The Importance of Nutrients for Microbial Priming in a Bog Rhizosphere

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Abstract

Wetlands host anaerobic microbes which convert organic carbon into methane (CH_4), a powerful greenhouse gas. Wetland plants can influence which carbon compounds are available for microbial processing by exuding freshly fixed carbon from their roots. Notably, exudation of carbon from plant roots can trigger microbial priming: the process of new carbon stimulating the microbial community into processing more soil carbon than they otherwise would have. This study utilized high resolution Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS) analysis to probe the chemical composition of soil organic compounds from the rhizosphere of *Carex aquatillis*, a common wetland sedge, which is known to have stimulated microbial priming within a peat soil. The goal was to identify what types of molecules are created or lost during microbial priming in the wetland rhizosphere and thus advance mechanistic understanding of the process. FT-ICR-MS analysis demonstrated that more microbial transformations of carbon occurred among water-soluble compounds than among hydrophobic compounds, but that some hydrophobic compounds were processed as well. Crucially for understanding microbial priming, the root exudates triggered increased processing

of high molecular weight molecules regardless of nutrient content but processed low molecular weight compounds only if they contained essential nutrients. The pattern of which molecules are processed and which are not is evidence for a selective priming effect in which some types of carbon compounds are used at an increased rate, while others are not.

Introduction

Wetlands host anaerobic microbes which convert organic carbon into methane (CH_4), a powerful greenhouse gas. Globally, this wetland-generated CH_4 is responsible for approximately a third of total CH_4 emissions (Ciais et al. 2013), and CH_4 in the atmosphere is responsible for 15-19% of total greenhouse gas radiative forcing (Intergovernmental Panel on Climate Change 2014). This substantial contribution to the greenhouse effect is dynamic and responds to environmental changes; wetland CH_4 emissions are the primary driver of interannual variability in global emissions (Ciais et al. 2013). The microbial communities that produce CH_4 are sensitive to a variety of variables including temperature (Westermann 1993; Yvon-Durocher et al. 2014), pH (DeLaune et al. 1986; Kotsyurbenko et al. 2007), redox environment (Fetzer and Conrad 1993; Fritz et al. 2011; Boye et al. 2017), and the composition of the organic carbon available to them (Updegraff et al. 1995; Girkin et al. 2018). Two of those variables can in turn be dynamically impacted by the presence of plants: redox environment through gas transport effects and carbon availability through root exudation. As such, influencing the microbial environment makes plants a key factor in wetland CH_4 emissions.

The types of carbon molecules present in the soil is an important variable influencing wetland CH_4 emissions because it determines what metabolic processes microbes can conduct. It was once thought that the structure of a molecule determined whether it was “labile” or “recalcitrant” to microbial degradation (Sollins et al. 1996; Hedges et al. 2000; Lützow et al. 2006), and authors proposed various means by which the “recalcitrance” could be established from the molecular structure (Lützow et al. 2006; D’Andrilli et al. 2015). Over time, the scientific consensus has shifted to recognize that all carbon compounds are ultimately capable of being microbially processed, albeit at different rates, and that the order of microbial preference can depend on a variety of factors including the composition of the microbial community and the

redox state of the environment (Schmidt et al. 2011; Lehmann and Kleber 2015). While authors continue to use the language of “recalcitrance” and “lability”, sometimes acknowledging the changing understanding of these terms (e.g., Zhang et al. 2018) and sometimes not (e.g., Barcellos et al. 2018), these studies still provide valuable insights into situations where certain classes of molecules are more or less bioavailable to the microbes present.

The situational bioavailability of carbon compounds can depend on traits of the molecules as well as on environmental factors. One fundamental constraint on microbial use of carbon is the limited pore size of cell walls, which dictates that any molecule that is either too large or not water soluble must be degraded by exo-enzymes before the microbe can take it up (Benz and Bauer 1988; Fenchel et al. 2012). However, microbes have also been shown to preferentially utilize high molecular weight compounds in a variety of environments despite the extra step of extracellular degradation (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012). As microbes use large compounds they metabolize them into smaller compounds, thus shifting the size distribution of molecules (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018). Another property that has been studied is aromaticity. More aromatic compounds have historically been thought to be less bioavailable (Lützow et al. 2006), but new evidence indicates that is likely only true in oxic environments; when oxygen is limiting, thermodynamic limitations dominate and aromaticity is not controlling (Pracht et al. 2018). In those oxygen-limited or highly reduced environments, the nominal oxidation state of carbon (NOSC) can be the most important factor (Keiluweit et al. 2016; Boye et al. 2017).

Wetland plants can influence what carbon compounds are available for microbial processing by exuding freshly fixed carbon from their roots in the form of sugars, carbohydrates, organic acids, amino acids, and a variety of other small, water-soluble compounds (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012). These compounds stimulate the growth of beneficial microbial and fungal communities (Broeckling et al. 2008). Plants also introduce higher molecular weight compounds to the soil through sloughing of root cells and release of mucilage, but these compounds generally are less utilized by the soil microbial community (Bais et al. 2006). Studying root exudates in soil is challenging

because root exudates often are quickly utilized by microbes and thus these compounds do not remain in the soil for measurable periods of time. Analytical techniques can only measure what remains in the soil. This under-detection of quickly metabolized compounds may bias results towards compounds that are less bioavailable in the rhizosphere.

Root exudates must provide some benefit to the plant, as they are a carbon cost (Marschner 1974). One way that root exudation can change the rhizosphere in a manner that potentially benefits plants is through microbial priming. Priming is the mechanism by which the addition of a new carbon source to soils stimulates microbes to degrade more of the existing soil carbon than they would have in the absence of the new carbon source (Fontaine et al. 2007; Kuzyakov 2010; Ruirui et al. 2014; Ye et al. 2015). Priming can occur because the fresh carbon causes changes to the microbial ecosystem, increasing the demand for carbon compounds and/or the ability of microbes to process carbon compounds (Fontaine et al. 2004; Kuzyakov 2010; Ye et al. 2015). Priming could benefit plants through nitrogen mining (N-mining), a process by which the addition of nutrient-poor carbon compounds causes microbes to degrade existing soil compounds which contain nitrogen (Craine et al. 2007; Ruirui et al. 2014). Wetland types such as bogs and some fens are often nitrogen limited (Aerts et al. 1992) despite the large amount of nitrogen bound in slowly decaying plant matter (Turunen et al. 2004; Moore et al. 2005; Drewer et al. 2010). These wetlands are therefore an environment where it is highly beneficial for plants to access the nitrogen already in the soil, using tactics such as N-mining. Other studies have also found evidence of other types of nutrient mining, for example for sulfur (Creamer et al. 2014). However, some recent studies have failed to find evidence for priming in peat soils, such as Girkin et al. (2018), though that study used root exudate analogs instead of actual plants.

This manuscript presents results from a plant-growth experiment that used peat from a boreal bog and a typical wetland sedge, *Carex aquatilis*. Waldo et al (2019) previously demonstrated, using isotopic tracers, that *Carex* in this experiment triggered microbial priming. That result was based upon the finding that in the presence of plants the emission of soil-derived CH_4 was increased by an order of magnitude, which was enough that in planted boxes there was more CH_4 being derived from soil carbon than derived from root carbon (Waldo et al.

2019). Here, we present the results of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis of organic compounds in root, rhizosphere, and unplanted soil samples from the Waldo et al. (2019) experiment. These results address the question of what soil organic compounds were processed when priming was active, with the goal of elucidating the factors controlling the occurrence of microbial priming. Understanding the causes and mechanisms of microbial priming will help researchers better predict the fate of wetland soil carbon in a future with predicted elevated boreal plant productivity.

