

# Methanogenesis and Methanogen Diversity in Three Peatland Types of the Discontinuous Permafrost Zone, Boreal Western Continental Canada

**Joseph B. Yavitt**

Department of Natural Resources, Cornell University, Ithaca, New York, USA

**Nathan Basiliko**

Department of Geography, University of Toronto at Mississauga, Mississauga, Ontario, Canada

**Merritt R. Turetsky**

Department of Plant Biology, Michigan State University, East Lansing, Michigan, USA

**Anthony G. Hay**

Department of Microbiology, Cornell University, Ithaca, New York, USA

Because recent patterns of permafrost collapse in boreal peatlands appear to enhance emissions of CH<sub>4</sub> to the atmosphere, we examined methanogenesis and methanogen diversity in peat soil from peatlands with and without permafrost in two peatland complexes situated in continental western Canada. Peat soil from the active layer of permafrost bogs had very low rates of CH<sub>4</sub> production (ca. 10 nmol g<sup>-1</sup> day<sup>-1</sup>), and we were unable to PCR-amplify 16S rRNA gene sequences using Archaea-specific primers in four peat samples. Surface peat soil from continental bogs with no permafrost supported moderate rates of CH<sub>4</sub> production (20–600 nmol g<sup>-1</sup> day<sup>-1</sup>), with maximum rates in soil located close to the mean water table level. Additions of ethanol stimulated CH<sub>4</sub> production rates, suggesting metabolic substrate limitations. Peat from internal lawns, which have experienced surface permafrost degradation in the past 150 years, had very rapid rates of CH<sub>4</sub> production (up to 800 nmol g<sup>-1</sup> day<sup>-1</sup>) occurring within the soil profile. Concomitant rates of anaerobic CO<sub>2</sub> production were greater in continental bogs (ca. 6 μmol g<sup>-1</sup> day<sup>-1</sup>) than in internal lawns (ca. 4 μmol g<sup>-1</sup> day<sup>-1</sup>) or in permafrost bogs (2.8 μmol g<sup>-1</sup> day<sup>-1</sup>). Analysis of the 16S rRNA gene for Archaea in the continental bog indicated mostly sequences associate with *Methanobacteriales* and RC-I with

a *Methanosarcinaceae* sequence in the deepest peat soil. In the internal lawn, *Methanosarcinaceae* were most common in peat soil with a *Methanoaetaceae* sequence in the deepest peat soil. This study showed that patterns of discontinuous permafrost and ongoing permafrost degradation in boreal regions create patchy soil environments for methanogens and rates of methanogenesis.

**Keywords** anaerobic carbon mineralization, boreal wetland, Canada, CO<sub>2</sub>, CH<sub>4</sub>, ecosystems, peat soils, discontinuous permafrost, Ribosomal-RNA sequences, trace metal

## INTRODUCTION

Near the southern limit of the discontinuous permafrost zone in boreal North America, wetlands with deep peat soils (peatlands) occur in three distinct settings: (1) as localized permafrost mounds that represent relict permafrost features from colder climatic periods of the Little Ice Age (Halsey et al. 1995), (2) as *Sphagnum* dominant bogs with no evidence of permafrost through the Holocene (hereafter: continental bogs), and (3) as *Sphagnum* dominant fens in which permafrost has degraded during the past 100–150 years (hereafter: internal lawns) (Beilman et al. 2001). Peat soils can be good habitat for methanogenic microorganisms (cf., Basiliko et al. 2003; Merila et al. 2006), and surveys of CH<sub>4</sub> emitted from peatlands into the atmosphere have found larger emission rates from internal lawns than from either permafrost mounds or continental bogs (Bubier et al. 1995; Liblik et al. 1997; Turetsky et al. 2002). If enhanced emission of atmospheric CH<sub>4</sub> from internal lawns turns out to be robust (cf., Payette et al. 2004; Bubier et al. 2005), then permafrost melting, accelerated in the face of regional climate warming (Vitt et al. 2000), would contribute to greenhouse gas induced

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Address correspondence to Joseph B. Yavitt, Department of Natural Resources, Cornell University, Ithaca, New York 14853-3001, USA. E-mail: jby1@cornell.edu

climate warming. These peatland types might also harbor distinct populations of CH<sub>4</sub>-Producing microorganisms (methanogens) that contribute to the different emission rates.

Northern peatlands show a great diversity of methanogen populations (Hales et al. 1996), even within individual peatlands (Basiliko et al. 2003; Galand et al. 2005). Despite the within-site diversity, methanogens that split acetate appear to dominate in nutrient-rich fens and in fens dominated by *Carex* sedge plants (Galand et al. 2005), and acetate fuels CH<sub>4</sub> production (aceticlastic methanogenesis) in these systems (Popp et al. 1999; Chaser et al. 2000). In contrast, H<sub>2</sub>/CO<sub>2</sub>-utilizing methanogens appear to carry out CH<sub>4</sub> production in nutrient-poor fens and bogs (Lansdown et al. 1992; Horn et al. 2003; Brauer et al. 2004; Galand et al. 2005). Many methanogens are capable of using H<sub>2</sub>/CO<sub>2</sub>, whereas aceticlastic methanogenesis occurs only with a few members of the *Methanosaetaceae* and *Methanosarcinaceae* (Zinder 1993; Garcia et al. 2000). Comparative studies also have found greater rates of CH<sub>4</sub> production in peat soils from nutrient-rich fens than bogs (Galand et al. 2005; Yavitt et al. 1997, 2005), suggesting better environmental conditions and/or supply of metabolic substrates enhance methanogenic activity in fens. However, the factors controlling methanogenic activity (quantity and type of metabolic substrates, nutrient elements, pH, temperature, extent of water saturation, antimicrobial properties of *Sphagnum* mosses found primarily in bogs) are confounded in many peatlands (Yavitt et al. 2005). Therefore it is not clear whether permafrost mounds and continental bogs, for example, have low rates of CH<sub>4</sub> production and, if so, for the same reasons.

