SOIL WARMING EFFECTS ON METHANE PRODUCTION PATHWAYS AND HOMOACETOGENESIS IN A NORTHERN MINNESOTA PEATLAND

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THESIS ABSTRACT

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Peatlands have sequestered one third of terrestrial soil organic carbon while simultaneously emitting the potent greenhouse gas methane, and the response of these ecosystem functions to climate change remains largely unknown. Gaining a mechanistic understanding of the processes underlying anaerobic methane cycling is imperative to this question, especially elucidating the relative importance of the acetoclastic and hydrogenotrophic methane production pathways. Homoacetogenesis, the reduction of carbon dioxide with dihydrogen to acetate, is a highly understudied process due to being viewed as thermodynamically unfavorable, but it may play an important role in anaerobic carbon cycling. Here we show that homoacetogens are strong potential competitors with hydrogenotrophic methanogens, and this effect is most pronounced in deeper and colder peat depths. Our results indicate that a better understanding of understudied processes may be essential in predicting the response of anaerobic carbon cycling in peatlands to climate change. This dissertation contains unpublished co-authored material.

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Dedicated to my late younger brother, Pete LeeWays

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CHAPTER I

INTRODUCTION

Wetlands and Carbon Storage

Wetlands are globally significant ecosystems with a variety of critical ecosystem functions, most notably storing considerable amounts of carbon[1,2]. This is due to the prolonged inundation of the soil, which greatly reduces the availability of oxygen and lowers subsequent decomposition rates to a point where annual production of biomass is greater than decomposition[3]. This carbon accumulation is most extensive in peatlands, which comprise 4 million km^2 globally[4]. As a result, peatlands, covering approximately 2.7% of the earth's surface, contain roughly one third of the planet's terrestrial organic carbon stores, with estimates ranging from 455-612 Pg carbon[5–7]. As a consequence of anoxic conditions in the soil, peatlands also produce methane (CH_4), a potent greenhouse gas[8]. Wetlands are the largest natural source of atmospheric CH_4, and the response of their large carbon stores to future warming is still not well understood[9,10].

Sources of CH_4

CH_4 is the most abundant organic chemical in the atmosphere, contributing to about 20% of total radiative forcing[11]. Due to its molecular structure, CH_4 has a sustained-flux global warming potential of 45 over 100 year period, meaning when fluxes are sustained over time, the emission of 1 kg CH_4 would be offset by the sequestration of 45 kg carbon dioxide (CO_2)[8]. CH_4 emissions can come from a variety of anthropogenic and natural sources. Since 1980, natural sources have contributed between 33 and 54% of CH_4 emissions, while between 46 and 67% have been attributed to anthropogenic

sources[11]. The prominent natural sources include wetlands, freshwaters, and geological sources, whereas anthropogenic emissions are attributed to burning of fossil fuels, enteric fermentation, rice cultivation and others[10,12,13]. Of these, wetlands are the single largest natural source, although rice fields could be considered agricultural wetlands controlled by the same principles as natural wetlands. While the median wetland emission of CH_4 from published studies is 164 Tg yr^{-1}, about a third of total global emissions, wetlands also explain 70% of recent interannual variability in surface emissions (± 12 Tg of CH_4 yr^{-1}) [9,14]. The large and variable CH_4 fluxes from wetlands highlight the importance of understanding the processes that control CH_4 production in order to inform future climate models.

Anaerobic Controls on CH_4 Production

Heterotrophic microbial communities within soils utilize inputs of organic carbon from plant photosynthates for use as electron donors. This organic matter is broken down into simple substrates through a suite of microbial metabolic pathways. Within anaerobic soils, this process starts with degradation of complex polymers by microbial exoenzymes, followed by degradation by fermenters into organic acids, alcohols, and methylated compounds[15]. These compounds are further broken down into the substrates for the two major methanogenic pathways: hydrogenotrophic and acetoclastic methanogenesis (Figure 1).

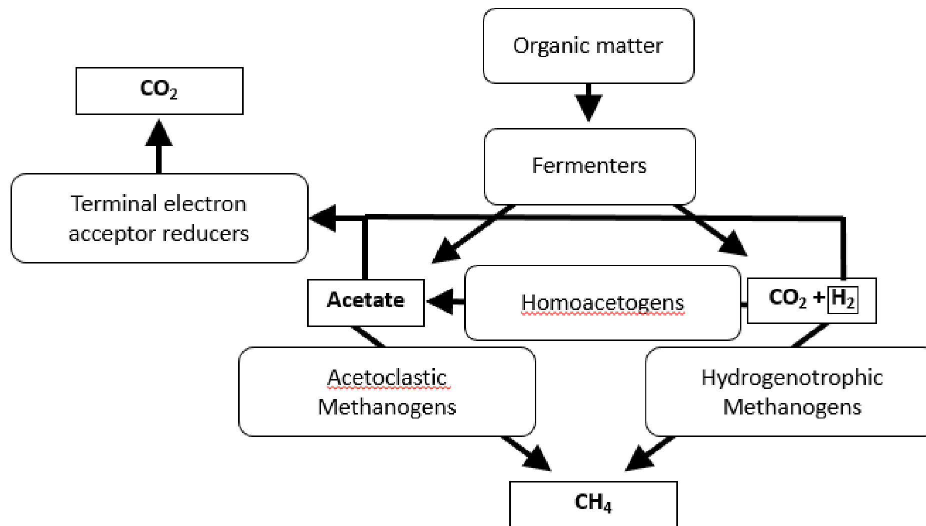


FIGURE 1 | **Conceptual diagram of CH_4 production and homoacetogenesis pathways.** The production of CH_4 in carbon-rich anaerobic ecosystems is controlled by a complex suite of microbial reactions. In particular, the relative importance of CH_4 production pathways as well as homoacetogenesis are poorly understood; homoacetogenesis has been traditionally discounted due to commonly being viewed as thermodynamically unfavorable. However, our findings demonstrate homoacetogenesis is an important pathway within anaerobic carbon and acetate cycling and may consequentially impact how CH_4 producing systems respond to climate change.

