

RESEARCH ARTICLE

Constraints on microbial communities, decomposition and methane production in deep peat deposits

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Abstract

Peatlands play outsized roles in the global carbon cycle. Despite occupying a rather small fraction of the terrestrial biosphere (~3%), these ecosystems account for roughly one third of the global soil carbon pool. This carbon is largely comprised of undecomposed deposits of plant material (peat) that may be meters thick. The fate of this deep carbon stockpile with ongoing and future climate change is thus of great interest and has large potential to induce positive feedback to climate warming. Recent *in situ* warming of an ombrotrophic peatland indicated that the deep peat microbial communities and decomposition rates were resistant to elevated temperatures. In this experiment, we sought to understand how nutrient and pH limitations may interact with temperature to limit microbial activity and community composition. Anaerobic microcosms of peat collected from 1.5 to 2 meters in depth were incubated at 6°C and 15°C with elevated pH, nitrogen (NH₄Cl), and/or phosphorus (KH₂PO₄) in a full factorial design. The production of CO₂ and CH₄ was significantly greater in microcosms incubated at 15°C, although the structure of the microbial community did not differ between the two temperatures. Increasing the pH from ~3.5 to ~5.5 altered microbial community structure, however increases in CH₄ production were non-significant. Contrary to expectations, N and P additions did not increase CO₂ and CH₄ production, indicating that nutrient availability was not a primary constraint in microbial decomposition of deep peat. Our findings indicate that temperature is a key factor limiting the decomposition of deep peat, however other factors such as the availability of O₂ or alternative electron donors and high concentrations of phenolic compounds, may also exert constraints. Continued experimental peat warming studies will be necessary to assess if the deep peat carbon bank is susceptible to increased temperatures over the longer time scales.

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Introduction

Owing to their cool, saturated conditions, northern peatlands serve as an extensive carbon (C) sink storing approximately one third of the world's terrestrial C [1–3]. In these systems, peat profiles accumulate largely undecomposed plant material to several meters deep representing thousands of years of C accumulation [4,5]. Although it has long been established that the peatland C balance is sensitive to anthropogenic disturbance [6], there remains considerable uncertainty about how these systems will respond to changes in climate [7]. Warming trends are expected to be greatest at high latitudes [8] and there has been an increased effort to understand how C cycling processes in northern peatlands will respond to these predicted changes. Much of this effort has focused on the acrotelm, the shallow peat that experiences a fluctuating water table, where it is expected that warmer and drier conditions will stimulate C mineralization [1,9–11]. The catotelm, saturated and anoxic deep peat that may extend meters below the surface, is not likely to experience the drying that may occur in the shallow peat. However temperature-induced changes in microbial community composition or function in the catotelm layer could dramatically alter the C balance in these systems over time.

As part of the effort to understand ecosystem-level responses to climate changes, the Spruce and Peatland Responses Under Changing Environments experiment (SPRUCE; <http://mnspruce.ornl.gov>) was designed to achieve whole ecosystem warming of a boreal peatland system [12]. Located in the Marcell experimental Forest (Minnesota, USA), this regression-based experiment began with a 13-month deep peat heating treatment, where experimental plots were heated up to +9°C above ambient conditions from the surface down to a depth of 2 m. Although surface CH₄ flux was significantly correlated with deep peat heating, results indicated that activity in the surface acrotelm peat, not deep catotelm peat, was responsible for increased CH₄ production [13]. Additionally, Wilson et al. [13] found that microbial communities and C decomposition did not respond to the 13 months of *in-situ* warming. Findings from this first year of the SPRUCE experiment suggested that the deep peat carbon pool may remain stable despite increased temperature. Ambient temperatures in the deep peat remain relatively stable throughout the year, averaging 6–7°C between 1.5 to 2 m at the SPRUCE site. The finding that microbial communities in the deep peat did not respond to even the highest (+9°C) treatment was somewhat surprising given that numerous studies have shown a shift in community structure in response to elevated temperature [14–17]. The structure of peat microbial communities not only determines the functional mechanisms responsible for C decomposition [18], but may also influence temperature sensitivity of respiration rates [17]. Understanding what factors may lead to, or limit, shifts in microbial community structure should aid in constraining C balance of the system and in forming predictions as to the trajectory of these systems under a warming climate.

With low availability of electron acceptors, fermentation and methanogenesis are the primary C decomposition processes in the anoxic catotelm. These processes lead to the accumulation of organic acids and a correspondingly low pH. Ombotrophic bogs typically have a pH ≤ 4.5, well below the reported optimum pH of 5.5 to 6 for methanogenesis [19,20]. Consequently, acidic conditions force physiological constraints on microbial communities and their activities, such as CH₄ production. Rain-fed ombotrophic bogs are also characteristically low in nutrients and previous work has indicated that microbial communities can be limited by low nitrogen (N) and phosphorus (P) availability in these systems. Bragazza et al. [21] saw that *Sphagnum* decomposition rates were correlated with atmospheric N deposition across a European gradient of N deposition, and Tfaily et al. [22] found that N is immobilized in the highly humified deep peat in the same Minnesota peatland under study here. Using a combination of NMR, enzymatic assays, and metagenome sequencing, other previous work has also found

evidence suggesting N and P limitation [23,24]. Hence, nutrient and pH limitations may serve to protect the C bank from elevated temperatures. Conversely, additional perturbations to peatland nutrient cycles, such as N deposition, could have the potential to alter this C balance in peatland systems. Given these uncertainties, the objective of our study was to identify factors that may be limiting decomposition and microbial community change in deep peat deposits. We conducted a 70-day microcosm incubation of deep peat (originating from 150–200 cm depth) at 6 and 15°C to mimic ambient and +9°C SPRUCE project experimental conditions, with added treatments including elevated pH and the addition of N and P in a full factorial design. Microcosms were monitored for CO₂ and CH₄ production, and microbial community dynamics were assessed using qPCR and amplicon sequencing of the bacterial, archaeal, and methanogen communities. We expected that alleviation of each of these potential constraints (pH, N, and P) would lead to increased decomposition as monitored by CO₂ and CH₄ production, as well as shifts in microbial community abundance and composition.