Materials and Methods

Sample Collection

This study utilized samples collected from a previously described experiment (Waldo et al. 2019). Briefly, we grew *Carex aquatilis*, a common boreal wetland sedge, for ten weeks in rhizoboxes filled with bog peat collected from Interior Alaska. The bog site was described in detail by Neumann et al. (2019). Control boxes consisted of peat with no plants. We monitored CH_4 emissions throughout the experiment. During weeks 5 and 10 of the experiment, we exposed 4 plants each to $^{13}CO_2$ by placing a clear fluxing hood on each rhizobox and injecting 15 mL of 99 atom% $^{13}CO_2$ into the headspace over a period of five consecutive days. This $^{13}CO_2$ was photosynthesized and isotopically labeled the plants. Following labelling, we anaerobically collected root and soil samples, destroying the plants.

For both box types (real plants and control) we collected soil samples from the same locations, but the method of sample collection depended on box type. For planted boxes, we collected roots simultaneously with the rhizosphere soil. We collected the samples at depths of approximately 5 cm, 20 cm, and 35 cm. At each depth, we took three samples: one in the center and one six cm from either edge of the box. For planted boxes, we cut root sections from each location. We sonicated the root sections in phosphate-buffered solution (PBS) then moved the root to a new container. All the soil that fell off the root during sonication we considered rhizosphere, which is an operational definition of the rhizosphere which has been used previously (White et al. 2015). We saved both roots and rhizosphere soil for analysis. For unplanted control boxes, we sampled cubes of soil from the 9 standard locations. We stored all

samples at -20 °C before shipping them to the Environmental Molecular Sciences Laboratory in Richland, WA, USA, where they were stored at -80 °C until analysis. In total, we analyzed 73 rhizosphere samples from 9 planted boxes (4 harvested after week 5 and 5 harvested after week 10), 43 bulk soil samples from 5 unplanted boxes, and 6 root sections from 4 plants.

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS)

A 12 Telsa FT-ICR-MS (Bruker Daltonics) was used for analyzing the extracts of carbon compounds from the soil samples, based on the methods of Tfaily et al. (2015). We performed three sequential extractions on each soil sample to extract compounds that dissolve in different solvents: a water extraction for hydrophilic compounds, methanol extraction for compounds of intermediate hydrophobicity, and chloroform extraction for hydrophobic compounds. The extractions were performed sequentially in the stated order.

To perform the extractions, we thawed the samples at room temperature. We then centrifuged (VWR Symphony 4417) samples at a rotational centrifugal force of 2720g for 5 min and collected the supernatant containing the water-soluble compounds. To extract the remainder of the water-soluble compounds, we used four cycles of centrifugation in which we added 1 mL of water to the sample, shook it for 30 min, centrifuged it, and collected the supernatant. The combined supernatantfrom the five centrifugations (the initial porewater and four water additions) was acidified to pH 2 using nitric acid. This solution contained all of the water-soluble compounds in the sample, in addition to unwanted salts. The water extraction was coupled with a solid-phase extraction (SPE) to remove salts from the solution that interfered with FT-ICR-MS readings according to Thorsten et al. (2008). We activated SPE columns (PPL, 500 mg, Varian Mega Bond Elut, Varian Inc.) with 1 mL of methanol, then ran the acidified water samples through the column. Salts moved through the column while carbon compounds were retained. We then washed each column three times with 10 mM hydrochloric acid and dried it by blowing nitrogen gas through the column. Last, we eluted the carbon compounds off the column using 2 mL of methanol.

The methanol extraction was performed on the pellet of peat remaining after the final centrifugation of the water extraction. We added 1 mL of methanol to each sample, shook it for

two hours, centrifuged it and collected the supernatant. After the methanol extraction, we immediately added 1 mL of chloroform to the remaining peat pellet and let it sit quiescent overnight at 4 °C. We then centrifuged the samples and collected the supernatant as with the previous extractions. Pure chloroform does not run well in the ICR-MS instrument, so after the extraction was completed the chloroform extracts were blended with methanol in a 1:1 ratio so that they could be run directly on the instrument.

The water extraction contained water-soluble compounds, the methanol extraction contained compounds on intermediate solubilities, and the chloroform extraction contained compounds which were not soluble in water. All three extractions were then analyzed on a FT-ICR-MS. We used a Bruker electrospray ionization source in negative ion mode with the following settings: 0.1 s ion accumulation time, 200 scans, range of 98-900 m/z, resolution 260k at 400 *m/z*. Negative ion mode was selected because it has been shown to be more effective for ionization of organic molecules (Stenson et al. 2003). The instrument was flushed with a mixture of water and methanol between samples, and methanol blanks were run at the beginning and end of each session to check for carryover from the samples. The instrument was calibrated weekly with a standard solution containing $C_2F_3O_2$, $C_6HF_9N_3O$, $C_{12}HF_{21}N_3O$, $C_{20}H_{18}F_{27}N_3O_8P_3$, and $C_{26}H_{18}F_{39}N_3O_8P_3$.

We processed the data using DataAnalysis software (v4.2, Bruker Daltonics) to convert the raw spectra into mass to charge (*m/z*) ratios, which we then assigned molecular formulas using a Compound Identification Algorithm (Kujawinski and Behn 2006; Minor et al. 2012). We assigned formulas to peaks with a signal to noise ratio of at least 7, and mass measurement error of less than one ppm. The only atoms we allowed to be assigned were the most common isotope for each of C, H, O, N, S, and P, plus ^{13}C. Formulas were only assigned which included at least one each of C, H, and O, which had no more than 100 C, 200 H, 30 O, 20 N, 10 S, and 6 P, and which had at least four O per P.

Laser ablation electrospray ionization mass spectroscopy (LAESI-MS)

To analyze compounds present on and in roots, we used laser ablation electrospray ionization mass spectroscopy (LAESI-MS) (Stopka et al. 2017; Stopka et al. 2019). We mounted

2-4 cm long root sections on microscope slides using double-sided tape and ablated the surface of the root using focused 7-nanosecond pulses from a mid-IR laser (IR Opolette HE 2731) set to a 2.94 µm wavelength. The ablated material was carried into a Thermo Fischer Velos Orbitrap Pro MS using an ion spray generated from a solution of 50% methanol (in DI water), 0.1% acetic acid . The spray flowrate was at 500 nL/min minute and aligned on-axis to the MS orifice. Both positive ion mode and negative ion mode were used to analyze the ions captured. We sampled roots using a grid scan where we ablated the surface of the root in a rectangular grid with between 300 and 400 µm between the centers of each ablation target. All raw spectra were assigned molecular formula using the HMDB 4.0 database (Guo et al. 2017) through the METASPACE platform (Palmer et al. 2017). This system only assigned formulas to m/z ratios which correspond to an identified metabolite in the HMDB 4.0 database.

Soil Extraction Data Organization for Analysis

We combined FT-ICR-MS formula lists from all extractions of each soil sample to form a single list of all compounds extracted from that sample. Due to variability in ionization efficiency, we only considered compound presence, not intensity, in our analysis. We then compiled compounds from all the rhizosphere soil samples to form one list of compounds present in the rhizosphere, and we compiled compounds from all un-planted soil samples to form a second list of compounds present in unplanted soil. By comparing these two lists and removing any compounds which appeared on both, we isolated those compounds which were unique to either the rhizosphere or unplanted soil. Using the assumption that the unplanted soil was comparable to the initial condition of the soil before the influence of plants, we considered the compounds unique to the rhizosphere to have been created and those compounds unique to the unplanted soil to have been lost in the presence of roots. Compounds which were common to both the rhizosphere and unplanted soil were classified as persistent. For each sample we further identified compounds in the water-extracted fraction and in a lumped category of hydrophobic compounds containing both methanol extracted, and chloroform extracted compounds. Within the water-extracted or hydrophobic fraction of each sample we identified the created, lost, and persistent compounds based on the master lists.

