds; ~29% versus ~22% in recharge), more highly condensed organic matter (i.e., coal-, soot-, charcoal- and black-carbon-like compounds; ~38% versus ~25% in recharge), and notably less microbially derived organic matter (i.e., lipid-, protein-, amino-sugar-like compounds; ~13% versus ~30% in recharge). The remaining proportion of compounds did not overlap with a known class.

The detected composition of the mobilized SOC signifies it was predominately composed of plant- and fire-derived organic matter that had not yet been processed by microbes. Similarly, Bailey et al. (2017) found that soluble carbon in smaller, less accessible soil pores had proportionately more lignin-, tannin- and condensed hydrocarbon-like compounds and proportionately fewer lipid-like compounds than soluble carbon in larger, more accessible soil pores. Traditionally, lignins, tannins and condensed hydrocarbons were considered molecularly recalcitrant (Lehmann and Kleber, 2015; Lutzow et al., 2006; Schmidt et al., 2011), and thus their presence was historically interpreted as resulting from negative enrichment (i.e., microbes preferentially decomposing other compounds). However, in both the Bailey et al. (2017) study and in the Neumann et al. (2014) incubation (see section 3.4), these terrestrially derived and highly condensed organic compounds were readily degraded. Thus, the presence of these compounds indicates instead that they were protected from *in situ* microbial attack, an interpretation that aligns with contemporary understanding that physical and ecological factors control carbon bioavailability rather than molecular structure.

Of all the compound classes presented in Figure 2h, tannin- and condensed hydrocarbon-like compounds have the highest nominal carbon oxidation state (NOSC) while lipid- and protein-like compounds have the lowest NOSC (Boye et al., 2017; Keiluweit et al., 2016). The relative enrichment of tannin- and condensed hydrocarbon-like compounds and depletion of lipid- and protein-like compounds in mobilized SOC (Figure 2h) explains the positive skew of the NOSC distribution for mobilized SOC relative to recharge DOC (Figure 2f). The median NOSC value for mobilized SOC was +0.10 while the median value for DOC in pond and rice field recharge was -0.56 and -0.32, respectively (Table 1). Not surprisingly, Bailey et al. (2017) similarly found that soluble carbon from the smaller, less accessible soil pores had a higher NOSC than soluble

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carbon from the larger, more accessible soil pores, though the difference was not statistically significant. In the anaerobic conditions of the Bangladeshi aquifer, where highly energetic electron acceptors like oxygen and nitrate are absent (Swartz et al., 2004), it is more thermodynamically favorable to oxidize compounds with high NOSC (Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011). Thus, the presence of these more oxidized, thermodynamically favorable carbon compounds within mobilized SOC further supports the idea that this carbon pool evaded microbial processing within the aquifer.

Notably, in mobilized SOC and in recharge DOC, roughly half of all identified molecules had sulfur heteroatoms (47% in pond recharge, 59% in rice field recharge, and 51% in SOC) (Figure 2a; Table 1). Organosulfur compounds form in organic, sulfate-reducing environments where reduced sulfide species can react with various types of organic matter (Heitmann and Blodau, 2006; Perlinger et al., 2002; Schouten et al., 1993). As such, organosulfur compounds have been detected in various anaerobic environments (Brown, 1986; Urban et al., 1999). In an aquifer system in Rifle, CO, sulfur heteroatom abundance increased as DOC was sampled from more chemically reduced zones (Evert, 2015). Sediment and water samples obtained for the Neumann et al. (2014) incubation study similarly came from chemically reduced environments. Groundwater at the depth from which the sediment was collected contained ~7 mg/L-S as sulfate and 64 mg/L-S as sulfide (Swartz et al., 2004). Sulfurization of organic matter is an abiotic process that occurs early in sediment diagenesis at ambient temperatures and pressures (Kohnen et al., 1991; Schouten et al., 1993). However, biologically-mediated reactions can also involve organosulfur compounds (Brosnan and Brosnan, 2006; Madigan et al., 2003; Thauer, 1998). In the later incubation period, new organosulfur compounds were detected in biotic incubations but not in abiotic incubations, indicating that microbes either generated sulfide (via dissimilatory sulfate reduction) that abiotically reacted with DOM during this time period or they directly synthesized organosulfur compounds (see section 3.4).

3.3 Carbon transformations between day 1 and day 18 of incubation

At the onset of the incubation experiment, 1 day after recharge waters were mixed with aquifer sediment, DOC concentration and composition were dominated by sediment porewater. Average DOC concentrations were 140-300 mg/L (Figures 3a-d; Table S2), which Neumann et al. (2014) showed were explained by dilution of mobilized SOC in sediment porewater (1059 ± 186 mg/L) with recharge water (17 ± 7 mg/L and 30 ± 3 mg/L, in rice field and pond recharge, respectively). Characterization by FT-ICR-MS showed that DOC in these initial incubation samples was more similar to mobilized SOC in sediment porewater than to DOC in aquifer recharge water (Figure S1). Both mass balance and chemical characterization indicate that the primary source of soluble organic carbon in the incubation experiment was that mobilized from the aquifer sediment.

