

Soil metabolome response to whole-ecosystem warming at the Spruce and Peatland Responses under Changing Environments experiment

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In this study, a suite of complementary environmental geochemical analyses, including NMR and gas chromatography-mass spectrometry (GC-MS) analyses of central metabolites, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) of secondary metabolites, and lipidomics, was used to investigate the influence of organic matter (OM) quality on the heterotrophic microbial mechanisms controlling peatland CO₂, CH₄, and CO₂:CH₄ porewater production ratios in response to climate warming. Our investigations leverage the Spruce and Peatland Responses under Changing Environments (SPRUCE) experiment, where air and peat warming were combined in a whole-ecosystem warming treatment. We hypothesized that warming would enhance the production of plant-derived metabolites, resulting in increased labile OM inputs to the surface peat, thereby enhancing microbial activity and greenhouse gas production. Because shallow peat is most susceptible to enhanced warming, increases in labile OM inputs to the surface, in particular, are likely to result in significant changes to CO₂ and CH₄ dynamics and methanogenic pathways. In support of this hypothesis, significant correlations were observed between metabolites and temperature consistent with increased availability of labile substrates, which may stimulate more rapid turnover of microbial proteins. An increase in the abundance of methanogenic genes in response to the increase in the abundance of labile substrates was accompanied by a shift toward acetoclastic and methylotrophic methanogenesis. Our results suggest that as peatland vegetation trends toward increasing vascular plant cover with warming, we can expect a concomitant shift toward increasingly methanogenic conditions and amplified climate–peatland feedbacks.

peatland | metabolome | climate change | metagenomics | elevated methane and carbon dioxide

Peatlands contain an estimated one-third (1–3) or more (4) of global soil organic matter (OM). While much of this OM is locked away in frozen permafrost, thawing and nonpermafrost peatlands have also historically been significant net C sinks (5). Following its deposition in peatlands, microorganisms mediate the decomposition of soil OM to both CO₂ and CH₄. Because CH₄ has a much greater global warming potential compared with CO₂ (6, 7), whether peatlands exert a net warming or net cooling effect on climate depends on the balance between CO₂ uptake by primary producers and CO₂ and CH₄ emission from heterotrophic respiration (5). The relative production of CO₂:CH₄ from microbial respiration depends on the availability of terminal electron acceptors (TEAs) which promote CO₂ over CH₄ production, while under TEA-depleted conditions, peatland systems can approach a

1:1 production of CO₂ and CH₄ (8, 9). Hydrology plays a role, in that permanently inundated areas are more anoxic and the low availability of TEAs facilitates methanogenic conditions leading to higher CH₄ emission rates (5, 10). Active microbial consortia at a given place and time may also “tune” the CO₂:CH₄ production ratio by partitioning TEA pathways, speeding the rates of some reactions relative to others via production of enzyme catalysts, leading to heterogeneous C-gas production via the buildup of intermediates.

Climate warming and the resulting ecosystem response, including lowered water tables and changes in vegetation community composition and production rates and composition, influence OM decomposition and the relative production of CO₂ and CH₄.

Significance

While peatlands have historically stored massive amounts of soil carbon, warming is expected to enhance decomposition, leading to a positive feedback with climate change. In this study, a unique whole-ecosystem warming experiment was conducted in northern Minnesota to warm peat profiles to 2 m deep while keeping water flow intact. After nearly 2 y, warming enhanced the degradation of soil organic matter and increased greenhouse gas production. Changes in organic matter quality with warming were accompanied by a stimulation of methane production relative to carbon dioxide. Our results revealed increased decomposition to be fueled by the availability of reactive carbon substrates produced by surface vegetation. The elevated rates of methanogenesis are likely to persist and exacerbate climate warming.

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Increasing primary production will increase the availability of fresh OM through increased litter inputs and elevated root exudates (e.g., ref. 11). Radiocarbon evidence from a number of peatlands shows that dissolved OM (DOM) contains more important substrates for methanogenesis than solid peat at any given depth (12–16). Thus, increases in the quantity of DOM input at the surface can increase CH₄ production at depth. The quality of OM available for decomposition, defined as energy yield upon oxidation under anoxic conditions, may also influence the rate and pathways of CH₄ production (12, 14). For example, sedge-dominated wetlands contain higher-quality OM for decomposition relative to *Sphagnum*-dominated bogs and, in turn, exhibit higher rates of CH₄ production and higher relative rates of acetoclastic methanogenesis (12, 14, 17, 18). Aside from altering vegetation and hydrology, temperature could exert a simple kinetic effect on microbial activity in peat, with warmer conditions leading to higher CO₂ and CH₄ production rates. Since CH₄ production has a higher Q₁₀ (Q₁₀ represents the proportional increase in metabolic rate or growth rate with a 10 °C increase in temperature) than CO₂ production, there would be a differential kinetic effect with CH₄ production being stimulated more than CO₂ for a given temperature increase (19–21).

The Spruce and Peatland Responses under Changing Environments (SPRUCES; <https://mnspruce.ornl.gov/>) experiment is a whole-ecosystem warming and elevated air partial pressure of CO₂ (eCO₂) experiment in a regression design to test the impacts of climate drivers on ecosystem response in a nonpermafrost, undrained peatland. The SPRUCES group previously reported on an earlier phase of the experiment, referred to as deep-peat heating (DPH), in which the peat was heated at varying temperatures up to +9 °C above ambient before aboveground warming and eCO₂ started (22). During DPH, surface peat decomposition was increasingly methanogenic with temperature treatment, but there was no response of the peat below 25 cm to heating (22). In addition, no change in peat-associated microbial communities was observed with warming (22). Following the ~1-y DPH treatment, aboveground warming from 0 to 6 m was added to the DPH experiment to achieve whole-ecosystem warming (23). Temperature treatment enclosures are duplicated and elevated air CO₂ concentrations (~900 parts per million by volume [ppmv]) are applied in one of each of the temperature treatments. Under these conditions, surficial soil temperatures are higher than those measured during DPH which may exacerbate warming effects on CO₂ and CH₄ production (22). DOM in near-surface peat may become more concentrated and change composition with drying or shifts in the vegetation community (24, 25), which will also influence CO₂ and CH₄ production rates. Additionally, lagged responses in deep peat to warming became apparent only after more than 13 mo of DPH (26), leading us to examine the mechanisms mediating CO₂ and CH₄ production rates since the initiation of whole-ecosystem warming.

This study assesses changes in CO₂ and CH₄ production with experimental warming driven primarily by changing OM quality and methanogenic activity. We hypothesized that increasing OM quality and microbial processing rates would facilitate increasingly methanogenic conditions. Multiple complementary and novel, high-resolution analytical approaches were employed to characterize the DOM. Each of these analyses provides molecular-level detail to elucidate both unique and overlapping subsets of molecular compounds comprising the DOM. While each technique presents its own limitations, their collective strengths can be leveraged in an integrated approach to provide a complementary analysis. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) allows semiquantitative analysis of large-molecular-weight (>~100 to 1,000s Da) metabolites. One key advantage of this technique is the capability to resolve novel compounds and reveal key metabolic pathways (e.g., ref. 27). Gas chromatography-mass spectrometry (GC-MS) is capable of identifying smaller-molecular-weight

compounds (typically <400 Da) in a semiquantitative way, allowing us to probe changes in critical small metabolites such as sugars. However, derivatization and drying during sample preparation can result in the loss of volatile compounds (28). To identify and quantify such volatile compounds (e.g., alcohols) and a range of carbohydrate compounds that are difficult to quantify using MS-based methods, ¹H liquid-state NMR was used. While the NMR method is truly quantitative, quantification requires standardization relative to a standard database (as does the GC-MS method). Thus, compound identification is limited to known compounds for which standard curves have already been developed. NMR is also less sensitive than many of the other techniques and cannot reliably identify compounds with concentrations less than ~1 μM. Nevertheless, the quantitative nature of this technique provides valuable insights into the changes in some of the most metabolically important compounds (e.g., glucose, pyruvate). Metabolomic and metaproteomic analyses were coupled with analyses of microbial community composition and genomic potential (determined with 16S ribosomal RNA [rRNA] gene sequencing and shotgun metagenome approaches) to elucidate dominant methanogen taxa and pathways of methanogenesis. The results differentiate between upstream (e.g., increased substrate supply) and downstream (e.g., microbial inhibition) controls on CO₂ and CH₄ production dynamics in nonpermafrost, undrained peatlands during their response to warming.