Compound Classification and Bioavailability Metrics – FT-ICR-MS and LAESI-MS

Compounds from both soil (FT-ICR-MS data) and roots (LAESI-MS data) were classified according to their molecular formula using two different classification schemes. The first, a frequently used atom-ratio classification scheme (Kim et al. 2003; Mopper et al. 2007; Minor et al. 2012; D'Andrilli et al. 2013; D'Andrilli et al. 2015), involved sorting compounds using only O-to-C and H-to-C ratios, producing classes with properties similar to lipids, lignins, proteins, condensed hydrocarbons, amino-sugars, unsaturated hydrocarbons, carbohydrates, tannins, and "other" undefined compounds. The second was a more recently described multidimensional stoichiometric compound classification (MSCC) scheme that takes into account molecular size and heteroatom (N, S, and P) composition (Rivas-Ubach et al. 2018). The compound classes described in the MSCC are lipids, proteins, amino-sugars, carbohydrates, nucleotides, phytochemicals, and "other" undefined compounds. Classification resulting from both schemes does not indicate the compound is necessarily a lipid, lignin, protein, etc., but rather that the compound has properties similar to that of a lipid, lignin, protein, etc.

We used two metrics to describe chemical properties: aromaticity index (AI) (Koch and Dittmar 2006; Koch and Dittmar 2016) and nominal oxidation state of carbon (NOSC) (LaRowe and Van Cappellen 2011). Both metrics are unitless. Calculations for these metrics are given in Equations 1 & 2, with the elemental symbols representing the number of atoms of that element present in the molecular formula. Aromaticity Index values calculated below zero are defined to be zero, because negative aromaticity has no physical meaning (Koch and Dittmar 2006).

$$1)\ AI = \frac{1+C-0.5*O-S-0.5*(N+P+H)}{C-0.5*O-S-N-P}$$

$$2)\ NOSC = 4 - \frac{4*C+H-3*N-2*O+5*P-2*S}{C}$$

Statistics – Compound Class and Metrics

For the heteroatom and compound classifications, we analyzed the total count of each classification within each soil sample (all three extractions combined). Box and whisker plots of these data show the quartiles for total counts within samples. For metrics that calculate values per compound (AI, NOSC, and atom ratios), we separated hydrophobic and water-extracted

compounds into separate lists and took the mean values for analysis. Therefore, each sample produced six values: the mean of created, lost, and persistent compounds in both the water extraction and hydrophobic extractions. For all statistical comparisons we used a mixed-effects ANOVA with the box from which the sample came as a random effect variable, and the treatment type (planted or unplanted) as the fixed effect, which we tested for significance. We performed this test using the "fitlme" function in MATLAB (R2018b).

Microbial Metabolisms and Transformation - FT-ICR-MS

To assess involvement of identified compounds in microbial metabolic pathways in the soil we compared assigned molecular formulae from the FT-ICR-MS data to the KEGG database (release 86.1) to identify metabolic pathways involving the detected compounds (Kanehisa and Goto 2000). We used the full list of KEGG compounds detected in our samples, not just created or lost ones as with other analyses. This choice was made because we wanted to assess the full pathway, and some compounds may be present on multiple pathways. Because metabolic pathways can include members of various hydrophobicities, we combined all three extractions for KEGG analysis.

We also identified biochemical transformations in the soil using the methodology of Breitling et al. (2006) modified with an updated list of transformations from Graham et al. (2017). This technique finds the difference in mass between all pairwise combinations of mass peaks in the sample and compares them to a list of mass changes caused by known biochemical transformations (Table S1). Because the transformation analysis relies on the relationships between compounds/peaks we used all data and did not use the created/lost/persistent categorization used for other analyses. If an observed mass difference was within 10^{-4} Da of a transformation on the list then we counted it as a transformation. We analyzed the number of transformations in each extraction normalized to the number of peaks and performed a principal component analysis (PCA) on the percentage of total transformations that each individual transformation accounted for within each extraction. We performed the PCA using the "pca" function in MATLAB (R2018b). This procedure was repeated using only transformations involving nitrogen atoms and transformations involving sulfur atoms. For the

nutrient-specific analysis of number of transformations, results are expressed as a percentage of total transformations.

We also performed a variation of transformation analysis by comparing all peaks in unplanted samples with all peaks in rhizosphere samples. This identified which transformations converted compounds present in the unplanted soil into the compounds present in the rhizosphere. The sign (positive or negative) of the mass difference was used to determine whether the transformation represented the addition or removal of a group.

Results

Results describing the characteristics of persistent, lost, and created compounds are described individually first and comparisons between these groups are made at the end of the results section.

Persistent compounds

Using the traditional classification scheme, the carbon compounds which were present in both unplanted and rhizosphere soil consisted largely of lipid-like and lignin-like compounds, with these two categories comprising a mean of over 50% of compounds (Fig 1, uncategorized compounds not shown). The MSCC only classified one quarter of persistent compounds as part of a set category and placed three quarters of persistent compounds in an “other” category (SI Fig S1). The MSCC is intentionally designed to be more precise about class assignments (Rivas-Ubach et al. 2018), but the large number of unclassified compounds demonstrates how, in

complex environmental media where compounds degrade and are reprocessed, many molecules fail to fall within the stringent categories.