Between day 1 and day 18, average DOC concentrations increased by 13±5 to 28±16 mg/L in incubations conducted with pond recharge (Figures 2a-b; Table S2) and average concentrations did not noticeably change in incubations conducted with rice field recharge, after accounting for variability between replicates (Figures 2c-d; Table S2). During this time period, the number of compounds identified by FT-ICR-MS increased in all treatments. In the two pond incubations

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~4000 new compounds were detected (Figures 2e–f), and in the two rice field incubations ~2000 new compounds were detected (Figures 2g–h). Across all four treatments, the compositional character of newly detected compounds on incubation day 18 matched that of DOC in initial incubation water (i.e., from day 1), which, in turn, was similar to SOC mobilized into sediment porewater (SI Figure S2). This correspondence can be explained by abiotic desorption of SOC from aquifer sediment into incubation water between day 1 and day 18 of the incubation, with newly detected compounds representing those desorbed from aquifer sediment. In support of abiotic desorption, during this time period there was a proportionately greater detection of large compounds (Figure 4, SI Figure S3, SI Figure S4), which can be explained by slower diffusive partitioning rates for larger compounds. If this interpretation is correct, then increases in DOC concentrations between day 1 and day 18 of the incubation can be attributed to desorption of SOC.

Fewer compounds were lost between day 1 and day 18 than were newly detected, and the type of recharge water used in incubation had no impact on the number of lost compounds (Figure 2e–h). Instead, use of sterilized versus native sediment controlled compound loss. Treatments with native sediment lost ~1500 compounds (Figures 2f,h) while treatments with sterilized sediment lost ~800 compounds (Figures 2e,g). Greater loss of compounds in incubation sets conducted with native sediment indicates that microbial transformation of organic carbon occurred during this early incubation period. Figures 4 and S3 show that compounds in the CHO heteroatom group with high O-to-C ratios and high NOSC were lost in proportionately greater numbers in biotic than in abiotic incubations. These compounds had lower H-to-C ratios, were predominately smaller than 600 Da, and were identified as aromatic (Figures 4 and S3).

The preferential loss of aromatic compounds observed in the biotic anaerobic incubation (Figures 4 and S3) contrasts with that typically observed in aerobic incubations. In aerobic conditions, microbes do not initially target aromatic compounds. While aromatic compounds eventually get oxidized, in the initial phases of aerobic degradation, they are actually selectively preserved (Lutzow et al., 2006; Schmidt et al., 2011). In both aerobic and anaerobic conditions, the rate of carbon oxidation depends both on the thermodynamic driving force driving the reaction (i.e., the net difference between energy gained from the reaction and energy needed to synthesize ATP) and on the ability of microbial communities to acquire and process reactants (Jin and Bethke, 2003). When oxygen is the electron acceptor, the thermodynamic driving force of carbon oxidation does not depend on NOSC (Keiluweit et al., 2016). Thus, no carbon form is more or less energetically favorable to oxidize. Instead, non-thermodynamic factors, such as enzyme kinetics, influence the rate of microbial utilization. In contrast, with anaerobic electron acceptors, the thermodynamic driving force rapidly drops as NOSC decreases (Keiluweit et al., 2016). Thus, in anaerobic environments, compounds with high NOSC represent the most energetically favorable compounds to oxidize. These energy dynamics explain why aromatic compounds with high NOSC and high O-to-C ratios were initially targeted in the anaerobic incubations. Additionally, the <600Da size means these compounds had a high likelihood of being directly assimilated by microbes (Benz and Bauer, 1988; Nikaido and Vaara, 1985; Weiss et al., 1991).

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3.4 Carbon transformations between day 18 and day 85 of incubation

Between day 18 and 85, DOC was lost from incubations conducted with native sediment (Figure 3b,d), but not from those conducted with sterilized sediment (Figure 3a,c). Carbon mass balance calculations conducted by Neumann et al. (2014) indicated that DOC lost in the biotic incubations was transformed into methane. Accordingly, the number of identified compounds by FT-ICR-MS decreased in both of the biotic incubations (Figure 3f,h) but did not change in the abiotic incubations (Figure 3e,g). However, there was not a direct relationship between DOC concentration and number of compounds; both biotic incubations lost similar amounts of DOC by day 85 (Figure 3b,d; Table S2), but the decrease in the number of compounds was larger in incubations conducted with pond recharge than in those conducted with rice field recharge (Figure 3f,h).