Methods

The methods below are condensed, and more detailed experimental methods for all field collections and analyses are provided in an extensive *SI Appendix (SI Appendix, Methods)*.

Study Site. The SPRUCES experiment is located within the S1 bog of the US Department of Agriculture (USDA) Forest Service's Marcell Experimental Forest, 40 km northeast of Grand Rapids, MN, USA (47°30.4760N; 93°27.1620W) (29). The S1 bog is a perched ombrotrophic bog with a hummock/hollow microtopography where the water table typically fluctuates within the top 30 cm of peat during a typical year (30). The vegetation at the S1 bog is dominated by *Sphagnum* mosses (*Sphagnum angustifolium*, *Sphagnum fallax*, and *Sphagnum magellanicum*), black spruce (*Picea mariana*) and tamarack (*Larix laricina*) trees, ericaceous shrubs (*Rhododendron groenlandicum* and *Chamaedaphne calyculata*), and some graminoids and forbs.

A detailed technical description of the whole-ecosystem warming experiment can be found in ref. 23, and a graphical representation of the experimental treatment timeline as well as sampling dates is provided in *SI Appendix, Fig. S1*. Briefly, greenhouse-like walls (7 m tall by 12.8 m in diameter) are supported above the bog surface on helical piles that were inserted through the peat into dense glacial till and outwash sand below the peatland. The air and soil to 3-m depth of the open-topped chambers are warmed to +2.25, +4.5, +6.75, and +9 °C above the constructed control enclosure (+0 °C) temperatures at the site. Although elevated air CO₂ concentrations (+500 ppmv) are also applied at the site (23), the elevated CO₂ treatment was initiated only 2 mo prior to collection of our last set of samples (August 2016) and those effects will be addressed primarily in future studies.

Geochemistry, Metabolites, and Metaproteomes.

Sample collection and extraction. Porewater samples were collected three times (May, July, and August 2016; during the first full year after initiation of air heating) from depths of 25, 50, 75, 100, 150, and 200 cm below the bog surface in each of the 10 enclosures encompassing the +0, +2.25, +4.5, +6.75, and +9 °C treatments.

Solid peat samples were collected in August 2016 from each of the +0, +4.5, and +9 °C treatment chambers using a serrated knife at the surface and a Russian corer at depth. Cores were divided into depth sections and 15 mL of peat from each of 10- to 20- (15 cm), 40- to 50- (45 cm), and 75- to 100-cm (87 cm) depths was collected and extracted for metabolites, proteins, and lipids as described (31). The methanol layer was reserved for GC-MS analysis for central metabolite characterization and the chloroform layer was reserved for subsequent lipid analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Dissolved CO₂ and CH₄ concentrations and stable isotope analysis. Porewater concentrations and stable isotopes (δ¹³C) of CH₄ and CO₂ were obtained by

isotope ratio mass spectrometry after equilibration with a helium headspace. Stable isotope values were used to calculate $\alpha_c = [\delta^{13}\text{CO}_2 + 1,000]/[\delta^{13}\text{CH}_4 + 1,000]$, which provides an indication of the pathway of CH_4 production (32).

FTICR-MS analysis. August 2016 porewater DOM samples were analyzed by FTICR-MS, following dilution 2:1 with methanol, at the Environmental Molecular Sciences Laboratory using direct injection and a 12T FTICR-MS operating in negative-electrospray ionization (ESI). Molecular formulae were identified using Formularity software according to the Compound Identification Algorithm of ref. 27 as modified by ref. 33 using a signal:noise ratio greater than 7 and mass measurement error <1 parts per million (ppm). From the resulting FTICR-MS, we calculated the nominal oxidation state for C [NOSC (34, 35)] for each of the identified compounds. NOSC provides a thermodynamically relevant metric of the quality of OM available for decomposition (28, 35) under anoxic conditions.

NMR analysis. Solid OM (SOM) samples for NMR analysis were analyzed as described (31) (*SI Appendix, Methods*). NMR spectra were collected using a Varian Direct Drive 600-MHz NMR spectrometer equipped with a Varian 5-mm triple-resonance salt-tolerant cold probe. The one-dimensional ^1H NMR spectra of all samples were processed, assigned, and analyzed using Chenomx NMR Suite 8.3 with quantification based on spectral intensities relative to the d_6 -DSS [3-(trimethylsilyl)-1-propanesulfonic acid- d_6] internal standard (36). Two-dimensional ^1H - ^{13}C heteronuclear single-quantum correlation (HSQC) and ^1H - ^1H total correlation (TOCSY) spectra were collected to aid in metabolite assignment and validation.

GC-MS analysis. Polar metabolites were extracted from the SOM and derivatized as described previously (37) (*SI Appendix, Methods*). Fatty acid methyl ester (FAME; C8 to C28) calibration standards were prepared by diluting 100 mg FAME in 200 μL high-performance liquid chromatography-grade hexane. Samples and standards were analyzed in haphazard order in splitless mode with a constant port temperature of 250 $^\circ\text{C}$. Polar metabolites were separated using an Agilent HP-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) and analyzed on an Agilent GC 7890A coupled with a single-quadrupole MSD 5975C (Agilent Technologies). The FAME mixture was used for detection of retention indices prior to analysis of peat extracts.

The GC-MS raw data files were processed with MetaboliteDetector as reported previously (38), by matching GC-MS spectra and retention indices against a Pacific Northwest National Laboratory (PNNL) augmented version (>900 metabolites) of the FiehnLib (PubMed ID 19928838) library containing validated retention indices and spectral information (39). Peak areas were calculated for each compound identified and are reported relative to the integral of the ribitol peak. Compound identifications were manually validated by matching measured mass spectra with mass spectra from the National Institute of Standards and Technology Reference Database 14 (NIST14) GC-MS library.

Lipid analysis: LC. Lipids were extracted from the chloroform fraction of the SOM extracts resulting from the Folch extraction. To obtain high coverage of the lipidome, samples were analyzed in both positive- and negative-ionization modes using higher-energy collision dissociation and collision-induced dissociation. The LC-MS/MS raw data files were matched against the current LIQUID (40) reference database of over 25,000 unique lipid species. Duplicate lipids identified in both positive- and negative-ion modes were reduced to unique species for further analysis.