Despite the presence of frozen soils, psychrophilic methanogens (optimum growth at cold temperature) are not necessarily dominant. The ability to function over a wide range of temperatures should be favored, particularly in surface soil that is not thermally buffered. Temperature studies have found that homoacetogenic bacteria, which convert H<sub>2</sub> and CO<sub>2</sub> to acetate, out-compete methanogens for H<sub>2</sub> at cold temperatures (Fey and Conrad 2000; Kotsyurbenko 2005); hence, CO<sub>2</sub>-reducing methanogens must use a different electron donor, or the aceticlastic reaction fuels methanogenesis. There is evidence that cold temperatures select *Methanosaetaceae* (Chin et al. 1999).

We have used the mosaic of different peatland types across the landscape of boreal western Canada to investigate peat soil accumulation, plant material decomposition, and organic-C mineralization to CO<sub>2</sub> and CH<sub>4</sub> (cf., Turetsky et al. 2000, 2002; Turetsky 2005). In this study we examined rates of methanogenesis and the diversity of methanogen populations in peat soils from the three different peatland types. It represents a detailed assessment of methanogen presence and activity under different soil permafrost conditions. We also examined factors that favor methanogenesis, including rates of anaerobic C mineralization (CO<sub>2</sub> production), the addition of a metabolic substrate (ethanol), temperature, and metal content of the peat soils. This information will be useful in future studies designed to enrich for methanogens in permafrost zone wetlands.

## METHODS

### Field Sites

We sampled the surface soils of adjacent continental bogs, internal lawns, and permafrost mounds in two distinct peatland complexes in boreal western Canada. Our first sampling area was in north-central Alberta (56°00'N, 112°00'W), where mean annual temperature is -0.2°C, and mean annual precipitation is 472 mm. In this area, the continental bog, known locally as Sylvie's Bog, had vegetation dominated by ericaceous shrubs (*Ledum groenlandicum* *Chamaedaphne calyculata*) and cranberry (*Vaccinium oxycoccus*, *V. vitis-idaea*) with ground cover of *Sphagnum* mosses (*Sphagnum fuscum*, *Sphagnum angustifolium*). The permafrost mounds had a cover of feather mosses (*Pleurozium schreberi*) and a black spruce (*Picea mariana*) canopy. The internal lawn had *Sphagnum* mosses, cranberry as well as the sedge *Carex limosa* and the lily three-leaved false Solomon's seal (*Smilacina trifolia*). Further description is given in Turetsky et al. (2000).

We also sampled peat soils from sites located about 10 km south of Patuanak, Saskatchewan (55°51'N, 107°41'W), where mean annual temperature is -1.0°C, and mean annual precipitation is 444 mm. In this region, continental bogs have a sparse canopy of black spruce with Labrador tea (*L. groenlandicum*) shrub cover and ground cover of *S. fuscum* moss. Peat soil pH ranges from 3.56 to 3.81 (mean pH = 3.64). Isolated permafrost mounds occur with intact permafrost tables 60 to 80 cm below the surface vegetation, which is dominated by dense canopies of black spruce with ground cover of feather mosses (usually *P. schreberi* and *Hylocomium splendens*) and lichens. Peat soil pH ranges from 3.79 to 4.68 (mean pH = 4.04). Internal lawns are open fen peatlands dominated by *Sphagnum* mosses (*Sphagnum riparium*, *S. angustifolium*) and cottongrass (*Eriophorum vaginatum*). Peat soil pH ranges from 3.70 to 4.07 (mean pH = 3.83). See Turetsky (2005) and Turetsky et al. (2002) for further site description.

### Soil Sample Collection

In Alberta, two intact cores of peat soil were collected from each site using 10-cm diameter, 40-cm long PVC cylinders with a sharp bottom edge. In Saskatchewan, the two PVC cylinders were 1-m long. The cylinders were pounded into the soil ensuring the entrained soil did not compact. Peat cores were shipped on ice to the University of Alberta where they were frozen and cut into 2 cm increments using a band saw.

Peat cores from Alberta were divided into 2-cm depth intervals between 0 and 10 cm in the peat soil profile and 5-cm depth intervals between 10 and 40 cm in the peat soil profile. In the peat cores from Saskatchewan we used nine samples per peatland type taken 30 to 80 cm in the peat profile, carefully noting the sample depth in relation to the water table depth. The samples were sealed in individual plastic bags, placed within a second plastic bag, and shipped to Cornell University by overnight courier, where analyses occurred on the fresh samples. We were

careful to take samples only from the center of the bulk soil sample where exposure to ambient air was minimal.

### Peat Soil Analyses

We estimated rates of anaerobic CO<sub>2</sub> production and CH<sub>4</sub> production by incubating portions of each peat soil in vitro. A 20-g portion of peat soil (wet weight) and 20 mL of degassed, de-ionized water were placed into a 225 mL Mason jar that had a septum installed in the lid for sampling. The lid and septum were sealed with silicone sealant. The jar content was then flushed through four cycles of evacuating the headspace (5 minutes), then filling it with O<sub>2</sub>-free N<sub>2</sub>. Most incubations were performed in triplicate at 25°C without shaking.

