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Mapping substrate use across a permafrost thaw gradient

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ABSTRACT

Permafrost thaw in northern peatlands is likely to create a positive feedback to climate change, as microbes transform soil carbon (C) into carbon dioxide (CO_2) or methane (CH_4). While the microbiome's encoded C-processing potential changes with thaw, the impact on substrate utilization and gas emissions is less well characterized. We therefore examined microbial C-cycling dynamics from a partially thawed *Sphagnum*-dominated bog to a fully thawed sedge-dominated fen in Stordalen Mire (68.35°N, 19.05°E), Sweden. We profiled C substrate utilization diversity and extent by Biolog Ecoplates™, then tested substrate-specific hypotheses by targeted additions (of glucose, the short chain fatty acids (SCFAs) acetate and butyrate, and the organic acids galacturonic acid and p-hydroxybenzoic acid, all at field-relevant concentrations) under anaerobic conditions at 15 °C. In parallel we characterized microbiomes (via 16S rRNA amplicon sequencing and quantitative polymerase chain reaction) and C gas emissions. The fen exhibited a higher substrate use diversity and faster rate of overall substrate utilization than in the bog, based on Biolog Ecoplate™ incubations. Simple glucose additions (akin to a positive control) to peat microcosms fueled fermentation as expected (reflected in enriched fermenter lineages, their inferred metabolisms, and CO_2 production), but also showed potential priming of anaerobic phenol degradation in the bog. Addition of SCFAs to bog and fen produced the least change in lineages and in CO_2, and modest suppression of CH_4 primarily in the fen, attributed to inhibition. Addition of both organic acids greatly increased the CO_2:CH_4 ratio in the deep peats but had distinct individual gas dynamics and impacts on microbiota. Both organic acids appeared to act as both C source and as a microbial inhibitor, with galacturonic acid also likely playing a role in electron transfer or acceptance. Collectively, these results support the importance of aboveground-belowground linkages - and in particular the role of *Sphagnum* spp.- in supplying substrates and inhibitors that drive microbiome assembly and C processing in these dynamically changing systems. In addition, they highlight an important temporal dynamic: responses on the short time scale of incubations (which would reflect transition conditions in the field) differ from those evident at the longer scales of habitat transition, in ways consequential to C gas emissions. In the short term, substrate addition response reflected microbiome

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legacy (e.g., bog communities were slower to process C and better tolerated inhibitors than fen communities) but led to little overall increase in C gas production (and a high skew to CO_2). At the longer time scale of bog and fen thaw stages (which are used to represent these systems in models) the concomitant shifts in plants, hydrology and microbiota attenuate microbiome legacy impacts on substrate processing and C gas emissions over time. As habitat transition areas expand under accelerating change, we hypothesize an increased role of microbiome legacy in the landscape overall, leading to a lag in the increase of CH_4 emissions expected from fen expansion.

1. Introduction

Half of global soil organic carbon (SOC) is estimated to be frozen in permafrost (Tarnocai et al., 2009), much of it in peatlands (Hugelius et al., 2020), and its fate under climate change is uncertain (Swindles, Morris and Mullens et al., 2015). There is concern that stored SOC may be released into the atmosphere as carbon (C) gasses as permafrost thaws, generating a positive feedback to climate warming (Schuur et al., 2013). Depending on local geohydrology, thawing permafrost peatlands can produce wetlands (Moomaw et al., 2018; Natali et al., 2015). Under water saturated conditions, organic matter (from permafrost, the active layer, or new plant inputs) can serve as a substrate for production of CH_4 (Christensen et al., 2004), a radiative forcing gas with a ~33 times higher global warming potential than CO_2 at century timescales (Myhre et al., 2013). Therefore, the ratio of CO_2:CH_4 emitted from thawing permafrost systems, and the magnitude of old versus new C loss are critical parameters in defining the current and predicted magnitude of this positive climate feedback from these systems. These parameters, and their changes with thaw are determined by microbial processing of soil organic matter (SOM), which links microbiome composition and C substrate use to C gas emissions. While mapping thaw's impacts on potential microbiome C processing has progressed (e.g., Woodcroft and Singleton et al., 2018), a lack of functional linkage of active substrate utilization and emissions contributes to the uncertainties about the magnitude of climate feedbacks from these systems.

SOM degradation in thawing permafrost peatlands can be generalized by the following steps, which change with thaw and are mediated by dynamic microbial, biogeochemical, and plant interactions: (i) high-molecular-weight (HMW) organic compounds and/or polymers enter the soil from thawing permafrost C pools and from plants, as exudates and litter; (ii) extracellular enzymes break down HMW compounds and polymers into lower-molecular-weight (LMW) polymers and monomers; and (iii) microbes ferment these smaller molecules into fatty acids and alcohols (Bridgham et al., 2013).

When permafrost peatlands thaw into wetlands, plant-derived compounds shift as the ericaceous shrubs of palsas transition to lower pH *Sphagnum* moss-dominated bogs and/or higher pH sedge-dominated fens, with the most labile C compounds in the fens (Hodgkins et al., 2014; Wilson et al., 2022). *Sphagnum*-produced compounds persist in the peat (Hamard et al., 2019) and can inhibit microbial activity (Mellegård et al., 2009), while labile compounds can prime decomposition (Basiliko et al., 2012), even in anaerobic conditions (Lin et al., 2022). In aerobic conditions of palsas and in the upper unsaturated portion of bogs, the monomers are converted to CO_2 via aerobic respiration. In the fully water saturated fens and lower portion of bogs, terminal electron acceptors (TEAs) such as nitrate, manganese, iron, or sulfate - and, in peatlands, humic substances (Keller et al., 2009) - can allow anaerobic respiration to occur. If none of these are available, small organic compounds (e.g., acetate, formate, methanol, methylamines) or CO_2 can serve as TEAs via methanogenesis (Bridgham et al., 2013; Ye et al., 2012). The crux of plant-microbial interactions is the impact of plant-derived compounds on the microbiota: as substrate, inhibitor, or electron acceptor. Collectively, microbial and substrate interactions give rise to CO_2 and CH_4 emissions, with the latter increasing with thaw stage (McCalley et al., 2014; Hodgkins et al., 2014; Varner et al., 2022). As thaw accelerates in these systems (Varner et al., 2022), with cascading hydrologic and plant successional changes (Malmer et al., 2005), the consequences of microbial communities' composition, substrate utilization potential, and tolerance of inhibitors, for C gas production remains poorly constrained.

Therefore, in order to mechanistically connect substrates with microbes and C gas emissions, we asked (i) how does microbial substrate utilization change with thaw state from bog to fen, and (ii) how does substrate type impact microbial community composition and gas production? We hypothesized that (i) substrate utilization will increase (in both diversity and magnitude) from bog to fen given previous observations of higher diversity of C substrates and microbial activity (Wilson et al., 2022; Woodcroft and Singleton et al., 2018) and higher microbial cell numbers (Woodcroft and Singleton et al., 2018) with thaw, and (ii) the impact of specific substrates on microbial diversity and C gas emissions will reflect the microbiome's background exposure to them (i.e. bog vs. fen legacy effect), and the substrate's point of entry into microbial C processing (i.e. complex versus simple substrate, versus inhibitor).

2. Materials and methods

2.1. Site description

We focused on a climatically relevant bog-to-fen transition at Stordalen Mire in Abisko, Sweden (68° 20′N, 19° 03′E), where thaw-induced changes in hydrology drive changes in plant community composition (Malmer et al., 2005), SOM chemistry (Hodgkins et al., 2014; Wilson et al., 2022), microbial community composition (McCalley et al., 2014), and greenhouse gas production (Mondav et al., 2014). The bog is an intermediate-thaw habitat characterized by: (i) a seasonally thawed active layer overlying permafrost, *(ii)* a dominance of the *Sphagnum* spp.*, (iii)* acidic pore-water (pH = ~4.0–4.2; Hodgkins et al., 2014), and (iv) a seasonally variable water table that is perched above the local water table, receiving water and nutrients only by precipitation and surface runoff. The fen is a completely thawed feature (i.e., no detectable subsurface permafrost) and is characterized by: (i) *Eriophorum* spp. sedges, (ii) less acidic pore-water (pH = ~4.8–6.0; Hodgkins et al., 2014) and (iii) being waterlogged throughout the active season.

2.2. Sampling

On July 16, 2014 (bog) and July 17, 2014 (fen), two sites within the bog (bog site 1**:** 68° 21.1973′N 19° 02.8537′E; bog site 2**:** 68° 21.1968′N 19° 02.8319′E) and fen (fen site 1: 68° 21.1992′N 19° 02.8063′E; fen site 2: 68° 21.1959′N 19° 02.7992′E) habitats were sampled (within an area monitored via an autochamber system). Prior to sampling, pH and water table depth were measured, and pore water was collected for use in the laboratory incubations. One liter of pore water was collected from each of two depths at each site (~13 cm for the "mid" depth and ~33 cm for the "deep" depth) using a 60-mL syringe connected to a stainless-steel tube perforated along the bottom 4 cm and transported to the lab on ice. Peat cores were removed from the bog using a push corer (13 cm diameter) and from the fen using a Wardenaar Corer (10 × 10cm; Eijkelkamp, Netherlands). Cores were collected adjacent to the pore-water collection; within ~1m for the bog cores, and within ~3 m for the fen cores. Material for the incubations was collected from the side of each of two replicate core holes in each habitat, at "mid" (8–18 cm) and "deep" depths (28–38 cm; depths measured from the peat surface) and placed immediately in plastic bags and sealed. At both sites, mid-depth peat samples were collected from below the water table at time of sampling (directly below in the bog, and 10 cm below in the fen); average water table depth (June to September) for that year was −7.05 cm in the bog and +7.7 cm in the fen (with negative values indicating water table depths below the peat surface). Peat temperatures on the day of sampling ranged from 18 °C at the surface to 10.5 °C at 32 cm depth at the bog site, and 16 °C at the surface to 13.5 °C at 32 cm at the fen site. Peat was transported back to the field station on ice. Samples were used for incubations within 12 days of collection and stored at 4 °C until use.

2.3. C substrate utilization

We assessed microbial community C substrate utilization using two complementary methods: Biolog Ecoplate™ plates, and anaerobic incubations of peat soil with organic substrate additions (Fig. 1). Biolog Ecoplates™ (Biolog, Hayward, CA) provided rapid assessment of substrate use for 31 soil system-relevant substrates under aerobic conditions, but in a 96-well format requiring dilute aqueous suspensions of cells, which are less reflective of natural environmental conditions. Conversely, anaerobic incubations of the peat were amended with organic substrates and incubated at conditions tailored to reflect those *in situ* (discussed below). In the anaerobic incubations, SOC transformation was assayed via addition of a particular organic substrate followed by monitoring of CH_4 and CO_2 production in the headspace of the jar.

2.4. Substrate utilization: biolog plates

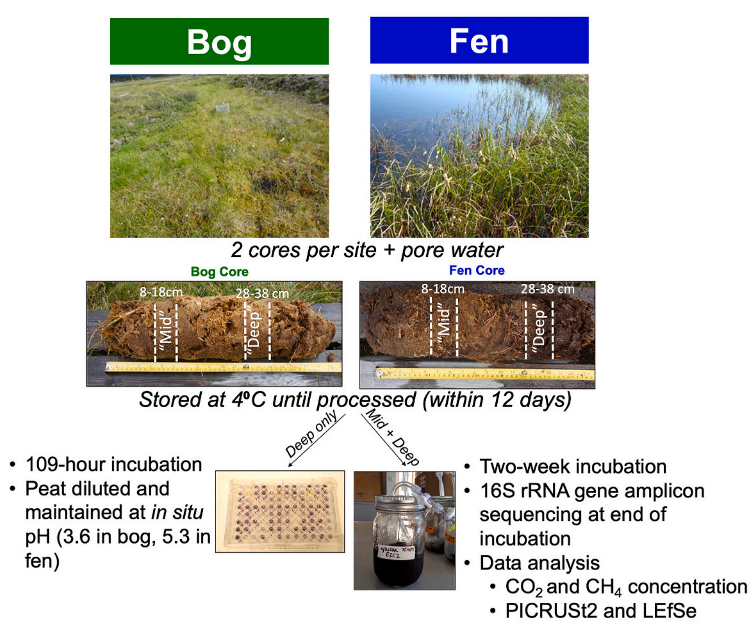
The organic substrates included in the 96-well colorimetric assay span diverse categories representative of compounds commonly present in soil organic matter: i) amines, ii) amino acids, iii) carbohydrates, iv) carboxylic acids, v) polymers, and vi) phenolic compounds (full list of substrates included in Table S1; Insam, 1997). Each 96-well plate contained the 31 substrates (Fig. 1) and a no-substrate negative control, each performed in triplicate wells under aerobic conditions. Biolog plates quantify metabolic activity by colorimetrically measuring cell respiration (NADH production), via reduction of a tetrazolium dye.

Deep (28–38 cm) peat (core 2) from both the bog and fen was tested for substrate use via the Biolog Ecoplate™ method (Preston-Mafham et al., 2002). For the initial generation of a soil/peat slurry for use in the plates, we used matched pore water from the same site and depth. Deep bog and fen peat samples were each combined with matched pore water at an initial 1:10 dilution. Subsequent dilutions of this initial suspension were made with phosphate buffer (50 mM K_2HPO_4) that was adjusted to match the respective bog or fen *in situ* pHs (3.6 and 5.3, respectively) using hydrochloric acid (HCl) or sodium hydroxide (NAOH) (Classen et al., 2003; Preston-Mafham et al., 2002). These pH-adjusted phosphate buffers were used to make 1:1000 dilutions of the initial soil suspension (i.e., 1:10,000 of the original pore water-saturated peat matrix); this 1:1000 dilution was empirically determined in trials to best produce a steady increase in color development rather than saturating the color development too quickly. Per the manufacturer's instructions, each Ecoplate well was inoculated with 135 μl of the diluted soil suspension. In parallel, the remaining soil suspension for both fen and bog was filtered with a 0.2-μm filter and inoculated into a second set of plates as a negative control, representing non-cellular effects, potentially including extracellular enzymes. All plates were incubated aerobically at 15 °C (empirically determined in trials to be the lowest temperature resulting in strong color development) except during the final days of incubation (between 84 h and 109 h), during which the plates were incubated at room temperature (~20 °C).

