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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 fermented 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

15 compositional changes during incubation, and advance understanding of how composition relates to bioavailability in anaerobic conditions. Mobilized SOC was more diverse and proportionately larger, more aromatic and more oxidized than DOC in surface recharge. In all samples, ~50% of identified compounds contained sulfur. After SOC was fermented into methane, new organosulfur compounds with high S-to-C ratios and high nominal oxidation state of carbon (NOSC) were detected. We conjecture these detected compounds were microbially synthesized to biochemically support methane

20 production or they formed abiotically following microbial sulfate reduction, which could have occurred during incubation but was not directly measured. Microbes transformed all carbon types during incubation, including those considered molecularly recalcitrant (e.g., condensed aromatics) and thermodynamically unfavourable to oxidize (e.g., low NOSC). While all compound types were eventually degraded, NOSC and compound size controlled the rates of carbon transformation. Large energy-rich compounds (e.g., aromatics with high NOSC) were targeted first while small energy-poor

25 compounds (e.g., alkanes and olefinics with low NOSC) 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 carbon degradation by microbes.

1 Introduction

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)).

5 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.

10 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 sorption to mineral surfaces, can limit microbial access to carbon (Baldock and Skjemstad, 2000; Petridis et al., 2014). Energetic constraints and metabolic capacity of microbial communities can control

15 the ability of microbes to process different chemical forms of OC (Fontaine et al., 2007; LaRowe and Van Cappellen, 2011; Trulleyova and Rulik, 2004). It is these factors that help explain the presence and contemporary processing of old OC (i.e., thousands of years old) in soils and sediments.

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

20 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.,

25 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.

Given the potential importance of SOC for fueling subsurface reactions, we sought 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. In anaerobic conditions, OC degradation occurs via multiple steps involving a

30 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 CO_2 and CH_4. 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

5   degradable OC (BDOC) experiment 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 Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS),

10   coupled with electrospray ionization (ESI) in negative ion-mode. FT-ICR-MS is capable of characterizing the thousands of different compounds contained in OC. The technique is not quantitative, as the ionization efficiency of compounds vary, making some compounds more difficult or impossible to identify (Sleighter and Hatcher, 2007). FT-ICR-MS resolves carbon compounds with unique mass-to-charge ratios (m/z). Ratios are then used to determine the elemental composition of compounds present. This technique has been applied to natural organic matter in a variety of environments (Chipman et al.,

15   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).

20   **2 Material and Methods**

2.1 Incubation

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

25   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-

30   impermeable bags, oxygen scavenging packets with oxygen indictor 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
5   frozen and later 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
10   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
15   has shown that organic matter is rich in carboxylic groups, as these gorups ionize best in negative mode (Stenson et al., 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.

Samples were introduced to the ESI source equipped with a fused silica tube (200 µm i.d) through a syringe pump
20   at a flow rate of 3.0 µL/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 °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
25   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: $C_2F_3O_2$, $C_6HF_9N_3O$, $C_{12}HF_{21}N_3O$, $C_{20}H_{18}F_{27}N_3O_8P_3$, and $C_{26}H_{18}F_{39}N_3O_8P_3$ with an m/z ranging between 112 to 1333 Da.

One hundred forty four individual scans were averaged for each sample and internally calibrated using OM
30   homologous series separated by 14 Da (–CH2 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

5 (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.

To further reduce cumulative errors, all sample peak lists within the entire dataset were aligned to each other prior

10 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

15 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.

2.3 Data processing

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

20 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 concentration 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 two recharge waters, sediment porewater, and incubation waters from day 1, days 17–20 (called day 18 throughout the manuscript), and days 80–91 (called day 85 throughout the manuscript). Combining replicates and

25 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 OC into 4 distinct heteroatom groups: 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

30 compound classification. For an organic compound $C_cH_hN_nO_oP_pS_s$, DBE was calculated as (Koch and Dittmar, 2006, 2016):

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

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

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

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

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

5   which identifies samples as alkanes (AI=0), olefinics (0<AI≤0.5), aromatics (AI>0.5), or condensed aromatics(AI≥0.67) (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.'