All compound types were microbially transformed, as evidenced by proportionally greater compound changes in biotic than in abiotic incubations between day 18 and 85 of the incubation, regardless of chemical index (Figures 5 and S5). Compounds traditionally considered molecularly recalcitrant (e.g., aromatics and condensed aromatics) were almost fully transformed in the biotic incubations, reinforcing current understanding that microbial communities can decompose a wide range of carbon types (Lehmann and Kleber, 2015; Schmidt et al., 2011). Additionally, compounds with low NOSC were lost from the biotic incubations (Figure 5 and S5). The loss of compounds with low NOSC in an anaerobic incubation conflicts with the recently presented idea that microbial degradation of these compound types is thermodynamically unviable in anaerobic environments (Boye et al., 2017; Keiluweit et al., 2016). The argument assumes that the thermodynamic driving force (i.e., net difference between energy gained from the reaction and energy needed to synthesize ATP) for oxidation of highly reduced carbon substrates approaches zero as less energetically rich, anaerobic electron acceptors get used in the redox reaction (Boye et al., 2017; Keiluweit et al., 2016). Boye et al. (2017) predicted that, in sulfidic conditions, compounds with NOSC below -0.3 are energetically unviable for respiring microbes. However, in the highly reducing environment of the incubation, where methanogenesis occurred, compounds with NOSC values as low as -2 were lost between incubation day 18 and day 85 (Figure 5, S5). This result indicates that microbial processing of these highly reduced compounds was not thermodynamically inhibited in the incubation despite the low energy yield associated with methanogenesis.

While all compound types were microbially transformed (i.e., were lost in greater proportion in the biotic than abiotic incubations), some compound types within the biotic incubations persisted between day 18 to day 85 (i.e., were common to both time points). Compound persistence was proportionately greater in the rice field incubation than in the pond incubation (Figures 5, S5, and S6). Our interpretation is that persistent compounds were not necessarily biologically inaccessible, but rather they were more slowly processed or were regenerated during degradation of larger compounds. One set of persistent compounds were those in the CHO heteroatom group with high H-to-C ratios, low NOSC, and small size (Figures 5, S5, S6). They were indexed as alkanes and olefinics. The persistence of CHO compounds with low NOSC directly contrasts with the proportionately greater loss of CHO compounds with high NOSC in the early incubation phase (Figures 4 and S3). While NOSC did not control thermodynamic viability in the incubation, it did affect relative rates of

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Deleted: Figure 4 and S6). They were indexed as alkanes and olefinics. The persistence of these compounds aligns with their low NOSC; in anaerobic conditions, the energetic potential associated with oxidizing these carbon compounds is small (Keiluweit et al., 2016). Similarly, the persistence of these CHO compounds

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compound transformation, with higher NOSC facilitating faster transformation, but only for CHO compounds. In other heteroatom groups, NOSC was not related to persistence (Figures 5 and S5). Instead, compound size predicted persistence, with the smallest compounds persisting in all groups (Figures 5, S5, and S6). While historically it was thought that larger, humified compounds resisted degradation, persistence of smaller compounds supports emerging understanding that organic carbon in the environment is continuously processed from larger to smaller molecular size (Lehmann and Kleber, 2015).

In contrast to persistence of smaller compounds, large compounds were lost and newly detected in both biotic and abiotic treatments in proportionately higher numbers than smaller compounds between day 18 and 85 (Figures 5, S5 and S6). These compound changes could reflect continued abiotic partitioning of large molecules between sediment and water, or, if the abiotic incubations did not stay completely sterile, it could reflect microbial processing of large compounds. However, if microbial processing occurred in the incubation conducted with sterilized sediment, it was slower than in biotic incubations and it did not alter DOC concentrations (Figure 3a,c).

A notable difference between incubations conducted with native and sterilized sediment was detection of new compounds in the CHO+S heteroatom group between day 18 and day 85 in native-sediment incubations (Figures 5, S5). The new CHO+S compounds had low H-to-C ratios, high S-to-C ratios, and high NOSC. They had a range of O-to-C ratios and spanned all aromaticity indices. Other studies have directly connected presence of organosulfur species with microbial activity (D'Andrilli et al., 2013; Gonsior et al., 2011). We reason that these detected compounds either formed due to microbial sulfate reduction and sulfide generation during this period, which was not directly measured, followed by abiotic sulfurization of organic matter, or they were microbially synthesized. Both pathways are plausible. Sulfurization of organic matter is well documented (Brown, 1986; Heitmann and Blodau, 2006; Kohnen et al., 1991; Urban et al., 1999), and microbes can synthesize a wide array of organic sulfur compounds (Brosnan and Brosnan, 2006; Madigan et al., 2003; Thauer, 1998).