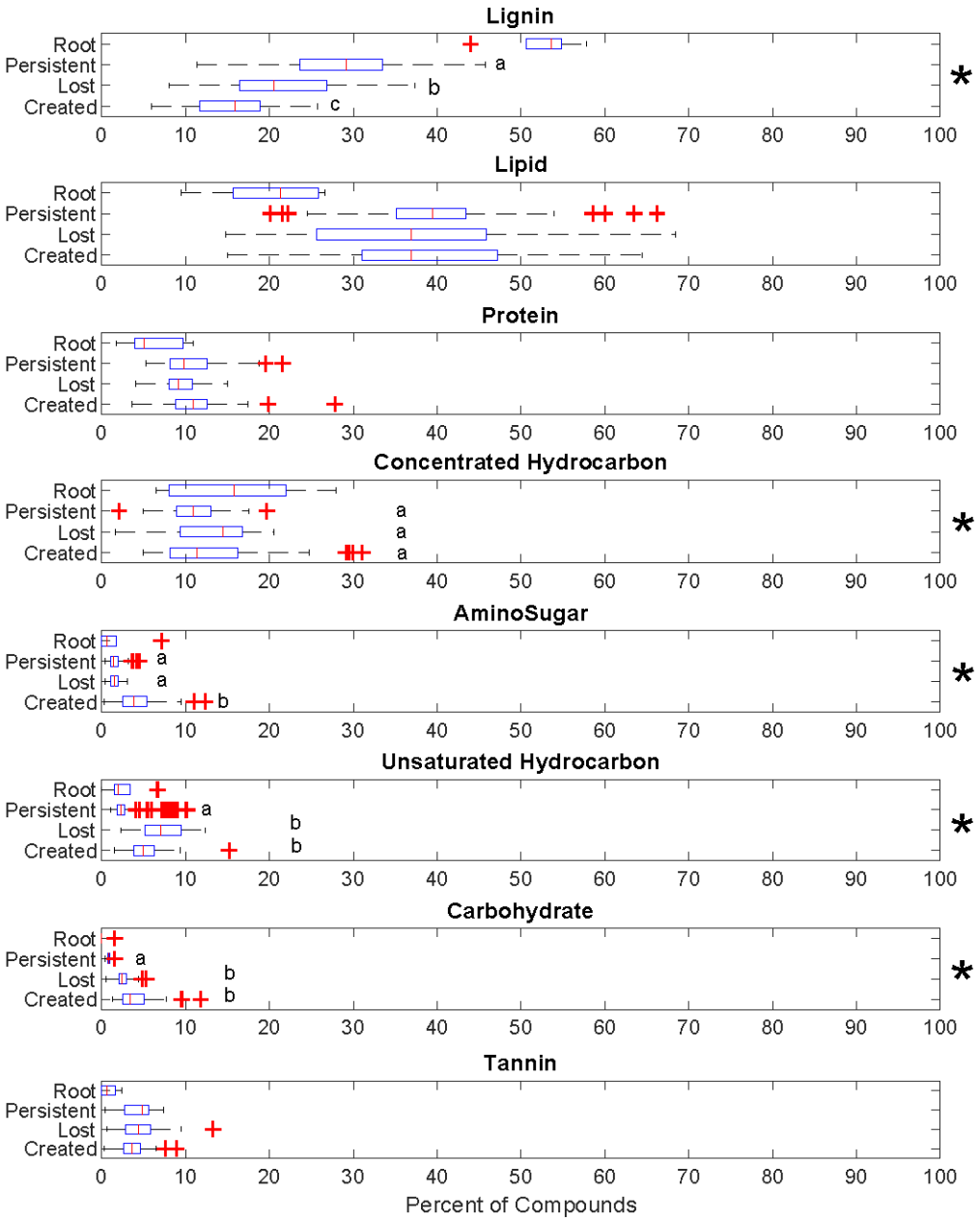


Figure 1. Atom-ratio compound classifications for persistent, lost, and created soil compounds as well as root surfaces. Boxes show median with upper and lower quartiles, and tails show all data within 2.7σ of the mean. Outliers outside 2.7σ are marked with "+". Created n=73, lost n=40, persistent n=112, and roots n=6. The asterisks (*) indicate that soil compound category (persistent, lost, or

created) is a statistically significant ($p < 0.05$) effect in a mixed-effects model. The letters indicate which categories are significantly ($p < 0.05$) different or not according to a Kruskall-Wallis ANOVA. Root compounds were not included in the statistical analysis. Uncategorized compounds not shown.

The persistent compounds had few heteroatoms, and were mostly comprised of only carbon, hydrogen, and oxygen (Fig 2). Graphs showing the ratio of each heteroatom to carbon can be found in SI Figure S2. Persistent compounds had significantly higher NOSC and AI in the water-soluble fraction than the hydrophobic fraction (Fig 3). The median mass was 436 Da for water-extracted persistent compounds and 615 Da for hydrophobic persistent compounds.

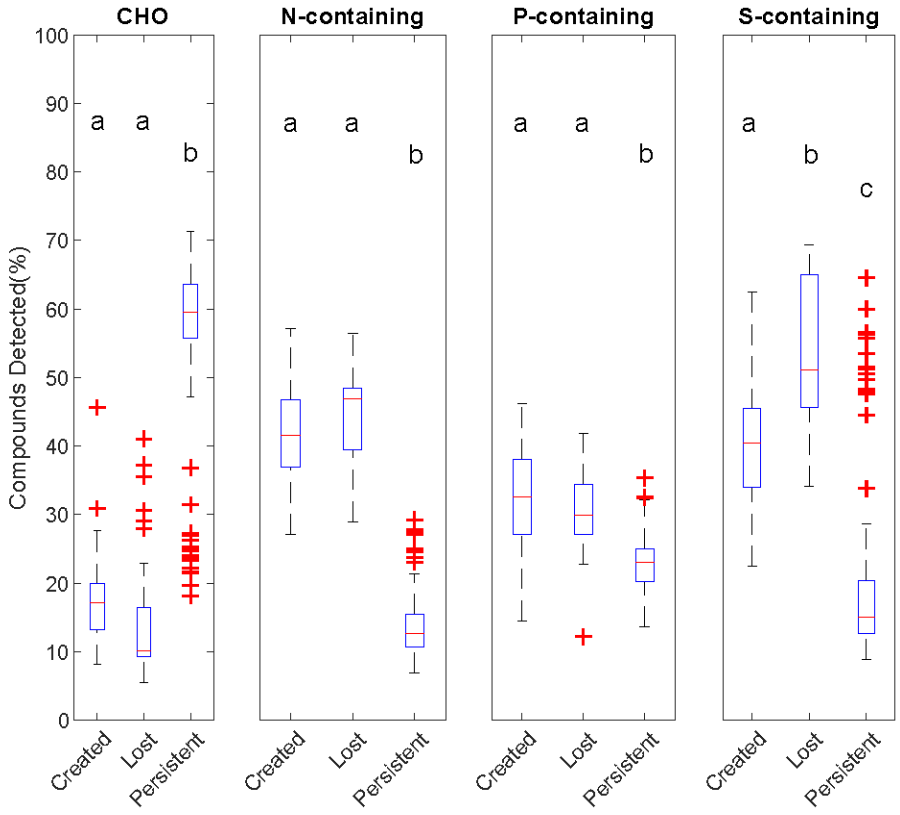
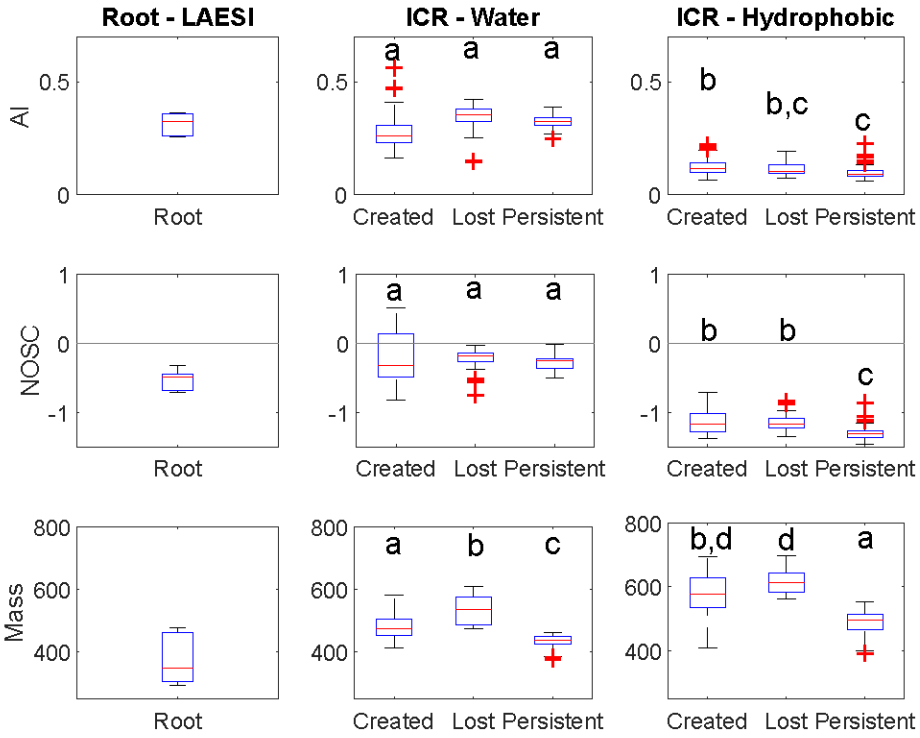


Figure 2, number of compounds in each sample that fall into different heteroatom groups for persistent, lost, and created soil compounds. All heteroatom groups had significant ($p < 0.05$) differences; differences within each type are marked with letters. Distributions are of the means of individual samples. Boxes show median with upper and lower quartiles, and tails show all data within 2.7σ of the mean. Outliers outside 2.7σ are marked with "+". For created n=73, for lost n=39, and for persistent n=112.