Samples from Alberta were incubated for 40 days. After that, 5 mL of an anaerobic stock solution of 0.3% ethanol was added to each jar, the headspace was flushed again through four cycles with O<sub>2</sub>-free N<sub>2</sub>, and the sample was incubated for an additional 20 days. Samples from Saskatchewan were incubated for 60 days. A second set of samples from Saskatchewan were incubated for seven days; one portion per sample was given 5 mL of degassed, deionized water, and a second portion was given 5 mL of the anaerobic ethanol solution. We also used a third set of samples from Saskatchewan to examine the influence of temperature on microbial activity by incubating different portions of peat soil at 10, 20, 33, and 45°C.

We used ethanol as a metabolic substrate for methanogens (cf., Valentine et al. 1994) because it is easily converted to hydrogen and acetate by anaerobic bacteria, and both of these products subsequently fuel CH<sub>4</sub> production. Ethanol also has an advantage because it supplies acetate more gradually than in a direct acetate addition. At low pH values, acetate will become protonated and toxic to most microorganisms (Russell 1992), and thus only small amounts of acetate fuel CH<sub>4</sub> production (Brauer et al. 2004). We used 25°C (i.e., room temperature) for most of the incubations because it alleviates low temperature limitation of microbial activity and room temperature allowed us to study the largest number of samples, logistically.

We sampled gases in the jar headspace by filling a syringe with 10 mL of N<sub>2</sub>, injecting it into the jar, then taking a 10 mL sample with the same syringe. Gas samples were analyzed by gas chromatography (Perkin-Elmer, Sigma 3B, Wellesley, MA, USA) using a 3 m Porapak-Q (Waters Corporation, Milford, MA, USA) column (80/100 mesh) maintained at 50°C to separate gases and a thermal conductivity detector for CO<sub>2</sub> and a flame ionized detector for CH<sub>4</sub>. Rates of gas production were calculated between two consecutive samples, and are expressed as moles of gas produced per gram of dry soil per day.

In order to examine the recovery of microbial activity following persistent cold, two peat soil samples per peatland type from Saskatchewan were placed into plastic bags, which were sealed, and incubated at 2°C for 18 months. After that, two portions per sample were placed separately into jars and incubated in vitro as described above. On the second day of the incubation, 10 mL

of a 0.3% ethanol solution was added to one portion, and 10 mL of distilled water was added to the other, and the incubation continued. Gas samples were taken about every 10 days for 73 days and analyzed immediately for concentrations of CH<sub>4</sub> and CO<sub>2</sub>.

After incubation, soil pH was determined with a combination electrode. The volume of the jar headspace was measured by water displacement, and the soil was dried to quantify soil water content and dry mass of the sample. All rates of CH<sub>4</sub> production and CO<sub>2</sub> production are expressed as moles of gas produced per gram of dry peat per day.

The concentrations of selected chemical elements in the peat soils from Alberta were measured by inductively coupled plasma (ICP) spectroscopy (Spectro CIROS CCD Spectrophotometer, Kleve, Germany). Peat samples were dried, homogenized, and burned to ash in a muffle furnace (450°C, 4 hours). The ash was oxidized using 0.25 mL of 30% H<sub>2</sub>O<sub>2</sub> and reheated in the muffle furnace (450°C, 2 hours). Nitric acid was then added and diluted to a 10% solution for ICP analysis.

### DNA Extraction and PCR Amplification

Molecular analysis of methanogen diversity was done only in the peat soil samples from Saskatchewan. We used 17 different soil samples: four from permafrost mounds; six from internal lawns; and, seven from continental bogs. The permafrost mound samples were 35, 40, 40 and 70 cm deep. The internal lawn samples were 35, 65, 65, and 75 cm deep, all of which were below the 5–22 cm deep water table level. Three of the continental bog samples were at the 35 cm deep water table level, and the others 54, 80, and 80 deep. We extracted DNA with the FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA) using a MiniBeadbeater 8 instrument (Biospect Products, Bartlesville, OK, USA) for cell lysis. A 0.4 g (wet weight) portion was removed from each bag with a sterile spatula, placed in a Multimix 2 tissue tube, and extraction followed manufacture's instructions, except that DNA was washed four times with 500 µL guanidine thiocyanate (5.5 M) to remove humic substances and proteins, and DNA was eluted to a final volume of 100 µL. Extracted DNA was resolved by gel electrophoresis in 1% agarose stained with ethidium bromide, and the remaining solution stored at –20°C.

A fragment from positions 1 to 1100 (*E. coli* numbering) of the 16S rRNA gene was amplified using the archaeal-specific 1AF (5'-TCY GKT TGA TCC YGS CRG AG-3')-1100AR (5'-TGG GTC TCG CTC GTT G-3') set of primers (Hales et al. 1996). The PCR mixture contained the following components at its reactants concentrations per µL: 1× Taq buffer with 1.5 mM MgCl<sub>2</sub> (Eppendorf, North America, Westbury, NY, USA), 0.2 mM deoxy-nucleotide triphosphates (dNTP), 0.25 µM forward and reverse primers, 1.2 U of Taq Polymerase (Eppendorf North America, Westbury, NY, USA), 0.2 µg bovine serum albumin (BSA) and ~0.1–0.3 ng of extracted DNA. The PCR conditions were as described by Hales et al. (1996) with 30–35 amplification cycles. Amplification products

were examined by electrophoresis on 1% agarose gels for size verification.

### Enumeration of Microorganisms with MPN-PCR

We enumerated Archaea and bacteria with a most probable number-molecular amplification (MPN-PCR) (Chandler 1998). The 16S rDNA gene was initially amplified with a wide spectrum archaeal primer (Barns et al. 1994) followed by a second round of amplification with 1af and 1100ar to quantify *Archaea*. To enumerate Bacteria, the initial amplification was with a wide spectrum eubacterial primer (Wilson et al. 1990) followed by amplification with 1054F and 1492R. This enumeration technique was verified with standards of archaeal and bacterial DNA.