10   Compound changes were tracked between day 1 and day 18 and between day 18 and day 85 of the incubation. 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 'detected.'

3 Results and Discussion

3.1 Composition of DOC in aquifer recharge waters

15   Pond and rice field recharge collected for the Neumann et al. (2014a) incubation had similar numbers (same order of magnitude) of assigned formulas: 835 (pond) and 627 (rice field). The chemical composition of DOC in the two waters was proportionately similar (Figure 1, Table 1). Notable differences only existed in the H-to-C ratio distribution and in AI. Pond recharge contained proportionately fewer compound with H-to-C ratios <0.75 and proportionately more compounds with H-to-C ratios >1.25 compared to rice field recharge (Figure 1b). However, these ratio differences did not result in markedly

20   different compound classifications (Figure 1h; Table 1).

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) (Figure1g, 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,

25   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

30   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

5   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 1 h; 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

10   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 mass distribution for mobilized SOC was wider and skewed toward larger masses relative to DOC in recharge
15   (Figure 1d). The wider distribution aligns with the larger number, and thus, greater diversity of compounds detected in mobilized SOC relative to DOC in recharge. The dimensions of bacterial porin structures can lead to the exclusion of large organic solutes (e.g. >600Da), requiring extracellular enzymes to catabolize these larger molecules before they can be assimilated by microbial cells (Benz and Bauer, 1988; Nikaido and Vaara, 1985; Weiss et al., 1991). A majority of detected compounds in all waters were smaller than 600 Da, but given skew toward larger masses, mobilized SOC contained

20   proportionately more compounds that were larger than 600 Da (14% versus ~7% in recharge; Table 1). Thus, to facilitate microbial assimilation, ~700 compound types in mobilized SOC and only 40–60 compound types in recharge DOC likely required catabolism. In anaerobic environments, catabolism is often the rate-limiting step for degradation of organic matter (Freeman et al., 2001; Glissmann and Conrad, 2002; Reineke, 2001; Wu et al., 2001).

Mobilized SOC was more aromatic than recharge DOC. Similar to the mass distribution, the DBE distribution for
25   mobilized SOC was wider and skewed toward higher numbers relative to DOC in recharge (Figure 1e), signifying SOC was composed of a wider diversity of compounds, including those with more double bonds and rings. Based on aromaticity indices, aromatics accounted for the larger DBE (Figure 1g). SOC had 7 to 15% fewer olefinics (compounds with double bonds) but 9% more 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

30   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 compounds 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

5　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 than DOC in recharge water. Compound classification aligns with calculated NOSC values. Mobilized SOC contained fewer lipid-like and more tannin- and condensed hydrocarbon-like compounds relative to recharge DOC (Figure

10　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).

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 1; Table 1). Organosulfur compounds form in organic, sulfate-reducing environments where reduced sulfide species can react with various types of organic matter

15　(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. (2014a) 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

20　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, for example, coenzymes for methanogenesis contain sulfur heteroatoms (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 that abiotically reacted with DOM during this

25　time period or they synthesized organosulfur compounds to support fermentation or methanogenesis reactions (see *Carbon Transformations: Day 18 to Day 85*)

3.3 Carbon transformations between day 1 and day 18 of incubation

At the onset of the Neumann et al. (2014a) incubation experiment, 1 day after recharge waters were mixed with aquifer sediment, DOC concentrations and composition were dominated by sediment porewater. Average DOC concentrations were

30　140-300 mg/L (Figures 2a-d; Table S2), which could be explained by dilution of mobilized SOC in sediment porewater with recharge water (Neumann et al., 2014). At this early time point, the number of compounds identified in each incubation treatment was 3051 for pond recharge 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). Characterization showed that DOC in these initial incubation samples was more similar to mobilized SOC in sediment porewater than to DOC in aquifer recharge waters (Figure S1).