Hydrogenotrophic methanogenesis is a chemoautotrophic pathway that oxidizes di-hydrogen (H_2) to CH_4 using CO_2. Acetoclastic methanogenesis utilizes the dismutation of acetate, where the molecule is activated into acetyl-coenzyme A, from which it is cleaved to produce CO_2 and CH_4[16,17]. The fermentation products utilized by methanogens can also be utilized by other microbial groups that use a variety of terminal electron acceptors (TEAs) in their metabolism[18]. These processes include NO_3^- (denitrification),

Fe(III) (iron reduction), Mn(III, IV) (manganese reduction), SO_4^{2-} (sulfate reduction), and humic substance reduction[19]. According to thermodynamic theory, all of these reactions are more favorable than methanogenesis, and therefore CH_4 production would be competitively suppressed by TEA-reducing processes until those TEAs have been consumed. However, these processes have been shown occurring simultaneously in situ and in laboratory incubations[20]. Additionally, when carbohydrates or similar forms of organic matter are degraded, hydrogenotrophic methanogenesis should theoretically account for no greater than 33% of total CH_4 production; however, observational evidence shows that the hydrogenotrophic pathway dominates in ombrotrophic bogs[21–24]. This is one example of a large body of evidence that thermodynamics does not always control if microbial reactions occur, and that ecological and physiological factors may regulate microbial competition more than previously thought.

Homoacetogenesis

Within anaerobic environments, acetate is an important intermediate metabolite as it is utilized by various groups of microorganisms, including iron and sulfate reducers as well as methanogens[18]. Acetate is produced through fermentation of complex organic polymers or through homoacetogenesis. Homoacetogenesis, also known as chemoautotrophic acetogenesis, is defined as the reduction of CO_2 with H_2 to acetate through the acetyl Co-A pathway[25]. Therefore, the production of acetate serves as an important control in CH_4 dynamics because it is both a substrate for acetoclastic methanogens, and homoacetogens are a potential competitor for H_2 with hydrogenotrophic methanogens. Homoacetogenesis is generally considered a

thermodynamically unfavorable reaction due to low H_2 partial pressure in porewater as a result of H_2 consumption by TEA reducers and methanogens[26–28]. However, homoacetogens have been suggested to outcompete methanogens at low temperatures[29–32], and have been shown to do so across multiple wetland types[33]. A possible explanation for this competition could be homoacetogens utilizing interspecies H_2 transfer[34]. Additionally, anaerobic peatland soils with low concentrations of TEAs have been shown to accumulate acetate and produce little CH_4[22,24,35,36]. The mechanistic controls of these dynamics are not well understood, but one possibility is homoacetogens are outcompeting methanogens under certain conditions.

Recently, the importance of homoacetogenesis has become apparent in reaction-network models attempting to quantify rates of microbial carbon transformation in peatlands[37]. These models incorporate temporal changes in porewater concentrations and stable carbon isotopes of CH_4 and CO_2, as well as microbial community data suggesting abundance of homoacetogens, to predict rates of greenhouse gas production[38]. The inclusion of homoacetogenesis not only increases the convergence of models with field data, but the presence of the process and degree to which it is coupled to acetoclastic methanogenesis leads to the interpretation that hydrogenotrophic methanogenesis has less relative importance for total CH_4 production than previous models suggested.

Objectives and hypothesis

In this experiment we attempted, for the first time, to measure homoacetogenesis rates along with hydrogenotrophic methanogenesis in a northern Minnesota bog undergoing whole-ecosystem climate manipulation. The Spruce and Peatland Response

Under Changing Environments (SPRUCE) is a US Department of Energy funded project, which aims to assess the response of northern peatlands to increases in temperature throughout the soil profile and increased atmospheric CO_2 concentrations. We utilized the regression-based design of the SPRUCE experiment to generate a range of incubation temperatures corresponding to depths throughout the peat profile. We predicted that homoacetogenesis rates would be low in shallow peat and at high temperatures corresponding with larger rates of hydrogenotrophic methanogenesis, and, correspondingly, homoacetogenesis rates would be higher at deeper peat depths and lower temperatures with respect to hydrogenotrophic methanogenesis. Chapter II of this dissertation contains co-authored work.

CHAPTER II

MATERIALS AND METHODS

Coauthor acknowledgement

The experimental procedures described in the chapter were performed concurrently with Laura McCullough's research. While I was the primary contributor to my experiments, this project would not have been feasible without her assistance.

Site description

The SPRUCE (Spruce and Peatland Response Under Changing Environments) experimental field site is in the 8.1 ha S1 Bog (47º30.476' N, 93º 27.162' W) located within Marcell Experimental Forest (MEF) in northern Minnesota, USA. S1 Bog has been the subject of extensive scientific investigations and has been thoroughly described previously[39–42]. The subhumid continental climate at MEF has an average annual air temperature of 3.3 ºC, with daily mean extremes of -38 and 30ºC and mean annual precipitation of 768 mm[43]. S1 Bog is an ombrotrophic (i.e., precipitation-fed) peatland with minimal groundwater influence due to a perched water table. The bog soil is primarily the Greenwood series (Typic Borohemist, http://websoilsurvey.nrcs.usda.gov) with average peat depths of 2 to 3 m[44], and a surface soil pH of ~4.1 that increases with depth to ~5.1 at 2 m. The vegetation is dominated by *Picea mariana* (black spruce) and *Larix laricina* (larch) with low-stature ericaceous shrubs and a nearly 100% cover of *Sphagnum* mosses.