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323 Figure 3. Boxplots of molecular indices. The rows show: aromaticity index (AI), nominal oxidation state
324 of carbon (NOSC), and the molecular mass in Daltons. Letters indicate groups which are significantly
325 different ($p < 0.05$) from other groups for that index. The analysis spans the water and hydrophobic
326 classification, and does not include root compounds.

327

328 *Compounds processed in the rhizosphere — Created and Lost*

329 Compounds affected by microbial processing, those that were either created or lost,
330 were classified mostly as lipid-like and lignin-like compounds, similar to persistent compounds,
331 with these two categories comprising a mean of over 50% of compounds using the traditional
332 scheme (Fig 1), and were largely unclassified using MSCC (SI Fig S1). Unlike persistent
333 compounds, a majority of both created and lost compounds contained heteroatoms, with less
334 than 20% CHO compounds (Fig 2). Bogs are typically nutrient limited (Aerts et al. 1992), and it is

important to note that even though these heteroatoms existed they were not necessarily available to plants or microbes, as will be addressed in the discussion below.

Similar to persistent compounds, processed compounds had significantly higher NOSC and AI in the water-soluble fraction than the hydrophobic fraction (Fig 3). The median masses were between 475 Da and 615 Da. The masses varied both between water-extracted and hydrophobic fractions and between created and lost compounds (Fig 3), as will be discussed in the next sections.

Comparison between hydrophobic and water-extracted compounds

The hydrophobic compounds had lower AI and NOSC than water-extracted compounds across lost, created, and persistent compounds (Fig 3). For mass, each category in the water extraction was smaller than the corresponding category among hydrophobic compounds, e.g., lost compounds in the water extraction were smaller than lost compounds in hydrophobic, even though the lost water-extracted compounds were still larger than the persistent hydrophobic compounds.

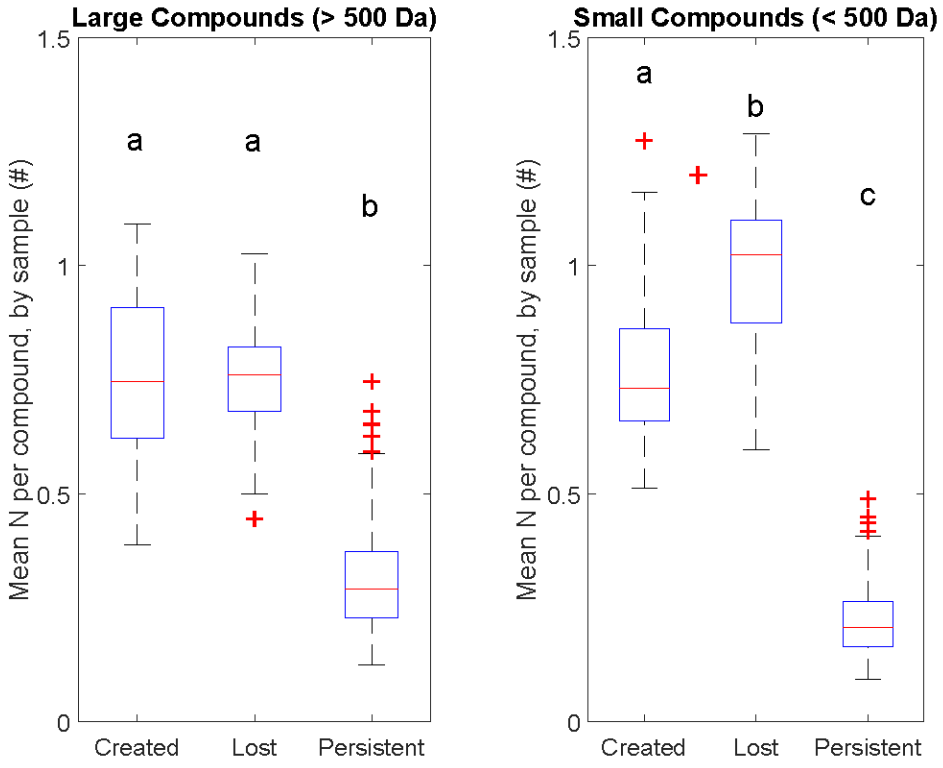
Comparison between processed and preserved compounds.

The compound classes were broadly similar between created, lost, and preserved compounds, but there were small yet statistically significant ($p < 0.05$) differences in lignins, amino-sugars, and carbohydrates (Fig 1). Preserved compounds had the highest percentage lignins, followed by lost then created. Created compounds were comprised of more amino sugars than either persistent or lost compounds. Created compounds were also most likely to be comprised of carbohydrates, followed by lost compounds, and persistent compounds were the least likely to be carbohydrates.

Heteroatom groupings revealed significant ($p < 0.05$) differences between the persistent and microbially processed molecules. The fraction of nitrogen- and sulfur-containing compounds was higher in both lost and created compounds than in persistent, with a smaller but still significant difference in phosphorus (Fig 2). Compounds comprised of only CHO had the opposite pattern; they were most abundant in persistent compounds.

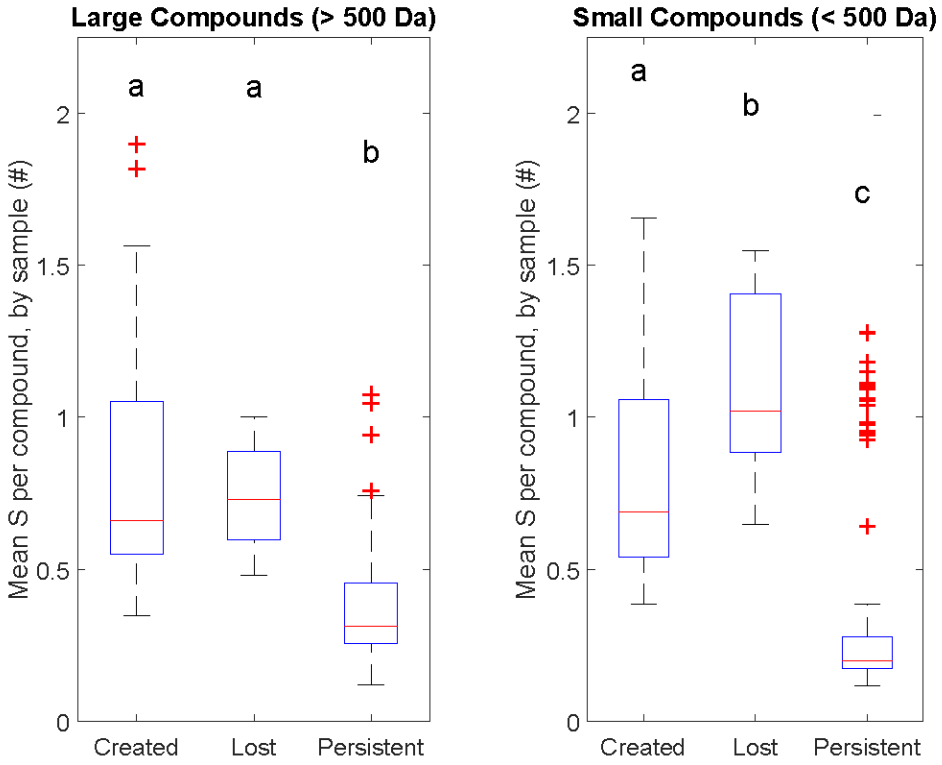
Of the three metrics shown in Figure 3, mass had the most salient differences across both processing type and hydrophobicity. In both hydrophobic and hydrophilic fractions, the lost compounds had the highest mass followed by created compounds with persistent compounds having the lowest mass. The NOSC data showed no statistically significant difference between preserved, created, and lost compounds in the water extracted fraction, but in the hydrophobic fraction the created compounds had higher ($p < 0.05$) NOSC values than persistent compounds, with lost compounds having an intermediate value not significantly different ($p > 0.05$) from either (Fig 3). There were statistically significant differences in AI; lost compounds in the water extraction had the highest AI, followed by persistent then created compounds. In the hydrophobic extraction the pattern was different: created compounds had the highest AI followed by lost then persistent compounds.