4 Conclusions

4.1 Sedimentary organic carbon

Collectively, FT-ICR-MS characterization of SOC mobilized off the aquifer sediment in the Neumann et al. (2014) incubation indicates it was a highly diverse and energetically favorable pool of organic carbon, predominately composed of terrestrial- and fire-derived organic matter not yet processed by microbes (Figure 2). Similarities in the compositional character between this pool of SOC and soluble carbon from smaller, less physically accessible pores in an intact soil core study (Bailey et al., 2017) imply that when organic carbon evades microbial attack, it remains highly diverse and composed of more oxidized, aromatic compounds (e.g., tannins and condensed hydrocarbons) relative to carbon that is readily accessible to microbial processing (e.g., DOC in recharge water or soluble carbon in larger soil pores).

Roughly half of the identified compounds in mobilized SOC (and in recharge DOC) contained sulfur (Figure 2). During incubation, after mobilized SOC was transformed into methane, new sulfur-containing compounds were detected in

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biotic incubations that had high S-to-C ratios and high NOSC values (Figures 5 and S5). These compounds were not detected in abiotic incubations. We reason that they either represent sulfurization of organic matter following microbial sulfate reduction, or were directly synthesized by the native microbial community.

As discussed in the Introduction, in the Neumann et al. (2014) experiment, SOC was initially mobilized due to sampling, homogenizing, and/or handling of the sandy aquifer sediment. While such physical perturbations to the subsurface would not occur *in situ*, geochemical perturbations to aquifers can and do occur, and geochemical perturbations hold potential to mobilize organic carbon off sediment into groundwater. For example, changes to pH or ion concentrations could desorb organic carbon while reductive dissolution of carbon containing oxide minerals could release organic carbon into groundwater (Eusterhues et al., 2003; Fontaine et al., 2007; Gu et al., 1994b; Jardine et al., 1989; Kaiser and Zech, 1999; Mikutta et al., 2006). FT-ICR-MS and DOC concentration data (Figures 3–4, S2–S3) signify SOC was abiotically released from aquifer sediment during incubation. Release reflected desorption during equilibration of organic carbon between dissolved and sorbed phases. If it were to get mobilized within the anaerobic Bangladeshi aquifer, the characterized pool of SOC would represent a more energetically favorable carbon source than DOC transported into the aquifer with recharge water due to its higher NOSC (Boye et al., 2017; Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011) (Figure 2).

4.2 Microbial processing of organic matter in anaerobic environments

The direct link between microbial use and chemical character afforded by this study demonstrated that compound indices do not predict degradability. During incubation, native microbes successfully transformed all compound types (Figures 5 and S5), including those traditionally considered molecularly recalcitrant (e.g., aromatic and condensed aromatic compounds; (Lutzow et al., 2006)) and those currently considered thermodynamically unviable in anaerobic conditions (e.g., NOSC below -0.3; (Boye et al., 2017; Keiluweit et al., 2016)). The former result aligns with current understanding that under the correct conditions, microbes can process all carbon types (Lehmann and Kleber, 2015; Schmidt et al., 2011), but the latter result challenges the emerging idea that reduced carbon is thermodynamically unviable in anaerobic environments (Boye et al., 2017; Keiluweit et al., 2016).

Chemical indices did, however, give insight into rates of compound transformation. In the anaerobic incubation, NOSC controlled the rate of compound transformation. Aromatic CHO compounds with high NOSC were microbially transformed first (Figures 4 and S3), while alkane and olefinic CHO compounds with low NOSC persisted (i.e., were transformed last; Figures 5 and S5). Thus, while low NOSC values did not inhibit compound degradation, they did slow the rate of compound use. The more energetically favorable compounds with high NOSC were targeted first while the less energetically favorable compounds with low NOSC were targeted last. Compound size was also an important indicator. Larger compounds were actively transformed while smaller compounds persisted (Figures 5, S5, and S6).

Overall, these patterns are consistent with current soil science literature indicating that biotic communities eventually degrade and mineralize all forms of organic matter by continually converting larger energy-rich compounds into smaller energy-poor compounds (Lehmann and Kleber, 2015; Lutzow et al., 2006). Conceptually, the continual input and

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5 microbial conversion of carbon compounds coupled with the slower degradation rate of reduced carbon compounds in anaerobic conditions explains the apparent persistence of compounds with low NOSC in anaerobic environments (Boye et al., 2017). The explanation implies that degradation of highly reduced carbon compounds is thermodynamically viable in anaerobic conditions, which is in contrast to previous interpretations, but acknowledges the reaction is less energetically favorable than degradation of more oxidized carbon compounds, and thus, reduced carbon compounds are more slowly processed. In contrast to an incubation bottle, in an open system, where fresh carbon is continually supplied, the slower degradation rate of reduced carbon compounds would enable this portion of the organic carbon pool to build up in an anaerobic environment, skewing the NOSC distribution of soluble carbon to lower values. This interpretation aligns results from the incubation experiment, where every carbon type, regardless of NOSC, was transformed (i.e., lost; Figures 5, S5 and S6), with field observations indicating soluble carbon from anoxic sediments have lower NOSC values than that from oxic sediments (Boye et al., 2017).