Metaproteomics. The interphases resulting from the Folch extraction, containing the relevant proteins, were washed with cold methanol prior to solid-phase extraction. Following extraction, protein concentration was measured by bicinchoninic acid colorimetric assay. Extracts were centrifuged, and the sample pellet was dried before being resuspended in water to a final concentration of 0.1 μg peptide per microliter. Solutions were incubated at 60 $^\circ\text{C}$ for 30 min with dithiothreitol. A 100 mM NH_4HCO_3 :8 M urea solution was added to dilute the original solution 10-fold and then the sample was incubated again at 37 $^\circ\text{C}$ for 3 h with 1 mM CaCl_2 and porcine trypsin at a 1:50 enzyme:protein ratio. Liquid chromatography was used to separate the extracts. The resulting eluent was analyzed by ESI-MS collecting from 400 to 2,000 m/z with 1-ppm resolution at m/z 400 in a linear ion trap Orbitrap mass spectrometer. Peptides from the resulting metaproteomes were searched against a targeted database of functional annotations of short, 150-bp sequences from 83 metagenomes generated by the Joint Genome Institute (JGI; see *Metagenomics*). Duplicated proteins were removed. The data were filtered to a 1% false discovery rate. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the Proteomics Identifications (PRIDE) (41) partner repository with the dataset identifier PXD019912 and 10.6019/PXD019912.

Statistical analysis and metabolic network. Regression analyses were used to identify significant changes with temperature treatment for bulk geochemical

measures as well as individual metabolites. In this analysis, we relied significantly on regressions against temperature. Thus, the results depend on our choice of the relevant temperature metric against which to plot the data. Here we have chosen to plot against the temperature at the time of sampling as measured 50 cm below the peat surface at the time of sampling. We chose 50 cm as a representative depth below the mean annual variation in water table fluctuations where DOM resides and below which depth temperature variation with depth in each treatment is low [i.e., within a few degrees (22)]. However, it seems evident that biological units respond to the temperature integrated over some time period. Equally evident is that different units within the system will respond to temperature integrated over different timescales. For example, the concentration of labile substrates made available via root exudation may be influenced by temperature integrated over the growing season, while rates of microbial mineralization could be influenced by temperature integrated over a somewhat shorter timescale and could change with the actual temperature (depending on, e.g., optimal growth temperature). When considering the multiple interacting biological units in this study, the choice of integration time becomes even more complicated and, when looking at whole-ecosystem effects, it is tempting to suggest plotting against the assigned warming treatments. Here, we chose to plot against the temperature on the day of sampling rather than temperature differential because this approach limits biasing integration time toward any one step and, while this choice does neglect some temperature information, it nevertheless captures some seasonal effects which are likely crucial. The choice of a relevant time period over which to integrate temperature therefore is important yet challenging and should be carefully considered with reference to the specific goals of the study.

To evaluate changes in overall DOM composition, we calculated Pearson's linear correlation coefficients between temperature and FTICR-MS peak intensities. Correlation results were plotted as a van Krevelen diagram (molecular O:C vs. H:C ratios) to facilitate identification of major compound classes of DOM.

We constructed a dataset of the combined CO_2/CH_4 , FTICR-MS, NMR, GC-MS, LC-MS, and metaproteomics data (*SI Appendix, Methods*) and then conducted a principal-component analysis (PCA) of the resulting normalized dataset. We then queried the KEGG [Kyoto Encyclopedia of Genes and Genomes (42–44)] database for all pathways or modules in which any identified metabolites and enzymes occurred to identify potentially important metabolic processes occurring at the site.

Nucleic Acids.

16S rRNA gene sequencing. Samples for 16S rRNA gene sequencing were collected from porewater in June 2016 and from peat in June and August 2016. All samples used for amplicon sequencing were collected from 20- to 30-cm depth. Peat was sampled from hollow locations using a Russian corer. All samples were immediately frozen on dry ice and shipped to the Georgia Institute of Technology, where they were stored at -80°C until further processing. DNA was extracted from samples via whole filters (porewater) or 0.25 g of peat using the DNeasy PowerSoil Extraction Kit (Qiagen). Amplification and sequencing of the 16S rRNA gene were performed using the universal primer pair 515F and 806R (45) as described (21, 22, 26).

The primers and adapters were trimmed from the resulting sequencing reads using the program Cutadapt (version 1.18) (46) in paired-end mode. The trimmed reads were analyzed using DADA2 R package version 1.10.1 and workflow (47). The resultant amplicon sequence variants (ASVs) were taxonomically classified in DADA2 using the assignTaxonomy function and the SILVA SSU Ref NR99 v132 database (48). Chloroplasts, mitochondria, and eukaryotic ASVs were removed, as well as ASVs that were unclassified at the phylum level. Sequence counts were normalized by cumulative sum scaling using the package metagenomeSeq (49). Here we will only focus on archaeal methanogens. All raw sequences have been uploaded to the National Center for Biotechnology Information (NCBI) under BioProjects PRJNA640652 (porewater June 2016), PRJNA638786 (peat core June 2016), and PRJNA638601 (peat core August 2016). ASV feature tables and accompanying metadata are available in [Datasets S10](#) and [S12](#).

Metagenomics. Peat samples for metagenomic analyses were collected from each of the 10 experimental enclosures using Russian corers in June of 2015 and 2016. DNA was extracted from homogenized samples of each depth interval using the MoBio PowerSoil Extraction Kit (Qiagen). Six replicate 0.35-g extractions were combined and repurified with the MoBio PowerClean Pro Kit (Qiagen) and eluted in 50 μL of 10 mM Tris buffer. At the JGI, Illumina TruSeq metagenome libraries were generated for DNA extracted from the 10- to 20-, 40- to 50-, 100- to 125-, and 150- to 175-cm-depth increments (total of 83 metagenomes) using their standard protocols. Barcoded libraries were sequenced on an Illumina HiSeq 2500 instrument in 2 $\times$ 150-bp mode. Metagenomes are publicly available

on the JGI Genome Portal and NCBI Sequence Read Archive (SRA). SRA identifiers for each metagenomics dataset are provided in *SI Appendix, Tables S1 and S2*.

Metagenomic paired-end reads were merged using PEAR (50) (options: -p 0.001). All merged and nonmerged reads were then quality-trimmed with the SolexaQA package (51) (options: -h 17; $\geq 98\%$ accuracy per nucleotide position). Trimmed sequences were truncated to 150 bp to avoid read-length biases. Functional annotation of the trimmed short reads was performed as described (52).

Metagenome assembly, binning, and analysis of metagenome-assembled genomes (MAGs) were performed as described (52). To estimate the abundance of the MAGs in the environment, Bowtie 2 was used to align short-read sequences to assembled contigs (options: -very-fast) and SAMtools was used to sort and convert sequence alignment/map (SAM) files to binary alignment/map (BAM) format (53). Sorted BAM files were then used to calculate the coverage (mean representation) of each contig in each sample metagenome. These contig coverage values were multiplied by the contig length and summed for each MAG. These values were then divided by the total dataset size (bp) used in the original Bowtie 2 alignment, resulting in a relative abundance metric for each MAG, or the percent of DNA matches to each genome relative to all query DNAs (*SI Appendix, Table S5*).

Results

Geochemistry, Metabolites, and Metaproteomic Results.

Dissolved CO_2 and CH_4 concentrations and stable isotopes. Porewater CO_2 concentrations significantly increased with temperature (which is controlled by both season and temperature treatment) in all depths (P values are shown in Fig. 1A). Methane concentrations significantly increased with warming in only the 10- and 25-cm depths (Fig. 1B). However, after using a stable isotope-based model to correct for differential transport of CO_2 and CH_4 from peat (54), $\text{CO}_2:\text{CH}_4$ ratios were shown to significantly decline with warming in the 25-, 50-, and 75-cm depths (Fig. 1D). The declining $\text{CO}_2:\text{CH}_4$ ratio with temperature is consistent with the expected higher temperature sensitivity of methanogenesis (19–21) and findings during DPH that decomposition became more methanogenic with warming (22, 26). There was no significant change in the methanogenic pathways with temperature as indicated by α_{C} (Fig. 1C). The lowest CO_2 concentrations, highest $\text{CO}_2:\text{CH}_4$ ratio, and higher contributions of the acetoclastic methanogenesis pathway

(as inferred from α_{C} per ref. 32) were observed at the peat surface (10 and 25 cm).