Because molecular mass was different between the groups of molecules, and microbes break larger compounds down into smaller compounds (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018), it is useful to analyze high and low molecular mass compounds separately. Among low molecular mass compounds (<500 Da), in the water extraction, lost compounds had the highest average NOSC and AI, and the persistent compounds had the lowest values for both metrics, with the differences being statistically significant ($p < 0.05$) for both metrics (Fig S4). Among high mass (>500 Da) compounds there were no statistically significant differences in AI or NOSC in either water-extracted or hydrophobic compounds (Fig S5). Both high and low molecular mass compounds had largely similar classifications to the full data set (Figs S6 & S7). However, small created compounds had significantly less nitrogen and less sulfur than small lost compounds ($p < 0.05$, Fig 4 and Fig 5), a difference that did not exist among the large compounds ($p > 0.05$, Figs 4 and 5). This pattern was true among both hydrophobic and hydrophilic compounds (Fig S8). When examining compound classes by molecular size, the small created molecules had significantly ($p < 0.05$) more proteins, amino sugars, and carbohydrates than lost compounds (Fig S7).



388

389 Figure 4. Nitrogen to carbon ratios among both large (left) and small (right) compounds. Small
390 compounds created in the rhizosphere were depleted in nitrogen relative to those lost. No difference in
391 nitrogen content was detected between created and lost large compounds.



392

393 Figure 5. Sulfur to carbon ratios among both large (left) and small (right) compounds. Small compounds
394 created in the rhizosphere were depleted in sulfur relative to those lost. No difference in sulfur content
395 was detected between created and lost large compounds.

396

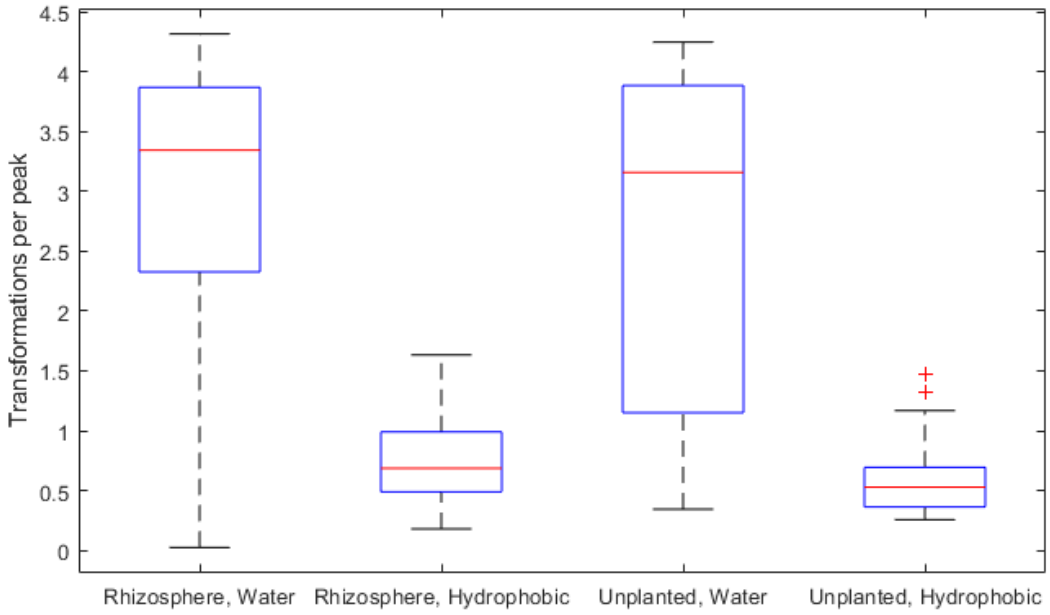
397 *Root compounds*

398 The compounds detected on the root surface by LAESI-MS are consistent with
399 fragments of structural lignins comprising the root itself. The traditional compound
400 classification scheme identified over half the compounds as lignin-like, with lipids and
401 concentrated hydrocarbons comprising most of the rest (Fig 1). As with the soil compounds,
402 most root compounds could not be classified with the MSCC (Fig S1). Around three quarters of
403 the detected compounds contained only C, H, and O (Fig S2), which is typical of lignins or other
404 structural molecules such as polysaccharides. The AI was high, which is again consistent with
405 structural lignins and polysaccharides that contain many aromatic rings.

406 Note that these measurements were made of the root material, not the root exudates
407 which are typically comprised of amino acids and sugars (Smith 1976; Lugtenberg et al. 1999;
408 Jones et al. 2009; Dommergues 2012) and are believed to stimulate microbial activity. However,
409 dead root cells sloughed into the soil may have been be introduced into the rhizosphere.

410 *Microbial Transformations*

411 Microbial transformation analysis involved all compounds present in a sample, not just those
412 identified as being persistent, created, or lost in the presence of roots. There were significantly
413 more ($p < 0.05$) microbial transformations among the water extracted compounds than in the
414 hydrophobic compounds, but there were no differences ($p > 0.05$) between the number of
415 transformations in rhizosphere or unplanted extractions (Fig 6). These results indicate that the
416 compounds in the water fraction were more involved in microbial metabolisms than those in
417 the hydrophobic fractions. Similarly, the PCA analysis of the transformation data showed that
418 the first two principle components together explained over 95% of variation and showed that
419 the water extraction was offset from hydrophobic compounds, but there was no difference
420 between rhizosphere and unplanted soil samples (Fig S8).



421

422 Figure 6. Comparison of number of microbial transformations in each extraction from both sample types
423 (rhizosphere and unplanted), including all compounds in each sample, not just lost or created. These

data show that more microbial transformations occurred with water-soluble compounds than with hydrophobic compounds. However, there is no difference in transformations between rhizosphere and unplanted soil.

Breaking the transformations down by nutrient showed that reactions involving nitrogen were similarly likely in rhizosphere and unplanted soil (Figure 7). However, transformations converting unplanted soil compounds to compounds found in the rhizosphere were more likely to involve nitrogen than the transformation within an extraction, whether the nitrogen was added during the transformation or removed (Figure 7).

Transformations involving sulfur were overall less common than those involving nitrogen, always accounting for less than 10% of all transformations. Unlike nitrogen, the PCA did reveal differences between the types of sulfur transformations happening within each extraction (Fig S10). Statistically significant ($p < 0.05$) differences in PC1 (explaining 49% of variation) and PC2 (explaining 16% of variation) point towards the extractions having unique transformation profiles. The strongest correlation for any one transformation is in PC1 where a sulfate for water substitution was four and a half times more predictive than any single other reaction. When transforming from unplanted soil to rhizosphere (The Removal and Addition categories in Figure 7), sulfur was significantly more likely to be removed than added ($p < 0.05$). Also, more removal and addition transformations involved sulfur than the transformations within any extraction ($p < 0.05$, Figure 7).