Data availability

15 Unprocessed FT-ICR-MS data are available at: <https://doi.pangaea.de/10.1594/PANGAEA.876660>

Pracht, Lara E; Tfaily, Malak M; Ardissono, Robert J; Neumann, Rebecca B (2017): FT-ICR-MS characterization of organic matter and further sample details of Bangladeshi aquifer sediment incubated with aquifer recharge waters. Dataset #876660 (DOI registration in progress)

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Supplement

Four tables, six figures and one section providing data and information supporting statements herein.

Author contributions

20 L.E. Pracht and R.B. Neumann designed the incubation experiment. L.E. Pracht and R.J. Ardissono carried out the incubation experiment. M.M. Tfaily conducted the FT-ICR-MS analysis. L.E. Pracht, R.J. Ardissono, M.M. Tfaily and R.B. Neumann processed the FT-ICR-MS data. L.E. Pracht and R.B. Neumann interpreted the FT-ICR-MS data and wrote the manuscript with editing input from M.M. Tfaily and R.J. Ardissono.

Competing interests

25 The authors declare they have no conflict of interest.

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We thank Rosalie K. Chu for analytical assistance with FT-ICR-MS, Jesse C. Turner for help with figure formatting, and Steve J. Burges for helpful comments that improved the manuscript. A portion of the research was performed using EMSL, a DOE Office of Science User Facility sponsored by the Office of Biological and Environmental Research (BER) and located at Pacific Northwest National Laboratory.

Figure Captions

Figure 1: Conceptual cartoon indicating where Neumann et al. (2014) collected aquifer sediment (marked with black circle) and aquifer recharge water used in the incubation experiment. Water vacuum As described in the text, recharge water was collected from beneath a rice field and from the sediments of a pond. At the field site, water from rice fields and ponds gets pulled into the aquifer to a depth of ~30-m where irrigation wells are screened. The cartoon is modified from Neumann et al. (2010).

Figure 2: Chemical characteristics of mobilized SOC and of DOC in pond and rice field recharge water. (a) Percent of identified compounds in heteroatom groups. Proportional distributions of (b) H-to-C ratios, (c) O-to-C ratios, (d) peak mass, (e) double bond equivalents, and (f) nominal oxidation states of carbon. Percent of identified compounds in groupings based on (g) aromaticity index (alkanes, AI=0; olefinics, 0<AI≤0.5; aromatics, AI>0.5; condensed aromatics, AI≥0.67) and (h) compound classifications (Table S4). Compound classifications are arranged in increases order based on NOSC, as obtained from Boye et al. (2017).

Figure 3: (a-d) Average concentrations of DOC and (e-h) number of organic compounds in incubation water 1, 18 and 85 days after initiation of the incubation experiment. In panels a-d error bars represent standard deviation around the mean (Table S2). In panels e-h, black circles indicate the total number of compounds present at a given time point. Bars between time points indicate the number of compounds that were identified at both time points, i.e., common (black), identified at the second time point but not at the first, i.e., newly detected (dark grey), and identified at the first time point but not at the second, i.e., lost (light grey).

Figure 4: Relative change in chemical indices between day 1 and day 18 for rice field recharge water incubated with native sediment and sterilized sediment. Plotted are the cumulative fractions of compounds that were identified at both time points, i.e., common (black), identified at the second time point but not at the first, i.e., newly detected (dark grey), and identified at the first time point but not at the second, i.e., lost (light grey). Compounds were separated by heteroatom group (CHO and CHO+S groups are shown) and characterized based on H-to-C ratio, O-to-C ratio, S-to-C ratio, nominal oxidation state of carbon (NOSC), peak mass, and aromaticity index.

Figure 5: Relative change in chemical indices between day 18 and day 85 for rice field recharge water incubated with native sediment and sterilized sediment. Plotted are the cumulative fractions of compounds that were identified at both time points, i.e., common (black), identified at the second time point but not at the first, i.e., newly detected (dark grey), and identified at the first time point but not at the second, i.e., lost (light grey). Compounds were separated by heteroatom group (CHO and CHO+S groups are shown) and characterized based on H-to-C ratio, O-to-C ratio, S-to-C ratio, nominal oxidation state of carbon (NOSC), peak mass, and aromaticity index.