Ecoplates were incubated in the dark and measured daily over five days using an absorbance spectrophotometer (Biotek ELx800 Absorbance Reader, Biotek, Winooski, VT) at a wavelength of 590 nm. To normalize for differences in inoculum density, substrate use can be quantified by average well color development (AWCD) averaged across replicates and can be measured repeatedly over time to allow the assessment of utilization during growth (Preston-Mafham et al., 2002; Stefanowicz, 2006; Garland, 1996; Lindstrom et al., 1998). AWCD from the control wells were subtracted from all amended wells before replicates were averaged for final AWCD.

2.5. Substrate utilization: CO_2 and CH_4 production in substrate-amended anaerobic incubations

For each site, 24 incubation jars (500 ml) were established, with peat



SUBSTRATE GROUP	# OF ECOPLATE SUBSTRATES (N=31; TRIPLICATES)	SELECTED SUBSTRATES (N=5 SUBSTRATES)
Carbohydrates	10	Glucose (100mM)
Carboxylic acids	7	Galacturonic Acid (150mM)
Short-Chain Fatty Acids	N/A	Acetate (351 uM) Butyric acid (11 uM)
Phenolics	2	P-hydroxybenzoic Acid (75 mg/ml)
Amines	2	N/A
Amino Acids	6	N/A
Polymers	4	N/A

Both incubated in dark at 15°C

Fig. 1. Experimental approach. Left: A bog and fen (top row) were cored and subsampled for "mid" (8–18 cm) and "deep" (28–38 cm) peats (middle row). Material was divided for use in Biolog Ecoplates™ and mason jar incubations (bottom row). 16S rRNA gene amplicon sequencing was done on initial field samples and t = final samples taken from the end of the mason jar incubation. Right: 31 and 5 substrates were assessed in the EcoplatesTM and mason jars, respectively. Grey dashed lines connecting Biolog and mason jar substrates indicate exact matches; 4-hydroxybenzoic is the conjugate base of p-hydroxybenzoic acid.

from two cores, at two depths, and 6 treatments (five substrates and an unamended pore water control) (Fig. 1). The organic C substrates (glucose, acetate, butyrate, galacturonic acid and p-hydroxybenzoic acid) were chosen to test specific aspects of C processing dynamics in the fen and bog habitats (Fig. 2). The addition of glucose tested whether gas production in the bog (with its inhibitory conditions and more recalcitrant SOM; Hodgkins et al., 2014) and fen would be equivalently fueled by a simple monosaccharide. Acetate and butyrate are short chain fatty acids (SCFAs) that are the products of microbial fermentation, and potential stimulants of methanogenesis, the former as a direct substrate and the latter via syntrophic butyrate oxidation (Liu et al., 2011). In some bogs, acetate may build up or be diverted to other processes (Duddleston et al., 2002). Adding these SCFAs tested the degree to which the bog and fen microbiomes could convert them to CH_4. Galacturonic acid, while commonly present in plants with pectin (Buston, 1934a,b), is especially abundant in *Sphagnum* (Spearing, 1972), the dominant bog species (Hough et al., 2020). It is a major component of the polymer sphagnan (Fudyma et al., 2019; Wilson et al., 2022; and 38% mol % reported in Ballance et al., 2007), which is responsible for *Sphagnum's* organic matter-preservation properties (Painter, 1991). Galacturonic acid may contribute to this by lessening microbial activity due to acidity (Clymo, 1964) and/or complexation with proteins (Painter, 1991; Hodgkins et al., 2014), although it is a substrate for some microbial species (Pankratov et al., 2008). Addition of galacturonic acid tested the potential for differing microbiome response in bog and fen based on prior exposure, since it is present in the bog at a higher abundance than in the fen due to *Sphagnum's* dominance in bog (Hough et al., 2020). P-hydroxybenzoic acid is an aromatic phenolic compound that is also found in *Sphagnum* moss, at a higher abundance than in the dominant fen vegetation and was selected due to its potential role as a decomposition inhibitor in the bog (Mellegård et al., 2009; Freeman et al., 2001), further allowing testing of the impact of prior exposure on microbiome response and gas production.

C substrates were dissolved by vigorous mixing in porewater from the matched sites and depths as the peat in each incubation. Final substrate concentrations in the porewater (Fig. 1) were as close as possible to those observed *in situ* in the same or similar sites, or used successfully in similar previous incubations, and were empirically determined in trials to be the lowest additions capable of producing C gas under these incubation conditions (anaerobic, 15 °C). Glucose (Sigma product D9434) was added at 100 mM, after testing at 75 mM (used by Degens and Vojvodic-Vukovic, 1999) and 100 mM. Acetate was added (as sodium acetate, Sigma product S2889) at 351 μM, ~6.6X the maximum observed previously at Stordalen Mire bogs and fens (53 μM; M. Hines, personal communication, April 2014), after testing at 53 μM and 351 μM (1 and 6.6X). Butyrate was added (as sodium butyrate, Sigma product B5887) at 11 μm, ~10X the 1.1 μM maximum observed previously at Stordalen Mire bogs and fens (M. Hines, personal communication, April 2014), after testing at 1 μM and 11 μM (~1.8 and 10X). These acetate and butyrate amendment concentrations are at the high end of concentrations observed across all habitats in Stordalen Mire (including in "collapsed palsa" thermokarst features, which reached concentrations 1-2 orders of magnitude higher than the maximum concentrations observed in bogs and fens, but rarely) (M. Hines, personal communication, May 2014). Galacturonic acid (Sigma product 48280) was added at 150 mM, compared to 100 mM used by Degens and Vojvodic-Vukovic (1999), after testing at 100 mM and 150 mM. P-hydroxybenzoic acid (Sigma product 240141) was added at 75 mg/ml, compared to 12.5 mg/g wet soil in acidic peatland (Mellegård et al., 2009), after testing 25 mg/ml and 125 mg/ml (2 and 10X).

Directly before the peat incubation setup, each peat sample was gently and thoroughly kneaded within its plastic bag to homogenize the material. To most closely emulate *in situ* conditions and communities, these incubations used fresh material, were relatively short in duration (two weeks), and did not include the pre-incubation period common to many such anaerobic studies to develop fully anaerobic conditions (e.g., in Ye et al., 2012, Keller and Takagi, 2013, and Wilson et al., 2017, pre-incubations ranging from six days to two weeks). This likely resulted in some residual oxygen (O_2) being present, however both bog and fen material were collected from fully saturated depths. In addition, the unamended fen incubations produced CO_2:CH_4 at ~1:1, suggesting a limited cumulative impact of any residual O_2.

45 g of wet homogenized peat was added to each mason jar along with 160 mls of C substrate porewater solution, resulting in ~50% headspace in the jar (as in Hodgkins et al., 2014). The jars were mixed to evenly distribute the C substrate porewater solution throughout the saturated peat. The headspace of the jars was flushed with nitrogen gas three times, with 30 s of flushing and then 30 s of shaking between flushes (final flush was 1 min) (as in Hodgkins et al., 2014). Jars were then incubated in a dark incubator at 15 °C for two weeks (Fig. 1).

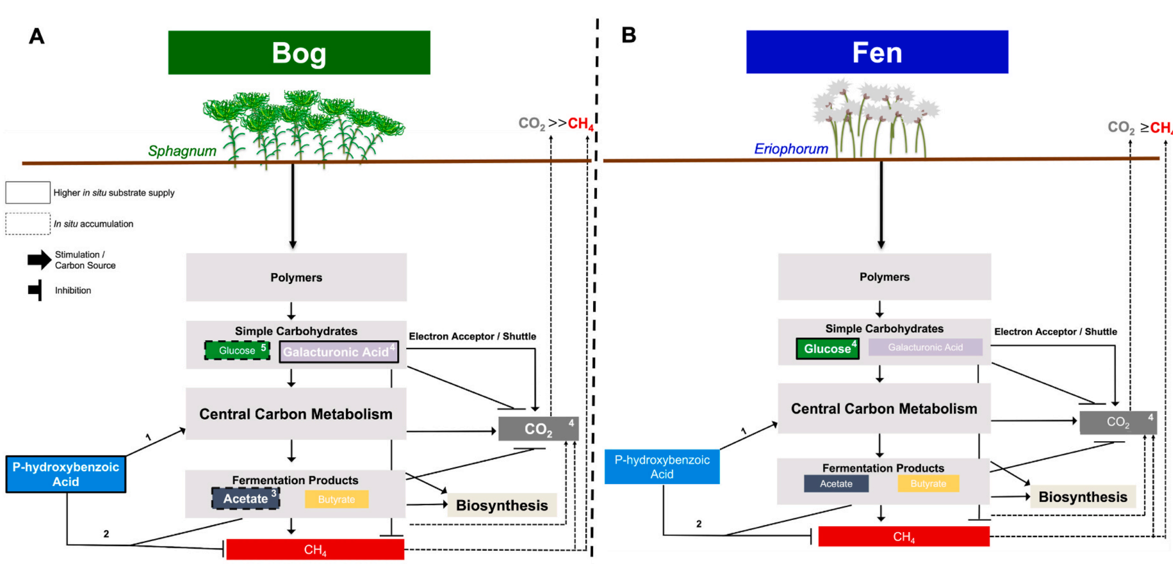


Fig. 2. Simplified conceptualization of microbial soil carbon processing of incubation-amended substrates (glucose, acetate, butyrate, galacturonic acid and p-hydroxybenzoic acid) in the bog (A) and fen (B) habitats. Solid outlines of substrate text boxes indicate higher inferred use, while dashed outlines denote accumulation under ambient conditions. References for *in situ* chemical abundances or roles: [1] Fuchs et al. (2011), [2]Mellegård et al. (2009), [3]Duddelston et al. (2003), [4]Hodgkins et al. (2014), [5]AminiTabrizi et al., 2020.

2.6. Sampling and analysis of microcosm headspace

After the start of the incubation, the headspace of the jars was sampled at least once every three days throughout the 2-week incubation. 5 ml each for CO_2 and CH_4 analysis were collected in separate syringes, with simultaneous headspace replacement using N_2 gas. CH_4 was measured using a gas chromatograph with an FID detector (GC-FID, Shimadzu GC-2014), and CO_2 was measured using an infrared gas analyzer (LI-6200). The GC-FID detector response was calibrated with a 2010 ppm or 2.5 ppm CH_4 standard depending on the concentration in the sample. The infrared gas analyzer was calibrated by creating a standard response curve with dilutions of a 1% CO_2 standard into a continuous flow of zero air. CH_4 and CO_2 production rates were calculated based on a linear change in headspace concentration over time, per gram of dry peat. At the end of the incubations, peat samples were dried and weighed to determine dry weight. For statistical analyses, depths were combined to provide 4 replicates per treatment per habitat (due to low replication based on peat availability). An ANOVA was used to test for significant impact of substrate on gas production relative to the controls.

2.7. Microbial community characterization: 16S rRNA gene quantification and amplicon sequencing

Samples (1 ml) for microbial community analyses were collected from the bog and fen material before the incubations began ("time-initial"), and for all control and substrate-amended jars at the end of the 2-week incubation ("time-final"). Samples were immediately frozen at −80 °C, and after completion of the experiment were shipped frozen back to Tucson, Arizona, United States for processing. DNA was extracted using the MoBio PowerSoil kit, single tube protocol (Mo Bio Laboratories, Carlsbad, CA, USA). qPCR of 16S rRNA was performed at the Australian Centre for Ecogenomics, as described in Woodcroft et al. (2018), using the 16S 1406F/1525R primer set designed to amplify bacterial and archaeal 16S rRNA genes: F - GYACWCACCGCCCGT and R - AAGGAGGTGWTCCARCC. 16S rRNA V4-region amplicon sequencing was performed at Argonne National Labs using the early Earth Microbiome Project protocol (Caporaso et al., 2011, 2012), using primers 515f; 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806r 5′-GGACTACHVGG GTWTCTAAT-3′, with paired-end sequencing on an Illumina MiSeq. Amplicon sequence data was processed using QIIME (Caporaso et al., 2010) version 1.9.1 to generate operational taxonomic units (OTUs, at ≥ 97% identity) and counts per sample with rarefaction to 10,000 sequences.

Community alpha diversity was calculated as richness (number of OTUs observed) and as Shannon diversity, and beta diversity was visualized by non-metric multidimensional scaling (NMDS) of OTU relative abundance using the Bray-Curtis dissimilarity, in the R vegan package (Oksanen et al., 2020). Genus-level lineages that differed significantly by relative abundance between each treatment and the control were identified using the LEfSe (Linear discriminant analysis Effect Size; Segata et al., 2011), using a significance threshold of 2.0 LDA score and p-value < 0.05. Microbial functional potentials were inferred from 16S rRNA sequence data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) tool (Langille et al., 2013), with differentially abundant pathways in each treatment identified using LEfSe (LDA >2.0; $p < 0.05$).

2.8. Data analyses and statistics

For Biolog Ecoplates, an ANOVA was performed to identify significant differences in substrate utilization (i) by site, (ii) use over time, (iii) and interaction effect between site and time (initial vs. final). For qPCR data, the effect of substrate and site on copy number was assessed by ANOVA, and then a Tukey HSD test was performed to identify treatments that were statistically different from controls.