### Cloning, Sequencing, and Phylogenetic Analysis

We constructed 16S rRNA gene clone libraries as described by Basiliko et al. (2003). Six clone libraries were constructed for samples from 35 to 75 cm depth in the continental bog and internal lawn. Using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and m13 primer screening, 60 clones per library were selected for restriction analysis with *Hae*III and *Hha*I enzymes (New England Biolabs, Ipswich, MA, USA). Clones displaying unique restriction patterns were sequenced with an ABI 3730 automated sequencer (P-E Applied Biosystems, Foster City, CA, USA) and Big Dye Terminator chemistry using the 1100AR primer in cycle sequencing reactions. We included sequence data

in further analysis when sequencing was successful at measuring >500 bp and truncated all of our sequences to 500 bp.

The CHIMERA\_CHECK program on the Ribosomal Database Project was used to identify potential chimeric sequences (Maidak et al. 2001). Similar sequences from environmental samples and cultured organisms were identified using the nucleotide-nucleotide BLAST tool in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and included in further analysis along with sequences from an "archaeal database" with 2500 complete and partial archaeal sequences (Jurgens 2002). Sequences were aligned using tools in the ARB software package and phylogenetic trees were estimated using the maximum likelihood method incorporated in the ARB package (The ARB Project, (<http://www.arb-home.de/>)).

Because we intended sequencing to serve only as a rapid means of coarsely identifying organisms in our samples and only used a single sequencing reaction for each clone, short (500bp) sequence fragments from cloned genes were added to the tree reference sequence tree using the parsimony tool in the ARB package without altering global tree topology.

## RESULTS

### Peat Soils from Alberta

We examined the top 35 cm of the peat deposit in each peatland type in Alberta. Samples from the continental bog and

TABLE 1  
Concentrations (mg kg<sup>-1</sup>) of several chemical elements in peat soil from three peatland types in Alberta.

	Continental bog	Permafrost mound	Internal lawn	Cool temperate bogs
A1	398 (1)	873 (1)	492 (1)	1000
B	5.5 (49)	64.7 (263)	47.0 (97)	1
Ca	1326 (26)	18787 (102)	5445 (52)	3000
Cd	0.1 (51)	0.23 (53)	0.15 (62)	n.d.
Co	0.20 (4.5)	0.69 (7.0)	0.26 (4.7)	0.30
Cu	1.7 (10.1)	1.9 (5.2)	1.6 (7.7)	5.0
Cr	0.23 (0.6)	1.24 (1.4)	0.26 (0.5)	n.d.
Fe	464 (2.1)	1031 (2.1)	1062 (3.8)	1000
K	461 (5.9)	376 (2.2)	320 (3.3)	400
Mg	342 (12)	1475 (24)	1030 (30)	600
Mn	87 (16)	507 (41)	126 (18)	30
Mo	0.20 (30)	0.18 (12)	0.60 (72)	1.0
Na	38 (1.4)	99 (1.6)	158 (4.6)	100
Ni	8.4 (30)	1.8 (2.9)	0.7 (2.0)	5.0
P	333 (74)	642 (65)	369 (67)	400
Pb	2.2 (11)	2.0 (5)	3.8 (16)	1.0
S	200 (51)	3799 (441)	416 (86)	1000
Ti	7.1 (0.3)	17.7 (0.3)	8.7 (0.3)	n.d.
V	0.38 (0.8)	1.08 (1.0)	0.37 (0.6)	n.d.
Zn	12 (24)	31 (28)	34 (55)	50

Values for cool temperate bogs are taken from Charman (2002). Enrichment factors (normalized to A1) for selected chemical elements in three peatland types are in parentheses.

permafrost mound incubated *in vitro* at 25°C for 40 days always had rates of CH<sub>4</sub> production <5 nmol g<sup>-1</sup> day<sup>-1</sup> between successive measurements of gas concentrations during the incubation period. Peat soils from the internal lawn also had similarly slow rates of CH<sub>4</sub> production, except in samples from the 25–30 cm depth interval, in which rates averaged 14.7 nmol g<sup>-1</sup> day<sup>-1</sup>. After the 40 day incubation, the addition of ethanol did not affect rates of CH<sub>4</sub> production in peat soils from the permafrost mound, and only peat from 22 cm depth in the continental bog showed a larger rate of CH<sub>4</sub> production (75 nmol g<sup>-1</sup> day<sup>-1</sup>) with added ethanol. However, the addition of ethanol did foster rates of CH<sub>4</sub> production in peat soils from the internal lawn, with values of 91 nmol g<sup>-1</sup> day<sup>-1</sup> in the 20–25 cm depth interval, 327 nmol g<sup>-1</sup> day<sup>-1</sup> in the 25–30 cm depth interval, and 87 nmol g<sup>-1</sup> day<sup>-1</sup> in the 30–35 cm depth interval.

Concomitant rates of anaerobic CO<sub>2</sub> production without added ethanol were between 2 and 8 μmol g<sup>-1</sup> day<sup>-1</sup> in peat soil throughout the depth profile in the continental bog. Peat soil in the permafrost mound and internal lawn had rates >10 μmol g<sup>-1</sup> day<sup>-1</sup> in the top 2 to 5 cm of the peat deposit, with rates <6 μmol g<sup>-1</sup> day<sup>-1</sup> in deeper peat. Overall the permafrost mound had the slowest rates of anaerobic CO<sub>2</sub> production through most of the peat deposit. Ethanol did not affect rates of anaerobic CO<sub>2</sub> production in peat soil from any of the sites.