FTICR-MS results. FTICR-MS of peat porewater identified 67,040 unique compounds present in the DOM across all the SPRUCE treatments and depths (*SI Appendix, Fig. S2*). Of the 7,985 compounds that were identified in five or more surface samples (≤ 25 -cm soil depth), 3,162 compounds were positively correlated with temperature, 2,553 compounds were negatively correlated with temperature, and 2,270 did not correlate strongly with temperature ($-0.10 < \rho < +0.10$; *SI Appendix, Fig. S2*). In this analysis, compounds that are negatively correlated with temperature disappear, or are consumed, at high temperatures, while those that are positively correlated appear, or are produced, at high temperatures. Compounds that increased with temperature were largely lignin and other phenolic-like compounds. Compounds that declined with warming were largely carbohydrates, peptides, and compounds with low O:C ratio. Microbially associated metabolites such as protein-like and lipid-like compounds declined with temperature. Carbohydrates also declined with temperature, while other hydrocarbons increased with warming. Average NOSC in surface peat DOM declined significantly with temperature, consistent with expectations during enhanced decomposition ($P = 0.0031$; Fig. 2). The decline in DOM NOSC indicates that residual DOM is more decomposed at warmer temperatures than at lower temperatures. In addition, simple electron balance dictates that decomposition of lower-oxidation-state OM will increasingly favor methanogenesis over CO_2 production, consistent with our measurements of $\text{CO}_2:\text{CH}_4$ ratios (Fig. 1) (22, 26).

NMR results. Twenty-seven compounds involved in central carbon metabolism were detected by NMR. Of these, 13 were of sufficient concentration to be quantified. Methanol, which can be used by methylophilic methanogens to produce methane, declined slightly, though not significantly, with temperature (*SI Appendix, Fig. S4*). As with previous analyses (15, 55), depth explained a significant amount of variation in many compounds identified by NMR (*SI Appendix, Table S6*). Several compounds were present only at 15 cm but not at 45 and 87 cm (*SI Appendix, Table S6*),

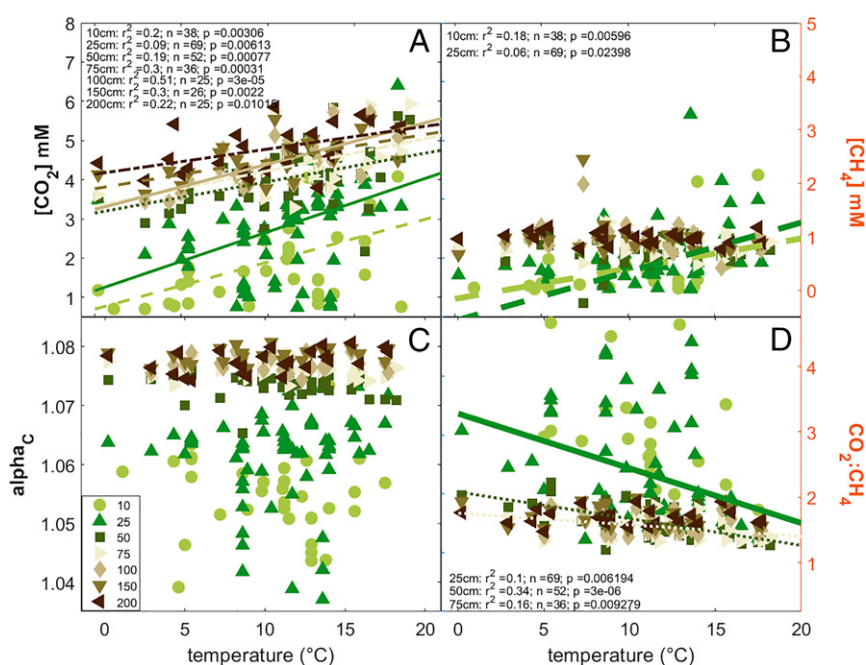


Fig. 1. Porewater CO_2 and CH_4 concentrations (A and B), α_{C} (C), and $\text{CO}_2:\text{CH}_4$ ratios (D) across all seasons plotted against measured temperature in each enclosure (at 50 cm below the peat surface). Different colors denote different depths. Lines indicate significant regressions for each depth; r^2 and P values for significant regressions are indicated in each panel.

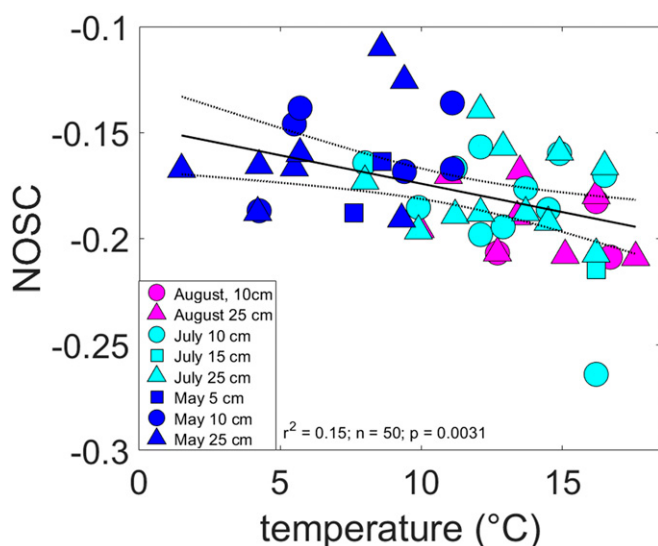


Fig. 2. Average carbon nominal oxidation state in surficial FTICR-MS-identified compounds declines with peat temperature in each enclosure (measured at 50 cm below the peat surface). r^2 and P values indicate regression results (solid line $\pm 95\%$ CI in dashes) with temperature. Symbol colors indicate different sampling months; shapes indicate depth.

leading us to focus on changes in surface concentrations with temperature.

GC-MS results. Ninety-two compounds were observed by GC-MS. Of these, 49 were matched to compounds available in the database including important fermentation metabolites such as glucose, pyruvate, succinate, and several ($n = 10$) primary amino acids. Seven of the identified compounds were also observed by NMR. Similar trends with temperature were observed using the two different techniques (SI Appendix, Fig. S3), providing a cross-check validating the approach. Sugars and amino acid concentrations in the surface (15-cm) SOM, as measured by GC-MS, varied across temperature treatment, with trends typically toward higher sugar concentrations and lower free amino acid concentrations with increasing temperature (Fig. 3). Glutamine was a notable exception among the amino acids, with a positive trend observed in concentration versus temperature treatment (Fig. 3). In addition, compounds related to plant stress response (oleic acid, inositol, galactinol, and 4-hydroxy-3-methoxybenzoic acid) increased with temperature (SI Appendix, Fig. S5).

Lipid results. A total of 209 unique lipids, including isomers, were observed across three lipid categories and 13 subclasses (SI Appendix). The main classes identified within glycerophospholipids included glycerophosphate (PA), glycerophosphocholines (PCs), glycerophosphoethanolamines (PEs), and glycerophosphoglycerol (PG). Among glycerolipids, we identified digalactodiglycerols (DGDGs), diacylglycerolstridylglycerols (DGs), and other glycerolipids (DGTSAs). Ceramides (Cers) within the sphingolipids class were also identified.