The Shannon index, was calculated for substrate use diversity with the following equation:

$$H = -\sum_{i=1}^{N} p_i ln\ pi_i$$

where H is the Shannon index, N is the number of substrates (in this case 31), and p_i is the ratio of the use of a particular substrate "i" (calculated as the average absorbance of three replicates) to the AWCD (Stefanowicz, 2006).

R studio version 4.0.2 (R Studio Team, 2020) was used for all statistical analyses and illustrations. P-values < 0.05 were deemed to be significant in this study.

3. Results

Via the parallel application of dilute, aerobic substrate-use profiling (Biolog plates), and intact, anaerobic substrate additions with monitoring of gas production and microbial community composition (incubations), we found that peat source, recent microbiome exposure to substrates (which we refer to here as "microbiome legacy effects"), and substrate entry point to microbial C processing all impact biotic responses and system outputs.

3.1. Substrate utilization: biolog plates

The fen showed greater substrate utilization than the bog on Biolog plates, by all metrics. Substrate utilization patterns of 28–38 cm deep peat showed a consistently greater color development in the fen than the bog (Fig. 3A). Diversity of substrate use (Fig. 3B) was also greater in the fen than the bog (Shannon-Weaver index of $H = 0.71$ and 1.19 at the start and end in the fen, versus $H = 0.37$ and 1.03 in the bog).

Substrate use was consistently greater in the fen than in the bog, significantly so for amines ($p < 0.001$; Fig. 4, Fig. S3), carbohydrates ($p < 0.001$) and carboxylic acids ($p = 0.023$) (Fig. 4). Substrate use increased over time, with a significant temporal trend in both the bog and fen in the same three classes that had significant differences by habitats: for the amines ($p < 0.001$, average standard deviation (SD) at 109hrs = 0.06 for bog and 0.31 for fen), carbohydrates ($p < 0.001$, SD at 109hrs = 0.46 for bog and 0.57 for fen) and carboxylic acids ($p = 0.005$, SD at 109hrs = 0.33 for bog and 0.36 for fen). There was no significant interaction effect of site and time (Table S2). The rate of the substrate use exhibited three different patterns. There was a steady increase in the use of carbohydrates and polymers for both bog and fen. Metabolism leveled off in both bog and fen for amines, carboxylic acids, and amino acids, though for the latter to a greater extent in bog than fen, and in the fen only for phenolics. Lastly, although not significant, a lag in usage was observed in the bog for phenolics from 0 to 44 hrs., though they reached a similar cumulative utilization as the fen by the end (usage of individual phenols in Fig. S2).

3.2. Substrate additions: CO_2 and CH_4 production

We measured gas production in the incubations to monitor the impact of substrate addition on CO_2 and CH_4 flux (Fig. 5, Table S3, Fig. S5). In the control incubations, the CO_2:CH_4 ratio was higher in the bog than fen (bog mid = 35, deep = 17, versus fen mid = 2, deep = 1).

Glucose-amended peats yielded an increase in CO_2 production (μmol/day/g dry soil) in both sites and in both depths (bog: mid = 60% increase, deep = 71%; fen: mid = 111%, deep = 80%) with a 58% and 38% increase in CH_4 production (μmol/day/g dry soil) in the bog mid and deep depth, respectively. However, the fen's CH_4 production decreased by 41% (i.e., 59% proportion of the control production) in the mid depth samples and stayed similar to the control in the deep depths with only a 4% increase. SCFAs (acetate and butyrate) generally showed no drastic deviations from the control. However, in the SCFA

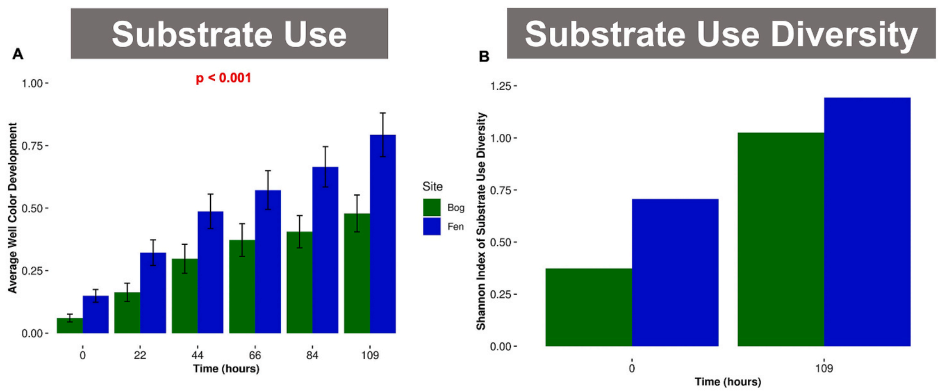


Fig. 3. Differences in overall substrate use (for 31 different substrates) in Biolog™ Ecoplates in the fen (blue) and bog (green) over time (109 h). (A) Average well color development (minus control wells, and normalized among plates; see Methods) in aerobic Biolog™ Ecoplate incubations. Error bars are±standard errors (n = 3 replicates for each substrate); p-value < 0.001 denotes a significant difference (ANOVA) between bog and fen overall substrate utilization. **(B)** Diversity (Shannon Index) of substrate use across a whole plate for each habitat at initial and final incubation time points (Per Fig. 1, this assay was performed on deep peat only). Average well color development of individual substrates can be found in Table S1.

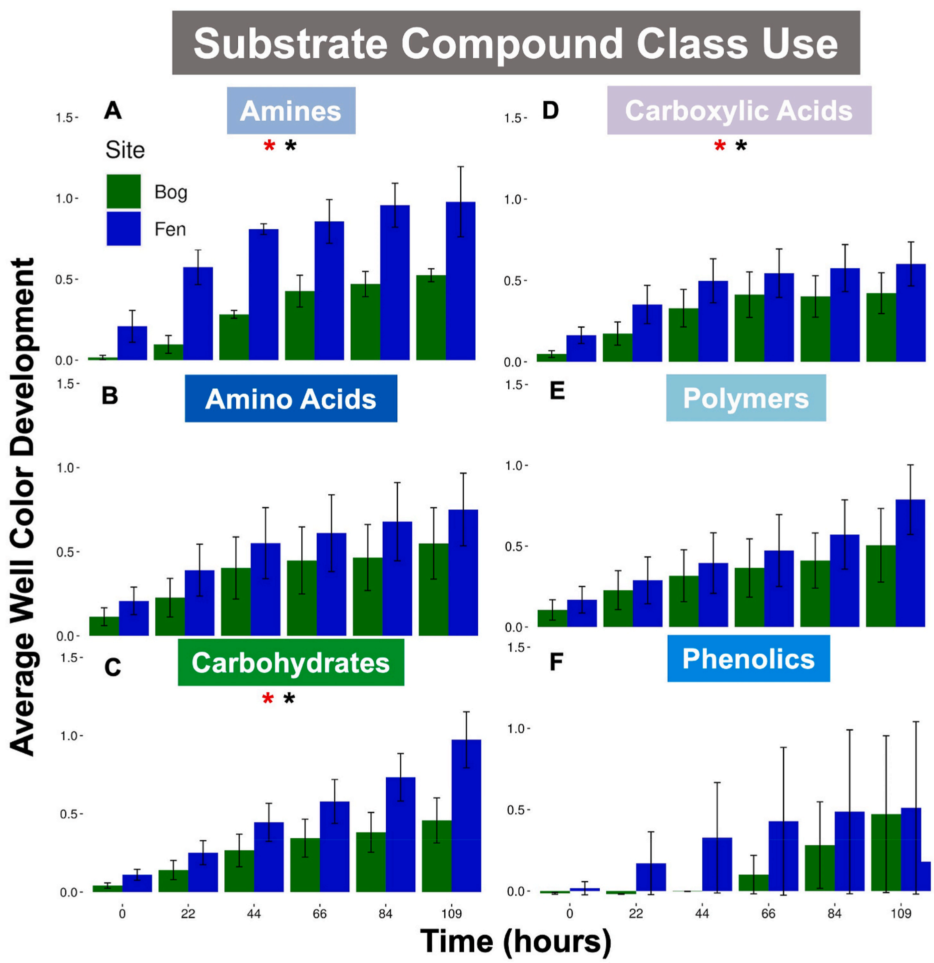


Fig. 4. Average substrate use (average well color development) by time and site for Biolog Ecoplate™ **incubations over 109 h of (A)** amines (n = 2) **(B)** amino acids (n = 6) **(C)** carbohydrates (n = 10) **(D)** carboxylic acids (n = 7) **(E)** polymers (n = 4) & **(F)** phenolics (n = 2), in bog (green) and fen (blue). Stars indicate a significant difference (p < 0.05; ANOVA) between site (red) or in use of substrate compound class over time across all time points (black).

amendments, CH_4 production exhibited a decrease in the fen (acetate: mid = 26%, deep = 29%; butyrate: mid = 61%, deep = 50%), whereas the bog's CH_4 production increased by 48% in the acetate mid, decreased 49% in acetate deep, and stayed similar for butyrate amended fen peats (~12% increase for both depths). The organic acids yielded the most notable change in gas CO_2 and CH_4 production. P-hydroxybenzoic acid depressed the CO_2 production at both depths in both the bog and fen while only depressing the CH_4 production in the deep depth of both sites (Fig. 5A–C), yielding a decrease in the CO_2:CH_4 ratio in the mid (bog = 93%, fen = 79% relative to the controls) and an extremely increased CO_2:CH_4 ratio in the deep (bog = 1,250%, fen = 1,850% relative to the controls). For galacturonic acid, CO_2:CH_4 ratios were greatest in the deep peats amended with galacturonic acid in both the bog (ratio = 590; 3,585% of the control) and fen (ratio = 668; a 66,846% of the control). Underlying these ratios, galacturonic acid-amended peats had similar average CO_2 production to that of the control jar (though the replicates were highly variable), while CH_4 production was virtually eliminated.

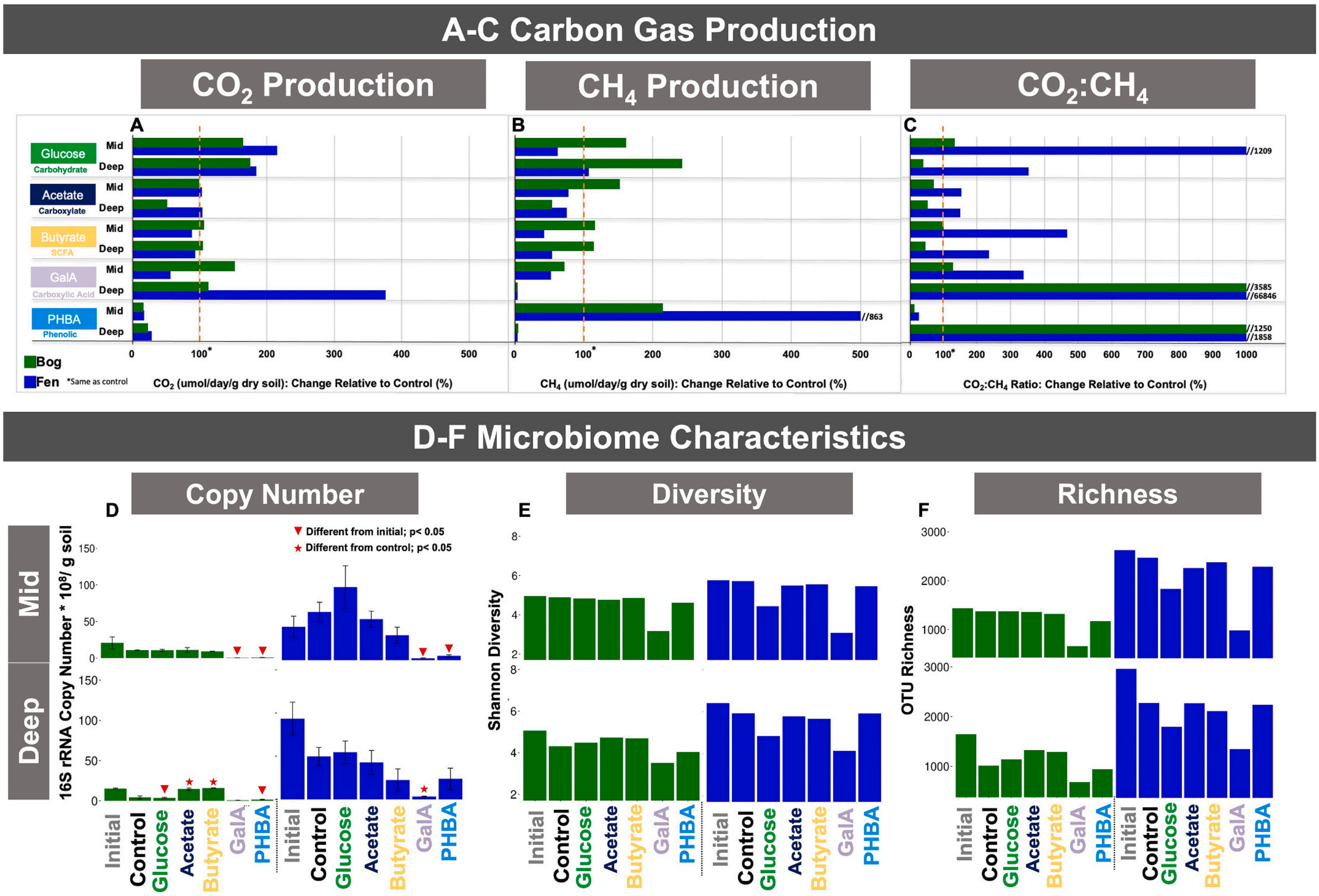


Fig. 5. Microbiome 16S rRNA copy number (A), richness (B), and Shannon's diversity (C) by amendment, site, and depth. Initial timepoint collected before incubations began, Control and amendments measured at the end of the two-week anaerobic incubation. (B) and (C) are assessed for 97%-sequence variant clusters (OTUs). Red symbols in (A) denote significant differences (p-value < 0.05; ANOVA) from the initial field samples (inverted triangle) or the control (star); for (B) and (C), low replicate number precluded statistical assessment of differences. **Production of (D) CO_2, (E) CH_4, and (F) CO_2:CH_4 relative to the control by amendment, site and depth. Red vertical dashed lines indicate no change relative to the control.** Hash marks indicate truncated bars which were done to display potential differences in smaller ratio amendments. For (D–F), ANOVA were run on depth-averaged data, but no significant differences were identified. Data for (A–F) are in Supplemental Table S3, absolute values are visualized in Fig. S5. Throughout, bog = green, fen = blue. Abbreviations: GalA = galacturonic acid; PHBA = p-hydroxybenzoic acid.