Peat soil from the continental bog had smaller concentrations of B, Ca, Fe, Mg, Na and Zn compared to soil from the other sites and only Ni had elevated concentration in the continental bog (Table 1). Peat soil from the permafrost peatland had markedly larger concentrations of Al, Cd, Co, Cr, Mn, P, S, Ti, and V. Therefore only Mo was enriched in peat soil from the internal lawn. Concentrations of K, Cu and Pb were similar in all three peatland types.

### Peat Soils from Saskatchewan

The peat soils from Saskatchewan extended deeper into the peat deposit. Samples incubated *in vitro* at 25°C for 60 days without added ethanol showed a 12-day lag period before the onset of CH<sub>4</sub> production (Figure 1). After that, the amount of CH<sub>4</sub> production varied widely, even among samples from different depths in the same peatland type. Rates of CH<sub>4</sub> production calculated between days 12 and 54 yielded rates >250 nmol g<sup>-1</sup> day<sup>-1</sup> in five of nine samples from the internal lawn, in two of nine samples from the continental bog, and in only one of six samples from the permafrost mound (Figure 2). The one permafrost mound sample with a large rate of CH<sub>4</sub> production, albeit collected deep in the peat soil profile, was still above the water table level in its collection site. Rates of anaerobic CO<sub>2</sub> production measured concomitantly were 2.5 to 7.5 μmol g<sup>-1</sup> day<sup>-1</sup> in peat soil from the permafrost mound (Figure 2). In the continental bog and internal lawn, the largest rates of anaerobic CO<sub>2</sub> production occurred in peat soil ca. 40 cm deep, and rates generally decreased monotonically with increasing depth in the peat deposit.

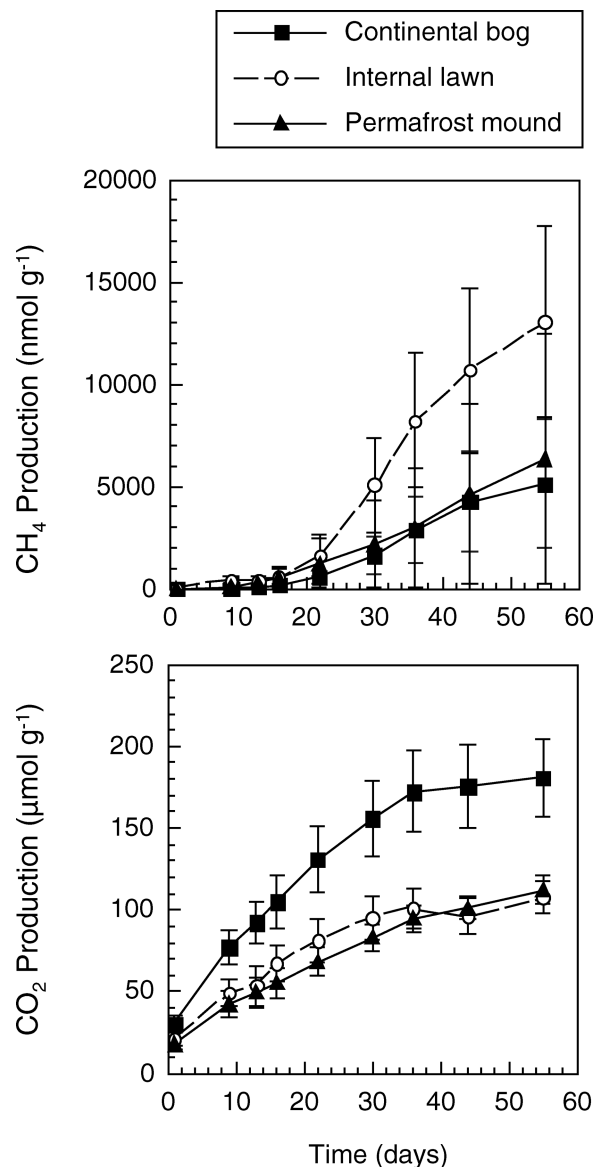


FIG. 1. Time course for CH<sub>4</sub> production (top panel) and anaerobic CO<sub>2</sub> production (bottom panel) in peat soils from three peatland types in Saskatchewan, Canada, incubated *in vitro* at 25°C for 54 days. Each point is the mean of samples from that peatland type regardless of depth in the peat soil profile; N = 9 for internal lawn and continental bog; N = 6 for permafrost mound. Error bar is 1 S.E.

In fresh samples, the addition of ethanol stimulated CH<sub>4</sub> production without the lag period observed in samples without added ethanol. As a result, the addition of ethanol during an ensuing 7-day incubation increased rates of CH<sub>4</sub> production up to 600-fold in peat soils from the continental bog and internal lawn, whereas ethanol added to peat soils from the permafrost mound affected the rate of CH<sub>4</sub> production only in the sample that produced CH<sub>4</sub> without added ethanol (Figure 3). Averaged across all samples, ethanol led to a 17-fold increase in rates of CH<sub>4</sub> production versus only a 17% increase in rates of CO<sub>2</sub> production (CO<sub>2</sub> data not shown). Notwithstanding, there was

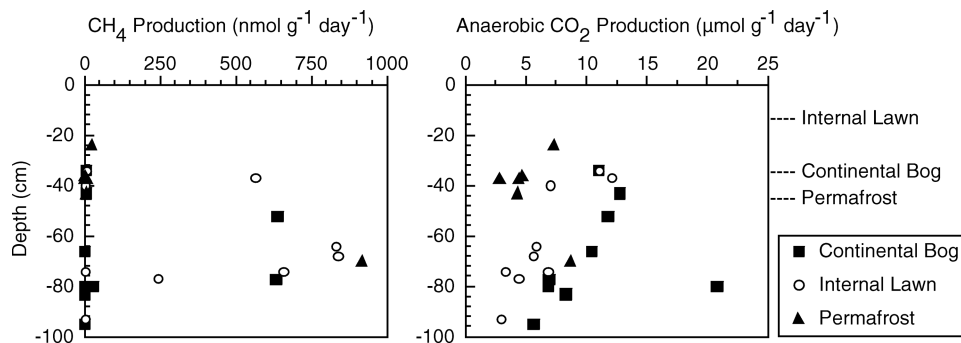


FIG. 2. Depth profiles for mean rates of  $\text{CH}_4$  production (left panel) and anaerobic  $\text{CO}_2$  production (right panel) in peat soils from three peatland types in Saskatchewan, Canada, incubated *in vitro* at  $25^\circ\text{C}$ . Rates for  $\text{CH}_4$  production were calculated after the 12-day lag period. The dashed line and peatland type indicates the water table height at the time the samples were collected.