Metaproteomics results. Total enzyme counts in peat extracts revealed a strong decline with depth (SI Appendix, Fig. S6). Approximately 90% of observed enzymes were found only in the shallowest depth (15 cm). However, there was no apparent change in total enzyme counts with temperature (t test, $P > 0.05$; SI Appendix, Fig. S6). Only 35 individual enzymes were significantly correlated with temperature using regression ($P < 0.05$; SI Appendix, Table S7). Proteases were among the enzymes with the strongest positive correlation with temperature. Oxidoreductases and some (though not all) hydrolases were among the enzymes that were negatively correlated with temperature (SI Appendix, Table S7).

Multivariate statistical analysis of combined metabolites and metaproteomics data. PCA of the combined metaproteomics, FTICR-MS, NMR, GC-MS, and lipidomics data revealed soil depth as a large driver of the variation among samples, accounting for $\sim 37\%$ of the total variability (Fig. 4). Prior investigations at SPRUCE have similarly found depth to be a strong driver of variation in both organic chemistry of the DOM (14, 55) and microbial community composition (56, 57). However, the envfit function in the vegan R package (58), which fits coordination results with environmental variables, identified a strong separation by temperature ($r^2 = 0.31$, $P < 0.08$) as well as depth ($r^2 = 0.83$, $P < 0.001$).

Surface peat was positively associated with valine, leucine, and isoleucine concentrations (Fig. 3), but also with the counts of the branched-chain amino acid transport system substrate-binding protein and the enzyme acetolactate synthase which catalyzes the first step in the production of these branched-chain amino acids (KEGG) (SI Appendix, Fig. S7). An important enzyme in glucose metabolism that was associated with surface peat samples was phosphofructokinase (SI Appendix, Fig. S7). The betaine lipids (DGTSAs; diacylglycerol- N,N,N -trimethylhomoserine and diacylglycerolhydroxymethyl- N,N,N -trimethyl- β -alanine), which are found in lichens, fungi, and mosses but not in flowering plants (59), were also associated with the surface samples (SI Appendix, Fig. S7). In contrast, many of the waxy lipid eukaryotic Cers were most correlated with the deeper samples (SI Appendix, Fig. S7).

While several enzymes were positively associated with the variation among temperature treatments in the PCA, those correlations were not strong, as indicated by low loading values. However, searching our identified proteins and metabolites against KEGG revealed several potential microbial pathways. We identified three out of four of the compounds in the gamma-aminobutyric acid (GABA) shunt module which conserves GABA via a closed-loop production process (60). We also identified many of the steps involved in the two main pathways of methanogenesis (Fig. 5), acetoclasty and hydrogenotrophy. In addition, we identified several intermediates and proteins involved in methylotrophic methanogenesis (Fig. 5), which has been a relatively understudied pathway for CH_4 production in peatlands until recent research (61).

Nucleic Acid Results.

16S rRNA gene sequences. The relative sequence abundances (16S rRNA gene) of the total, phylum-level microbial taxa in surface peat and porewater did not change significantly with experimental warming (SI Appendix, Fig. S8). Taxonomic classification of archaeal ASVs present in the dataset identified 166 ASVs belonging to known methanogen taxa. These ASVs were dominated by sequences classified as *Methanobacteriaceae* and *Candidatus methanoflorens* (rice cluster II), which collectively made up 1.1 ± 0.80 and $3.58 \pm 3.36\%$ of the total microbial community in peat and porewater, respectively (SI Appendix, Fig. S9). Methanogens in porewater exhibited more changes in response to temperature than in peat; however, these changes were not statistically significant ($r^2 = 0.11$, $P = 0.08$).

Metagenomics. Functional annotation of metagenome-derived short nucleic acid sequences revealed key genes involved in multiple pathways of methanogenesis (summarized by KEGG orthology terms) (SI Appendix, Fig. S10). For the 40- to 50-cm peat layer, we observed significant shifts in the relative abundance of methanogenic genes with warming. This evaluation was primarily based on KEGG modules M00567, M00357, M00356, and M00563, corresponding to methanogenesis from CO_2 , acetate, methanol, and methylamines, respectively (SI Appendix, Fig. S10).

Population genome binning led to the recovery of 10 nonredundant MAGs that were identified with the potential to mediate methanogenesis based on presence and completeness of the aforementioned KEGG modules. Taxonomic classification, source

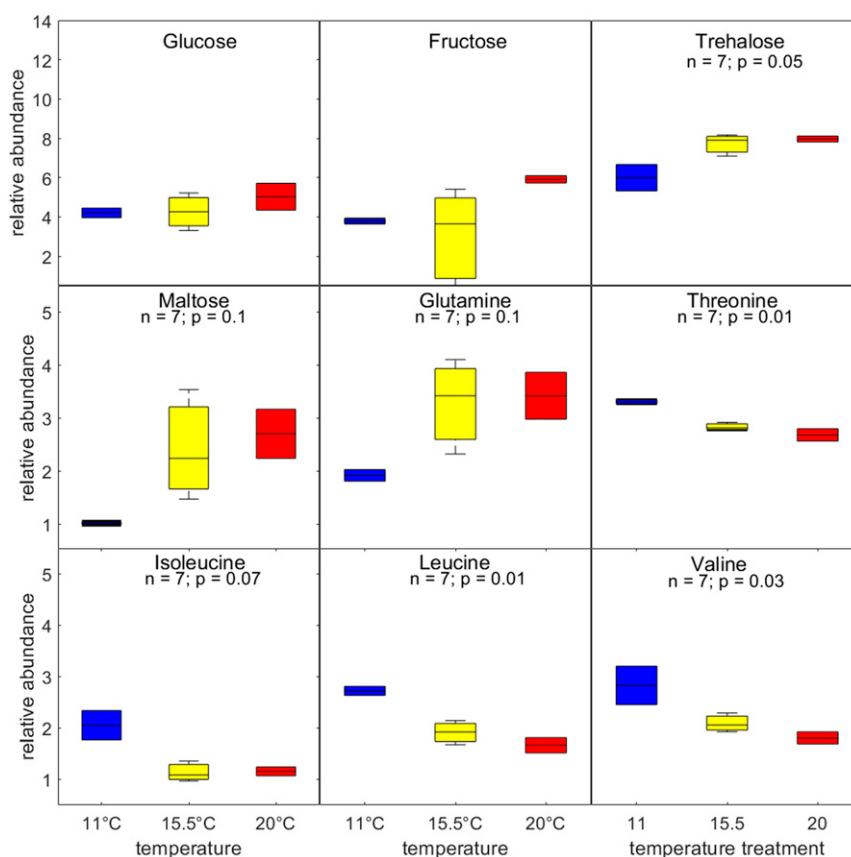


Fig. 3. Sugars and amino acids, as identified by GC-MS, from the peat extracts collected in August 2016 from the surface peat (15 cm) against the average measured peat temperature (at 50 cm) for a given temperature treatment. Values are reported in relative abundance from peak areas relative to an internal standard peak. The y-axis range for each row of panels is scaled as indicated. *P* values in each panel indicate regression results for that specific compound versus temperature; *P* values > 0.1 are not reported.

dataset SRA ID, basic genome statistics, and CheckM summaries for each MAG can be found in [SI Appendix, Table S3](#). The presence of key genes involved in methanogenesis pathways is provided in [SI Appendix, Table S4](#). When comparing the ASVs (derived from 16S rRNA gene sequencing) with the recovered MAG sequences, we found that $87.5 \pm 11\%$ of the methanogen ASVs acquired from peat samples were represented by the non-dereplicated MAGs, indicating that the MAGs adequately represented the more dominant methanogenic sequences obtained through this complementary approach ([SI Appendix, Fig. S12](#)). Methanogen ASVs acquired from porewater samples were also well-represented in the MAGs, albeit to a lesser extent than in peat ($67.4 \pm 15.4\%$).