3.3. Substrate additions: 16S rRNA copy number

Similar to field samples from bog and fen, the fen incubations had significantly higher copy numbers of the 16S rRNA gene than the bog (Fig. 5D, Table S3) at both depths in the control samples (p = 0.002 for both mid and deep, SD = 1.55 (mid) and 4.29 (deep) for bog, SD = 32.77 (mid) and 25.67 (deep) for fen). The incubation process did not significantly alter the 16S rRNA copy number in the controls when compared to initial field samples, but treatment did. In both habitats, at both depths, the 16S rRNA gene copy numbers decreased dramatically under galacturonic acid and p-hydroxybenzoic acid (Fig. 5D). This difference was significant with respect to the initial field samples (p < 0.001, initial SD = 20.22 (mid) and 1.60 (deep) for bog, SD = 45.54 (mid) and 93.5 (deep) for fen) for all but the mid-depth fen, where it was nonetheless significantly lower than the mid-depth control (p = 0.05, galacturonic acid SD = 1.65 (mid) and 1.11 (deep)) for the galacturonic acid treatment. Somewhat surprisingly, the glucose amendment also significantly reduced the 16S rRNA copy number 80% relative to the field in the deep bog (p < 0.001, SD = 1.60 for deep initial and 1.93 for deep glucose). In two other deep bog treatments, the addition of the fermentation products acetate and butyrate resulted in copy numbers increasing significantly (p < 0.05, SD = 3.1 for deep acetate and 0.84 for deep butyrate) relative to the controls. The fen demonstrated a greater decrease in 16S rRNA at both depths (p < 0.05) relative to the bog under the organic acid amendments (except p-hydroxybenzoic acid deep).

As observed in the field, diversity (Shannon index) was higher in the fen compared to the bog for all treatments (Fig. 5E). Galacturonic acid decreased community diversity relative to the control in both depths for the bog and fen. Richness also followed a similar pattern to diversity, with fen higher than bog (Fig. 5F). Richness reduction was most noticeable in the galacturonic acid amendment in both sites (in the bog, 52% reduction in the mid and 40% in the deep; in the fen, 61% in the mid and 41% in the deep) and with p-hydroxybenzoic acid in the bog compared to the unamended control (15% in mid, 17% in deep). Glucose-amended community richness exhibited a modest decrease in the fen at both depths (26% in mid, and 21% in the deep).

3.4. Substrate additions: community and inferred functional composition

Substrate impacts on microbial community composition were assessed relative to the control at the end of the 2-week incubations, via 16S rRNA gene amplicon sequencing of the incubated peat. Community composition (at the phylum level; Fig. 6A) was the most impacted by galacturonic acid treatment in both sites, with a decrease in relative abundance of *Acidobacteria* and an increase in *Euryarchaeota*, *Actinobacteria,* and unassigned species. P-hydroxybenzoic acid also caused a

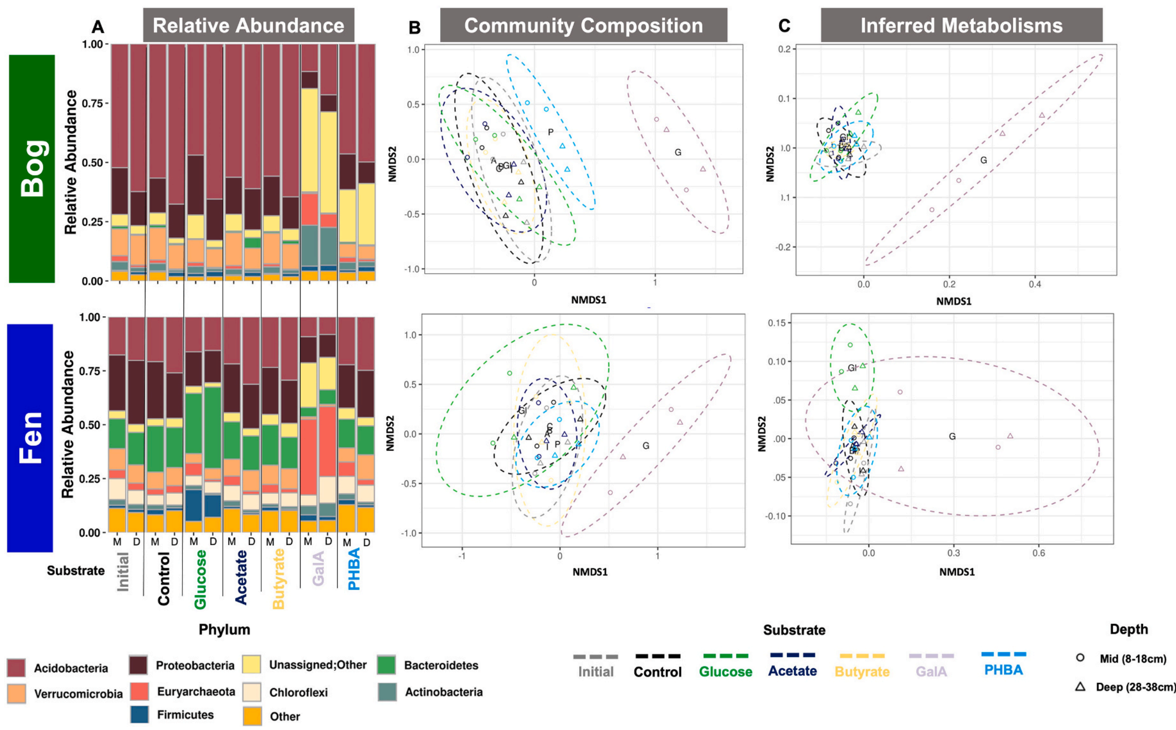


Fig. 6. Community dynamics demonstrated by (**A**) Ten most abundant phyla in bog (top) and fen (bottom), (**B**) Non-metric multidimensional scaling (NMDS) analysis of OTU-level community composition in bog (top) and fen (bottom), and (**C**) NMDS of PICRUSt2-inferred metabolisms (Kegg Orthologous (KO) genes) by treatment, site, and depth.

shift in the bog at both depths in increased relative abundance of unassigned lineages. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity showed that the galacturonic acid amended community composition was the most dissimilar to the control, initial, and all other amendments, in both sites (Fig. 6B). In the bog, the p-hydroxybenzoic acid-amended communities also diverged, though to a lesser degree than galacturonic acid. In the fen, glucose treatment also diverged from the control. Based on inferred community metabolisms (PICRUSt-inferred KEGG Orthologous genes; see Methods), the galacturonic acid treatment again exhibited the greatest dissimilarity

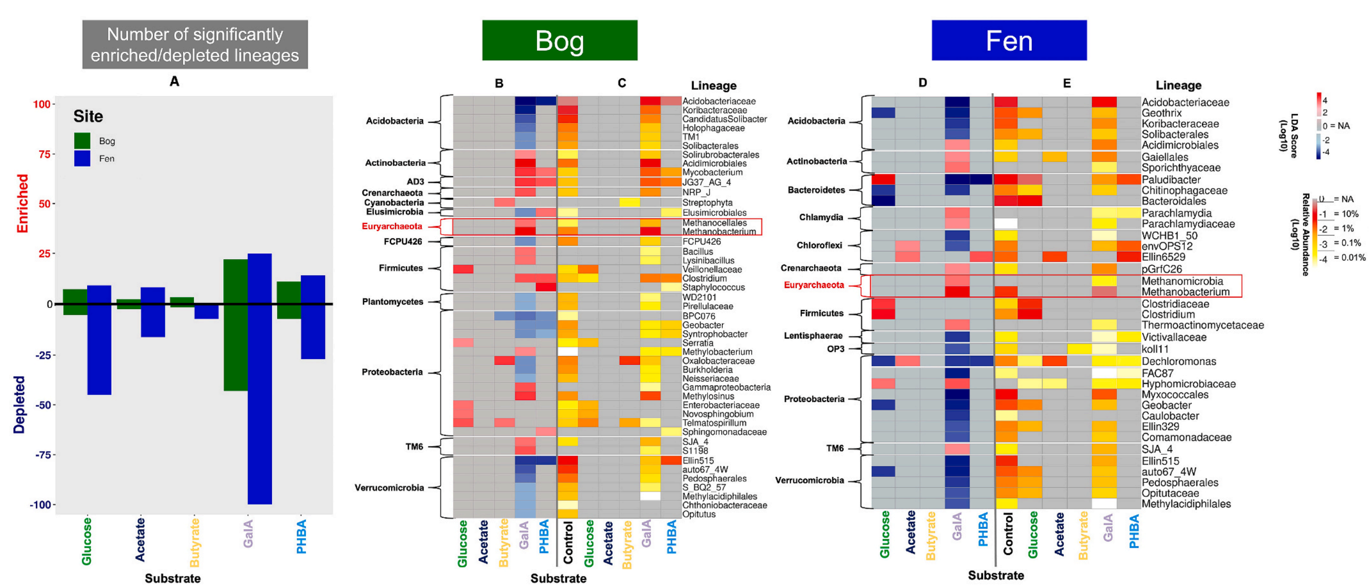


Fig. 7. Changes in significantly enriched or depleted lineages by each substrate amendment, for each habitat. (**A**) The number of lineages significantly (Kruskal-Wallace test, LDA > |2.0|, p < 0.05) enriched or depleted in each substrate amendment, relative to the control incubation; depths were combined for improved statistical power. (**B, D**) Significance of lineage change (LDA Scores), and (**C, E**) lineage relative abundance (Log10 scale). Bog lineages are shown in B and C, fen in D and E. Notably, only lineages with greater than 3.00 (bog) and 3.50 (fen) LDA scores, and p < 0.05, are shown for visual clarity; all lineages with an LDA greater than or equal to the established threshold of |2| and p < 0.05 (see Methods) are shown in Fig. S4. The LDA scale is discontinuous, with all LDA < |2.0| shown as grey (i.e., no significant enrichment or depletion), and the continuous red and blue color scales indicating enrichment (red) or depletion (blue) beyond the indicated threshold values. The relative abundance is shown on a continuous log scale with the associated untransformed relative abundances indicated next to the key. The lineages denoted by the red brackets and red boxing are methanogens.

compared to other treatments in both bog and fen (Fig. 6C). Overall, a stronger differentiation was observed taxonomically than metabolically, except for the galacturonic acid community.

3.5. Enriched or depleted lineages

Disproportionate impacts of substrate additions on particular genera were assessed for each habitat (Fig. 7, Fig. S4) using LEfSe (Segata et al., 2011), and combining depths for increased power to resolve habitat differences. Due to the number of significantly altered lineages, we highlight a few lineages here and refer readers to Fig. S4 for the full list (with many specific lineages discussed in Section 4). Here we report the number of lineages impacted under each amendment (summarized in Fig. 7A).

The glucose amendments to the bog significantly ($p < 0.05$) enriched 7 lineages (from 4 phyla) (Fig. 7A and 7B), and depleted 5 (from 3 phyla). Lineage depletions in the fen were much greater (45 total, Fig. 7A), with 14 phyla represented (Figs. 7D and S4), and glucose enriched 9 lineages from 3 phyla. *Telmatospirillum* was enriched in both habitats under glucose amendments. The strongest enrichment by glucose was of the *Paludibacter* lineage (within Bacteroidetes) in the fen (LDA = 5.41, $p = 0.02$), increasing 4-fold in relative abundance (Fig. 7E).

The SCFA-amended incubations exhibited few significantly altered lineages by relative abundances (Fig. 7A, B, and D). Acetate amendments only significantly altered the relative abundances of a small number of lineages in either habitat. Two lineages (from 2 phyla) in the bog and 8 (from 4 phyla) from the fen were significantly enriched, while 2 (from 1 phylum) were depleted in the bog and (16 from 7 phyla) were in the fen. The butyrate amendments also yielded just 2 enriched lineages in the bog (from 1 phylum) and none in the fen. Only one lineage was depleted in the bog, while 7 were in the fen (from 4 phyla).

Galacturonic acid-amended peat soils exhibited the greatest number of enrichments in both habitats (22 in the bog, 25 in the fen) and the greatest number of depleted lineages (43 in the bog, 100 in the fen), in both cases and habitats from diverse phyla (Fig. 7A, B, and D). Both sites demonstrated an enrichment of methanogens (*Methanobacterium* and *Methanocellales* in both bog and fen, and a *Methanomicrobia* in the fen).

P-hydroxybenzoic acid shifted the relative abundance of an intermediate number of lineages: 11 enriched (from 6 phyla) and 7 depleted (from 4 phyla) in the bog, and 14 enriched (from 7 phyla) and 27 depleted (spanning 11 phyla) in the fen (Fig. 7A, B, and D).