a positive correlation (Pearson  $r=0.48$ ) between the ethanol-induced rate of  $\text{CH}_4$  production and the ethanol-induced rate of  $\text{CO}_2$  production.

In our experiment, in which samples from each peatland type were stored for 18 months at  $0^\circ\text{C}$  then assayed for microbial activity, peat soils from the permafrost mound had very little  $\text{CH}_4$  production, except one sample that attained a  $\text{CH}_4$  production rate of  $126 \text{ nmol g}^{-1} \text{ day}^{-1}$ . Otherwise  $\text{CH}_4$  production rates were ca.  $7 \text{ nmol g}^{-1} \text{ day}^{-1}$ , and ethanol addition had no effect on rates in samples from the permafrost mound. In contrast, peat soils from the continental bog and internal lawn incubated without added ethanol initiated  $\text{CH}_4$  production 26 to 41 days after the incubation began. The addition of ethanol led to net  $\text{CH}_4$  production between 13 and 26 days after the ethanol addition. Peat soils from the internal lawn showed ethanol induced rates of  $\text{CH}_4$  production of  $165 \text{ nmol g}^{-1} \text{ day}^{-1}$  without ethanol. For the continental bog, ethanol induced rates of  $\text{CH}_4$  production were  $221 \text{ nmol g}^{-1} \text{ day}^{-1}$  versus  $20 \text{ nmol g}^{-1} \text{ day}^{-1}$  without ethanol. Concomitant rates of anaerobic  $\text{CO}_2$  production following ethanol addition were less in peat soil from the internal lawn ( $3.5 \mu\text{mol g}^{-1} \text{ day}^{-1}$ ) than from the permafrost mound ( $4.7 \mu\text{mol g}^{-1} \text{ day}^{-1}$ ) or the continental bog ( $4.9 \mu\text{mol g}^{-1} \text{ day}^{-1}$ ).

Peat soils from all three peatland types incubated for 10 days without added ethanol exhibited temperature optima for methanogenesis ca.  $33^\circ\text{C}$  (Figure 4). Temperature optima for anaerobic  $\text{CO}_2$  production was ca.  $10^\circ\text{C}$  in peat soil from the permafrost mound. Otherwise, the peat soil from the other two peatland types had temperature optima between 20 and  $33^\circ\text{C}$ . Essentially no  $\text{CH}_4$  or  $\text{CO}_2$  was produced at  $45^\circ\text{C}$ .

### 16S rRNA Phylogenetic Diversity

Analysis of the 16S rRNA gene revealed sequences associated with *Methanosarcinaceae* and *Methanosaetaceae* families and the *Methanobacteriales* and *Methanomicrobiales* (Figure 5). Sequences from the uncultured group Rice Cluster I (RC-I) were present in the continental bog. We were unable to isolate any archaeal 16S rRNA gene sequences from the permafrost peat soil.

In all of the samples, an MPN-PCR indicated that bacterial sequences accounted for  $>99\%$  of the DNA. We found  $10^4$  to  $10^5$  Archaea per gram of dry soil in samples from the continental bog and internal lawn. Samples from the permafrost mound had  $<1 \times 10^4$  Archaea per gram of soil.

In general, sequences associated with *Methanobacteriales* and RC-I were most common in the continental bog in soil close to the water table level, whereas we found a single *Methanosarcinaceae* sequence in the deepest sample below the water table level. We found more diversity of methanogens in the internal lawn with several sequences related to members of the *Methanosarcinaceae*, a sequence that grouped in the *Methanobacteriales* and a sequence in the *Methanomicrobiales* in peat soil close to the water table level. In addition, we found a single sequence associated members of the *Methanosaetaceae* in the deepest sample below the water table level in the internal lawn.

### DISCUSSION

Rates of  $\text{CH}_4$  production in the top 35 of the peat soils from all three peatland types in Alberta were much less than expected, especially, given the moderate rates of anaerobic  $\text{CO}_2$  production (cf., Yavitt et al. 1997 and values given therein). Therefore, anaerobic microorganisms were fairly active in the soil samples, but methanogens were not. There are several reasons why peat soils fail to produce  $\text{CH}_4$ , even when other anaerobic microorganisms are active. Most of the research has focused on water table level and chemical characteristics of the peat material, which are the two primary factors controlling  $\text{CH}_4$  production (Svensson and Sundh 1992). Water table level determines the amount of  $\text{O}_2$  exposure to anaerobic methanogens. These samples from Alberta would have been above the water table for, at least, part of the year. Nevertheless, since the 40-day incubation *in vitro* without  $\text{O}_2$  failed to initiate much  $\text{CH}_4$  production, we conclude that the samples harbor few methanogens. This contrasts methanogens in soils from rice paddy wetlands that survive even when the soil is dried completely, and they recover methanogenic activity when wetted (Conrad 2002). Therefore