Experimental Design

The SPRUCE experiment is a unique whole-ecosystem climate manipulative experiment that is examining how a northern bog responds to warming and elevated CO_2[42] . The experiment uses a regression-based design within 10 open-top enclosures (7 m tall, 12.8 m diameter): there are five warming treatments (ambient, 2.25, 4.5, 6.75, and 9.0 ^{o}C above ambient), each with an ambient and elevated CO_2 (eCO_2, ~ + 500 ppmv) treatment. Whole-ecosystem warming is achieved by a combination of deep peat heating to 3 m depth through an array of vertically installed below-ground electrical heaters and air warming from a propane-fired heat exchanger[42]. The experiment came on line in phases, with soil warming beginning in June 2014, whole-ecosystem warming in August 2015, and eCO_2 in June 2016.

Sampling

Peat was collected from each of the 10 enclosures in August and October of 2018. Using a Russian corer, we collected peat below the water table at depth increments of 30, 50, 75, 125, and 200 cm. Each depth increment was divided into three subsamples (see below). Samples above the water table were not utilized for this experiment. In the field within 10 minutes of sample collection, ~ 7 g wet peat was placed into 25 mL serum bottles that were sealed with 20 mm butyl rubber septa and immediately flushed with N_2 gas for 10 minutes to maintain an anaerobic environment.

Porewater was collected from piezometers installed at depths corresponding to the peat-sampling depths. The piezometers were open to the atmosphere, but their small diameter (< 1 cm) and pumping them out within 24 hr of sampling limited oxygen

exposure of the porewater. We used a peristaltic pump to collect porewater in syringes to eliminate bubbling, and the collected porewater was directly injected into N_2-filled serum bottles. Total sampling time for both peat and porewater took 3 days, during which samples already collected were stored on ice or at 4 °C. Peat and porewater bottles were then shipped on ice to the University of Oregon, weighed, and placed into incubators within 1 ºC of depth-specific, in situ temperatures over the prior week.

Sample preparation

In the laboratory, samples were processed in an anaerobic glove box filled with 98% N_2 and 2% H_2 gas (Coy Laboratory Products Inc., Grass Lake, MI, USA). Porewater corresponding to the plot and depth the peat was collected from was added to create an approximately 5 cm^3 headspace. The bottles were then capped and bubbled for 10 min with N_2. The samples were then pre-incubated at in situ temperatures for ~48 hr in the dark to allow buildup of hydrogen and carbon dioxide. After the pre-incubation, CH_4 and CO_2 concentrations were measured in the samples. The samples were then spiked with CH_4 to reflect typical in situ dissolved concentrations at each depth of 30 cm (0.25 mM), 50 cm (0.35 mM), 75 cm (0.45 mM), 125 and 200 cm (0.7 mM)[45]. Two of the three subsamples were spiked with 0.695 µCi of $NaH^{14}CO_3$ tracer to track pathways of homoacetogenesis and hydrogenotrophic methanogenesis. Samples were then incubated at in situ temperatures for 48 hr in the dark. A subsample of peat from each plot and depth at the SPRUCE site was dried at 60 °C for 3 d to determine moisture content.

CH_4 pathway, hydrogen and acetate production

Headspace CO_2 and CH_4 were quantified by gas chromatography using a flame ionization detector equipped with a methanizer (SRI Instruments, Torrance, CA, USA), and $^{14}CO_2$ and $^{14}CH_4$ production were measured separately with an in-line radioactive gas detector (LabLogic Systems Inc., Brandon, FL, USA). Hydrogenotrophic methane production ($CH_{4,hyd}$) was calculated according to Keller and Bridgham (2007)[24], as $CH_{4,hyd} = aC\alpha/Atg$. The recovered activity of $^{14}CH_4$ is represented by a, C represents the average ΣCO_2 pool size during the incubation, A is the amount of ^{14}C-labeled $NaHCO_3$ added, t is the incubation time, and g is the dry mass of peat in the slurry. For hydrogenotrophic methanogenesis, there is a ^{14}C:^{12}C isotope fractionation factor of 1.12, represented by α in the equation.

Hydrogen production was quantified by gas chromatography with a Peak Performer 1 (Peak Laboratories, Mountain View, CA, USA). From a third replicate set of samples incubated without $NaH^{14}CO_3$, porewater was collected and filtered with a Whatman GF/F glass fiber filter (Sigma–Aldrich, MO, USA) and PES Membrane 0.22 µm syringe filter (Millipore Express PLUS, Burlington, MA, USA). Acetate in filtered porewater was measured using a Dionex DX500 ion chromatograph system equipped with an HC-75 column (Hamilton Company, Reno, ND, USA) located at Chapman University (Orange, CA, USA).

Quantifying homoacetogenesis

We had previously measured rates of homoacetogenesis in soil samples using a $H^{14}CO_3^-$ tracer and ion chromatography with an in-line fraction collector to isolate

radiolabeled acetate[33]. However, this method is extremely laborious, so we developed a new rapid method (Figure 2) to measure putative homoacetogenesis by quantifying the conversion of $H^{14}CO_3^-$ to dissolved organic matter (DOM). This method is predicated on the assumption that after removal of radiolabeled microbial biomass, unreacted dissolved inorganic C (DIC), and CH_4 produced that the remaining ^{14}C in DOM is produced by homoacetogenesis. A variety of chemoautotrophic processes can occur under anaerobic conditions in nature that involve inorganic redox couples including sulfur, nitrogen, and iron compounds[46,47]. However, these reactions do not directly produce DOM and these compounds tend to be have very low concentrations in highly organic peats[24]. Hydrogenotrophic methanogenesis is also chemoautotrophic, but it too does not directly produce DOM. Thus, in short, dark, anaerobic incubations where microbial biomass turnover is minimized, the only known pathway to produced DOM is homoacetogenesis. There is one documented case where the chemoautotrophic production of propionate occurred in anaerobic excised rice roots[48]. Thus, there is some possibility that our putative homoacetogenesis measurements include the production of both acetate and propionate, which would not significantly change the ecological importance of this process.