Between day 1 and day 18, average DOC concentrations did not noticeably change in the Neumann et al. (2014a)
5 incubations conducted with rice field recharge (Figures 2c–d; Table S2), and concentrations increased by 13 to 28 mg/L in incubations conducted with pond recharge (Figures 2a-b; Table S2). During this time period, the number of identified compounds increased in all treatments. In both the biotic and abiotic pond incubations ~4000 new compounds were detected (Figures 2e–f), and in both the biotic and abiotic rice field incubations ~2000 new compounds were detected (Figures 2g-h). Across all four treatments, the compositional character of newly detected compounds matched that of DOC in initial
10 incubation water (i.e., from day 1) and 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 waters 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 3, SI Figure S3, SI Figure S4), which can be explained by slower diffusive partitioning rates for larger compounds. If this
15 interpretation is correct, desorption of SOC in pond recharge incubations was large enough to measurably increase DOC concentrations (Figures 2a–b), while desorption in rice field recharge incubations was not (Figures 2c–d).

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. Instead, use of sterilized versus native sediment controlled compound loss. Treatments with native sediment lost ~1500 compounds (Figures 2f,h) while treatments with
20 sterilized sediment lost ~800 compounds (Figures 2e,g). Greater loss of compounds in incubation sets conducted with native sediment indicates that microbial transformation of OC occurred during this early incubation period. Figures 3 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 3, S3).

25 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). Preferential loss of compounds with larger O-to-C ratios was similarly seen in a stream-water incubation (Kim et al., 2006). Larger O-to-C ratios correspond with higher NOSC, and thus, oxidation of these compounds was more energetically favorable. The fact that preferentially processed compounds were aromatic signifies thermodynamic accessibility was more important than molecular lability. This preference could reflect the
30 anaerobic conditions of the incubation; in oxic environments, aromatic compounds are secondarily processed instead of primarily processed (Lutzow et al., 2006). Notably, the thermodynamic driving force for carbon oxidation does not depend on NOSC when oxygen is the electron acceptor, but with anaerobic electron acceptors, the thermodynamic driving force rapidly drops as NOSC decreases (Keiluweit et al., 2016).

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 (Figures 2b,d), but not from those conducted with sterilized sediment (Figures 2a,c). Carbon mass balance calculations conducted by Neumann et al. (2014a) indicated that DOC lost in biotic incubations was fermented into methane. Accordingly, the number of identified compounds by FT-ICR-MS decreased in both biotic incubations (Figures 2f,h) but did not change in abiotic incubations (Figures 2e,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 (Table S2), but the decrease in the number of compounds was larger with pond recharge than with rice field recharge (Figure 2).

All compound types were microbially transformed, as evidenced by proportionally greater compound changes in biotic than in abiotic incubations, regardless of chemical index (Figure 4, S5). Compounds considered molecularly recalcitrant (e.g., condensed aromatics) and less thermodynamically favorable to oxidize (e.g., low NOSC) were, in many instances, almost fully transformed in biotic incubations. This result reinforces current understanding that microbial communities can decompose a wide range of carbon types (Lehmann and Kleber, 2015; Schmidt et al., 2011). However, some compound types did persist across the two time points. Compound persistence was proportionately greater in the rice field incubation than in the pond incubation (Figure 4, S5, 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 (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 contrasts with the proportionately greater loss of CHO compounds with high NOSC in the early incubation phase (Figure 3, S3). NOSC appears to predict relative rates of compound transformation, with higher NOSC facilitating faster transformation, but only for CHO compounds. In other heteroatom groups, NOSC was not related to persistence (Figure 4, S5). Instead, compound size predicted persistence, with the smallest compounds persisting in all groups (Figures 4, S5–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 other compounds between day 18 and 85 (Figure 4, S6). These compound changes could reflect continued abiotic partitioning of large molecules between sediment and water, or, if the abiotic incubations did not stay sterile, it could reflect microbial processing of large compounds. However, if microbial processing occurred in the abiotic incubations, it was slower than in biotic incubations and it did not alter DOC concentrations (Figure 2a,c).