The four most abundant MAGs were identified as members of the *C. methanoflorens* lineage, sharing high amino acid identity with a *Candidatus methanoflorens stordalenmirens* MAG recovered from a peatland in northern Sweden (62). Two of the MAGs exhibited species-level similarity with *C. m. stordalenmirens* (SPRUC2015.26.metabat2.21: 95.84%; SPRUC2015.15.metabat2.11: 96.36%), while the other two MAGs likely represent distinct organisms within the *C. methanoflorens* genus (SPRUC2016.33.metabat2.67: 79.24%; SPRUC2015.31.metabat2.23: 87.82%). Together, these four MAGs comprised 88% of the methanogenic MAG sequences and recruited an average of $0.59 \pm 0.77\%$ of the total metagenomic short reads ([SI Appendix, Fig. S11 and Table S5](#)). Although no methanogens have been cultivated from the *C. methanoflorens* group, interpretation of the nearly complete MAGs from previous work suggested CH_4 production from hydrogen and CO_2 (62, 63). In contrast, our *C. methanoflorens* MAGs contain

genes encoding key enzymes in the acetoclastic methanogenesis pathway ([SI Appendix, Table S4](#)). Confirming these metabolisms is beyond the scope of the present study and is likely to be inconclusive without cultivated representatives. The remaining methanogen MAGs were identified as members of the *Methanoregulaceae* ($0.01 \pm 0.03\%$ average relative abundance), *Methanomicrobiaceae* ($0.03 \pm 0.13\%$ average relative abundance), *Candidatus methanomethyliaaceae* ($0.03 \pm 0.05\%$ average relative abundance), and *Methanobacteriaceae* (0.02% average relative abundance; [SI Appendix, Table S5](#)). All of the MAGs exhibited strong depth stratification ([SI Appendix, Fig. S11](#)), although none presented a statistically significant response to experimental warming.

Discussion

Dissolved porewater CO_2 concentration increased significantly with temperature at all depths, suggesting increased rates of heterotrophic respiration throughout the peat profile (Fig. 1A), although r^2 values were weak, indicating low predictive power. Temperature explained the largest amount of variability in the 50- to 100-cm-depth interval, previously identified as a zone of high microbial C turnover (55). Porewater CH_4 concentrations also increased significantly with warming, but only in the surface peat (10 to 25 cm) (Fig. 1B). Increasing CH_4 concentrations with warming are consistent with changes in the abundance of core methanogenesis genes along with elevated methanogenic activity observed previously in surface peat ([SI Appendix, Fig. S10](#)) (22, 26). While both CO_2 and CH_4 concentrations increased, differential responses between the two gases led to declining $\text{CO}_2:\text{CH}_4$

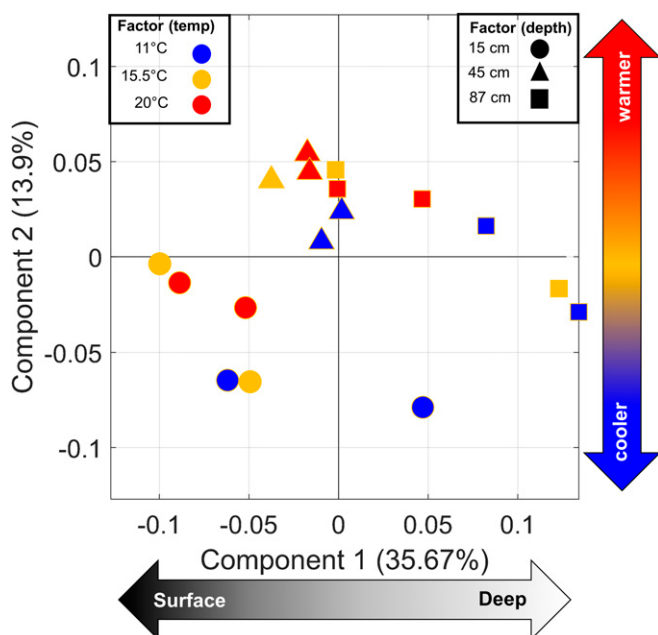


Fig. 4. PCA of combined geochemical data (FTICR-MS, GC-MS, NMR, LC-MS, and proteomics). Symbol colors indicate temperature treatments; shapes indicate sample depths.

ratios with warming at 25- to 75-cm depth (Fig. 1D), indicating that CH_4 production is more sensitive to warming than CO_2 . This result is concerning given the much higher warming potential of CH_4 compared with CO_2 (6, 7), exacerbating feedbacks with warming.

Previous results from SPRUCE and other bogs in both Canada and Sweden suggest that DOM, rather than the ancient peat, contains the primary substrates for microbial degradation, even several meters deep in the peat profile (12–16). This interpretation is based on the finding that DOM along with dissolved inorganic C and CH_4 up to several meters deep is thousands of years younger than the peat at the same depth (15, 16, 22). Exudates from plant roots coupled with vertical advection likely play a role in transporting DOM to support respiration at depth (22). Because of the importance of DOM in the production of CO_2 and CH_4 in peatlands, we leveraged FTICR-MS to gain the broadest view of the largest number of DOM compounds (>67,000 identified in this study). Additionally, NMR and GC-MS were used to quantify trends of either increasing or decreasing concentration with temperature (SI Appendix, Fig. S3). Many of the compounds we matched to KEGG modules were identified via GC-MS, while some were observed only by NMR, and a few of the larger metabolites in these pathways could only be inferred using the FTICR-MS dataset. This highlights the importance of using multiple complementary analytical approaches to elucidate a broader understanding of the metabolites present and how pathways might be changing in response to environmental perturbations.

As has been observed previously at this site (15, 22, 55), geochemical parameters displayed a strong stratification with depth. Highly labile compounds such as sugars and free amino acids declined with depth within the peat profile (SI Appendix, Table S6). Combined with increased counts of proteins like phosphofructokinase (SI Appendix, Fig. S7), this patterning suggests that declines in sugar concentrations with depth are the result of high processing rates in the surface that limit supply as DOM moves downward with advection to deeper peat depths.

The NOSC provides an effective metric for assessing the degradability (i.e., quality) of peatland OM such that high NOSC values represent low Gibbs free energy (GFE), indicating thermodynamically

favorable substrates. The declining NOSC with warming in the surface peat (Fig. 2) indicates enhanced decomposition which leaves behind compounds that are less thermodynamically favorable, that is, high GFE, in the remaining DOM pool. The surface DOM pool may be increasingly protected from microbial oxidation by thermodynamic limitations. As the oxidation state of the substrate shifts toward lower NOSC, more CH_4 must be produced during complete mineralization to account for the electron balance. Subsequent decomposition of the thermodynamically less favorable DOM (lower-oxidation-state OM) is likely to push production toward even more CH_4 relative to CO_2 (9, 34) as decomposition progresses, indicating a thermodynamic mechanism for increasingly methanogenic conditions in the surface peat.

The accumulation of lignin-like compounds with warming observed via FTICR-MS (SI Appendix, Fig. S2) could suggest increased leaching and/or hydrolysis of plant cell walls followed by the rapid consumption of liberated sugars and the accumulation of less-labile, lignin-like, and phenolic constituents of plant cell-wall degradation in the so-called island of stability (64, 65). This is especially likely in *Sphagnum*-dominated peatlands since *Sphagnum* cell walls are largely composed of glycosides whose degradation liberates glucose (observed in the GC-MS and NMR data) and phenols (observed in the FTICR-MS results) (66). Another possible explanation is elevated production of soluble lignin-like molecules from the degradation of larger, less soluble plant-derived metabolites at the surface. Most likely all of these processes are contributing to the pattern of DOM change we observe in Fig. 2 and SI Appendix, Fig. S2. Changes in vegetation type could exacerbate these effects. Recent results from the SPRUCE site indicate that warmer temperatures are associated with declining *Sphagnum* moss cover and an increase in vascular vegetation (24) which will likely result in higher inputs of labile DOM with warming (14, 18), thereby stimulating greenhouse gas production in peatlands.