Following gas measurements of the ^{14}C-radiolabeled samples, a 3 mL liquid aliquot from the bottles was passed through a 0.22 μm filter (Cole-Parmer, IL, USA) to remove microbial biomass, and briefly bubbled with air to strip out dissolved $^{14}CH_4$. Then 200 μL 1M NaOH was added to drive pH above 10 and convert all DIC to the carbonate form, and 100 μL 1M $BaCl_2$ was added to precipitate out excess ^{14}C-DIC as $BaCO_3$. After 24 hr, an aliquot without precipitate, where all ^{14}C should be in DOM, was

added to 4 mL liquid scintillation cocktail (Sigma-Alrich, St. Louis, MO, USA), and quantified on a Tri-Carb® 2810 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, USA) after at least 8 hr of equilibration in the dark. We did extensive preliminary experiments to optimize the conditions for this procedure that minimized loss of acetate by bubbling while removing the vast majority of CH_4 and DIC. Dead samples were run in all incubations and routinely had radioactivity < 0.237 nCi/mL, which was subtracted from live samples.

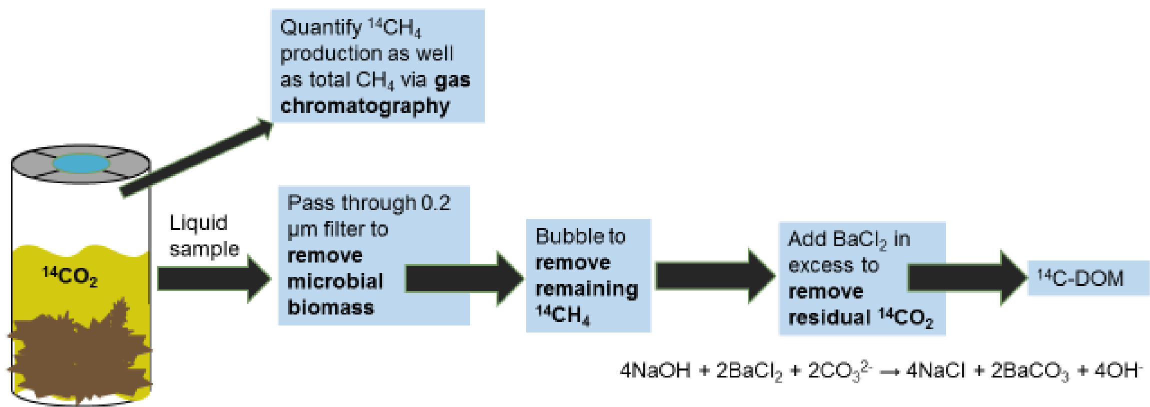


FIGURE 2 | **Conceptual diagram of the novel procedure to measure homoacetogenesis under in situ conditions.** After removing all other forms of ^{14}C, ^{14}C-DOM produced through presumptive homoacetogenesis was quantified on a liquid scintillation counter. Additionally, $^{14}CH_4$ production in the headspace was measured using a radiometric gas detector.

Rates of homoacetogenesis (H) were calculated according to Ye et. al (2014)[33], described originally by Hoehler et al. (1999)[26], as $H = aC\alpha / 2Atg$. The recovered activity of DOM produced through homoacetogenesis is represented by *a*, and the other variables are the same as described above. For every mole of acetate produced, 2 moles of CO_2 must be consumed, which is accounted for by the factor of 2 in the denominator.

Thermodynamic calculations

The Gibbs free energy of hydrogenotrophic methanogenesis and homoacetogenesis was calculated for conditions at the SPRUCE site, as well as our laboratory incubation conditions, according to Conrad and Wetter (1990)[29].

Statistical analyses

Log-transformed rates of hydrogenotrophic methanogenesis and homoacetogenesis, as well as the ratio between the two, were analyzed in R (version 3.6.0) using linear mixed effect models (Linear and Nonlinear Mixed Effect Models version 3.1-137) with temperature as a continuous variable, depth as a categorical variable, and plot as a random variable. If there was a significant interaction between depth and temperature, a Tukey's post hoc test (Least-Squares Means version 2.30-0) at $P < 0.05$ was performed to determine significant differences among depths. For significantly different depths or groups of depths, linear models with temperature as a response variable were created.

CHAPTER III

RESULTS AND DISCUSSION

Peatlands store globally significant amounts of carbon

Peatland ecosystems contribute significantly to the global carbon (C) cycle because of their vast C storage in organic soils and substantial impact on atmospheric greenhouse gases[6,9,49]. Anaerobic degradation of organic matter within these systems produces methane (CH_4), a potent greenhouse gas. Methane has a sustained global warming potential 45-times greater than CO_2 over 100 years[8], and currently accounts for ~20% of total radiative forcing[11]. Wetlands are the largest natural atmospheric CH_4 source and are currently responsible for about one-third of global CH_4 emissions[9,11]. Methane emissions from wetlands have provided important feedbacks in past climates[9], and whether climate change will increase CH_4 or CO_2 emissions from peatlands in a positive feedback mechanism is one of the most pressing questions in global change biogeochemistry[9,49–52].