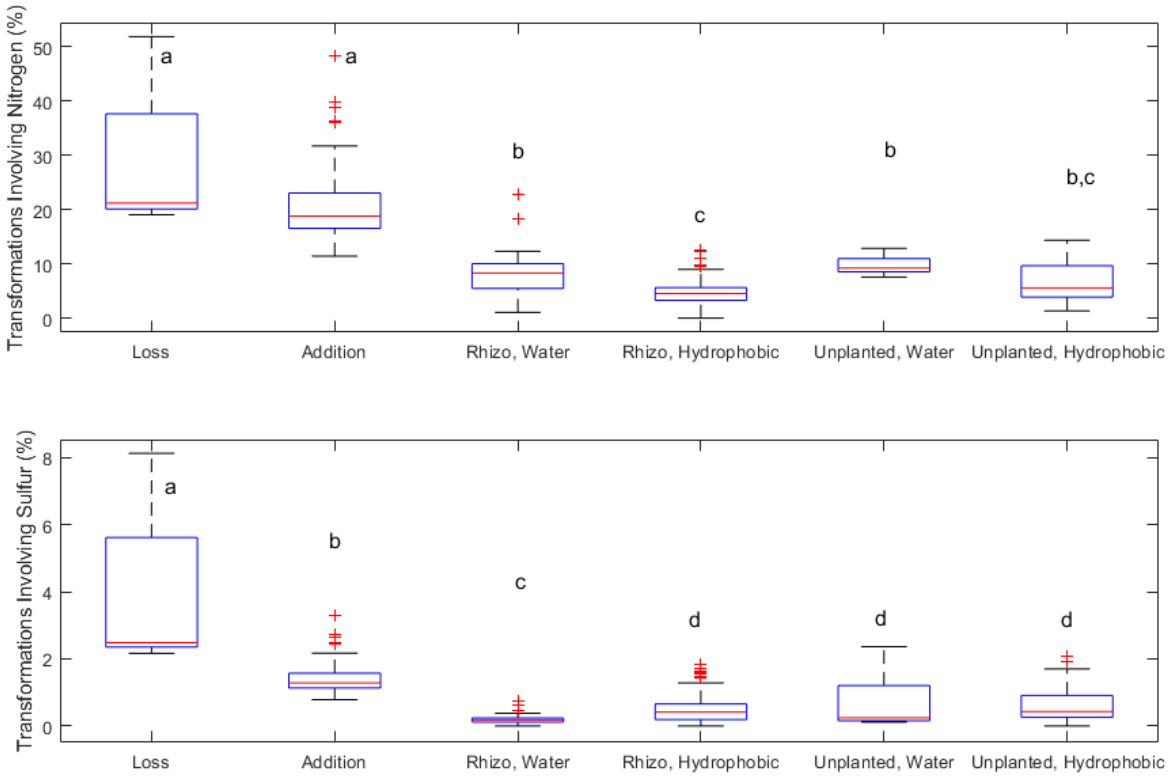


Figure 7. Comparison of the portion of transformations that involved nitrogen (top) and sulfur (bottom) among transformations withing and between carbon pools. The "Removal" category comprises transformations from unplanted soil to rhizosphere where a functional group is removed, the "Addition" category comprises transformations where a group is added. For both nutrients, the transformation type that was most likely to involve the nutrient was the loss of compounds from the unplanted soil.

KEGG pathway analysis revealed no significant difference ($p > 0.05$) in the number of metabolites or metabolic pathways between created and lost compounds. A PCA analysis of the pathways did reveal a statistically significant ($p < 0.05$) difference between created and lost compounds, but the difference was small. The first principle component explained 99.9 % of the variation, and its interquartile range among created compounds was .050 to .055, while among lost compounds the interquartile range was .052 to .060. There was no correlation between PC 1 and the flux of CH_4 out of the box ($p > 0.05$, $R^2 = .03$).

Discussion

A note on FT-ICR-MS data

Data obtained by FT-ICR-MS describe those compounds that were present in a sample during collection, not all those which ever existed. This distinction is important because those compounds which are most bioavailable and therefore used very rapidly may never show up in the results. In the context of this study, some root exudates fall into this category. Many types of root exudates may be added to the soil, but if some of those are processed quickly then only the less-bioavailable fraction will remain to be measured. Additionally, because the method only indicates whether compounds are present or not, there is no way to tell if a compound was exuded by a root or created by microbes in the soil.

Differences between Created and Lost Compounds

Compounds lost in the presence of roots must have been processed in a way that does not occur in the absence of roots. Compounds created in the presence of roots could have come from either root-influenced processes (e.g., metabolic by-products of microbes that utilize root exudates) or been exuded from the roots themselves. However, direct detection of root exudates is unlikely for several reasons. Some of the sugars and amino acids that comprise root exudates (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012) were too small to be detected consistently using the FT-ICR-MS technique (<100 Da). Additionally, sugars and amino acids are water-soluble and typically have high NOSC values, making them highly bioavailable in anoxic systems (Keiluweit et al. 2016). Because the created compounds were similar to lost and preserved compounds in terms of NOSC and proportion of protein- and amino-sugar-like compounds (Figs 1 and 3), we conclude that the exudates themselves were not directly detected. Working from the interpretation that the compositional changes we observed in the rhizosphere were *not* due to the direct detection of root exudates, we can draw conclusions about what kind of microbial processing could explain the observed changes in the rhizosphere.

The experiment from which the analyzed samples were obtained demonstrated that, based on greater CH_4 emissions from planted than unplanted boxes, the rhizosphere housed a

more active microbial population (Waldo et al. 2019). However, neither the KEGG database nor microbial transformation analysis (Fig 6) techniques detected a difference between rhizosphere and bulk soil samples, as those techniques have in other studies (Graham et al. 2017). This lack of difference could indicate that these metabolite-based methods are not very sensitive to differences in the population or activity of microbes in two systems that are similar (e.g., the same highly organic soil material), or that, in the rhizosphere, key metabolites were cycled so quickly that they did not exist long enough to be detected. This rapid cycling would impact the other metrics used to understand soil carbon by biasing the measurements against the most biologically active molecules.

For both hydrophobic and hydrophilic compounds, the lost compounds were higher MW than the created compounds (Fig 2), leading to a net decrease in average compound size across the rhizosphere. This decrease in mass is consistent with the breakdown of higher MW molecules into smaller ones, which aligns general understanding of microbial processing. The fact that plants were facilitating the mass decrease aligns with the findings of Chanton et al. (2008) who found smaller compounds in *Carex* dominated bogs than in those where vascular plants were absent. In this experiment, the group with the smallest mean mass was the preserved compounds, indicating that microbial and/or abiotic transformations did not target these small compounds and that they were also not taken up by plants.