A notable difference between incubations conducted with native and gamma-irradiated sediment was detection of new compounds in the CHO+S heteroatom group between day 18 and day 85 in native-sediment incubations (Figures 4, 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

5   activity (D'Andrilli et al., 2013; Gonsior et al., 2011). We conjecture these detected compounds formed due to microbial sulfate reduction during this period, which was not directly measured, followed by abiotic sulfurization of organic matter, or they were microbally synthesized to support methane production, which was measured and did occur over this time period (Table S3). Both pathways are plausible. Sulfurizaiton of organic matter is well documented (Brown, 1986; Heitmann and Blodau, 2006; Kohnen et al., 1991; Urban et al., 1999), and methanogenesis involves multiple different organosulfur

10 compounds, including some with high S-to-C ratios (e.g., coenzyme M has an S-to-C ratio of 1). Biochemical investigations have revealed that organosulfur compounds are concomitantly oxidized as other carbon substrates are reduced into methane (Thauer, 1998). Such transformations could theoretically explain detection of sulfur-containing compounds with high NOSC (Figures 4, S5).

4 Conclusions

15 **4.1 Broader perspective for sedimentary organic carbon**

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.

20 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

25 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

30 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 contained sulfur. During incubation, after mobilized SOC was fermented into methane, new sulfur-containing compounds were detected in biotic incubations that had high S-to-C ratios and high NOSC values (Figures 4, S5). These compounds were not detected in abiotic incubations. We conjecture they either represent sulfurization of organic matter following sulfate reduction, or a form of OC used by microbes to biochemically

5   support degradation and mineralization of SOC to methane.

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

10   chemical indices did, however, give insight into rates of compound transformation.

In the anaerobic incubation, NOSC rather than molecular structure controlled the rate of compound transformation. Aromatic CHO compounds with high NOSC were microbially transformed first (Figures 3, S3), while alkane and olefinic CHO compounds with low NOSC persisted (Figures 4, S5). This result is in contrast to that observed in aerobic incubations where molecular structure controls the rate of compound use (Lutzow et al., 2006). The difference confirms that

15   thermodynamic favorability of carbon oxidation is an important factor in anaerobic conditions (Keiluweit et al., 2016).

Compound size was also an important indicator. Larger compounds were actively transformed while smaller compounds persisted (Figures 4, S5, 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).

20   **Data availability**

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

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25   **Supplement**

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

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Author contributions

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

5   manuscript with editing input from M.M. Tfaily and R.J. Ardissono.

Competing interests

The authors declare they have no conflict of interest.

Figure Captions

Figure 1: Chemical characteristics of mobilized SOC and 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)

15   nominal oxidation states of carbon. Percent of identified compounds in groupings based on (g) AI (alkanes, AI=0; olefinics, $0<AI\leq0.5$; aromatics, AI>0.5; condensed aromatics, AI≥0.67) and (h) compound classifications (Table S4).

Figure 2: (a-d) Average concentrations of DOC and **(e-h)** number of organic compounds 1, 18 and 85 days after initiation of incubation experiment. In panels a-d error bars represent standard deviation around the mean (Table S2). In panels e-h, black symbols indicate the total number of compounds present at a given time point. Symbols plotted between time points indicate the number of compounds that

20   were (blue) identified at both time points, i.e., common, (red) identified at the second time point but not at the first, i.e., newly detected, and (grey) identified at the first time point but not at the second, i.e., lost.