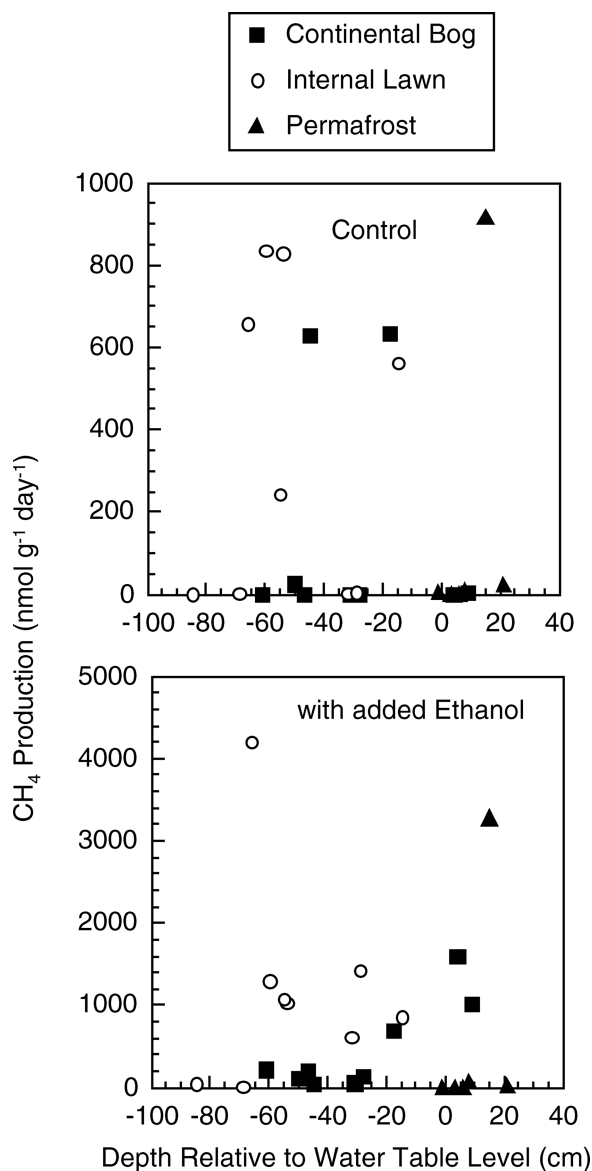


FIG. 3. Relationships between rates of  $\text{CH}_4$  production in peat soils from three peatland types in Saskatchewan, Canada, incubated *in vitro* at  $25^\circ\text{C}$  either without (top panel) or with added ethanol (bottom panel) relative to the water table level at the time of sample collection.

at least some wetland methanogens are inhibited but not killed by  $\text{O}_2$  exposure.

Water table level also determines the extent that peat material decomposes under oxic (above the water table level) versus anoxic (below the water table level) conditions. Decomposition under oxic conditions preferentially consumes carbohydrates leaving polyphenolic Klason lignin in the residue. When exposed to anoxic conditions, Klason lignin decomposes slowly, resulting in a limited supply of metabolic substrates to fuel methanogen activity (Yavitt et al. 2005). Turetsky et al. (2000) showed that peat material is distinctively richer in Klason lignin in the permafrost mound and internal lawn than in the continen-

tal bog. However, the availability of metabolic substrates did not appear to be the reason for little  $\text{CH}_4$  production because the addition of ethanol to alleviate any limitation by supply of metabolic substrates, increased  $\text{CH}_4$  production primarily in the three deepest samples in the internal lawn. Therefore, we conclude that the availability of metabolic substrates was not limiting  $\text{CH}_4$  production in the other samples.

The analysis of chemical element content in the peat soils points to a different reason for the lack of  $\text{CH}_4$  production in each peatland type. All of the peat soils had notably small concentrations of Cr, Ti, and V (Table 1). Both Cr and V are required for methanogen growth (Jarrell and Kalmokoff 1988), and the availability of trace metals can be small enough to limit  $\text{CH}_4$  production, in particular, in peat soils from remote regions that rely on atmospheric deposition for all chemical element input (Basiliko and Yavitt 2001). Although we do not know the specific concentrations of chemical elements required for methanogen growth in these peat soils, we can assess the relative supply of each chemical element by calculating its enrichment factor, after Gorham and Janssens (2005). Enrichment factors are concentrations of any particular chemical element normalized to Al in the peat soil. Aluminum is used, because it is well known to be derived from windblown mineral soil, not accumulate by plants, and measured precisely. Enrichment factors (EF) are:

$$\frac{[\text{M}] \text{ sample}/[\text{Al}] \text{ sample}}{[\text{M}] \text{ soil}/[\text{Al}] \text{ soil}}$$

where concentrations of any given element, M, and Al in soils are median values given in Bowen (1979). An  $\text{EF} = 1$  if windblown mineral soil is the only source of a given element. Values greater than 1 imply a local source of pollution and deposition on the peat soil. This calculation shows that Cr, Ti, and V have EF close to, or below 1.0, indicating very little supply of these elements to support growth of methanogens. Whether the concentrations of these trace metals control  $\text{CH}_4$  production *per se* needs to be validated experimentally. Notwithstanding, these data show that the peat soils have very low concentrations of metals, suggesting that resident methanogens are adapted to low ionic strength conditions for growth. Indeed, a medium that mimics peat chemistry is necessary to cultivate the resident methanogens (cf., Brauer et al. 2006).

In contrast, most of the samples from Saskatchewan showed a 10- to 12-day lag period before the onset of  $\text{CH}_4$  production when incubated *in vitro* under anoxic conditions. The lag suggests either 1) a small population of methanogens and perhaps methanogens that were slow to respond to anoxic conditions, or 2) a large concentration of electron acceptors that fueled anaerobic respiration at the expense of  $\text{CH}_4$  production, i.e., the initiation of anaerobic  $\text{CO}_2$  production with no lag in the same samples (Figure 1).