Methods

Site description and sampling methods

The SPRUCE experiment field site (<http://mnspruce.ornl.gov>) is located at the Marcell Experimental Forest S1-Bog (N 47°30.4760'; W93°27.162') in northern Minnesota, USA. Soil in this 8.1 ha ombrotrophic peatland has been characterized as the Greenwood series, a Dysic, frigid Typic Haplohemist, (<http://www.websoilsurvey.nrcs.usda.gov>) with an average depth of 2 to 3 m and pH in the range of 3 to 4.5 that varies with depth. The overstory vegetation consists primarily of *Picea mariana* (Mill.) Britton, Sterns & Poggenburg (black spruce) and *Larix laricina* (Du Roi) Koch (larch) while the understory is dominated by ericaceous shrubs and intermittent herbaceous species with a thick sphagnum surface layer. Sphagnum species vary in with microtopography but *Sphagnum magellanicum* or *S. angustifolium* generally dominate hummocks and *S. fallax* dominate in hollows [25]. The S1-Bog and the SPRUCE experiment has been extensively studied with complete above and belowground characteristics described in previous publications and data products [13,22,26–28]. The SPRUCE experiment includes three raised metal boardwalks extending 88, 112, and 92 m into the S1-Bog. The ends of these transects are ~80 m apart and served as non-treatment areas to sample the deep peat used in this experiment. In August 2016 two cores were taken from each of these three transects with a Russian peat borer (Eijkelkamp, Netherlands) with deep peat deposits (150–200 cm below surface) from each transect serving as separate replicates for the experiments below. Prior characterization of peat from this depth in the S1 bog has shown it to be typically largely hemic, ~6000–7000 years old, with ash content of ~10% and bulk density of ~0.15 g/cm³ [28–30]. Peat from each transect was homogenized in the field, distributed into one liter Nalgene bottles, topped off with porewater taken from the same depth to a zero headspace, then capped and stored at 4°C in a coldroom until laboratory microcosm construction.

Microcosm construction

To examine whether the temperature response of deep peat communities was limited by other environmental factors, a full factorial experimental design with elevated temperature, N, P, and pH treatments was employed with samples from each transect serving as replicates ($n = 3$ for each condition and time point; see below). Each treatment consisted of two levels, control (approximately ambient) and elevated. At our study depth of 150–200 cm, there is low spatial and temporal variation in peat properties [28] with N, P and pH values averaging 24 g N and 650 mg P per kg dry peat, and a pH of ~4.5 [22,28,29,31]. Because we wanted to eliminate potential N or P limitations with minimal secondary effects on respiratory processes such as

denitrification, 2.54 M NH_4Cl was used to increase available N by 24 mg N per g dry peat and 29 mM KH_2PO_4 was used to increase P availability by 0.65 mg P per g dry peat, effectively doubling N and P without adding potential electron acceptors. Peat pH was adjusted to 5.5 using 0.5 M NaOH for the elevated pH treatment in an effort to alleviate potential pH constraints on CH_4 production [32]. Initial 10 day single variable trials were conducted to ensure that nutrient additions did not influence pH and that pH adjustments were stable over time. pH was also verified to be within 0.2 units of target pH on samples taken at 35 & 70 day destructive samplings (S4 Fig). Homogenization, nutrient additions, and microcosm setup were conducted in a glovebag under anoxic conditions and microcosms were constructed with 5 grams of peat distributed into 120 ml serum vials with butyl rubber stoppers. In total, 96 microcosms were constructed to account for the 16 treatment combinations (N x P x pH x temperature), 2 destructively sampled time points (35 and 70 days), and 3 replicates for each time point. Although all peat samples were essentially saturated at the time of sampling, sterile Milli-Q water was used to equalize the water content after processing to ensure equal water content across sample vials representing the treatments and transect replicates. Temperature treatments were maintained at 6°C, to mimic the SPRUCE ambient plot temperatures, and 15°C to mimic the SPRUCE +9°C treatment, with Precision Refrigerated Incubators (Thermo Scientific Inc.). At this depth, *in-situ* peat is largely buffered from temporal temperature fluctuations and remains near these target temperatures year-round [12,28]. Microcosms were placed inside styrofoam coolers within incubators to reduce the chance of temperature fluctuation and incubators were maintained within 0.5°C of target temperatures.

Quantification of CO_2 and CH_4 production

Production of CO_2 and CH_4 was determined by sampling headspace gas in the three replicate microcosms on days 10, 20, 35, 52, and 70. To avoid introducing biases associated with differing sample sizes, on days 10, 20, and 35, these three replicates for gas sampling were chosen at random (from the six available microcosms), and then on days 52 and 70 were done on the three remaining microcosms after destructive harvest for DNA analyses (below). Gas sample measurements were conducted using a SRI 8610C gas chromatograph with a methanizer and flame ionization detector (SRI Instruments) using methods detailed in [33]. Total CO_2 and CH_4 production was calculated as the combined headspace and dissolved gas concentrations (assuming equilibrium) and are reported as $\mu\text{g CO}_2\text{-C}$ or $\text{CH}_4\text{-C}$ per g dry peat.

DNA extraction, quantitative PCR, and rRNA gene amplicon sequencing

Total DNA was extracted from untreated peat samples at the time of setup (time zero) and on samples from destructively sampled microcosms at 35 and 70 days. MoBio PowerSoil DNA extraction kits (formerly MoBio, now Qiagen) were used with a slightly modified protocol that included a 30 minute incubation at 65°C immediately after the bead beating step. Duplicate 0.25 g samples were extracted and combined during an additional cleanup step using the MoBio PowerClean Pro cleanup kit (MoBio). Cleaned DNA extracts were quantified using a NanoDrop ND-1000 (Thermo Scientific).

Abundance of bacterial, archaeal, and methanogen community members was determined by quantitative PCR (qPCR) of bacterial and archaeal 16S rRNA genes, and methyl coenzyme M reductase (*mcrA*) genes using previously described methods [13]. Briefly summarized, Bacterial 16S was targeted using Eub338 and Eub518 primers [34,35], Archaeal 16S was targeted with 915F and 1059R primers [36], and *mcrA*_F and *mcrA*_R [37] were used to target the methanogen specific Methyl-coenzyme M reductase (*mcrA*) functional gene. Triplicate reactions were performed on a CFX96TM Real-Time PCR Detection System (Bio-Rad

Laboratories) with iQ SYBR Green Supermix (Bio-Rad, CA, USA). Ct values were then compared to standard curves for each gene constructed from *Escherichia coli* and *Methanococcus maripaludis* S2 genomic DNA, corrected for copy number and genome size, and are reported as gene copies per gram of dry peat.