Microbial pathways controlling CH_4 dynamics

Predicting the response of peatland C stores and CH_4 fluxes to future climate forcing is challenging due to the mechanistic complexity of processes controlling CH_4 emissions. The two major microbial metabolic pathways that produce CH_4 in freshwater anaerobic systems are hydrogenotrophic and acetoclastic methanogenesis[16,17] (Figure 1); however, the respective contribution of these pathways to CH_4 production in peatlands is not fully known[36,53–55]. Understanding the relative importance of these pathways is

crucial to informing Earth System Models (ESMs), which often do a poor job of capturing the large variation in rates of CH_4 emissions among wetlands[56–58].

Homoacetogenesis, a microbial metabolic process in which CO_2 is reduced with H_2 to acetate through the acetyl Co-A pathway[25,59], potentially contributes to CH_4 dynamics through competing for substrates with hydrogenotrophic methanogenesis and producing acetate to fuel acetoclastic methanogenesis. Yet this pathway is almost totally unstudied in natural systems, because it is methodologically difficult to quantify and it is considered thermodynamically unfavorable under low H_2 porewater partial pressures[60], which are typical in peatlands[61]. However, in natural systems, processes are not solely driven by thermodynamics; ecological and physiological factors can be highly influential. At lower temperatures homoacetogenesis appears to be more competitive than methanogenesis for H_2[29–32], and has been shown to do so across multiple peatland types[33]. In this study, we elucidate the importance of homoacetogenesis relative to hydrogenotrophic methanogenesis and their response to future climate change, and analyze the implications of this research in modeling CH_4 dynamics. We hypothesized that hydrogenotrophic methanogens would consume more H_2 relative to homoacetogens in shallow peat corresponding with higher temperatures, and homoacetogens would be more competitive for H_2 at deeper peat depths corresponding with lower temperatures.

Anaerobic C pathways in a peatland manipulative climate change study

This study was part of the Spruce and Peatland Response Under Changing Environments (SPRUCE; http://mnspruce.ornl.gov) project located in the ombrotrophic (i.e., ran-fed) S1 Bog in Marcell Experimental Forest in northern Minnesota, USA. SPRUCE is the first field experiment to warm an entire bog, including vegetation and peat to at least 3 m depth in 115 m^2 open-topped chambers[42]. It uses a regression-based design with five warming treatments ranging from +0 to +9 ºC above ambient at +0 and +500 ppmv atmospheric CO_2 (eCO_2). In August and October of 2018 (3 and 5 years after initiation of eCO_2 and warming, respectively), we sampled anaerobic peat and porewater from the SPRUCE plots from depths ranging 25 to 200 cm below the surface. Samples were incubated anaerobically within 1 degree C of in situ temperatures, as well as at in situ CH_4 concentrations, along with the addition of a $NaH^{14}CO_3$ radio-tracer to track pathways of hydrogenotrophic methanogenesis and homoacetogenesis.

Following a 48-hr incubation, hydrogenotrophic methanogenesis rates were quantified via gas chromatography and a radioactive gas detector, and calculated according to Keller and Bridgham (2007)[24]. Rates of homoacetogenesis were quantified through a procedure developed by us, and calculated according to Ye et al (2014)[33]. Our novel method, described in more detail within the Methods section, estimates putative homoacetogenesis through quantifying the conversion of $H^{14}CO_3^-$ to dissolved organic matter (DOM). After removal of radiolabeled microbial biomass, unreacted $H^{14}CO_3^-$, and $^{14}CH_4$, homoacetogenesis is the only chemoautotrophic pathway currently known to produce DOM within a dark, anaerobic environment. There is one documented case of chemoautotrophic propionate production seen in washed rice roots[48]; however, the

occurrence of this process would not substantially change the ecological significance of our results.

Rates of hydrogenotrophic methanogenesis decreased exponentially with depth and increased linearly with temperature in all but the 50 cm depth increment (Figure 3, Supplementary Table 1). These rates are similar to other measurements of hydrogenotrophic methanogenesis from similar systems[24,62]. Rates of putative homoacetogenesis decreased sharply with depth and linearly increased with temperature (Figure 4). As stated above, homoacetogenesis rates have rarely been measured in natural ecosystems, and this is the first study to examine temperature and depth responses under relatively in situ conditions. There was no significant correlation of eCO_2 treatment with either rates of hydrogenotrophic methanogenesis or homoacetogenesis (Supplementary Table 1).