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Table 1. Chemical Character of Initial Waters used in Neumann et al. (2014a) Incubation*

	Pond Recharge	Rice Field Recharge	Sediment Porewater
% CHO	26.7	22.0	9.6
% CHON	9.9	7.3	17.6
% CHO+P	16.0	11.8	21.3
% CHO+S	47.3	58.9	51.4
H-to-C ratio; <u>median</u> , mean (stdev)	<u>1.33</u> , 1.238 (0.558)	<u>1.18</u> , 1.100 (0.614)	<u>0.92</u> , 1.019 (0.528)
O-to-C ratio; <u>median</u> , mean (stdev)	<u>0.286</u> , 0.354 (0.279)	<u>0.333</u> , 0.404 (0.301)	<u>0.375</u> , 0.435 (0.299)
DBE; <u>median</u> , mean (stdev)	<u>7.5</u> , 8.4 (5.0)	<u>8.0</u> , 8.7 (5.0)	<u>13.0</u> , 13.3 (7.1)
NOSC; <u>median</u> , mean (stdev)	<u>-0.56</u> , -0.23 (1.33)	<u>-0.32</u> , 0.14 (1.56)	<u>0.10</u> , 0.07 (0.86)
Peak Mass (Da); <u>median</u> , mean (stdev)	<u>341.1</u> , 379.6 (155.6)	<u>352.9</u> , 391.3 (162.4)	<u>437.0</u> , 454.6 (134.5)
% Compounds >600 Da	6	8	14
Aromaticity Index			
% Alkane	30.9	28.6	26.5
% Olefinic	43.7	35.3	28.6
% Aromatic	6.5	6.6	15.5
% Condensed Aromatic	19.0	29.6	29.5
Compound Classification			
% Lipid-like	14.6	14.5	3.9
% Protein-like	15.4	12.1	5.8
% Amino Sugar-like	1.8	3.0	3.2
% Cellulose-like	3.8	2.2	4.3
% Lignin-like	18.3	17.1	16.9
% Tannin-like	1.9	2.1	7.4
% Condensed Hydrocarbon-like	24.1	25.7	38.0
% Other	20.0	23.3	20.4
% Terrestrially derived (cellulose, lignin, tannin-like)	24.0	21.4	28.6
% Microbially derived (lipid, protein, amino sugar-like)	31.8	29.6	12.9

*percentages reflect only those compounds detected by the FT-ICR-MS analysis method.

persisted. Preferential use of aromatic compounds, which are typically considered molecularly recalcitrant, demonstrates that in the anaerobic conditions of the incubation, thermodynamic favourability of carbon oxidation rather than molecular structure controlled the rate of

Organic carbon (OC) in aquifer sediments can impact groundwater quality, both positively and negatively, by fueling microbial reactions that remove and contribute dissolved contaminants to groundwater (e.g., natural attenuation of nitrate (Korom, 1992), mobilization of geogenic arsenic (Postma et al., 2007), methane production (Parkin and Simpkins, 1995)). Ultimately, the bioavailability of sedimentary OC (SOC) will dictate the extent to which it can fuel subsurface reactions. In aquifers, OC is often considered to have low reactivity relative to that in soils and marine sediments (Christensen et al., 2000; Hartog et al., 2004; Jakobsen and Postma, 1994), with increasing age frequently corresponding with decreased reactivity (Chapelle et al., 2009; Jakobsen and Postma, 1994; Postma et al., 2012). Over time, more bioavailable OC is thought to be preferentially degraded, leaving more molecularly recalcitrant and/or inaccessible forms behind.

Studies in soils and sediments demonstrate that OC bioavailability is controlled by multiple factors. Molecular structure, as well as ambient conditions, such as pH, ORP, temperature and nutrient concentrations, can control rates at which microbes process OC (Arndt et al., 2013; van Bergen et al., 1998; Keiluweit et al., 2016; McDowell et al., 2006). Physical protection mechanisms, such as OC

Neumann et al. (2014a) previously conducted an incubation experiment using sediment and waters from Munshiganj, Bangladesh, designed to evaluate the ability of OC in aquifer-recharge waters and aquifer sediment to fuel microbial reduction reactions. Groundwater at the site is contaminated with arsenic and supersaturated with methane (Harvey et al., 2002). The incubation uncovered a pool of bioavailable OC in aquifer sediments, equating to 0.33 ± 0.06 mg OC g⁻¹ sediment, or $8.8 \pm 0.7\%$ of total SOC (SI Section 1). The native microbial community fermented the mobilized SOC into methane. The tested sediment was a fine-grained sand and lacked peat, (Chapelle, 2000a) silt, and clay components often implicated as sources of SOC fueling subsurface reactions (Aravena and Wassenaar, 1993; Chapelle, 2000b; Desbarats et al., 2014; McMahon and Chapelle, 1991). The experiment demonstrated that old (~5000 years old), sandy aquifer sediments can contain notably large amounts of microbially-available OC.

(2014a) experiment and to track chemical compositional changes as SOC was converted into methane. In anaerobic conditions, OC degradation occurs via multiple steps involving a consortium of microbes (Megonigal et al., 2003). Hydrolytic exo-enzymes depolymerize OC into monomers. Monomers are then fermented into low molecular weight alcohols and volatile fatty acids, which are eventually mineralized into CO₂ and CH₄. Our effort not only characterizes a previously undocumented pool of bioavailable OC in

sandy aquifer sediments but also advances understanding of how OC chemical composition relates to bioavailability in anaerobic environments.