3.6. Inferred metabolism via PICRUSt2

Community metabolism was inferred for the lineages present (PICRUSt2, see Methods) and analyzed using LEfSe to identify differentially abundant pathways. Like the observed lineage-related patterns described above, the fen exhibited more enriched pathways than the bog (125 versus 95) (Fig. 8 and Table S6; across all treatments relative to controls). Except for the galacturonic acid treatment, numbers of enriched pathways within each treatment were greater in the fen as well.

Within the glucose treatment for both sites, no pathway enrichments were distinct in rank (all LDAs ~ 2.0, p-value < 0.05). The 12 bog-enriched pathways were involved in fermentation (hexitol, acetylene), amino acid synthesis, amine and polyamine biosynthesis, carboxylate degradation, N-acetylneuraminate catabolism, metabolic regulator biosynthesis and glutaryl-CoA degradation. Depletions in the bog were minimal: GDP-mannose biosynthesis, mannosylglycerate biosynthesis I and dTDP-β-L-rhamnose biosynthesis. Fen-enriched pathways were more numerous (81 compared to 12 in the bog). Top enriched pathways were within the categories of cobamide biosynthesis, fermentation to pyruvate, starch and glycan degradation, amino acid biosynthesis, peptidoglycan biosynthesis and glycolysis II (from fructose 6-phosphate). Depletions in the fen were greater than the bog as well (62 versus 3). Top pathway depletions included aerobic respiration I, fatty acid and lipid biosynthesis, enzyme cofactor biosynthesis, sulfur compound metabolism and assimilatory sulfate reduction.

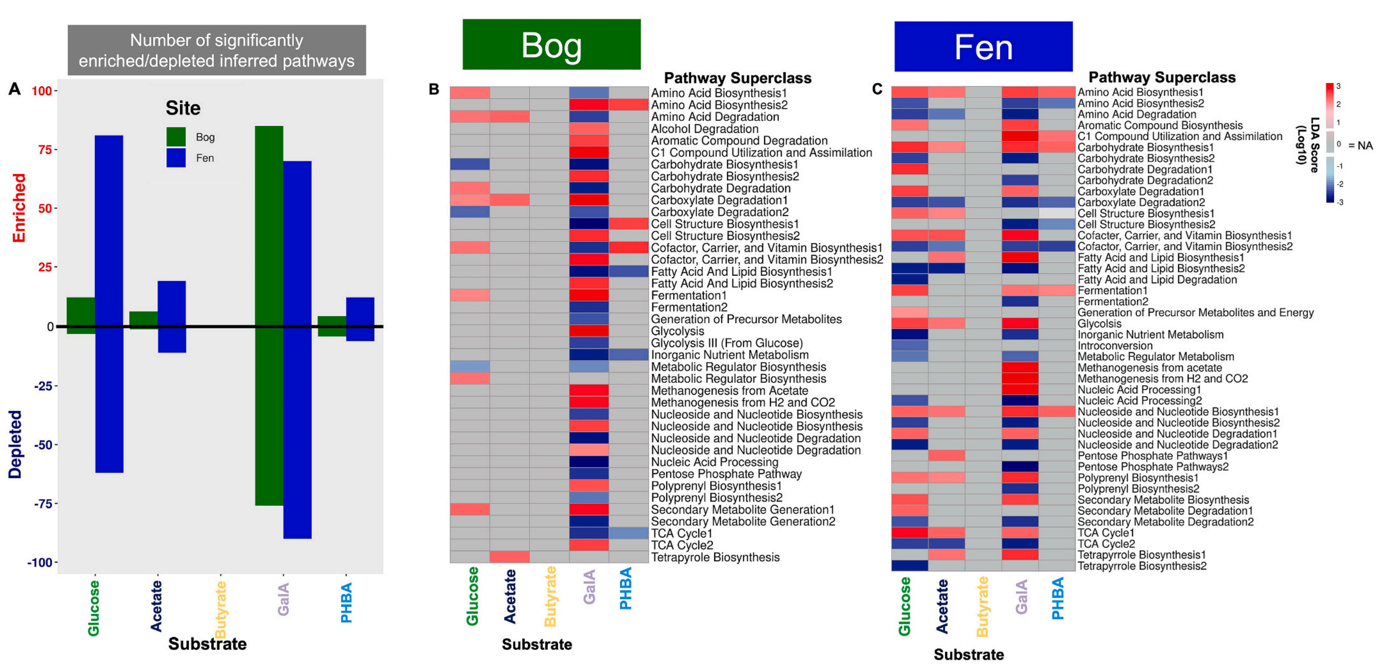


Fig. 8. Mapping of significantly enriched or depleted inferred pathway super classes by each substrate amendment, for each habitat, with depth. **(A)** The number of inferred pathways significantly (Kruskal-Wallace test, LDA > |2.0|, $p < 0.05$) enriched or depleted in each substrate amendment, relative to the control incubation; depths were combined for improved statistical power. **(B)** bog inferred pathways and **(C)** fen inferred pathways. The LDA scale is discontinuous, with all LDA < |2.0| shown as grey, and the continuous red and blue color scales indicating enrichment (red) or depletion (blue) beyond the indicated threshold values. Numbers following super pathway names denote enriched (1) or depleted (2) groups of pathways. Depths were combined for improved statistical power. The enrichments and depletions for all PiCRUST-inferred MetaCyc pathways represented by these super classes are listed in Table S3.

Acetate-amended bog soils were enriched in some inferred pathways related to acetate utilization for growth. In the fen, however, more pathways fell under nucleoside/nucleotide biosynthesis, peptidoglycan biosynthesis and utilization of precursor metabolites (pentose phosphate pathway (non-oxidative branch) I). Depletions in the fen were seen in fatty acid and lipid biosynthesis. A depletion in the TCA cycle VII (acetate-producers) was also observed in fen acetate amended soils. Butyrate exhibited no significantly enriched or depleted inferred pathways.

Similar to the patterns of lineage enrichment/depletions, galacturonic acid yielded the greatest number of differentially impacted inferred pathways. A range of pathways were identified in both the bog and fen galacturonic acid treatment. The most enriched inferred pathways (the subset with LDA >3.0) shared in both the bog and fen were involved in the generation of precursor metabolites, C1 compound utilization and assimilation and most notably, electron carrier biosynthesis. The bog was also enriched in glycolysis (from fructose 6-phosphate and from glucose 6-phosphate) and autotrophic CO_2 fixation via the reductive TCA cycle. Depletions also varied within each site. The bog was most depleted in fatty acid biosynthesis, sugar biosynthesis and degradation, while the fen was most depleted in biosynthesis pathways (fatty acid, unsaturated fatty acid, liposaccharide, and sugar nucleotide), nucleic acid processing (Queuosine Biosynthesis and Salvage) and aerobic respiration.

Under galacturonic acid amendment, methanogenesis from both acetate (acetoclastic) and from H_2 and CO_2 (hydrogenotrophic) were enriched in the bog and fen as well. Acetoclastic methanogenesis was most enriched in the bog (LDA = 2.72; $p < 0.05$) followed closely by hydrogenotrophic methanogenesis (LDA = 2.70; $p < 0.05$). The fen's enrichment of these pathways was flipped, with the hydrogenotrophic pathway more enriched than the acetoclastic pathway (LDA = 3.19 versus LDA = 2.98; $p < 0.05$).

Overall, there were fewer enriched/depleted lineages in the p-hydroxybenzoic acid-amended peat soils. The bog-enriched pathways (4 total; Table S6) were involved in electron carrier biosynthesis and nucleoside/nucleotide degradation. Depletions in p-hydroxybenzoic acid-amended bog soils were the general biosynthesis (fatty acids, lipids, lipopolysaccharides) pathways, generation of precursor metabolites (via TCA cycle) and assimilatory sulfur reduction. Fen enrichments (12 pathways) included fermentation to pyruvate, autotrophic CO_2 fixation, and biosynthesis of sugar nucleotides and several amino acids. Depleted pathways in the fen include biosynthesis (cell wall, single C carrier, amino acid), metabolic regulator biosynthesis (ppGpp metabolism) and sugar acid degradation of D-Galacturonate.

4. Discussion

This study examined microbiome C substrate use in peats from a thawing permafrost peatland's partially thawed bog and fully thawed fen. We quantified (i) diverse substrate utilization potential (by Biolog Ecoplate™) and (ii) the impact of selected substrate amendments on greenhouse gas emissions and microbial community composition (by anaerobic microcosms, with 16S rRNA amplicon sequencing). The thaw progression from bog to fen increased use of substrates overall and in each compound class, as well as diversity of substrates used, as profiled by Biolog Ecoplate). Amendments with 5 specific substrates had varying impacts on CO_2 and CH_4 production and microbiome composition. The observed patterns were dictated by the substrate's point of entry in microbial C processing (e.g., as substrate for fermentation or methanogenesis, etc), the microbiome's prior environmental exposure to the substrate (i.e., its legacy), and additional consequences (i.e., inhibition or electron acceptance) of particular substrates.

4.1. Habitat and amendment type influence microbial 16S rRNA copy number, diversity, and richness

The higher extent and diversity of substrate utilization in the fen than the bog (Fig. 3) is likely due to: (i) its higher cell densities (Woodcroft and Singleton et al., 2018; Wilson et al., 2021), (ii) more diverse initial microbiomes (Mondav et al., 2017) and thus metabolic potential (Woodcroft and Singleton et al., 2018), and/or (iii) the fen's greater geochemical diversity (e.g., of dissolved organic matter compounds; Hodgkins et al., 2014; Wilson et al., 2022). These features are interconnected; lineage richness and diversity can correspond to greater metabolic diversity in the microbiome (Raczka et al., 2021), and reciprocally, higher substrate diversity can fuel greater metabolic diversity (Zak et al., 1994). These bog vs fen differences in the field are maintained in microcosms; here, fen incubations maintained higher cell densities, diversity, and richness than the bog over time for most amendments (Fig. 5D–F). Furthermore, a recently published study of anaerobic incubations from the same field material confirmed that fen peat retained higher geochemical diversity than bog over a much longer incubation period than performed here (100 days, Wilson et al., 2021).

Amendments with organic acids (galacturonic acid and p-hydroxybenzoic acid) decreased the 16S rRNA copy number, Shannon's diversity, and richness (Fig. 5 D-F; all three metrics with galacturonic acid, 16S copy for p-hydroxybenzoic acid, and richness and diversity modestly in bog only with p-hydroxybenzoic acid). System pH has been known to be strongly correlated to changes in microbiome cell abundance (Wang et al., 2020) and structure; galacturonic acid contributes to system acidity (Clymo, 1964; Mitsch and Gosselink, 2000) due to greater proton release in systems with pH above its dissociation constant (pKa = 3.51) (Huisjes et al., 2012). This observation is also consistent with both bog and fen microbiota's lower usage - and leveling off of use over time - of carboxylic acids in general (Fig. 4) and galacturonic acid in particular (Fig. S1), in comparison to most other substrate classes, in the Biolog assays. P-hydroxybenzoic acid can impact microbial survival due both to its role as a phenolic acid (pKa = 4.54) and its breakdown production of phenol, which can be inhibitory (Rauha et al., 2000; Mellegård et al., 2009) though may not be in some wetland microbiomes (McGivern et al., 2021). Phenols are difficult to degrade in some microbial systems (Almendras et al., 2018), while they can also be a C source for specialized microorganisms (Fuchs et al., 2011). In addition, the lower phenolic usage observed in the Biolog Ecoplates™ and drop in 16S rRNA copy number in the incubations further corroborated the idea of phenolic acid microbial toxicity.

4.2. Habitat effects and microbiome legacy shape community responses to specific substrates

The thaw-associated shift from dominance by *Sphagnum* to sedges, with a concomitant change in plant C input type, quality and complexity (Hodgkins et al., 2014; Wilson et al., 2022), shapes differences in microbiome metabolic degradation potential (Woodcroft and Singleton et al., 2018). The fen microbiota successionally derives from the bog, and bog-enriched lineages and metabolisms persist, though at lower numbers, in the fen (Mondav et al., 2017). *Sphagnum* spp. persist in the fen in smaller numbers (Hough et al., 2020) such that microbiota continue to be exposed to lower levels of *Sphagnum*-derived substrates. However, the two habitats' microbiomes responded quite differently to substrate amendments. The substrates' specific differential impact on the number of altered lineages, and on inferred metabolisms of those enriched and depleted lineages, can be used to illuminate the environmental and C processing mechanisms at play under thaw-associated ecosystem change.