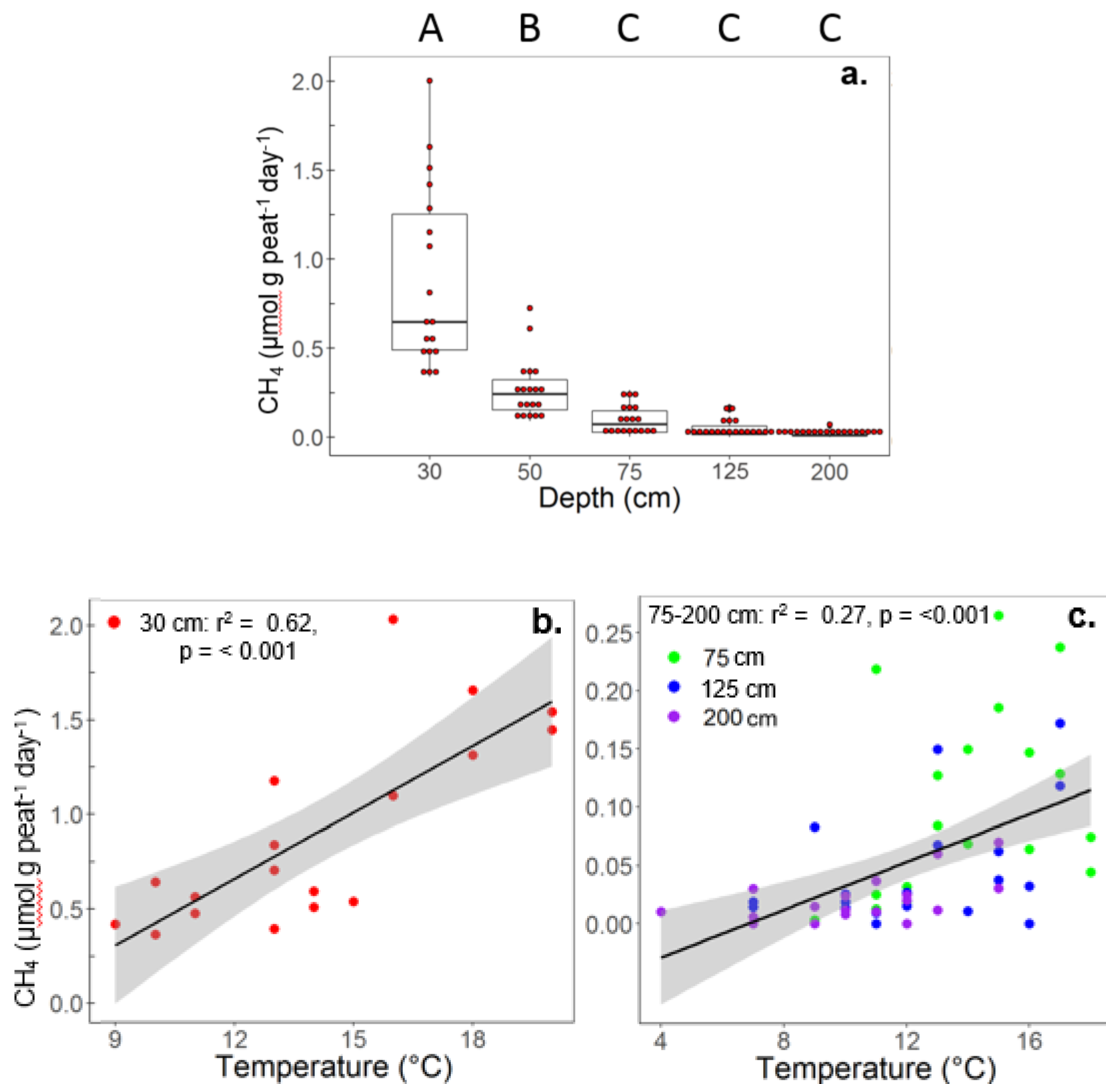


FIGURE 3 | **Hydrogenotrophic methane production from anaerobic incubations. (a)** Box plot of hydrogenotrophic methanogenesis rates in samples anaerobically incubated within 1^{0}C of in situ temperatures. Different letters indicate significant differences among depths. **(b)** Within the significantly different depth increments, positive correlations with temperature occurred at 30 cm and **(c)** 75-200 cm. Linear regressions with 95% confidence intervals are depicted in black and gray, respectively. Note: The temperature effect of the 50 cm depth increment is not shown because the linear regression was not significant ($p = 0.55$, $r^2 = 0.02$).