In addition, we characterized DOC in the experimental aquifer-recharge waters. The waters were collected from underneath a rice field and from pond sediments (Neumann et al., 2014a). Rice fields and ponds are the two primary aquifer recharge sources at the site (Harvey et al., 2006), and pond recharge evolves into high-arsenic groundwater while rice field recharge evolves into low-arsenic groundwater (Neumann et al., 2010). Prior to the Neumann et al. (2014a) incubation, a biologically degradable OC (BDOC)

Page 3: [5] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

conducted in aerobic conditions indicated that ~30% of DOC in pond recharge and ~0% of DOC in rice field recharge was degradable (Neumann et al., 2010). Molecular characterization of DOC in these two waters provides additional insight into relationships between chemical composition and bioavailability, and enables a comparison of carbon compounds found in aquifer sediment to those found in surface recharge.

To characterize OC, we utilized

Page 5: [6] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

Neumann et al. (2014a) thoroughly describe the incubation experiment from which FT-ICR-MS samples were collected. Briefly, incubation materials were collected from Munshiganj, Bangladesh. Aquifer sediment was from 9.1 m depth, where conditions were anoxic. Sediment was homogenized and half of it was dried and sterilized by gamma irradiation. Prior to incubation, aliquots of aquifer sediment taken from the half that was not dried and sterilized were vacuum-filtered (0.2 μm) to collect sediment porewater. Filtered (0.45 μm) aquifer-recharge waters were collected from underneath a rice field and from pond sediments. Aquifer sediment (8.2 g, dry weight) was incubated with both recharge waters (10 mL) in clean sterile serum vials that were destructively sampled 1, 17–20, 80–81, 91, 184 and 273 days after initiation (Neumann et al., 2014a). Samples were kept anoxic during all collection, handling and processing steps through the use of an anaerobic chamber, gas-impermeable bags, oxygen scavenging packets with oxygen indicator tabs, glass BOD bottles, and glass serum vials sealed with butyl rubber stoppers (Neumann et al., 2014a). Dissolved organic carbon (0.2 μm filter) was analyzed in all waters (Sievers TOC analyzer). Headspaces from a few incubation bottles were analyzed for methane (GC-FID). Previously published (Neumann et al., 2014a) DOC and methane concentrations for all replicates and time points are presented in SI Tables S1–S3.

2.2 FT-ICR-MS analysis

Aliquots (100–200 μL) of vacuum-filtered water samples from destructively sampled incubation bottles were immediately frozen and later packed in dry ice for shipment to the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory in Richland, WA.

Page 8: [7] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

Based on AI, DOC in pond recharge had a higher percentage of olefinic compounds (44% vs. 35% in rice field recharge) and lower percentage of condensed aromatic compounds (19% vs. 30% in rice field recharge) (Figure 1g, Table 1). This difference indicates that DOC in pond recharge was more molecularly labile than that in rice field recharge. Olefinics are unsaturated hydrocarbons that contain at least one carbon double-bond and are easily decomposed by hydrolase enzymes, while condensed aromatics contained fused aromatic rings and are the polymer class considered most resistant to degradation (Lutzow et al., 2006). The aromaticity difference between the two waters aligns with the previously conducted BDOC assay discussed in the *Introduction* that detected, in aerobic conditions, more biologically degradable DOC in pond recharge than in rice field recharge (Neumann et al., 2010).

3.2 Composition of mobilized sedimentary organic carbon

Concentration and character of OC mobilized off aquifer sediment into sediment porewater differed from DOC in aquifer recharge. Sediment porewater, which was shown by Neumann et al. (2014a) to have a higher DOC concentration than recharge waters (1059 ± 186 mg/L vs. 17 ± 7 mg/L and vs. 30 ± 3 mg/L, in rice field and pond recharge, respectively), also had a higher total number of assigned formulas (5263 vs. 627 and vs. 835 in rice field and pond recharge, respectively). Mobilized SOC in sediment porewater and DOC in recharge waters had similar O-to-C ratio distributions (Figure 1c), but different H-to-C ratio distributions (Figure 1b). Mobilized SOC had a greater proportion of compounds with H-to-C ratios between 0.5 and 1.25 (Figure 1b), corresponding with ~5% and ~18% more compounds getting classified as tannin- and condensed hydrocarbon-like, respectively (Figure 1h; Table 1), and it had a smaller proportion of compounds with H-to-C ratios between 1.25 and 2 (Figure 1b), corresponding with ~8% and ~10% fewer compounds getting classified as protein- and lipid-like, respectively (Figure 1h; Table 1). These differences in compound classification imply that relative to DOC in recharge, mobilized SOC was composed of proportionately more terrestrially derived organic matter (i.e., cellulose, lignin and tannin-like compounds; ~29% versus ~22% in recharge), more highly condensed organic matter (i.e., coal-, soot-, charcoal- and black-carbon-like compounds; ~38% versus ~25% in recharge), and notably less microbially derived organic matter (i.e., lipid-, protein-, amino-sugar-like compounds; ~13% versus ~30% in recharge). The remaining proportion of compounds did not overlap with a known class.