Surprisingly, neither AI nor NOSC was able to explain why some compounds were used and others were not, even though those metrics have been useful in other studies (e.g., Keiluweit et al. 2016; Boye et al. 2017; Pracht et al. 2018). Transformation analysis (Fig 6) indicates that the water-extracted compounds were more actively used in microbial metabolisms, but there were no significant differences among created, lost, and preserved compounds in the water-extracted AI or NOSC. There were, however, differences in AI and NOSC between water-extracted and hydrophobic compounds, indicating that these indices were still differentiating between molecules with important properties such as hydrophobicity. One possible explanation for the apparent lack of influence of NOSC is that while there was no detectable oxygen in the soil (Waldo et al. 2019), the flux of oxygen from the roots could have nonetheless generated enough electron acceptors that NOSC was less of a salient factor than in

516 the highly reduced conditions studied by Keiluweit et al. (2016) and others (e.g., Boye et al.
517 2017; Pracht et al. 2018). Instead, molecular mass and nutrient content had more apparent
518 control over microbial processing (Figs. 3 and 4), an observation that is best explained by
519 microbial priming.

520 *Microbial Priming*

521 We demonstrated in our previous analysis of isotopic data from this plant-growth
522 experiment that microbial priming was occurring (Waldo et al. 2019). Priming is broadly defined
523 as an increase in microbial use of soil carbon due to the addition of a fresh carbon source. In
524 this analysis, changes in molecular characteristics such as size are used as proxies of microbial
525 activity. Most notably, higher MW compounds were broken down into smaller compounds in
526 the presence of roots. The roots could have simply accelerated a microbial process which also
527 occurred in the unplanted soil, a situation that is consistent with the hypothesis that priming is
528 caused by increased populations of microbes needing more food (Ye et al. 2015).

529 However, the microbes could have also engaged in N-mining. The N-mining hypothesis
530 predicts that microbes break down molecules that would otherwise be less bioavailable in
531 order to obtain nitrogen (Craine et al. 2007; Ruirui et al. 2014). N-mining could explain why the
532 low molecular mass compounds that were created had significantly less ($p < 0.05$) nitrogen than
533 those that were lost (Fig 4). There was a net removal of nitrogen from small compounds in the
534 presence of roots. Plants likely encouraged this N-mining in two ways: they added nitrogen-
535 poor root exudates which provided microbes with extra energy but not extra nutrients, and
536 they also would have absorbed some of the small nitrogen-containing compounds, decreasing
537 the pool of available nitrogen in the soil. Among large compounds, the created and lost
538 compounds had similar nitrogen content (Fig 4), indicating that these compounds were not
539 being mined for nitrogen. Rather, large compounds were processed for energy regardless of
540 nitrogen content. The molecules that remained and accumulated in the rhizosphere were small,
541 nitrogen-poor compounds that did not meet the needs of the plants or microbes.

542 Sulfur-containing compounds also followed a similar pattern to nitrogen-containing
543 ones (Fig 5), indicating that S-mining may be occurring as well as N-mining. In fact, the

transformation analysis provided additional evidence for S-mining; significantly more sulfur-containing groups were removed than added, a difference that appeared to exist for nitrogen transformations, but was not statistically significant (Figure 7). In addition, the transformation analysis found differences in the types of transformations between rhizosphere and unplanted soil in reactions involving sulfur. The single transformation which was most increased in the rhizosphere was a sulfate for water substitution. The fact that sulfate is the primary form of sulfur taken up by plants (Leustek and Saito 1999) indicates that plant uptake may be ultimately driving the sulfate use in the rhizosphere. This observation is consistent with previous studies that have shown that addition of root exudates or other simple substrates can increase microbial sulfur utilization in a variety of soils (Vong et al. 2003; Vong et al. 2003; Creamer et al. 2014). Studies of the interaction between soil carbon and nutrient availability have focused on nitrogen because it is both a key nutrient and one with high anthropogenic influence (Creamer et al. 2014). However, the concept can be applied to any limiting nutrient in the soil.

Priming of water soluble versus physically bound carbon

The FT-ICR-MS data analyzed here advances understanding of the mechanisms of priming by demonstrating that the presence of plants triggered increased processing of both large molecules (regardless of nutrient content, Fig 3) and small nutrient-containing molecules (Figs 4&5). Interestingly, none of the evidence for mechanisms of priming varied between hydrophobic and hydrophilic compounds. Both rhizosphere and unplanted soil samples had more microbial transformations and more transformations involving nitrogen in the water-soluble compounds (Fig 6). For all other evidence of microbial activity (e.g., created compounds of smaller size than lost compounds), the same patterns were exhibited between hydrophobic and water-soluble compounds (Fig 3 and Fig S8). This finding is notable because Graham et al. (2017) found that the addition of water-soluble, thermodynamically favorable carbon decreased microbial processing of carbon compounds in the chloroform extraction, referred to as the “physically bound” fraction in that study.

While such preservation of the physical bound, hydrophobic fraction did not occur in our system, the nature of our evidence for priming indicates that the results of Graham et al.

572 (2017) do not necessarily contradict priming, but instead elucidate how it occurs. We propose
573 that priming is a selective effect which often increases total carbon use, but which may increase
574 *or decrease* the microbial use of specific carbon pools such as water-soluble versus physically
575 bound or compounds with specific heteroatoms. This selectivity could be the result of several
576 factors. At its most basic level, it could be that priming exacerbates the already-present
577 preferences, such as for soluble compounds (Boye et al. 2017; Graham et al. 2017).
578 Alternatively, selective priming could be the result of favoring the growth of one community of
579 microbes over other communities, or the result of encouraging microbes to change their
580 metabolisms and produce a different set of enzymes. The nutrient-mining hypothesis, which
581 this study strongly supports, is premised upon the idea that microbes can use different pools of
582 carbon for different purposes, i.e., some compounds are utilized for energy and others for
583 nutrients. That effect is demonstrated here by the fact that created and lost large compounds
584 had similar nitrogen content, while smaller compounds had significant amounts of nitrogen
585 removed when processed (Fig 4). This study also found evidence of a similar effect for sulfur,
586 another plant nutrient. At least some portion of the nutrients that disappeared from the ICR
587 data were taken up by plant roots, and as plants removed nutrients from the soil system
588 microbes needed to process more nutrient-containing compounds to ensure their own supply.
589 If root exudates are more favorable for microbes to use for a certain purpose then native
590 compounds in the soil which microbes used for that purpose may be protected, a possible
591 explanation for the results of Graham et al. (2017).

592 **Conclusions**

593 This study built upon prior analysis of Waldo et al (2019), which established that
594 microbial priming occurred in the presence of plants and led to increased CH_4 production and
595 emissions. The two metrics that were most useful in differentiating between compounds
596 processed and preserved in the presence of plants were size and nitrogen content in water-
597 soluble compounds. It is well established that microbes break higher MW molecules down into
598 smaller molecules during processing of organic carbon (Amon and Benner 1996; McArthur and
599 Richardson 2002; Antony et al. 2012; Pracht et al. 2018), and here we demonstrated that the
600 presence of plants led to increased processing of those large molecules, presumably due to the

addition of root exudates. This increase in processing of large molecules is an example of priming caused by a more active microbial community utilizing more total soil carbon. Small molecules were also processed to a greater extent in the presence of plants, but only if they contained nutrients, lending support to the nutrient-mining hypothesis (Craine et al. 2007; Ruirui et al. 2014; Creamer et al. 2014) and possibly expanding it to indicate sulfur-mining as well. This selective priming implies a shift in the carbon processing capabilities and/or nutritional needs of the microbial community in the presence of highly preferable compounds. Because boreal bogs tend to be nutrient-limited environments, this laboratory study implies that such nutrient-mining may be favorable in similar systems around the world and the strong priming effect demonstrated in the companion study (Waldo et al. 2019) could occur in natural systems. Changes to the global climate and nutrient cycles are connected (Greaver et al. 2016), and so determining how they interact in this key ecosystem will help us understand future changes to soil carbon storage in northern peatlands, which represents an important climate feedback (Yu 2012).

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