FTICR-MS and GC-MS results both reveal that many amino acids were negatively correlated with temperature (Fig. 3). In contrast, results for individual sugars increased with warming in the GC-MS results (Fig. 3). Results from FTICR-MS are not as clear with regard to carbohydrates. This discrepancy could be due to the generally poor resolution of carbohydrates by ESI-FTICR-MS methods or, more likely, it reflects differences between di/monosaccharide abundances (identified by GC-MS) versus larger carbohydrate molecules (identified by FTICR-MS). Lower abundances of large carbohydrates relative to di/monosaccharides could indicate increasing hydrolysis (or other mechanisms) breaking down larger carbohydrates (e.g., plant cellulose) into their substituent monomers at warmer temperatures. This interpretation is also consistent with the observed increase in soluble lignin-like compounds (Fig. 3), both of which can be formed during the breakdown of plant cell walls. However, hydrolytic enzymes did not show a consistent correlation with temperature: Some were positively correlated while others declined with increasing temperature (SI Appendix, Table S7). Thus, the increase in monosaccharides may also be due to increasing exudation of sugars from roots, particularly glucose, fructose, and maltose, which are known to comprise a large portion of potential sugar exudates (67). Consistent with this hypothesis, vanillic acid (4-hydroxy-3-methoxybenzoic acid), which can support microbial respiration (68), also increased with temperature (SI Appendix, Fig. S5). This suggests the possibility that this degradation pathway was slowed or inhibited at elevated temperature perhaps via inactivation of enzymes or a shift in the microbial community (69), or because there was an increasing availability of alternative more labile compounds present. This suggests a top-down control on the rising CO_2 and CH_4 production rates with temperature: Increasing abundance of simple sugars stimulates fermentation and subsequent methanogenesis.

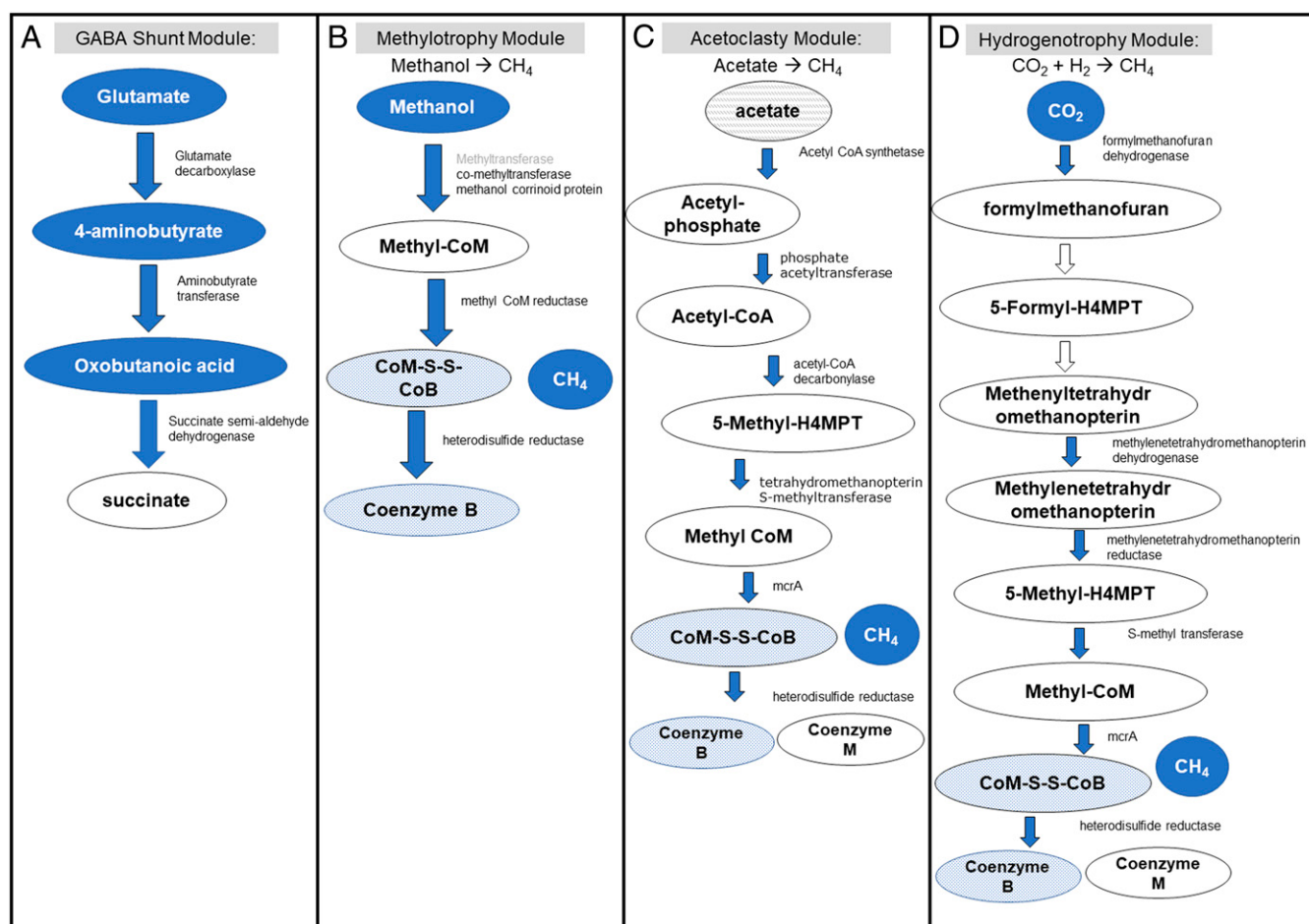


Fig. 5. Results of matching compounds and enzymes to the KEGG module database. We found three of the four compounds and at least one subunit from each of the enzymes involved in the GABA shunt pathway (A; https://www.genome.jp/kegg-bin/show_module?M00027) as well as many of the enzymes and molecules involved in different pathways of methanogenesis (B–D) including methylotrophic methanogenesis (https://www.genome.jp/kegg-bin/show_module?M00356), acetoclastic methanogenesis (https://www.genome.jp/kegg-bin/show_module?M00357), and hydrogenotrophic methanogenesis (https://www.genome.jp/kegg-bin/show_module?M00567). Filled ovals indicate compounds that were identified in our dataset by NMR or GC-MS; hatched pattern ovals indicate compounds for which a consistent *m/z* was found in the FTICR-MS data. Although not quantified by other techniques, the presence of acetate was inferred from low α_C values in the surface consistent with acetoclastic methanogenesis indicating that acetate was present as a substrate. Filled arrows indicate the enzyme catalyzing that step was found in the proteomics dataset, while open arrows indicate the enzyme was not found.