Sequencing of 16S rRNA genes followed the two-step approach described previously [38] using frameshifting nucleotide primers and barcode tag templates. However, we used a modified primer mixture to increase phylogenetic coverage of bacteria and archaea. The primer mixture consisted of 10 forward and 7 reverse 515F and 806R primer variants [39] that were combined in equal concentration at 0.5 μ M to amplify the V4 region of 16S rRNA gene (S1 Table). A 0.7 to 1 ratio of Agencourt AMPure XP beads (Beckman Coulter) were used to clean PCR products prior to the secondary PCR with barcoded reverse primers. Experimental units were then pooled based on agarose gel band intensity to make several replicate libraries and again purified with Agencourt AMPure XP beads. Library quality was then analyzed using a 2100 Agilent Bioanalyzer (Agilent Technologies) and the library with the highest quality was selected for sequencing. After quantification with Qubit (Invitrogen), the amplicon library was adjusted to a final concentration of 9 pM, spiked with 15% PhiX and paired-end sequenced using an Illumina MiSeq (Illumina, San Diego, CA, USA) with 500 (2 x 250) cycles.

Data analysis

Data were checked for outliers and normality prior to statistical analysis using R v.3.2.4 [40]. The qPCR data required square root transformation while all other data were analyzed without transformation. Three-way ANOVAs blocked by sampling site (transect) with Tukey HSD mean comparisons were used to test the effect of temperature, nutrient additions, and pH treatments on alpha diversity, taxa abundance, qPCR gene copies, and cumulative gas production after 70 days. Because the aim of this study was to examine which factors have the ability to influence the structure and function of deep peat microbial communities, results were focused primarily on comparing incubation treatments against the control incubations (6°C, no addition, ambient pH). All figures were constructed using R package ggplot2 [41].

Sequence data were processed using a combination of UPARSE and QIIME (v 1.9.1) pipelines [42,43]. Paired-end sequences were joined, demultiplexed, and screened for quality using the `split_libraries_fastq.py` command with stringent quality standards ($p = 0.95$, $r = 1$, $q = 19$). Forward and reverse primers were trimmed using Cutadapt [44]. The UPARSE pipeline was then used to remove low quality sequences and chimeras prior to 97% OTU clustering with the `pick_open_reference_otus.py` command that performs closed-reference OTU picking prior to de novo OTU picking. Taxonomy was assigned using the BLAST algorithm against the Greengenes (v 13.9) database [45,46]. The resulting OTU table was converted into BIOM format [47] and singleton and contaminants (mitochondria and chloroplasts) OTUs were removed. To better assess the starting communities, the three time zero samples were sequenced to a greater sequence depth than the 96 incubated samples, ranging from 2–3 million sequences per sample compared to 6–60 thousand sequences). Because of this, the time zero samples were separated from the incubation samples prior to threshold filtering and community analysis. Bokulich threshold filtering was employed at 0.005% across the OTU table [48] and samples were rarefied to 10,000 sequences and represented 687 OTUs. Representative sequences from each OTU were aligned using PyNAST [49] and a phylogenetic tree was constructed from aligned sequences using FastTree [50]. Alpha and beta diversity metrics were calculated using the `alpha_diversity.py` and `core_diversity.py` scripts in QIIME. Subsequent plotting and statistical analyses of alpha-diversity metrics were carried out in R v.3.2.4 [40] using the previously described blocked, three-way ANOVA. The R package Vegan v2.3–5 [51]

was used to construct principal coordinate analysis (PCoA) plots with the weighted and unweighted UniFrac distance matrices [52] and to test for community responses to treatments using PERMANOVA analyses with Bray-Curtis distance measures. There were no significant differences between 35 and 70 day harvests in microbial community structure, therefore for the results are only discussed for 70 day samples (see supplemental figures S2–S4 Figs for 35 day data). All non-sequence data are available via DOI at the CDIAC data archive (<http://dx.doi.org/10.3334/CDIAC/spruce.040>) and all DNA sequence data are available in the NCBI Bioproject Archive (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA362203>).

Results

CO₂ and CH₄ production

The cumulative amount of CO₂-C released after 70 days was significantly greater in microcosms incubated at 15°C ($p < 0.001$) although neither nutrient additions or pH ($p > 0.1$) influenced the amount of C released as CO₂ (Fig 1). The cumulative amount of CH₄-C released after 70-days was also greater in the 15°C incubations ($p < 0.001$). Although CH₄-C released was not influenced by pH ($p = 0.518$), there was a significant effect of nutrient addition ($p < 0.001$) that was driven by a marked decrease in CH₄-C production in the treatments with nitrogen addition (N & NP) ($p < 0.001$ for both addition treatments). The ratio of CH₄-C to CO₂-C also increased with elevated temperature ($p = 0.016$) but decreased with N and NP additions ($p < 0.001$ for both). There were no significant interaction effects in either the CO₂ or CH₄ analysis.

Microbial abundance measures with qPCR

Fig 2 shows the overall response of microbial community members to incubation temperature, nutrient additions, and pH treatments. Data were log transformed prior to analysis with a blocked ANOVA to account for the variation in abundances among the original transect samples that served as replicates in our experiment. Bacterial 16S rRNA gene abundance was not influenced by incubation temperature ($p = 0.181$) but increased under elevated pH conditions ($p < 0.001$). Compared to controls, bacterial abundance decreased with N and NP additions under ambient pH ($p < 0.001$ for both additions). However, under elevated pH conditions, bacterial abundance in N and NP additions were not significantly different from control incubations ($p = 0.999$ for both). Under ambient pH conditions archaeal 16S rRNA gene abundance decreased with elevated incubation temperature ($p = 0.011$). However at 15°C, elevated pH incubations had significantly increased archaeal abundance compared to unamended controls ($p = 0.009$). The archaeal response to nutrient additions mirrored that of the Bacteria in that incubations receiving N (and NP) addition had significantly lower copy numbers compared to controls ($p < 0.001$ for both N and NP), while N and NP additions combined with elevated pH were not significantly different from control ($p = 0.891$ and $p = 1.000$). Methanogen abundance, measured by quantifying the *mcrA* gene copy number, followed a similar pattern as the archaeal 16S gene abundance. Elevated incubation temperature led to a lower abundance of methanogens under ambient pH conditions ($p = 0.001$) while methanogen abundance was greater than controls under elevated pH conditions ($p < 0.001$ for both temperatures). Decreased methanogen abundance was also seen with N and NP additions at ambient pH ($p < 0.001$ for both additions), but this effect was not evident under elevated pH conditions ($p = 0.482$ and $p = 0.599$). Although both bacterial and archaeal abundances decreased with N and NP additions (see above), the ratio of Archaea to Bacteria increased only with N addition ($p = 0.038$). Elevated incubation temperature reduced the ratio of Archaea to Bacteria ($p = 0.001$) suggesting that higher incubation temperatures favor bacterial