The glucose-amended incubations represent the baseline case of adding easily digestible C to the resident microbiomes. Biolog assay-based carbohydrate usage (glucose 1-phosphate included) was linear with time in both bog and fen (and significantly lower in bog; Fig. 4), unlike the leveling-off observed in most other compound classes, reflecting carbohydrates' rapid breakdown and use by microbes generally (Gunina and Kuzyakov, 2015). However, in the incubations, glucose usage did not lead to a rise in inferred cell numbers in bog or fen

(Fig. 5C). While this could imply steady usage by the community overall without cell division, glucose amendments did significantly enrich a small number of lineages, in bog and fen (Fig. 7A), which unsurprisingly were dominated by known fermenters. In the bog, glucose strongly enriched known fermenters *Veillonellaceae*, *Telmatospirillum* and *Serratia* (metabolically defined in Esquivel-Elizondo et al., 2017, Hausmann et al., 2018, and Degelmann et al., 2009, respectively) (Fig. 7), while in the fen, numerous fermenters were enriched including *Telmatospirillum, Clostridiaceae*, *Clostridium, Pelosinus and Paludibacter* (metabolically defined in Uz and Ogram, 2006, Moe et al., 2012, and Ueki, 2006*)*. For *Veillonellaceae* and *Clostridiaceae*, this is consistent with their reported enrichment in glucose-amended acidic German mire soils (Hunger et al., 2015). The largest enrichment (4-fold increase, LDA score = 4.9, $p = 0.02$; Fig. 7) was in *Paludibacter*; it was not significantly enriched in the bog or any other fen amendment, making the "bottle effect" (sensu Pernthaler and Amann, 2005) unlikely. Notably, in dilute mono- and poly-saccharide-amended bioreactors created with peat from these same fen sites, *Paludibacteriacea*e were also enriched (G. Tyson and B. Woodcroft, personal communication, June 2021). Not only were more lineages enriched in the fen by glucose, but far more phylogenetically inferred metabolisms (amino acid, fatty acid, carbohydrate, and nucleotide/nucleoside biosynthesis) were enriched in the fen than the bog (Fig. 8). Collectively, these results suggest a general stimulation by glucose of the fen's more dense, diverse, and active microbiome, supported by its higher pH and lower aromatic and recalcitrant C sources (fens in general, Lin et al., 2012 and Rui et al., 2009, and this fen in particular, Hodgkins et al., 2014). The bog's fewer glucose-enriched lineages, and lower utilization of labile carbohydrates in the Biolog assays, suggest that bog microbiota's growth has not been limited just by access to labile C sources (Rui et al., 2009; and see *Gas Production* section below), but also by environmental inhibition (potentially pH and plant-derived inhibitors, per above) and/or initial microbiome effects (lower cell numbers, diversity, and richness in the initial bog microbiome compared to the fen; Fig. 5). Indeed, evidence for sugars' slow uptake and resulting accumulation in the bog due to inhibitory conditions is just emerging (AminiTabrizi et al., 2020). Additionally, while there were lineages that were depleted under glucose amendment in the bog and fen (with many more in the latter), they and their inferred metabolisms were diverse; this implies their apparent depletion was likely due to slower relative growth since inferred cell numbers were stable.

Acetate and butyrate, as SCFAs produced by anaerobic decomposition, are key C molecules in mires. Indeed, C tracing of glucose fermentation in microcosms from a German bog and fen revealed 24 and 40% of added C became acetate and butyrate, respectively (Hunger et al., 2015). The lack of phylogenetic or functional change in acetate-amended incubations (Figs. 7 and 8) likely has different explanations in the two habitats and may be associated with the previously established idea of acetate accumulation in the bog (Ye et al., 2012). Acetoclastic methanogenesis and other acetate-utilizing processes (such as syntrophic acetate oxidation (Hädrich et al., 2012; Dyksma et al., 2020), and iron and sulfate reduction (Hädrich et al., 2012)) may be inhibited by the acidic and inhibitory bog chemistry (Hines et al., 2008). The resulting imbalance between fermentation-based production of acetate and its consumption may cause the acetate accumulation observed in the bog. Acetate accumulates more in shallow depths, where both labile precursors and inhibitors are highest, derived from the overlying *Sphagnum* (Duddleston et al., 2002). Once accumulated, acetate itself can also suppress microbial growth (Schink, 1997). Thus, in the already high-acetate bog, the resident microbiota is those that can tolerate high acetate and thus adding more would not shift their composition, as observed in this study. In the deeper bog peat, the 16S rRNA-inferred cell density increased significantly with acetate addition (Fig. 5D), while the composition didn't shift (Fig. 6A), suggesting a stimulation of the already-present acetate utilizers (which we hypothesize did not occur in the shallow peat due to the higher levels of plant-derived inhibitors). In fens, acetate has a high turnover rate due to its consistent production and rapid use by microbes (King et al., 1983; Wellsbury et al., 2002; Hädrich et al., 2012). Concomitantly, acetate addition did not cause a substantial change in the overall fen microbiome (Fig. 6), though did result in more significant changes in specific lineages and metabolisms than in the bog (Figs. 7A and 8). Notable enriched lineages include *Dechloromonas*, which uses acetate as an electron donor (Sun et al., 2009), and a lineage of *Syntrophaceae*, a family that uses fermentation products (including acetate, e.g., *Smithella propionica*, Gray et al., 2011) in syntrophic association with methanogens. More lineages in the fen were depleted than enriched (Fig. 7A), consistent with their potential suppression by high acetate in a normally low acetate-concentration environment. In addition, the fermenter *Telmatospirillum*, which was enriched by glucose, was depleted by acetate, potentially due to inhibition by excess product.

Butyrate, once produced by fermentation, may be slow to be further utilized, due to: i) the endergonic nature of its degradation (Schink, 1997); ii) the syntrophy required between butyrate-oxidizers and methanogens to overcome that energetic barrier (Liu et al., 2011); and iii) since acetate is frequently produced in tandem with butyrate, and then subsequently from butyrate, the inhibitory effects as it accumulates can further slow butyrate use (Schink, 1997; Zhang et al., 2004). As with acetate, 16S-inferred cell density only increased in the deep bog peat (Fig. 5D), potentially similarly due to the simultaneous presence in the bog of microbes capable of using fermentation products, and the lower level of plant-derived inhibitors at depth. Also, like acetate, butyrate-amended incubations did not change the overall community composition in either bog or fen (Fig. 6) and yielded only a small number of changed lineages and inferred pathways (Figs. 7, 8, S4). Interestingly, the facultative anaerobic fermenter *Telmatospirillum* was among those enriched by butyrate, which seems counterintuitive as we might expect inhibition of fermentation by addition of butyrate. However, in ^{13}C-butyrate incubations with acidic fen peat, *Telmatospirillum* was labeled, leading the authors to posit it might be involved in butyrate oxidation (Schmidt et al., 2016). In addition, a *Telmatospirillum* metagenome-assembled genome from a high-iron habitat encoded iron reduction (Gagen et al., 2019). While mires are typically considered low-iron environments, recent work at this site has revealed geochemical and microbial evidence for iron cycling (Patzner et al., 2020), which may be induced or accelerated under warmer conditions (AminiTabrizi et al., 2021) - potentially impacting butyrate processing.

Galacturonic acid (synonymous with galacturonate) is a major component of *Sphagnum* cell walls (Kremer et al., 2004), making it a common substrate in our *Sphagnum*-dominated bog and present in smaller concentrations in the fen. This sugar acid produced by far the greatest change in microbiomes, at the level of phylum, OTU, and inferred metabolisms (Fig. 6), and caused the largest number of lineage and pathway enrichments/depletions (Figs. 7 and 8), and the largest impact on diversity metrics (Fig. 5). Overall, we present three independent mechanisms by which galacturonic acid impacts these microbiomes: (i) as a C substrate, (ii) via acidification, (iii) as an extracellular electron acceptor or shuttle.

Enriched lineages and inferred metabolisms support the impact of galacturonic acid as a C source. Upon addition both bog and fen were significantly enriched in fermenters. In the bog, the enrichment of the fermenter *Clostridium* occurred in tandem with *Lachnospiraceae*, which can engage in metabolic cross-feeding with *Clostridium* by using the galactose it produces from galacturonic acid (Larsen et al., 2019). Galacturonic acid-amended bog peats were also enriched in *Bacillus*, which can use organic acids such as galacturonic acid as a C source (Schilling et al., 2007). In addition, the fen was enriched with *Actinomycetales*, a member of the Actinomycetes order known to produce pectinolytic enzymes (Brühlmann et al., 1994). Interestingly, both bog and fen were depleted in many lineages of *Acidobacteria*, a phylum commonly found in *Sphagnum*-dominated habitats and with diverse lineages capable of degrading galacturonic acid (Pankratov et al., 2008); this could reflect poorer competition in the incubation setting with other fast-growing lineages able to use galacturonic acid (Kielak et al., 2009). Enrichment of inferred glycolysis and fermentation pathways (Fig. 8) corroborates the role of galacturonic acid as a C source, potentially via galactose (dissociated galacturonic acid) entry into central C metabolism (Berg et al., 2002).

Since the change induced by galacturonic acid was so much greater than that of the other major sugar glucose, the most significant mechanism of its impact may be its contribution to system acidity (described in Clymo, 1964) rather than as a C source. Indeed, *Mycobacterium*, a common soil bacterium that thrives in acidic, cold, and wet soil conditions (Walsh et al., 2019), was significantly enriched in both bog and fen (Fig. 7) under galacturonic acid amendment. The large number of lineage depletions, across a range of taxonomic groups, may also be due to the impacts of galacturonic acid's acidification of the environment. This may be particularly impactful in the fen which has a baseline higher pH (3.6 in bog v 5.3 in fen), and thus more lineages intolerant of the higher acidity (or the sudden change), and indeed there were roughly twice as many depletions in the fen than the bog (Fig. 7, Fig. S4; although it was a similar portion of the habitat's lineages overall, since fen richness is roughly twice that of the bog; Fig. 5F). The importance of acidification from galacturonic acid is also corroborated strongly by the significant reduction in 16S rRNA-inferred cell numbers, and large reduction in richness and diversity, observed with this amendment (Fig. 5).

The third potential way galacturonic acid - a sugar acid that is abundant in pectin and is commonly present in the acid hydrolyzates of humic acids - could be influencing the microbiome is by serving as an external electron acceptor. Humic substances can accept electrons from cells and may serve as electron shuttles to other acceptors (Keller and Takagi, 2013; Wilson et al., 2017; Lovley et al., 1996). Like other humics, galacturonic acid is known to be redox active (Protzko et al., 2018). While the canonical electron-accepting moiety in humics is quinones, which galacturonic acid lacks, that role has expanded to include non-quinone redox functional groups (Hernández-Montoya et al., 2012; Ratasuk and Nanny, 2007). These groups can account for a large portion (25–44%) of microbial extracellular electron acceptance (Hernández-Montoya et al., 2012), and encompass a much broader range of humic-associated molecules, including galacturonic acid (for which the redox active functional group is the carboxylic acid). Extracellular electron transfer can occur directly, via nanowire pili (Rotaru et al., 2014) and/or direct-membrane-to-membrane electron transfer (McGlynn et al., 2015), or indirectly, via redox-active shuttle molecules. Interspecies electron transfer (IET; sometimes called "direct IET" i.e., DIET) is the syntrophy of electron donor microbes and recipient methanogens, potentially fueling methanogenesis (Rotaru et al., 2014). Adding conducting molecules, either organic or inorganic, to IET co-cultures or communities increases transfer and associated methanogenesis (Rotaru et al., 2014; Chen et al., 2015). Of the known IET-associated lineages in our incubations, electron donor lineages *Geobacter* (Rotaru et al., 2014), *Syntrophobacter* (Chen et al., 2020), and *Dechloromonas* (in the fen only; Guo et al., 2021) decreased significantly in the presence of galacturonic acid, while known recipient methanogens were enriched (*Methanobacterium*, Guo et al., 2021; *Methanocella,* Fu et al., 2018; and *Methanosarcina*, Rotaru et al., 2014, which was abundant but not enriched). The changes in inferred metabolic pathways could support a role for galacturonic acid as an external electron acceptor or shuttle: the bog was highly enriched in inferred electron carrier biosynthesis (all within the menaquinol- 8,11, and 13 super pathways; Table S6), which is unlikely due just to increased fermentation, since electron carrier biosynthesis increased far less in the glucose treatment (where fermenters are enriched). In addition, the fen was enriched in methanogen-associated redox pathways (Table S6), specifically archaeal flavin biosynthesis and the flavin-derivative factor 420, also known as co-enzyme F420. Overall, the lineage and metabolism results are somewhat contradictory; given the increasing evidence for widespread use of external electron acceptors in the microbial world (Lovley et al., 1996), we interpret these results as suggesting that galacturonic acid is being used by not-yet-identified IET lineages, which may or may not be syntrophic with methanogens (which increase, though net CH_4 concentration does not, see section 4.3). These observations show that galacturonic acid may be a facilitator of microbial redox interactions in Mire environments.

Lastly, galacturonic acid was the only substrate that significantly enriched methanogens in the bog, and it led the largest increase in methanogens in the fen (with only p-hydroxybenzoic acid causing any other enrichment in methanogens) (Fig. 7, Fig. S4); these lineage enrichments were also among the highest LDA scores in any treatments. We hypothesize these enrichments of methanogens may be caused by all three of galacturonic acid's possible roles: as C source, acidifier, and electron carrier. Increased fermentation would provide more substrates for methanogens, and both acetoclastic and hydrogenotrophic inferred pathways were enriched in both bog and fen (Fig. 8). However, only hydrogenotrophic *lineages* were enriched in either habitat. Hydrogenotrophic methanogens have tolerance for lower pH than acetoclastic methanogens (e.g., *Methanobacterium* activity at pH 4.7 and as low as 3.8, Bräuer et al., 2004 and Kotsyurbenko et al., 2007, respectively), and can tolerate sharp drops in pH while acetoclastic methanogens cannot (Wang et al., 2020). The hydrogenotrophic lineages *Methanocellales* and *Methanobacterium* were significantly enriched in both bog and fen by galacturonic acid, with a larger effect and stronger significance in the fen for each (Fig. S4 and Table S5). In addition, another unclassified lineage of the hydrogenotrophic *Methanomicrobia* was significantly enriched in the fen. Although inferred acetoclastic methanogenesis pathways were enriched in both bog and fen, resident facultatively acetoclastic lineages (i.e., *Methanosarcina* and *Methanosaeta*, at the field site per McCalley et al., 2014, and present in these samples; Table S4) were not enriched *or* depleted in the presence of galacturonic acid. Collectively, the stronger enrichment in the fen of hydrogenotrophic methanogenesis (more enriched lineages, higher significance of lineages and pathways) may reflect the larger impact of acidification on the fen, with a shift from its acetoclastic-favoring environment (McCalley et al., 2014) to an acidic hydrogenotrophic-favoring condition, causing a proliferation of hydrogenotrophic methanogens while the acetoclasts merely persisted. Lastly, as noted in the preceding paragraph, galacturonic acid may have stimulated methanogens as a mediator of syntrophic electron transfer; this is supported by the fact that p-hydroxybenzoic acid (next section) also enriched for methanogens, although not for methanogen-associated redox pathways.