Figure 3: Relative change in chemical indices between day 1 and day 18 for rice field recharge water incubated with native sediment and sterilized sediment. The cumulative fraction of compounds that were (black) identified at both time points, i.e., common, (dark grey) identified at the second time point but not at the first, i.e., newly detected, and (light grey) identified at the first time point but not at the

25   second, i.e., lost, were separated by heteroatom groups (CHO and CHO+S 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 (alkanes, AI=0; olefinics, $0<AI\leq0.5$; aromatics, AI>0.5; condensed aromatics, AI≥0.67).

Figure 4: Relative change in chemical indices between day 18 and day 85 for rice field recharge water incubated with native sediment and sterilized sediment. The cumulative fraction of compounds that were (black) identified at both time points, i.e., common, (dark grey)

30   identified at the second time point but not at the first, i.e., newly detected, and (light grey) identified at the first time point but not at the second, i.e., lost, were separated by heteroatom groups (CHO and CHO+S 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 (alkanes, AI=0; olefinics, $0<AI\leq0.5$; aromatics, AI>0.5; condensed aromatics, AI≥0.67).

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

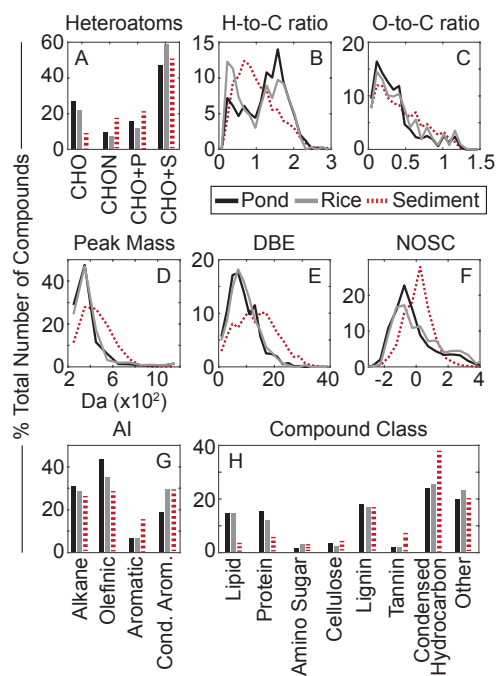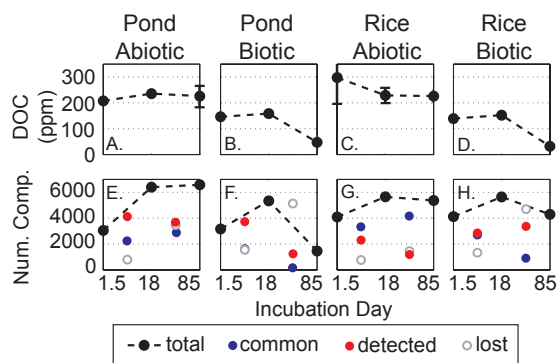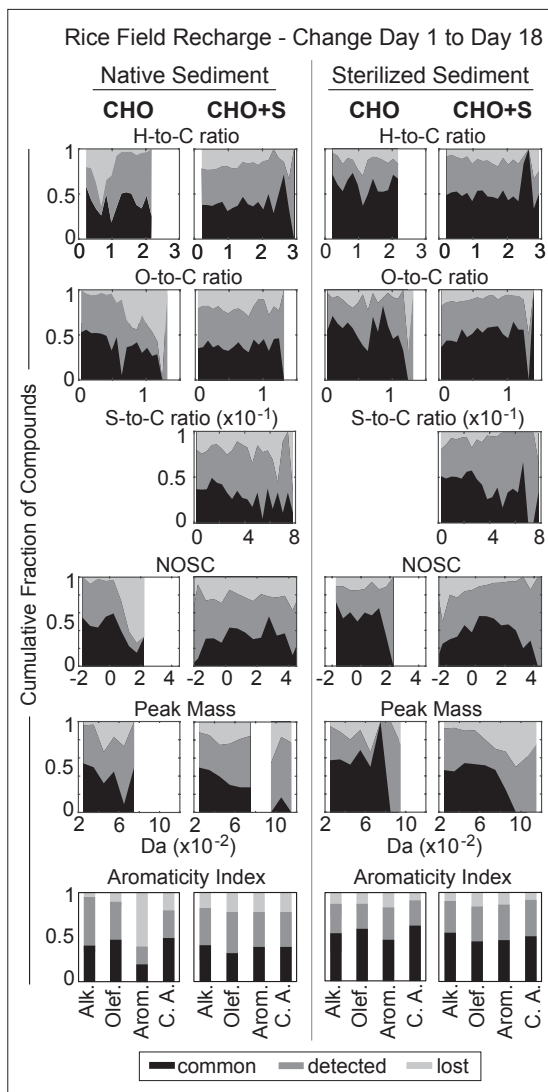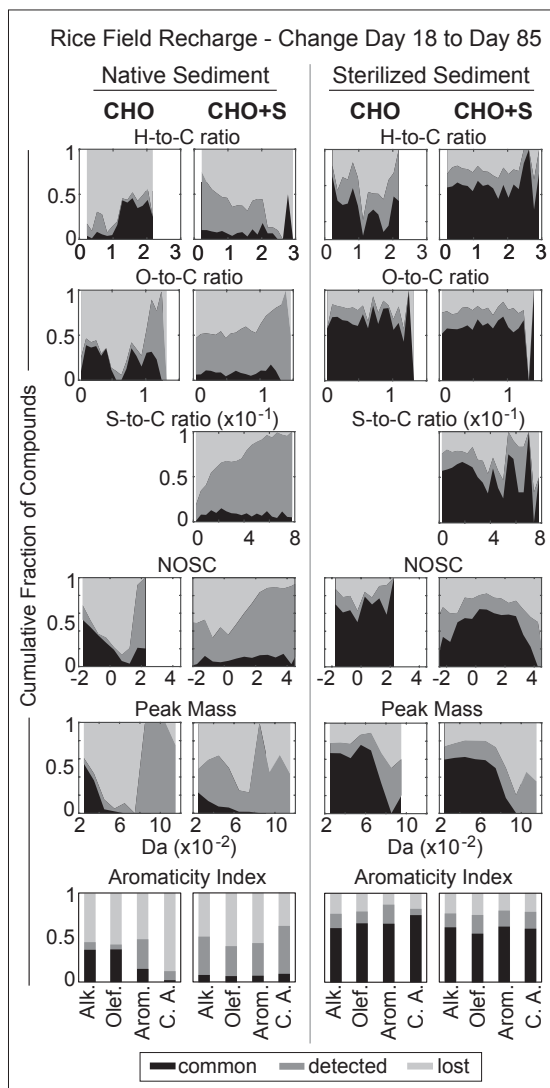|	Pond Recharge	Rice Field Recharge	Sediment Porewater
% CHO	26.7	22.0	9.6
% CHON	9.9	7.3	17.6
% CHO+P	16.0	11.8	21.3
% CHO+S	47.3	58.9	51.4
H-to-C ratio; median, mean (stdev)	1.33, 1.238 (0.558)	1.18, 1.100 (0.614)	0.92, 1.019 (0.528)
O-to-C ratio; median, mean (stdev)	0.286, 0.354 (0.279)	0.333, 0.404 (0.301)	0.375, 0.435 (0.299)
DBE; median, mean (stdev)	7.5, 8.4 (5.0)	8.0, 8.7 (5.0)	13.0, 13.3 (7.1)
NOSC; median, mean (stdev)	-0.56, -0.23 (1.33)	-0.32, 0.14 (1.56)	0.10, 0.07 (0.86)
Peak Mass (Da); median, mean (stdev)	341.1, 379.6 (155.6)	352.9, 391.3 (162.4)	437.0, 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

FIGURE 1

FIGURE 2

FIGURE 3

Biogeosciences
Discussions

FIGURE 4