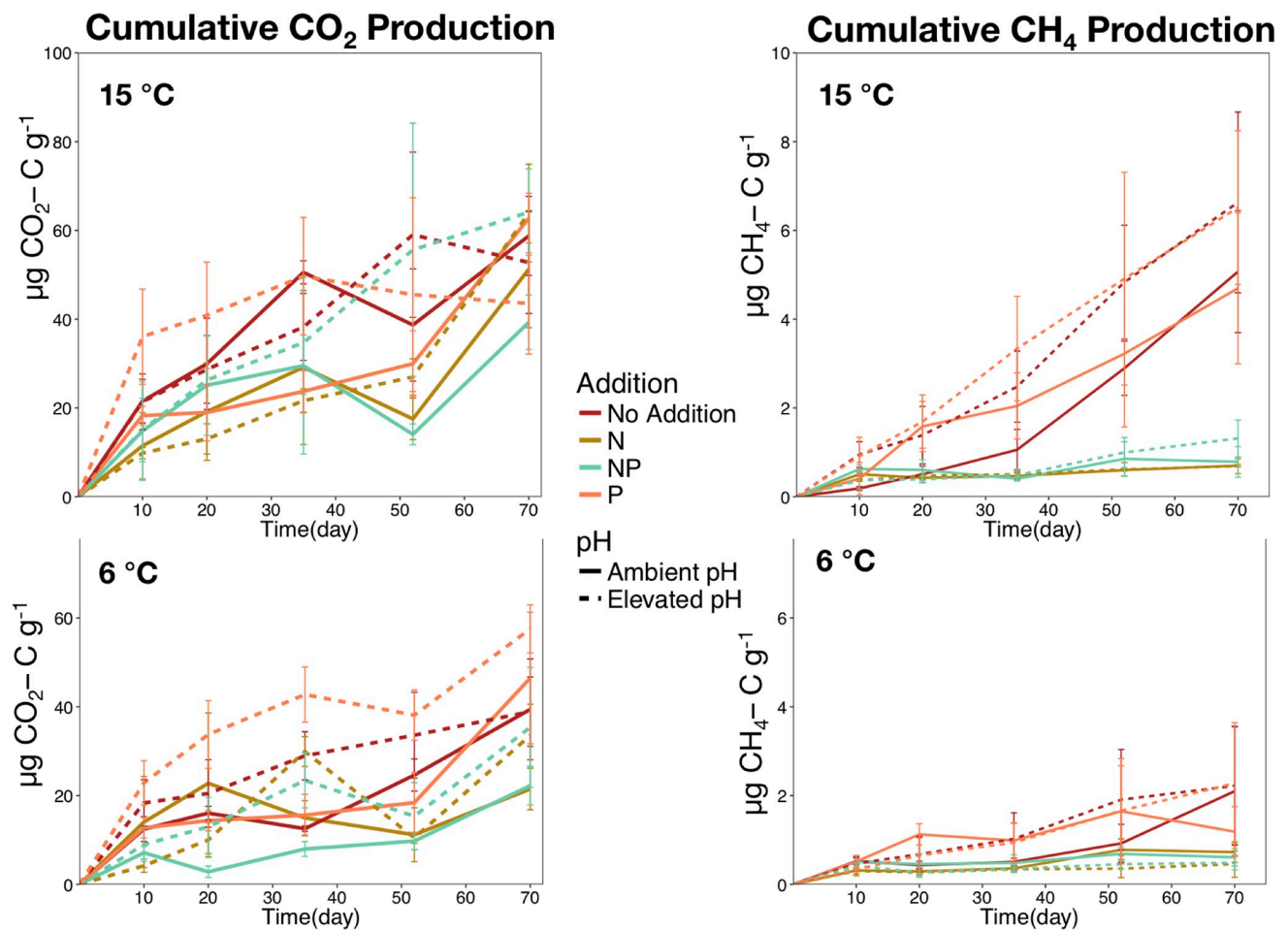


Fig 1. Cumulative production of CO₂ (left) and CH₄ (right) in peat microcosms incubated at at 6 (bottom) and 15°C (top). Nutrient addition treatments are coded by color and the elevated pH treatment is represented by dashed lines.

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populations; however as mentioned above, the overall bacterial abundance increases with temperature were not significant ($p = 0.181$). The addition of P alone did not significantly influence the abundance of bacterial, archaeal or methanogen community members.

Microbial community composition with rRNA-gene sequencing

Both weighted and unweighted PCoA ordinations (Fig 3) show significant clustering by transect, which was confirmed by PERMANOVA analysis ($p < 0.001$ for both). Despite the distinct transect clustering pattern within the unweighted ordination, pH also had a significant influence on community composition in both the weighted ($p = 0.001$) and unweighted ($p = 0.002$) ordinations and tends to separate along PC1. Driven by the N and NP additions, nutrient addition was a significant grouping factor in the weighted ordination ($p = 0.001$), although there was not a significant effect of nutrient addition in the unweighted ordinations. Both ordinations and PERMANOVA tests indicated that the community structure was not influenced by temperature or incubation time. After 70 days of incubation, microcosm alpha diversity metrics (Chao1 and Observed OTUs) were significantly lower in the elevated pH