Phenolic acids - including p-hydroxybenzoic acid - are another major component of *Sphagnum* (Mellegård et al., 2009; Tetemadze et al., 2018), and are known as strong microbial inhibitors (Cho et al., 1998; Rauha et al., 2000; Mellegård et al., 2009). They can have multiple mechanisms of inhibition: decreasing extracellular enzyme activity (Wetzel, 1992) by covalent interactions with active sites, in the "enzymatic latch" or "polyphenol lock" paradigm (Freeman et al., 2001; Fenner and Freeman, 2020); direct toxicity (Pletzer and Weingart, 2014; Zoetendal et al., 2008); damaging microbial membranes (Miklasińska-Majdanik et al., 2018); limiting nutrient availability (via decreased nitrogen mineralization; (Northup et al., 1995); and in the case of phenolic acids such as p-hydroxybenzoic acid, contributing to acidity (Stalheim et al., 2009). In these roles, they can be potentially important regulators of SOC processing, slowing remineralization (Min et al., 2015; Pinsonneault et al., 2016). Indeed, these compounds are one of the causes of long-term preservation of human bodies in northern peat bogs ("bog bodies", Stankiewicz et al., 1997). However, phenols including p-hydroxybenzoic acid can also be used as C sources by specialized microbes or communities (Fuchs et al., 2011; Wilhelm et al., 2020), a process increasingly recognized to occur under anaerobic conditions, not just aerobic ones (e.g., McGivern et al., 2021). *Sphingomonadaceae,* a family with a genus capable of degrading phenolic acids including p-hydroxybenzoic acid (*Sphingomonas oligophenolica* spp.*;* Ohta et al.,

2004) was enriched in the bog. Several other statistically enriched lineages (*Staphylococcus*, *Clostridium*, and *Syntrophorhabdaceae*) can degrade phenols generally (Senthilvelan et al., 2014; Guo et al., 2015; Qiu et al., 2008, respectively), but lack reported p-hydroxybenzoic acid degradation specifically. In this study, while overall phenolic use by the microbiome in the Biolog plates was lower than other compound classes (Fig. 4), consistent with inhibition and/or slow degradation, the compound p-hydroxybenzoic acid was actively and substantially used in both bog and fen (of 31 compounds tested, p-hydroxybenzoic acid had higher maximum inferred usage than 21), with a lag in the bog, and high leveling-off in the fen (Fig. S2). In the amended incubations, an intermediate number of lineages and inferred pathways were significantly altered relative to other substrates, but there were a smaller number of significant changes in the bog than the fen. We hypothesize that, similar to galacturonic acid, this is due to the bog microbiome's legacy of higher baseline prior exposure due to more *Sphagnum*-derived inputs (Tetemadze et al., 2018, and in this bog in particular, Hodgkins et al., 2014), and acidity leading to a greater persistence of phenolic acids (Bai et al., 2019)**.** The bog's lower community response and the lag in usage (seen in the Biolog data) could also be due to its relative acidity, since phenol-degrading enzymes function better at a more neutral pH (Pind et al., 1994; Sinsabaugh, 2010). The fen's greater extent of enrichments and depletions may thus be a result of its lower prior exposure, with both stimulatory and inhibitory effects as consumers were able to proliferate in a non-substrate-limited condition, including potentially dormant consumers becoming active, and lineages for which p-hydroxybenzoic acid is toxic were impacted by its higher concentration.

4.3. CO_2 and CH_4 gas dynamics from changing C inputs

Under purely anaerobic conditions in the absence of inorganic terminal electron acceptors (such as iron, sulfate, etc) with abundant cellulosic C, methanogenesis should dominate and produce stoichiometrically equal CO_2 and CH_4 (Tarvin and Buswell, 1934; Lovley et al., 1982; Hines et al., 2008). In many bog environments, including ours, this prediction does not hold, with appreciably more CO_2 produced than CH_4 (Hines et al., 2008; Yavitt and Seidman-Zager, 2006, for bogs generally; at Stordalen, Hodgkins et al., 2014; McCalley et al., 2014). There are many potential mechanisms proposed for the bog's deviation from 1:1, spanning inhibition of methanogenesis or promotion of non-methanogenic CO_2 production. *Sphagnum*-derived compounds may directly inhibit methanogens via humic toxicity (Hines et al., 2008; Duddelston et al., 2003), and the phenol subset of humics may accumulate in bogs due to lower activity of phenol oxidases (Freeman et al., 2001). Sustained elevated anaerobic non-methanogenic production of CO_2 may occur in bogs via (i) microbial electron transfer to various extracellular organic matter compounds (Wilson et al., 2017), (ii) HMW OM fermentation (Keller and Bridgham, 2007; Tfaily et al., 2013; Vile et al., 2003), (iii) respiration with alternative electron acceptors (e.g., sulfates, iron, manganese, nitrate, or humics) (Keller and Bridgham, 2007; Lovley et al., 1996), and (iv) abiotic C transformations (Fudyma et al., 2021). The fen's close-to-equimolar ratio reflects more favorable methanogen conditions (more labile C and less *Sphagnum*-derived inhibitors) (Hodgkins et al., 2014; Hornibrook et al., 1997, 2000).

Overlain on these bog vs fen differences in gas emissions are the impacts of substrate amendments. While the amendments caused statistically significant shifts in microbial community composition, described above, the changes in gas production were not statistically significant (Table S3; likely due to low replication requiring the combination of shallow and mid depth data, though that was also true for the microbiome data). Some of the gas emission changes were nonetheless appreciable and help inform our understanding of peat response to substrates. Glucose amendment resulted in an increase in CO_2 production at both sites and depths (Fig. 5A), consistent with the general stimulation of fermentation in the bog and of the overall community in the fen indicated by the community profiles (Figs. 7 and 9). Corroborating this interpretation, Duddleston et al. (2002) identified fermentation as responsible for the majority of CO_2 production in an Alaskan peat bog incubation experiment. In our incubations, only in the bog, but not the fen, was the CO_2 increase associated with a CH_4 increase (indeed, in the shallow fen CH_4 production decreased). The lack of CH_4 production change in the fen with glucose may reflect a lack of substrate

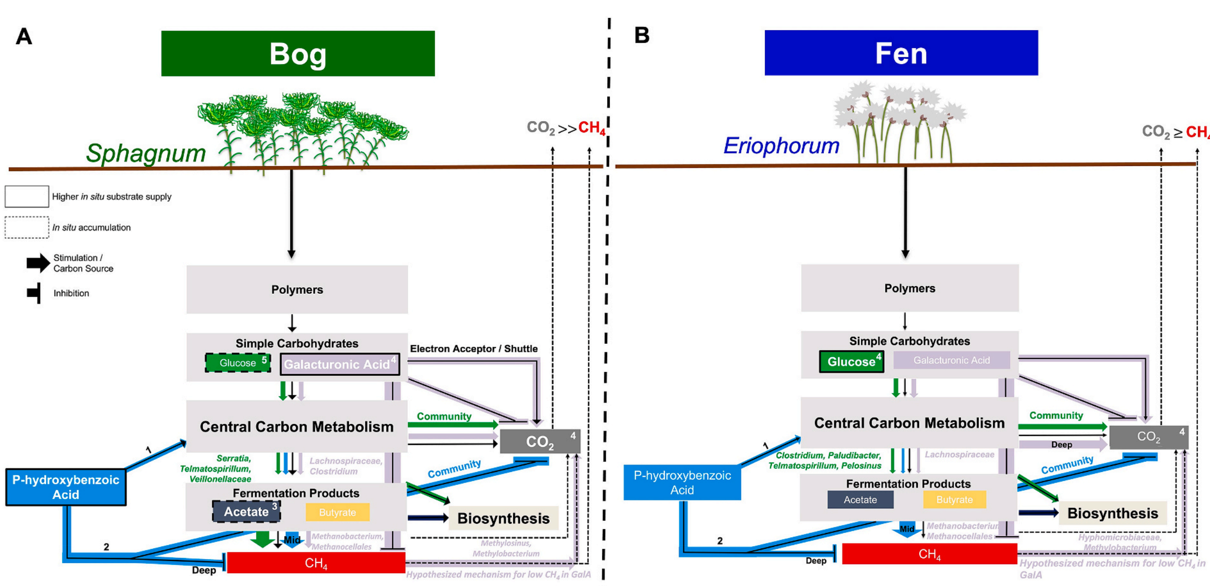


Fig. 9. A simplified conceptualization of microbial soil carbon processing (as in Fig. 2), **overlain with the carbon gas results and inferred carbon processing mechanisms from amending incubations with the five substrates** (glucose, acetate, butyrate, galacturonic acid and p-hydroxybenzoic acid) in the bog (A) and fen **(B)** habitats. Substrates, and the arrows indicating their inferred impacts on subsequent carbon processing, are color-coded: glucose (green), acetate (navy), butyrate (gold), galacturonic acid (purple), and p-hydroxybenzoic acid (blue); black is the original unamended structure. Arrow thickness reflects the magnitude of the effect. Arrow ends indicate either stimulation (pointed) or inhibition (flat). Solid outlines of text boxes indicate higher inferred use (for substrate), while dashed outlines denote accumulation (for substrate) under ambient conditions. [1]Fuchs et al. (2011), [2]Mellegård et al. (2009), [3]Duddelston et al. (2003), [4]Hodgkins et al. (2014), [5]AminiTabrizi et al., 2020.

limitation given the fen's abundance of simple carbohydrates (Hodgkins et al., 2014), and a microbiome well-adapted to utilizing them in diverse ways (Woodcroft et al., 2018) as indicated by the enrichment of diverse metabolisms (above). The lack of change in CH_4 could also be underlain by a shift from hydrogenotrophic to acetoclastic methanogenesis, due to the increase in fermentation byproducts produced by the numerous fermenters that were enriched in the fen after glucose amendment (acetoclastic methanogens were not enriched so a shift would be due to changing activity).

On the other hand, in the bog, the increase in CH_4 production after glucose addition may reflect prior substrate limitation, i.e., be driven by an increase in fermentation by-products implied by significantly increased fermenters, however the recent observation of sugar accumulation in the bog would refute this (AminiTabrizi et al., 2020). As described above, the microbiome and Biolog data indicate that the bog microbes are impacted by inhibitors. The addition of exogenous free glucose may be fueling the degradation of inhibitors, allowing greater methanogenesis. Specifically, phenols (which can be toxic to methanogens; Vasta et al., 2019) are more rapidly degraded under anaerobic conditions when glucose is added (Tay et al., 2001). The occurrence of such priming of detoxification in the bog glucose amendment is corroborated by the significant enrichment of *Novosphingobium*, a genus known to degrade phenolics and humics (Kumar et al., 2017; Wang et al., 2018), and of OTUs within *Burkholderiaceae*, some lineages of which degrade phenolic acids (Wilhelm et al., 2020). Complex *Sphagnum*-derived compounds, including phenolics, accumulate with depth in bogs (Tfaily et al., 2014), and the higher CH_4 production from the deep bog is consistent with the glucose-based priming of phenol degradation having a greater alleviation-of-toxicity effect in deeper bog peat. Furthermore, in addition to degradation of inhibitors, microorganisms can use strategies to withstand them, and glucose can fuel the energy production and enzyme biosynthesis required to do so (Bore et al., 2017).

Lastly, it is possible that the lack of increased CH_4 concentrations in the fen glucose incubations is due to some methanotrophy occurring, and none, or less, occurring in the bog. Lags in CH_4 production (also seen in control; see Fig. S6) are common in peat incubations (including in other glucose-amended anaerobic incubations, of an acidic fen with 19–34-day lags; Hädrich et al., 2012). These lags may be due to prerequisite consumption of all residual O_2, although since anaerobic microsite methanogenesis is now recognized (Angle et al., 2017) the lag could also be due to residual waning aerobic methanotrophy. It could also be caused by anaerobic oxidation of CH_4, but no known responsible lineages occur in our community profiles (Fig. S4 and Table S5), nor have they been reported at this bog or fen in other studies (Woodcroft and Singleton et al., 2018; Wilson et al., 2019). In addition, in our fen incubations, glucose significantly enriched the proposed aerobic methanotroph *Hyphomicrobiaceae* previously discovered at this site (Singleton et al., 2018) (Fig. 7). This lineage was proposed to be adapted to low O_2 conditions based on its presence in deeper peats and its closest cultured relatives being known microaerobes (Singleton et al., 2018). Glucose's differential impact on gas emissions by gas type and habitat highlights the need to consider external factors unique to each habitat which may modulate microbiome response to the addition of a simple sugar.