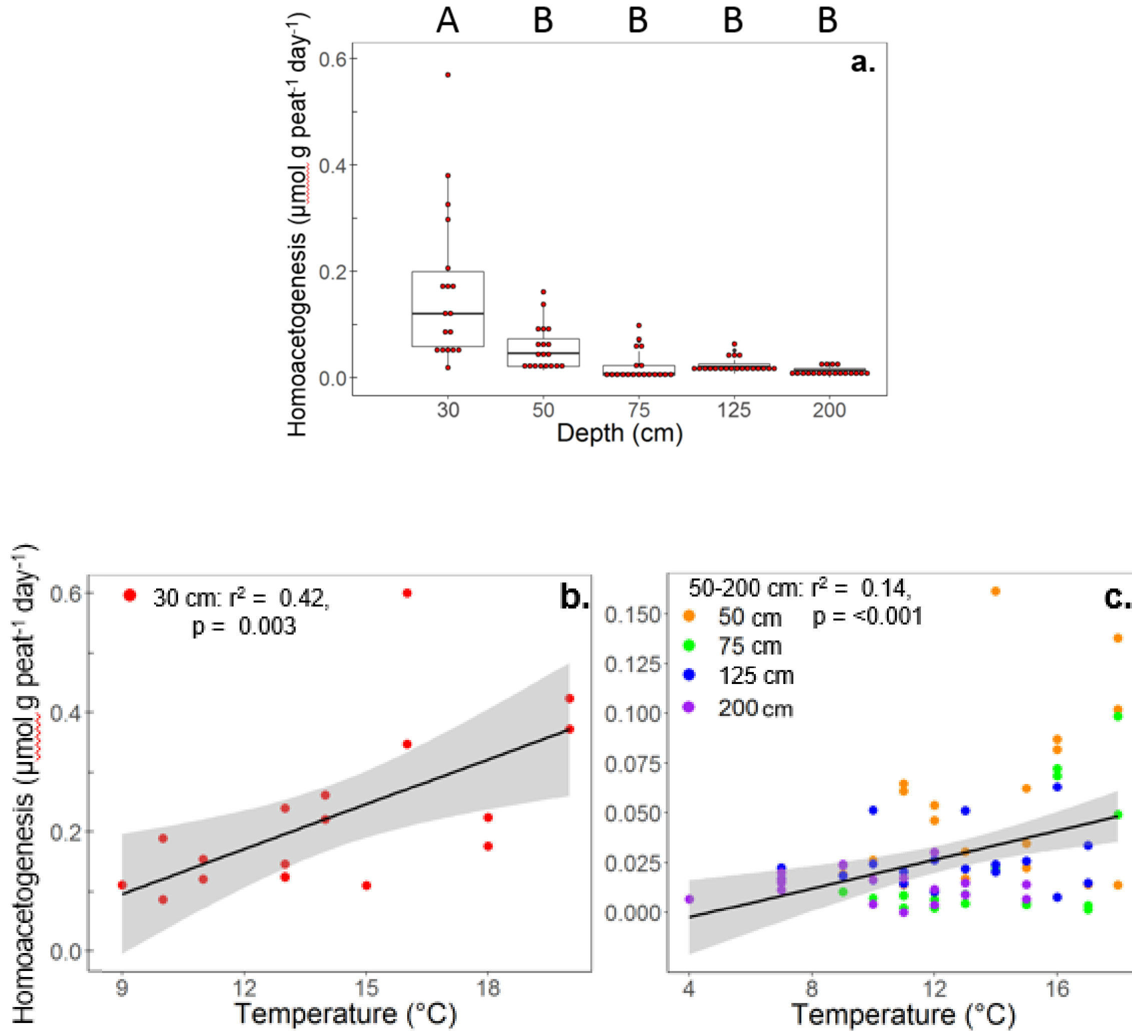


FIGURE 4 | **Homoacetogenesis rates from anaerobic incubations.** (**a**) Box plots of rates of homoacetogenesis in samples anaerobically incubated within 1^0C of in situ temperatures. Different letters indicate significant differences among depths. (**b**) Positive correlations with temperature occurred at 30 cm and (**c**) 50-200 cm depth increments. Linear regressions and 95% confidence intervals are shown in black and gray, respectively.

The hydrogenotrophic methanogenesis and homoacetogenesis pathways utilize H_2 at a stoichiometrically equivalent ratio, allowing them to be directly compared with respect to their H_2 use. The ratio of homoacetogenesis to hydrogenotrophic methanogenesis (Figure 5) increased with depth to a maximum at 100 cm and decreased with increasing temperature. Under the relatively in situ conditions of our experiment, rates of H_2 consumption by homoacetogenesis at several depths were close to and at certain points exceeded that of hydrogenotrophic methanogenesis, suggesting substantial competition for H_2 is occurring between these processes. Additionally, our results show this competition is sensitive to temperature, with increasing warming shifting the system away from homoacetogenesis and towards methane production via hydrogenotrophic methanogenesis.

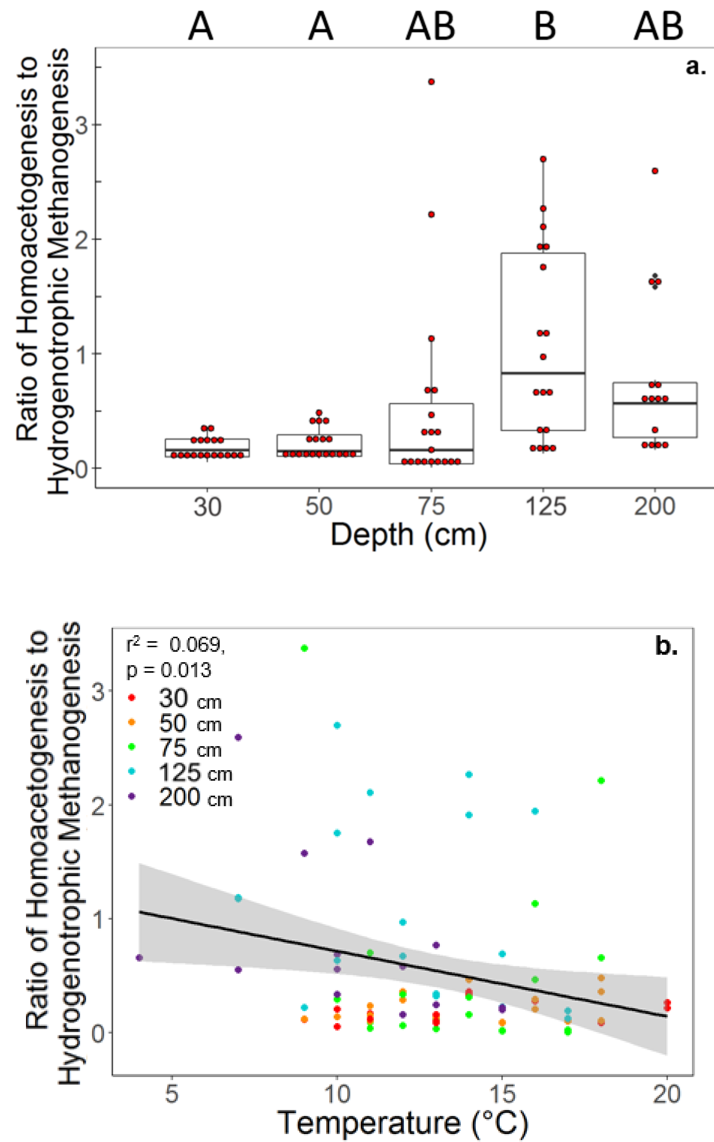


FIGURE 5 | **Ratio of homoacetogenesis to hydrogenotrophic methanogenesis.** The use of H_2 at stoichiometrically equivalent ratios by homoacetogenesis and hydrogenotrophic methanogenesis allows for a 1:1 comparison of rates with respect to H_2 consumption. This ratio increases with depth (**a**) and decreases with temperature (**b**). Boxplots and linear regressions are in the same style described previously.