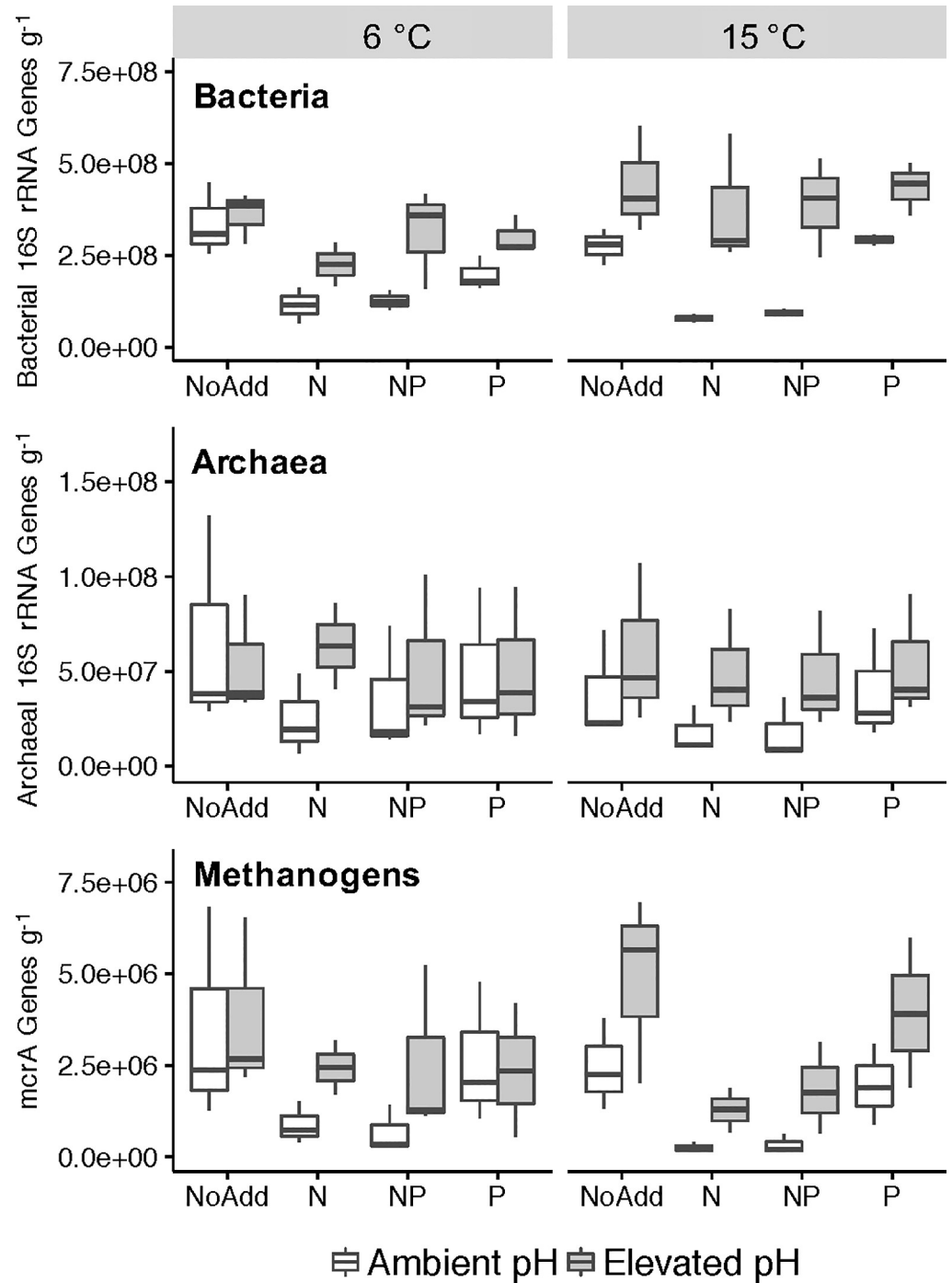


Fig 2. Bacterial, Archaeal, and methanogen abundance measured by qPCR after 70 days of microcosm incubations. Bacterial and archaeal population sizes were determined by quantifying 16S rRNA gene copies and methanogens were assessed by quantifying *mcrA* gene copies. pH adjustment treatments are shown in grey, and unadjusted treatments are shown in white. Abundance is presented as gene copies per g dry peat.

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treatments ($p < 0.001$ for both) but did not vary significantly among nutrient addition treatments (Fig 4). There was also significant spatial variation in alpha diversity metrics (Chao1 $p = 0.022$ and observed OTUs $p = 0.001$). Microcosm replicates with peat originally from

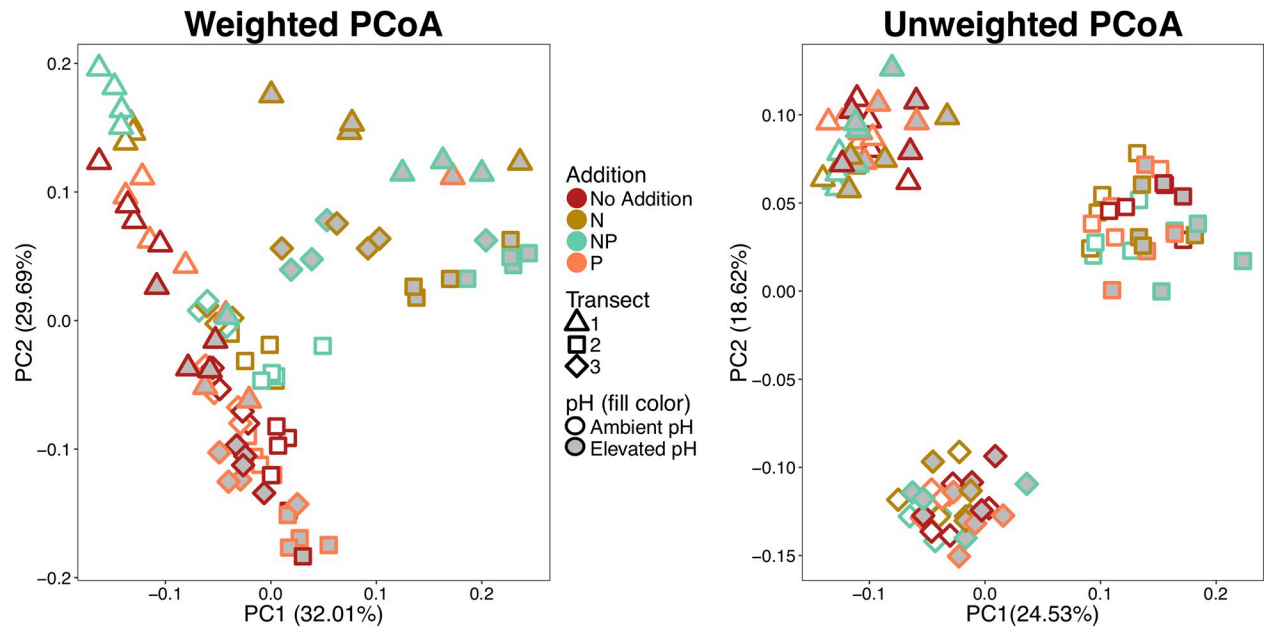


Fig 3. Weighted and unweighted UniFrac PCoA ordinations of microcosm microbial communities. Each point represents an individual microcosm. Color, shape, and fill are used to code for by nutrient addition, transect (origin location), and pH treatment, respectively. Ordinations include samples from both temperature treatments and time points (not denoted) as they did not significantly differ.