aromatics than DOC in recharge waters (Table 1). The percentage of condensed aromatics in SOC was larger than that in pond recharge, but matched that in rice field recharge (Table 1). Aromatic rings increase molecular recalcitrance (Lutzow et al., 2006), and thus, AI differences imply that mobilized SOC consisted of proportionately more molecularly recalcitrant carbon compounds than DOC in recharge. This result aligns with classical perceptions that surface-derived OC is more chemically reactive than sedimentary-derived OC (Jakobsen and Postma, 1994). However, given sediment porewater contained ~35-times more

DOC than recharge (Table S1), the pool of molecularly labile OC was actually larger for mobilized SOC than for recharge DOC.

A majority of

Page 9: [9] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

in mobilized SOC had a higher nominal oxidation state of carbon (NOSC, Eq. 2) than compounds in recharge DOC (Figure 1f). NOSC distributions had medians of 0.10 for mobilized SOC, -0.56 for pond recharge and -0.32 for rice field recharge (Table 1). For OC redox reactions, the Gibbs energy associated with the oxidation half reaction is inversely correlated with NOSC (LaRowe and Van Cappellen, 2011), and thus, oxidation of organic carbon becomes more thermodynamically favorable as NOSC increases. This trend is particularly relevant in anaerobic conditions where redox reactions depend on less energetic terminal electron acceptors (Keiluweit et al., 2016; LaRowe and Van Cappellen, 2011). The more positive NOSC values for mobilized SOC indicate it was more thermodynamically accessible to microbes

Page 9: [10] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

recharge DOC (Figure 1h). Carbon in lipids have negative nominal oxidation states, while carbon in tannins and condensed hydrocarbons have positive nominal oxidation states (Keiluweit et al., 2016).

Page 10: [11] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

mixed with sterilized sediment (i.e., pond abiotic), 3162 for pond recharge mixed with native sediment (i.e., pond biotic), 4089 for rice field recharge mixed with sterilized sediment (i.e., rice field abiotic), and 4120 for rice field recharge mixed with native sediment (i.e., rice field biotic) (Figures 2e-h).

Page 10: [12] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

did not noticeably change in the Neumann et al. (2014a) incubations conducted with rice field recharge (Figures 2c–d; Table S2), and concentrations

Page 13: [13] Deleted **Rebecca Neumann** **12/20/17 11:37 AM**

In the Neumann et al. (2014a) experiment, SOC was initially mobilized due to sampling, homogenizing, and/or handling of the sandy aquifer sediment. Incubation demonstrated the carbon was microbially-available; most of it was fermented into methane. If this pool of SOC were destabilized *in situ*, it could fuel microbially-mediated subsurface reactions with consequences for groundwater quality.

Groundwater in Bangladesh contains high concentrations of dissolved methane, and this methane gets directly emitted to the atmosphere when groundwater is pumped for rice irrigation (Neumann et al., 2014b). FT-ICR-MS and DOC concentration data (Figures 2–3, S2–S3) imply SOC was abiotically released from aquifer sediment during incubation. Release reflected desorption during equilibration of OC between dissolved and sorbed phases. Desorption of SOC may be occurring at the field site where incubation materials were collected. Field data have indicated DOC age increases with depth while DOC concentrations remain constant (Harvey et al., 2002); a situation that is only possible if SOC was released along groundwater flow paths as younger DOC was mineralized or sequester into sediment (Neumann et al., 2010).

FT-ICR-MS showed the mobilized SOC was highly diverse. It was composed of a large number of chemically distinct compounds likely originating from terrestrial and microbial sources. Compound classification based on H-to-C and O-to-C ratios implied that SOC was composed of proportionately more terrestrially derived organic matter, more highly condensed organic matter, and less microbially derived organic matter than DOC in surface recharge water. Additionally, it was proportionately larger, more aromatic and more oxidized than DOC in surface recharge (Figure 1).

Roughly half of the identified compounds

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4.2 Chemical indices and bioavailability

The direct link between microbial use and chemical character afforded by this study demonstrated that compound indices describing molecular lability (e.g., aromaticity) and thermodynamic favorability (e.g., NOSC) do not predict degradability. During incubation, native microbes successfully transformed all compound types (Figures 4, S5). The chemical indices did, however, give insight into rates of compound transformation.

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(alkanes, AI=0; olefinics, 0<AI≤0.5; aromatics, AI>0.5; condensed aromatics, AI≥0.67).

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(alkanes, AI=0; olefinics, $0 < AI \leq 0.5$; aromatics, $AI > 0.5$; condensed aromatics, $AI \geq 0.67$).

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