Dihydrogen concentrations increased substantially at 75 cm depth and below and at cooler temperatures (Supplementary Figure 1). These levels were weakly correlated with homoacetogenesis rates ($p = 0.04$, $r^2 = 0.05$), but not with hydrogenotrophic methanogenesis ($p = 0.26$, $r^2 = 0.02$). Additionally, acetate accumulation occurred during the incubation, and this accumulation was correlated with rates of hydrogenotrophic methanogenesis ($p = 0.002$, $r^2 = 0.09$) as well as homoacetogenesis ($p = 0.035$, $r^2 = 0.04$).

We calculated Gibbs free energy of hydrogenotrophic methanogenesis and homoacetogenesis for our incubations as well as field conditions. In all cases hydrogenotrophic methanogenesis was more negative, and homoacetogenesis often had thermodynamically unfavorable positive values. A possible explanation for these two processes to be occurring simultaneously is interspecies H_2 transfer[34,63,64] (Conrad and Babbel, 1989, Thauer et al, 2008, Stams and Plugge, 2009).

Implications of results

Acetoclastic methanogenesis is typically the dominant CH_4 production pathway in anaerobic systems[21,24], and increasing CH_4 emissions are associated with a shift from the hydrogenotrophic to acetoclastic pathway[65]. Homoacetogenesis has been suggested to account for a considerable portion of acetate production in anoxic sediments[33,66]. Thus, to the extent this process is important in natural systems, its contribution of substrate to acetoclastic methanogenesis could have significant implications for global CH_4 production.

The different methanogenesis pathways and CH_4 oxidation have relatively strong isotopic discrimination that has often been used for decades to estimate in situ the relative

importance of the acetoclastic versus hydrogenotrophic pathway of methanogenesis and rates of methanotrophy[67–69]. However, homoacetogenesis has a similar isotopic fraction signature to hydrogenotrophic methanogenesis[70], so the degree to which it is important may overestimate hydrogenotrophic methanogenesis. This has been demonstrated in reaction-network modeling of microbial carbon transformation in peatlands, where incorporation of homoacetogenesis into models increases the convergence of the model with field data when microbial community data is considered[37,38]. Additionally, CH_4 stable isotopes are used with atmospheric inverse modeling to estimate the wetland contribution to global CH_4 emissions and their spatial distribution[14,71]. These models analyze atmospheric stable isotopic signatures with an assumption about the relative importance of the CH_4 production pathways[72]. To the extent that these assumptions are incorrect due to the contribution of homoacetogenesis will make the global model assumptions incorrect.

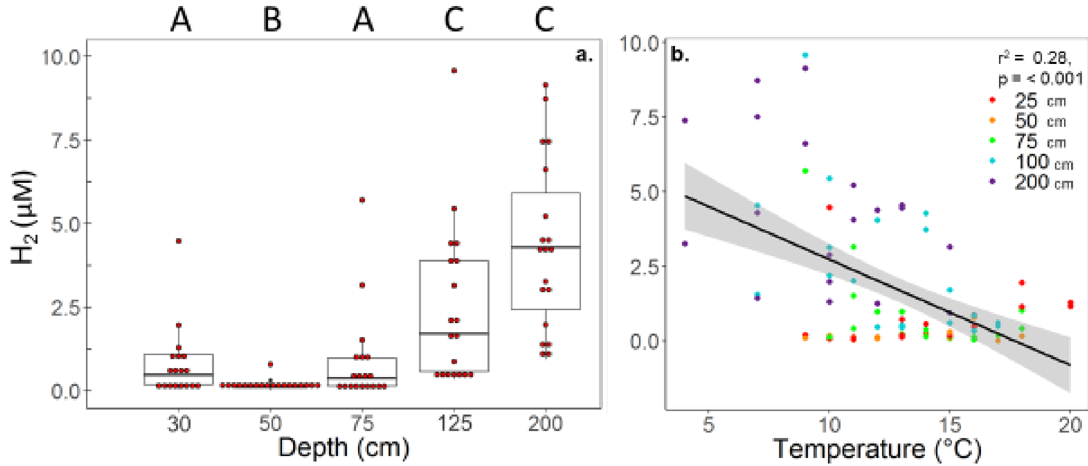
APPENDIX: SUPPLEMENTARY FIGURES

Linear mixed effect model	Analysis	Degrees of freedom	P-value
Hydrogenotrophic methanogenesis rates	Temperature	1	<0.0001
	Depth	4	<0.0001
	Temperature:Depth	4	<0.0001
	CO_2 treatment	1	0.23
Homoacetogenesis rates	Temperature	1	<0.0001
	Depth	4	<0.0001
	Temperature:Depth	4	0.007
	CO_2 treatment	1	0.86
Ratio of homoacetogenesis to hydrogenotrophic methanogenesis	Temperature	1	0.007
	Depth	4	<0.001
	Temperature:Depth	4	0.50
	CO_2 treatment	1	0.83

SUPPLEMENTARY TABLE 1 | **Table of linear mixed effect model results.**

Linear mixed effect models of hydrogenotrophic methanogenesis rates, homoacetogenesis rates, and the ratio of homoacetogenesis to hydrogenotrophic

methanogenesis were performed with temperature, depth, their interaction, and CO_2 treatment as predictor variables, with plot as a random variable.



SUPPLEMENTARY FIGURE 1 | **Hydrogen levels from anaerobic incubations.** Hydrogen increased with depth (**a**) and decreased with temperature (**b**) following anaerobic incubations. Different letters indicate significant differences among depths. Linear regressions and 95% confidence intervals are shown in black and gray, respectively.

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