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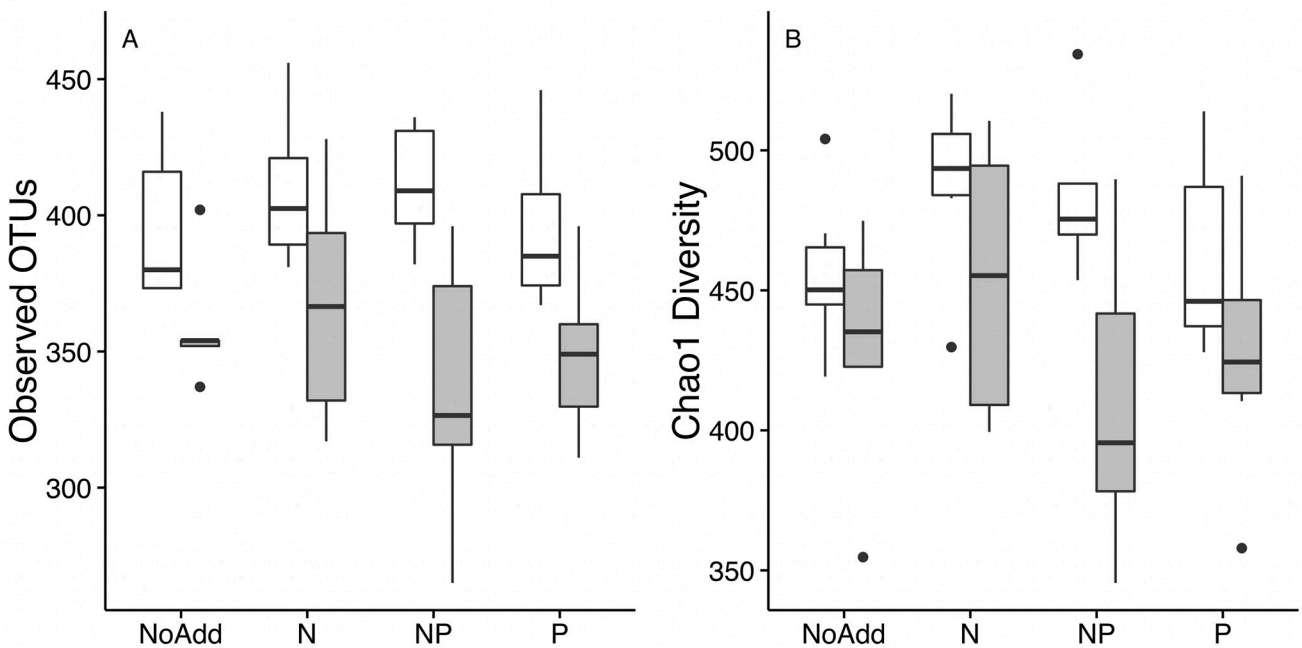


Fig 4. Alpha diversity metrics of microcosm communities after 70 day incubations. pH adjustment treatments are shown in grey, while unadjusted treatments are shown in white. Treatments incubated at 6 and 15°C were not significantly different and are combined for simplicity, thus each bar represents 6 microcosms.

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Table 1. Mean percent relative abundance (and standard deviation) of dominant microbial phyla and Proteobacteria classes after 70 day anaerobic incubation. Data represent combined values for 6 and 15°C temperature treatments as temperature had little effect on overall microbial community composition and Bacteroidetes was the only taxa to have significant temperature response ($p = 0.04$). Asterisks denote significant difference from the control samples (no addition, ambient pH) determined by Tukey adjusted p-values denoted as ***, **, and * for $p = 0.001, 0.01,$ and $0.05,$ respectively.

	No Addition		N Addition		N & P Addition		P Addition	
	Ambient pH	Elevated pH	Ambient pH	Elevated pH	Ambient pH	Elevated pH	Ambient pH	Elevated pH
Crenarchaeota	3.12 (2.06)	2.25 (1.28)	3.12 (2.05)	2.54 (1.77)	3.17 (2.36)	1.87 (0.98)	2.80 (1.27)	1.59 (0.83)
Acidobacteria	19.01 (4.29)	21.7 (4.86)	16.1 (2.74)	10.1 (3.04)**	16.0 (2.21)	8.39 (4.01)***	20.3 (3.10)	18.6 (5.36)
Actinobacteria	0.54 (0.14)	0.38 (0.05)	0.81 (0.27)	3.59 (6.69)	0.75 (0.23)	2.16 (2.66)	0.61 (0.16)	0.33 (0.12)
Bacteroidetes	7.74 (1.70)	7.56 (1.11)	6.84 (1.05)	2.86 (1.15)**	7.63 (1.73)	4.04 (2.56)*	7.50 (2.78)	6.25 (2.53)
Chloroflexi	1.61 (1.04)	1.30 (0.70)	2.43 (1.19)	1.32 (0.95)	2.70 (1.21)	1.01 (0.45)	1.57 (0.90)	0.93 (0.45)
Fibrobacteres	1.09 (0.64)	1.53 (0.77)	1.91 (0.58)	0.85 (0.35)	2.01 (0.59)	0.62 (0.38)	1.47 (0.75)	1.00 (0.91)
Firmicutes	6.23 (3.34)	6.26 (5.55)	6.22 (1.32)	2.95 (1.27)	5.68 (1.53)	2.63 (2.39)	4.15 (1.41)	6.70 (4.17)
Proteobacteria	53.5 (7.03)	53.8 (5.91)	53.3 (5.74)	71.5 (7.90)**	52.4 (8.44)	75.8 (9.42)***	54.4 (3.33)	60.8 (10.4)
Alpha	7.66 (2.37)	7.15 (2.75)	6.78 (1.75)	3.08 (1.05)	8.50 (3.47)	4.41 (4.60)	9.14 (2.21)	7.03 (5.00)
Beta	35.9 (10.0)	37.7 (6.67)	34.8 (8.17)	16.8 (6.45)*	32.5 (11.5)	17.9 (11.4)	35.0(5.52)	34.2 (14.5)
Delta	7.61 (4.13)	6.78 (1.53)	7.13 (2.99)	4.25 (2.16)	7.36 (3.18)	3.86 (2.37)	7.10 (3.15)	6.27 (4.41)
Gamma	2.34 (1.78)	2.21 (0.95)	4.68 (2.25)	47.4 (17.8)***	4.05 (0.63)	49.6 (25.8)***	3.17 (1.81)	13.3 (25.3)
Verrucomicrobia	3.28 (1.12)	2.24 (0.88)	4.12 (1.39)	1.73 (0.94)	3.84 (1.29)	1.43 (0.84)	3.09 (1.09)	1.67 (0.68)

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transect 1 had significantly greater diversity than the other two transects, even after 70 days of incubation. This trend is also seen in peat samples that were harvested at time zero, although only one sample from each transect was sequenced (S2 Fig). Similar results were seen at 35 days of incubation (S3 Fig) with diversity in transect 1 being higher than the other transects and elevated pH treatments having lower diversity than ambient pH. Similar to the community composition measures, these alpha-diversity metrics did not change with incubation temperature.