In addition to changes in primary C processing, we also observed changes in secondary metabolites associated with plant drought stress in response to warming. Galactinol and scylloinositol both increased with temperature treatment (*SI Appendix, Fig. S5*). These compounds are involved in the synthesis of many polysaccharides including those produced in plants in response to desiccation stress (70); their production may have been stimulated by the warmer, dryer conditions under warming. In contrast to most amino acids examined (Fig. 3), glutamine increased with warming. Glutamine is central to microbial metabolism as an important component of nucleic acids, proteins, and the initial product following ammonium uptake (71), potentially signaling increased N demand by microorganisms. Kolton et al. (21) suggested increased microbial demand for protein and membrane repair occurs at higher levels of experimental warming, which could lead to increasing ammonium uptake while also depleting the availability of other free amino acids under warming conditions. In parallel, CO₂ concentrations may increase as microbial respiration increases to meet the higher energy demands. We identified three of the four compounds and enzymes catalyzing each of the steps involved in the glutamate–glutamine, GABA shunt module (Fig. 5). In this

pathway, glutamate is decarboxylated to GABA, which then gets converted to succinate. Although we could not unequivocally identify succinate in the NMR data due to a low signal:noise ratio, it was observed in the GC-MS results. The relative abundance of succinate did not change significantly with temperature; however, this metabolite is involved in many metabolic pathways, including the tricarboxylic acid cycle, and therefore the controls on its concentration in the soil are likely complex. The GABA shunt pathway has been observed in plants as a response to temperature shock and in bacteria as a response to acid stress and is thought to be a potential source of nitrogen for bacteria (72). Though not conclusive, finding three of the four major compounds as well as the enzymes linking them provides convincing evidence that this reaction pathway plays a key role at our site. Further, it supports other results that suggest plants are responding to temperature stress and that microorganisms are responding with high N uptake to support elevated protein turnover and membrane repair rates induced by higher temperature treatments (21).

Given the importance of methanogenesis in peatlands (73), it is not surprising to find many of the compounds and enzymes involved in methanogenic pathways (Fig. 5). It was interesting to

note, however, that all of the enzymes and four out of five compounds in methylotrophic methanogenesis were detected. This is consistent with the slight shift toward methanogenesis from methylated substrates observed in the isotopically derived alpha indices as a function of temperature. Consistent with enhanced methylotrophic methanogenesis, we observed a decline in methanol concentrations (*SI Appendix, Fig. S4*). In addition, both the 16S rRNA ASVs and metagenomics datasets provide evidence for the presence of known methylotrophic methanogens in SPRUCE peat (*SI Appendix, Figs. S9 and S11*), with two MAGs containing key genes involved in methylotrophic methanogenesis pathways (*SI Appendix, Table S4*). These MAGs exhibited strong depth stratification, indicating that members of the *Methanobacteriaceae* may be more important methylotrophs in the surface (10 to 20 cm), whereas *C. methanomethyliaaceae* become dominant in deeper peat (100 to 175 cm). Typically, it has been assumed that the majority of CH₄ in peatlands is produced via the hydrogenotrophic and acetoclastic pathways and only recently has the potential importance of methylotrophic methanogenesis gained attention (61, 74, 75). Further, methanol is a degradation product of lignin, and the accumulation of lignin-like compounds in the DOM (*SI Appendix, Fig. S2*) may indicate that methylotrophic methanogenesis will become an increasingly important pathway as warming continues.

Consistent with the porewater CH₄ and multiomics results presented in this study, rate measurements along with in situ CH₄ fluxes quantified at SPRUCE clearly show that methanogens in SPRUCE peat are responding to warming (26). In contrast, no significant change in the absolute abundance of methanogens, as determined by qPCR, was observed (26). We interpret this as a more rapid, pronounced change in the physiology or activity of methanogens in response to temperature, whereas a change in abundance or biomass is more muted and difficult to detect beyond natural heterogeneity. Indeed, the growth of methanogens is expected to be slow given the low thermodynamic yield of methanogenesis under in situ conditions that prevail in northern peatlands (73). Further, in other long-term warming experiments conducted in an aquatic environment, CH₄ production rates increased to a much larger extent in comparison with changes in the abundance or community composition of methanogens (76). Methanogens thus appear to increase their efficiency, resulting in an increase in the CH₄:CO₂ ratio. In addition, changes in abundance or community composition are more difficult to detect due to limited replication of our sequence datasets related to the inability to perform extensive, destructive core sampling at the SPRUCE experiment.

Overall, methanogens previously detected in peat soils and described as hydrogenotrophic (*Methanoflorens*, *Methanoregulaceae*) predominated at all depths at SPRUCE. Consistent with previous work (21, 22, 26), methanogen communities were stratified with peat depth. Throughout the peat column, methanogen ASVs and MAGs with a high sequence identity to *C. methanoflorens* predominated and peaked in relative abundance at 40- to 50-cm depth. Members of the *Methanobacteriaceae*, which are among the most metabolically versatile of methanogen groups, showed the highest relative abundance at the surface 10- to 20-cm depth and were virtually absent at deeper depths. Members of the *Methanoregulaceae*, *Methanomicrobaceae*, and *Methanomethyliaaceae* were detected below 100-cm depth at lower relative abundances. The *Methanomethyliaaceae* are known methylotrophs, producing CH₄ from methylated substrates (63).

Consistent with earlier results from the SPRUCE site (15, 22, 55), depth within the peat profile was the largest driver of geochemical variation. Moreover, detailed examination of small metabolites also revealed changes with experimental warming that differ from those observed with depth. This suggests that

warmer temperatures did not simply enhance decomposition but likely stimulated production of substrates used for downstream fermentation and methanogenesis. Overall, the general trend that emerged was that plant-derived metabolites and simple sugars increased with warming while substrates for microbial biosynthesis generally decreased with warming. The decline in NOSC of DOM compounds with temperature should promote CH₄ production over CO₂ production due to electron balance, thereby helping to explain increasingly methanogenic conditions with warming. Plant-derived metabolites involved in heat and drought stress appeared to increase with warming. Water stress may ultimately reduce CO₂ uptake by plants, via reduction of stomatal conductance (70), which could have adverse implications for C storage in drought-stressed peatlands. Our results add to the accruing body of evidence that warming peatlands become more methanogenic. This shift has significant implications for peatland-climate feedbacks since CH₄ is a much stronger greenhouse gas than CO₂. In agreement with previous models of microbial C-use efficiency, it could be speculated that elevated decomposition rates may represent a transient response to warming that will eventually slow in the future as potential substrates become depleted. However, our detailed analysis of metabolic pathways suggests that this is not the case. Rather, increasing rates of heterotrophic respiration, and especially methanogenesis, are fueled by increasing availability of labile sugars produced by surface vegetation. Thus, elevated rates of methanogenesis are likely to persist even as additional mechanisms of CH₄ production appear to be “turned on” (methylotrophy), further exacerbating climate warming.

Data Availability. All data presented in this manuscript and the supporting files, including figure source data, are publicly available. The mass spectrometry proteomics data have been deposited in the MetaproteomeXchange Consortium via the PRIDE (41) partner repository with the dataset identifiers [PXD019912](https://www.ebi.ac.uk/pride/archive/study/019912) and [10.6019/PXD019912](https://www.ebi.ac.uk/pride/archive/study/019912). All raw 16S rRNA gene sequences have been uploaded to the NCBI under BioProjects [PRJNA640652](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA640652), [PRJNA638786](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA638786), and [PRJNA638901](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA638901). Metagenomes are publicly available in the JGI Genome Portal and NCBI SRA. SRA identifiers for each metagenomics dataset are provided in *SI Appendix, Table S5*. Metabolomics data have been provided in the *SI Appendix* and are publicly available from the SPRUCE long-term repository (<https://doi.org/10.25581/spruce.083/1647173>). Metagenome and proteome sequence data have been deposited in the NCBI, JGI, and PRIDE (see manuscript text and supporting information). All study data are included in the article and/or supporting information.

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