Acetate and butyrate amendments exhibited the least change in both CO_2 and CH_4 production (relative to unamended controls; Fig. 5). Although this is consistent with these substrates' minimal impact on the microbiota (Figs. 7 and 8), it is in contrast to the typical expectation for adding syntrophic substrates into methanogenic habitats (Schmidt et al., 2016). The small decrease in CH_4 in the fen, with no change in CO_2 production, reflects these SCFA selectively affecting processes downstream of fermentation such as methanogenesis. The fen site hosts more acetoclastic methanogens (McCalley et al., 2014; Woodcroft and Singleton et al., 2018), which are more susceptible to various inhibitors than hydrogenotrophic methanogens (Shin et al., 2019; Angelidaki and Ahring, 1993; Penning and Conrad, 2006). Despite SCFAs being substrates for methanogenesis, adding high concentrations could have reduced methanogenic activity by inducing pH changes (Wang et al., 2020; Shin et al., 2015; Bräuer et al., 2004) or via substrate inhibition of the acetate kinase or acetyl-CoA synthetase enzymes (though not assessed yet for these enzymes that we could find, substrate inhibition may impact ~25% of known enzymes; Kokkonen et al., 2021). Indeed, acetate additions have decreased CH_4 production in other peat bog incubations (Bräuer et al., 2004), a rumen-associated methanogenic consortium (Kessel and Russell, 1996), and wastewater treatment sludge (Alves et al., 2009). Butyrate addition to an anaerobic sludge digester similarly decreased CH_4 production (Xu et al., 2018). Mechanistically, butyrate also requires multiple conversions for methanogenic use (as described previously; Schink, 1997; Zhang et al., 2004) and as described above (*Microbiome legacy* section) the lack of enrichment of requisite syntrophic butyrate oxidizers (except potentially *Telmatospirillum*, in the bog) in our incubations may lead to the accumulation of undissociated forms of butyrate that can become inhibit methanogens (Pavlostathis and Giraldo-Gomez, 1991). Further supporting the idea that these SCFAs may have been at toxic concentrations in these incubations, they were added at ~7-10X field concentrations (based on preliminary experiments showing that was required for any shift in gas production). Although both acetate and butyrate are common methanogenic precursors, these results emphasize how their increase under natural conditions does not necessarily lead to increased CH_4 and can in fact suppress its production.

The organic acid-amended (galacturonic acid and p-hydroxybenzoic acid) peats exhibited the most dramatic changes in gas production, although in quite distinct ways (Fig. 5). Both organic acids can serve as microbial C sources or inhibitors, while galacturonic acid may also act as an electron shuttle and p-hydroxybenzoic acid may act as an enzymatic latch (Fig. 9). First, the gas production trends with galacturonic acid differed by gas (CO_2 vs. CH_4), habitat, and depth. The bog's minimal change in CO_2 relative to the fen is consistent with a greater microbial capacity for galacturonic acid use as a C-substrate where fresh *Sphagnum* inputs are higher (Hodgkins et al., 2014; Hough et al., 2020). In the fen, mid depth CO_2 decreased, and given the large depletion of lineages described above, this may reflect galacturonic acid's inhibitory role as a weak acid as it dissociates (Huisjes et al., 2012). In contrast, the deep fen CO_2 production increased >3-fold, potentially reflecting two mechanisms. Humic substances' electron accepting potential typically decreases with depth (Kappler et al., 2004), and their use as electron acceptors fuels microbial respiration (Keller et al., 2009; Wilson et al., 2017). Therefore, the impact on CO_2 production of adding fresh unreduced humic substances - in this case galacturonic acid - should be larger at depth, especially in the fen where there's a much lower input of *Sphagnum*-derived humics. In addition, it is possible that the large cell die-off evidenced by the 16S rRNA copy numbers (which decreased appreciably in all galacturonic acid treatments; Fig. 5) may have further fueled fermentation or respiration due to a large release of labile C.

Unlike CO_2, CH_4 followed a similar pattern of response in both habitats to galacturonic acid addition (Fig. 5). With the observed enrichment of methanogens, one would expect an increase in CH_4 production in these amended microcosms, however, we observed the opposite, especially at depth. Methanotrophs were also significantly enriched by galacturonic acid (*Methylosinus* and *Methylobacterium* in the bog, *Methylobacterium* and *Hyphomicrobiaceae* in the fen; Fig. 7); galacturonic acid was the only substrate eliciting such generalized methanotroph enrichment. This was not driven by just shallow or deep samples (lineage abundance data Table S4), nor was there any evidence of these anaerobic incubations having leaks. Ivanova et al. (2016) reported methanotroph enrichment and methanotrophy upregulation by the addition of pectin (which is primarily composed of galacturonic acid) to hypoxic bog incubations; the authors hypothesized this was due to the release of methanol - a stimulant of methanotrophy - during the breakdown of pectin. Here, this mechanism would require sufficient residual O_2 to fuel appreciable methanotrophy, which seems unlikely. Alternatively, methanogenesis may have been circumvented by other favorable terminal electron acceptors including the galacturonic acid itself (and possibly oxidized forms of sulfur and iron present in these systems (Hausmann et al., 2018; Patzner et al., 2020) and potentially more available in these incubations due to galacturonic acid's high reactivity), with C flowing mainly towards CO_2 production (Keller et al., 2009; Keller and Takagi, 2013). However, since CO_2 only increases appreciably in the deep fen, while CH_4 production decreases in both habitats and depths and with similar magnitude in deep bog and fen, electron acceptance by humics cannot be a major responsible mechanism. While the mechanism(s) underlying this dramatic drop in CH_4 thus remain unclear, it is striking that the deep bog and fen galacturonic acid treatments produced by far the highest CO_2:CH_4 ratios of any amendments (Fig. 5), with elevated CO_2:CH_4 in the mid-depth fen as well. Since galacturonic acid is derived from fresh *Sphagnum*, it makes sense that its impact would be strongest at depth, and in the fen. Given the central role of galacturonic acid in bogs, these patterns are consistent with bog habitats' characteristically high CO_2:CH_4 production ratio generally and speaks to the importance of this compound in driving that imbalance, since it made the fen's mid-depth ratio more similar to that of a bog. This provides yet more evidence that *Sphagnum*-supplied humics are shaping the microbiome, with additions producing the least impact in the bog and most in the fen, especially at depth.

The second organic acid, p-hydroxybenzoic acid, caused an opposite effect for CO_2 and CH_4 compared to galacturonic acid: the CO_2 was much lower in both bog and fen, at both depths, than for any other amendment (as CH_4 was for galacturonic acid), and the CH_4 had different responses by habitat and depth (as the CO_2 had for galacturonic acid). The marked decrease in CO_2 implies that p-hydroxybenzoic acid is mainly acting as a microbial inhibitor in this system (Fig. 9), concordant with the dramatic decrease in cell numbers in all treatments except fen-mid (Fig. 5D), and with previous observations of an inverse relationship between phenol concentration and CO_2 production (Poirier and Chapleur, 2018). P-hydroxybenzoic acid's role as a C source appears less important (given the reduced CO_2), but still relevant; Biolog data showed active use of the compound in both bog and fen deep. Only one lineage of a known potential p-hydroxybenzoic acid degrader - a *Sphingomonadaceae* - was enriched by p-hydroxybenzoic acid, and only in the bog. A lineage of *Syntrophorhabdaceae* was enriched in the fen, and this family includes a reported anaerobic phenol degrader obligately syntrophic with a hydrogenotrophic methanogen (Qiu et al., 2008). In addition, given the large overall community inhibition, resistant organisms may have been able to capitalize on now-available resources. Indeed, high phenol concentrations were previously found to inhibit phenol-degrading organisms but not methanogens, and ultimately yielded the highest CH_4 production (Wang et al., 1989). Consistent with that, the large increase in CH_4 production in the mid-depth of bog and fen occurred with reduced overall cell numbers, reduced CO_2 production, and - in the fen - modest enrichment of the methanogens *Methanosaeta* and *Methanocellales* and the syntroph *Syntrophorhabdaceae*. The lack of enrichment of methanogens in the bog, and of other methanogens in the fen, suggests that the surviving cells were able to be highly active but not rapidly dividing. Inversely, deep peat CH_4 production was virtually eliminated along with the deep peat CO_2, in both habitats (Fig. 5). The differential production by depth may be associated with the availability of, and competition for, precursor methanogenic substrates (Sela-Adler et al., 2017), which are higher near the surface due to supply from fermentation of plant inputs (Popp et al., 1999; Chasar et al., 2000; Duddleston et al., 2002), and potentially due to greater acetogens in shallower peats (e.g., Hädrich et al., 2012). Indeed, p-hydroxybenzoic acid depleted two lineages reported to compete with acetoclastic methanogens for acetate, the sulfate-reducing *Syntrophobacter* in the bog and *Desulfomonile* in the fen (Hädrich et al., 2012). In deeper peat where methanogenic precursors are less available (Hornibrook et al., 1997), the broad microbial inhibition by p-hydroxybenzoic acid may therefore limit substrate availability for methanogenesis, resulting in parallel large reductions in CO_2 and CH_4 on the timescale of these incubations.

While this study examined bacterial and archaeal lineages and potential roles in C processing, it is important to acknowledge that fungi contribute to peatland decomposition as well. Selective inhibition studies of peats from above the water table indicate that fungal contributions to community respiratory activity in sub-oxic incubations can approach that of bacterial (~20–70% as much as bacterial respiration across 9 diverse peatlands in Winsborough and Basiliko 2010 and Myers et al., 2012, and ~70% across 10 minerotrophic sites, mesotrophic and transitional sites in Amha et al., 2015) or even exceed it in some ombrotrophic bogs (566% averaged across 9 bogs in Amha et al., 2015). In this study, however, peats below the water table were used. In anaerobic peats, fungi are much less abundant by biomass than bacteria (fungal PLFAs were virtually absent in the catotelm of 5 European peatlands in Groß-Schmölders et al. (2020) and were ~0–20% the bacterial PLFA biomass in the catotelm across 8 peatlands in Juottonen et al., 2021), and are energetically constrained (being limited to fermentation, rather than diverse forms of anaerobic respiration). At this site, some typically fungal pathways of C processing may even be occurring via microbes; a type of xylose-degrading oxidoreductase formerly only known from fungi was found encoded and expressed (in both transcripts and proteins) in diverse Acidobacteria, Actinobacteria and Chloroflexi, together accounting for 13% of the bacterial community (Woodcroft and Singleton et al., 2018). Collectively, we believe that while fungal roles in anaerobic peat C cycling remain a knowledge gap, our source peats and experimental conditions make strong fungal contributions to our results unlikely. If fungi were strongly active in these incubations, it could impact our linkage of gas data to microbial profiles (since it would decrease their mechanistic relationship) but would not impact interpretation of substrate impacts on gas emissions or microbes separately.

5. Conclusion: ecosystems-to-genes interactions create differing C processing outcomes at different timescales

As climate changes, physicochemistry and biology of ecosystems shift in response, leading to a cascade of 'ecosystems-to-genes-to-ecosystems' feedbacks. In permafrost systems, this cascade starts with warming leading to thaw (Schuur et al., 2015), which induces changing geohydrology and plant community composition (Malmer et al., 2005; Hough et al., 2020), which determines substrate availability and plant-derived inhibitor concentrations (Hodgkins et al., 2014), which shape fundamental changes in the microbiome (Woodcroft and Singleton et al., 2018), and ultimately C gas emissions (Mondav et al., 2014). Often, these transitions are characterized separately, and in natural systems drivers co-vary, both of which reduce our ability to make mechanistic connections between drivers and outcomes.

In this paper, we tested previously hypothesized mechanisms connecting drivers and outcomes from substrates through microbiomes and emissions, in a bog and fen, at field-relevant substrate concentrations and controlled standardized conditions. Specifically, we profiled microbial utilization potential of a broad range of substrates, selected five field-relevant substrates spanning complexity, inhibitory potential, and point of entry into microbial C processing, and evaluated their impact on community composition, inferred metabolic potential, and greenhouse gas emissions, producing an integrated portrait of their associated C processing differences in the bog versus fen (Fig. 9).

On the short timescale of these incubations, the communities' habitat origins - i.e., legacy effects - dictated their substrate use abilities, and C gas emissions. The bog's lower 16S rRNA gene abundance, diversity, and richness gave rise to slower substrate use, with consistently fewer changes (enrichments and/or depletions) of specific lineages in the bog amendments than the fen. The fen's more diverse community and higher cell numbers were reflected in an ability to rapidly use a range of newly added substrates unless they were inhibitory. The differences in the bog and fen's substrate use diversity and speed confirmed prior genomic hypotheses of their C processing differences (Woodcroft and Singleton et al., 2018). The bog and fen microbiomes' susceptibility to inhibitory compounds reflected the habitats' different baseline levels, with the bog microbiome more resilient to them and potentially able to use them as substrates and electron acceptors. The bog microbiome converted the added C into gasses at similar ratios to the control, while the fen converted it disproportionately to CO_2 rather than CH_4. The exception was organic acids which caused large increases in CO_2:CH_4 in the deep bog, and even larger ones in the fen. Collectively, these results reveal a bog community with harsh *in situ* conditions, which was resistant to changing its composition, C processing, or gas emissions in response to field-relevant substrate perturbations. Methanogenic precursors are present in the bog, but their use was inhibited even with substrate additions. In the milder fen, methanogenic substrates were readily used (and thus more limiting), except under general community inhibition (such as by added p-hydroxybenzoic acid).

On the multi-decadal timescale of habitat transition at this site (Varner et al., 2022), the microbiomes holistically shift based on new conditions, with shifting tolerance for inhibitors and collective substrate use profiles. Thus, short term dynamics (here, responses to C inputs) - like those seen in transition states of ecosystems - differ from long-term dynamics, due to the lag in the historical community adjusting to new conditions; this can dictate the net climate forcing footprint (amount and ratio of CO_2 to CH_4) of the peat. The total area of the landscape that is undergoing transition can be appreciable, and not behave the way the well-studied established bogs or fens do; incubations such as these can shed light on potential transition zone dynamics. Established fens and bogs emit 60- and 13-fold more CH_4 than unthawed palsas, respectively (Varner et al., 2022). Our results suggest that in transition areas over the short term, added substrates (from plants, thawing permafrost, or lateral transport) can shift microbiomes but rarely appreciably increase C gas emissions, and shift the emissions ratios towards CO_2 rather than CH_4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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