Relative abundance of dominant phyla and Proteobacteria classes present at the end of the incubation period were primarily influenced by the interactive effects of nutrient addition and pH treatment (Table 1). The significant differences occur only with N or NP additions combined with Elevated pH, where the treatments a had a significantly lower relative abundance of Acidobacteria and Bacteroidetes that corresponds with a substantial increase in the relative abundance in Gammaproteobacteria. This is congruent with the community analysis where the N addition and Elevated pH samples were significantly clustered and divergent from other samples (Fig 3). Incubation temperature had little effect on the relative abundance of dominant taxa. In fact, the only phyla with a significant main-effect temperature response was Bacteroidetes, where mean relative abundance was slightly, but significantly, greater in samples incubated at 6°C, rather than samples at 15°C (6.87% vs. 5.64%, respectively; $p = 0.042$). Other than the significant interactions discussed above and presented in Table 1, no taxa were significantly influenced by nutrient addition alone. However, elevating pH had a significant negative effect on several taxa; including Chloroflexi, Fibrobacteres, Alphaproteobacteria, and Verrucomicrobia that all had lower relative abundances in incubations with elevated pH than incubations carried out at ambient pH (p -values < 0.001; S2 Table).

Discussion

In this laboratory study, we found that the mineralization of deep peat C to CO₂ and CH₄ was largely controlled by functional, rather than structural, responses of the microbial community to increased temperature. Elevated temperature increased production of both CO₂ and CH₄,

as well as the ratio of CH₄:CO₂ produced. Despite these physiological responses of increased C mineralization, elevated temperature had a relatively minor influence on the microbial community composition. qPCR analyses showed both overall archaeal and methanogen population sizes decreased with elevated temperature, although the size of the bacterial population showed no response. Similarly, the overall community structure was not significantly influenced by temperature. Contrastingly, elevating pH led to significant shifts in peat community structure, and effects of on CO₂ and CH₄ production trended higher, but were not statistically significant. The addition of N reduced CH₄ production and the size of bacterial and archaeal populations. Although population sizes were less impacted by N addition combined with elevated pH, the structure of the microbial community experienced a dramatic shift and CH₄ production remained significantly lower than the control microcosms.

Controls on CO₂ and CH₄

The well-established relationship between temperature and soil respiration [53] holds true in peatland systems with Yavitt et al. [54] describing temperature as the “master variable” controlling CO₂ production in *Sphagnum*-derived peat bogs. Numerous studies have shown similar results and while there is little doubt that elevating peat temperature will increase peatland CO₂ emission, the source of the C remains unclear. Dorrepaal et al. [9] reported elevated CO₂ emissions in an in-situ warming experiment and attributed 69% of the additional CO₂ to the deeper peat layers. However, Wilson et al. [13] found that only peat from the acrotelm produced more CO₂ under elevated temperatures. Yet in our microcosm study, increased CO₂ and CH₄ emissions were observed with elevated temperatures using catotelm peat sampled from the same bog system as Wilson et al. [13]. Hodgkins et al. [55] postulated that, following an exhaustion of labile carbohydrates with elevated temperatures, long-term responses of high latitude peat may nonetheless become limited by accumulated aromatic compounds, similar to tropical peatlands. Thus, coupled assessments of long-term emissions and changes in organic substrate composition (e.g., carbohydrates vs. aromatic compounds) should be performed in future studies of high latitude peat subject to elevated temperatures. It is estimated that over 75% of the C in the S1 bog ecosystem is held within the deeper catotelm layers below 50cm [28,29]. The competing C mineralization processes of fermentation and methanogenesis further complicate understanding of the C balance in peatland systems. While both CO₂ and CH₄ emissions increase with temperature [56], the relationship between CH₄ and temperature is not as strong as for CO₂, leading Yavitt et al. [54] to suggest that CH₄ production was limited by factors other than temperature. At the S1 bog, isotopic evidence indicates that acetoclastic methanogenesis dominates in shallow peat while hydrogenotrophic methanogenesis dominates in deeper peat [13]. Both of these pathways are inhibited under low pH conditions leading to increased CH₄ production in peat incubations with elevated pH [19,32]. Elevated pH treatments consistently trended toward greater CH₄ production (Fig 1), however likely due to the large differences between our replicates from that were taken from different parts of the bog, these were not significant. This agrees with the suggestion by Ye et al. [32] that factors other than pH can be responsible for the relatively low CH₄ production in ombrotrophic peatlands, compared to more minerotrophic systems. However, while both Williams and Crawford [19] and Ye et al. [32] observed significant increases in methanogenesis above pH 5, they also found peak production to be at pH 6.0 and 6.5 respectively, suggesting that further increases may be needed to better alleviate this constraint.

Although N limitation has been implicated in suppressing C decomposition in ombrotrophic peatlands, these studies have primarily focused on surface processes and the evidence is far from conclusive. Nutrient addition and gradient studies have shown that addition of

exogenous N increases decomposition and CO₂ emissions in peat bogs [21], while others found N additions have no or mixed effects [57–59], or even suppress decomposition [57,59,60]. The addition of N did not lead to increased CO₂ or CH₄ production in our study of deep peat deposits. In fact, N addition had a detrimental effect on the microbial community leading to decreased population sizes, altered community composition, and decreased C mineralization. We intentionally selected NH₄ as a N source to avoid stimulating denitrification, and known nitrate-dependent anaerobic methane and ammonium oxidizing organisms were not among those identified as shifting in rRNA-gene based analyses. The mechanism of this effect of N addition remains unresolved by our study, however primarily from the study of other systems such as anaerobic digestors and sewage plants, NH₄ is known to be an inhibitor of anaerobic processes and methanogenesis at high addition rates [61,62] and similar phenomena may have contributed to our observations as well. It is possible that the level of N addition used in this study was toxic to some members of the peat microbial community adapted for N limitation, and that a more subtle increase in N would have resulted in a differing response. Molecular and enzymatic data have suggested the possibility of P limitation in the S1 bog [23,24,31,63], and like most ombrotrophic bog ecosystems, peat and porewater samples from the SPRUCE site are low in N and P [28,29]. However, P did not seem to alleviate constraints on CO₂ or CH₄ production in our deep peat microcosms at the levels added here, as the addition of P had no significant effect on gas production or microbial community structure. Our findings show that overall, low nutrient conditions are not likely a primary factor limiting decomposition in the deep peat C bank, thus this large deep peat stock may be subject to accelerated decomposition under the increased temperature of the SPRUCE experiment given further time.

Role of microbial community

Similar to the findings of Wilson et al. [13], we did not see overall changes in community composition in response to elevated temperatures, although archaeal, including methanogen, population abundance decreased slightly in qPCR measures. Interestingly, these temperature suppressed methanogen populations were apparently offset by increased metabolic activity of others, resulting in significantly increased CH₄ production. Organic carbon mineralization to CO₂ was similarly uncoupled from microbial population levels as measured by qPCR. Other studies have found significant correlations of CH₄ production to methanogen population activity levels using RNA based analyses [64–66]. Although few studies have examined the responses of microbial communities to peat warming, especially from the deeper layers, many such experiments have been conducted in mineral soils. A meta-analysis of 75 manipulative experiments found that the response of soil communities to warming experiments were best explained by local climate and ecosystem type [67]. Although peat community structure hasn't responded to ex-situ warming in this experiment or in-situ warming in the early SPRUCE experiment, continued long-term warming of SPRUCE plots may lead to altered community structure much like the delayed response seen in other long term soil warming experiments in upland and tundra soils [15,68,69]. However, while peatland microorganisms could be considered 'slow growers' and thus need more time for observable shifts in community structure, we did witness significant shifts in community indicators with other experimental factors, suggesting a lag between physiological response and community structure that is specific to increased temperature.

Elevated pH had significant effects on the microbial population sizes, with greater abundances of all microbial groups examined using qPCR. Although pH did influence community structure, the largest treatment effect was seen in microcosms with elevated pH and N addition

(elevated pH + N and elevated pH +NP). The combined effect of elevated pH and N addition decreased the abundance of most community members while increasing a single genera, *Rahnella*. Sequences from this psychrotolerant Gammaproteobacteria comprised 40–50% of the sequence libraries from microcosms with elevated N and pH. Soil pH is known to be an important variable in determining microbial community structure [70,71] and thus a community response to pH was expected. In addition to constraining overall microbial communities, physiological adaptations allowing microbes to survive in this low pH, anoxic environment appear to come at the cost of lower biocatalytic activity compared to neutral pH anoxic sediments [72]. Microcosms with elevated pH trended toward elevated CO₂ and CH₄ production (Fig 1), however these results were not significant due to high variability between replicates. pH has been shown to be a key factor limiting phenol oxidase activity across multiple peatland systems [73]; thus, further analysis of its limiting effect on the decomposition of deep peat in our systems is warranted.

Although methanogen abundance and activity in the bog profile peaks around 30–50 cm in the acretolm [13], our findings show that elevated temperatures have the potential to increase CH₄ production in the deep peat. Whether CH₄ produced in the deep peat will diffuse to the surface and be released to the atmosphere, or perhaps be consumed by methane-oxidizing taxa, remains to be determined. Others have reported that *Sphagnum*-associated methane-oxidizers are ubiquitous across *Sphagnum* bogs and methane-oxidation rates increase with temperature [64,66,74]. However the ability of methane oxidation to reduce peatland CH₄ emissions is largely dependent on both water level and the extent of the temperature increase [75]. Additionally, it is possible that anaerobic methane oxidation (AOM) may also limit observable changes in methanogenesis in both our mesocosm experiments and natural systems [76–79]. However, evidence to date suggests these processes are primarily dependent upon either nitrate or sulfate as electron acceptors, and thus may be more important in freshwater wetlands and minerotrophic peatlands vs. more ombrotrophic systems like the S1 bog [76–78,80,81].

The majority of work examining microbial communities in peatlands has focused on *Sphagnum* and plant-associated microbes [74,82] and near surface peat [83–85]. Wilson et al. [13] examined peat communities down to 200 cm from across the SPRUCE experimental site and found that spatial heterogeneity was largely overshadowed by the strong depth stratification. Despite the relatively homogenous physical and chemical properties of deep peat at the S1 bog [22,29], pore water chemistry shows greater spatial variability at depth [28,30] thus suggesting that low lateral and vertical flow of porewater could limit microbial dispersal and contribute to patchy distributions [86]. Indeed, porewater movement, and thus substrate transport and availability, may be a key difference between our previous in-situ results and this microcosm study. The artificial conditions and homogenization of the peat matrix in our microcosm setup could have increased substrate availability, and may help explain increased CO₂ and CH₄ production in the absence of microbial community change. The aim of this study was not to examine microbial communities across the entire bog ecosystem, but rather to examine how peat communities respond to warming and which factors may limit a structural and/or functional response. However, peat from three separate transects was used for microcosm replicates and sequencing revealed distinct communities at each location. Further, the transect blocking variable was significant in the analysis of archaeal and methanogen populations and CH₄ production. Altogether, these findings suggest that although peatlands will contain sites with differential microbial composition capable of greater CH₄ production, distinct communities from each transect could have similar functional and structural responses to treatments.

While the surface peat may be most sensitive to elevated temperatures [13,19,87], our findings support Dorrepaal et al. [9] in showing that the deep C bank is indeed at risk under

elevated temperatures as we found that temperature is indeed a factor limiting microbial activity in deep peat reserves in the S1 bog. However, additional factors such as low initial population sizes, substrate availability, etc. are likely limiting in-situ decomposition of these deep peat layers, and how these respond to the longer-term SPRUCE manipulations, remains for additional study. Despite a relatively stable microbial community structure and population size, microorganisms inhabiting the deep peat seem to have the potential to become more active under elevated temperatures and thus accelerate the mineralization of the peatland C bank. These results show that elevating temperature can also increase the relative amount of C being mineralized to CH₄, thus having the potential of further accelerating the feedback loop of climate warming.

Supporting information

S1 Fig. Rarefaction curve and relative abundance of phyla in peat samples from each of the three transects harvested at time zero. Transect one had greater diversity and a higher relative abundance of Archaeal sequences.

(TIFF)

S2 Fig. Alpha diversity metrics of microcosm communities after 35 day incubations. Treatments incubated at 6 and 15°C were not significantly different and are combined for simplicity, thus each bar represents 6 microcosms. White bars represent ambient pH and grey bars elevated pH treatments.

(TIFF)

S3 Fig. Bacterial, Archaea, and methanogen abundance measured by qPCR after 35 days of microcosm incubations. Bacterial and archaeal population size was determined by quantifying 16S rRNA genes and methanogens were assessed by quantifying *mcrA* gene copies. Abundance is presented as gene copies per g dry peat.

(TIFF)

S4 Fig. Microcosm pH measured after destructively harvesting 35 and 70 days of incubations. Elevated pH conditions persisted throughout the duration of the experiment.

(TIFF)

S1 Table. Expanded list of primer mixtures applied for amplicon sequencing. Adapted for Illumina amplicon sequencing after Lundberg *et al.* 2014.

(DOCX)

S2 Table. Mean relative abundance (and standard deviation) for taxa with significant main-effect p-value for pH. Bold indicates taxa that had a main effect of pH with no addition interaction. Bacterioidetes was the only phyla with significant main effect temperature differences, and decreased in relative abundance from 6.87% at 6°C to 5.64% ($p = 0.04$).

(DOCX)

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