Therefore, a poor correlation between rates of  $\text{CH}_4$  production and anaerobic  $\text{CO}_2$  production characterized both the peat soils from Alberta (Pearson  $r = 0.01$ ) and the deeper peat soils

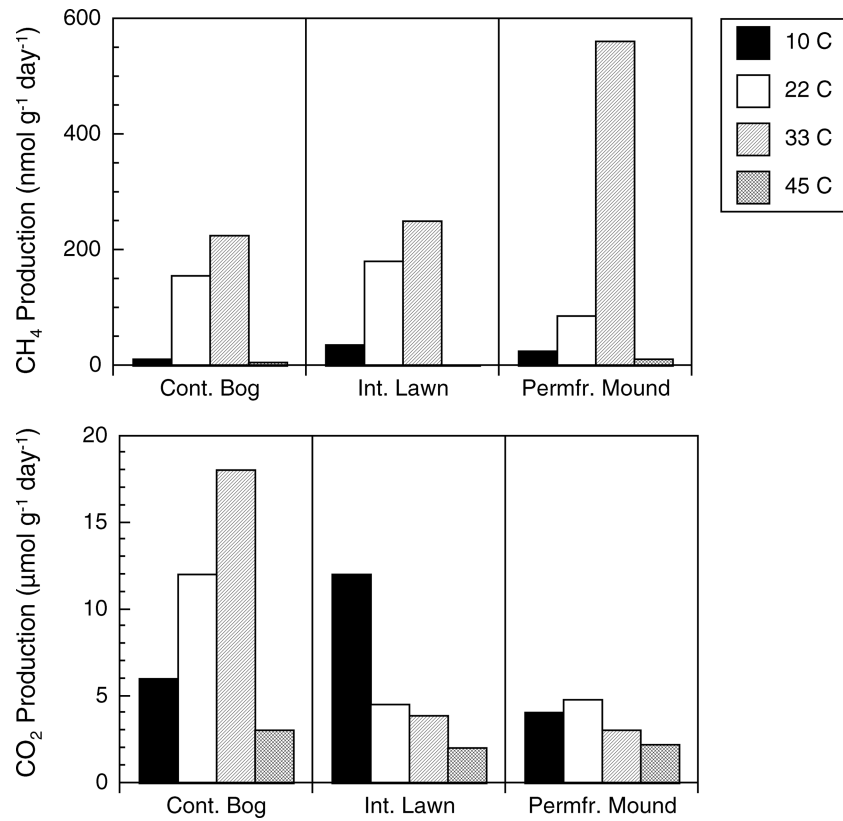


FIG. 4. Rates of CH<sub>4</sub> production (top row) and anaerobic CO<sub>2</sub> production (bottom row) in peat soils from three peatland types in Saskatchewan, Canada, incubated in vitro for 7 days across a range of temperatures.

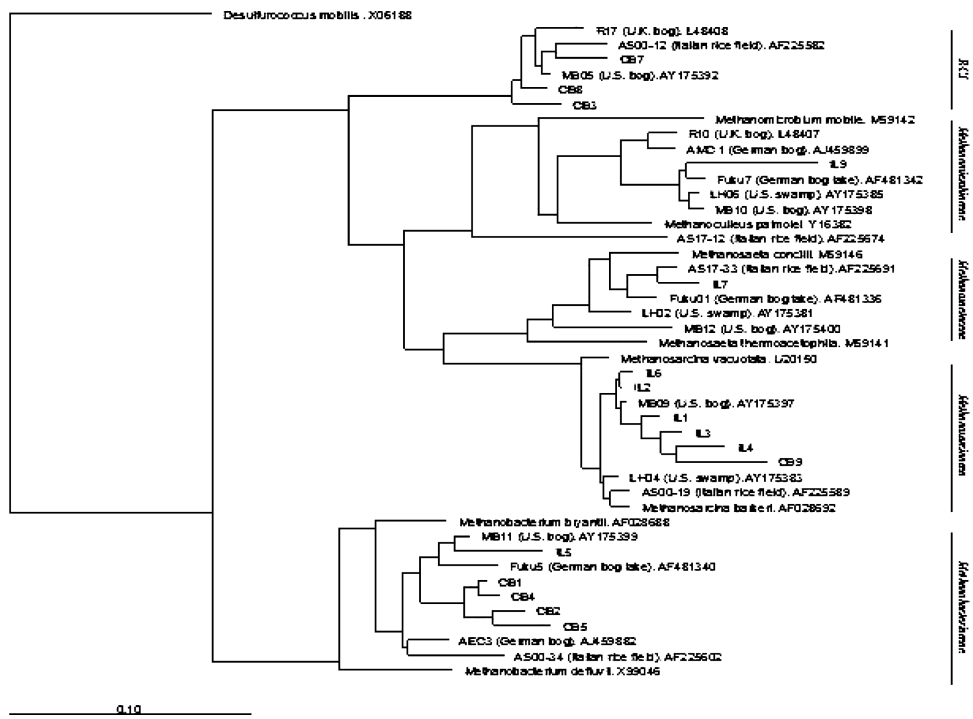


FIG. 5. Phylogenetic tree based on archaeal 16S rRNA gene sequences retrieved from continental bog (CB) and internal lawn (IL) peatlands in Saskatchewan, Canada and database sequences. The sources of similar environmental sequences are indicated in parentheses.

from Saskatchewan. When not constrained by temperature, rates of anaerobic CO<sub>2</sub> production in the peat soils are generally controlled by organic matter quality. The largest rates occur closer to the peat soil surface in least decomposed peat, and rates slow down with depth in deeper, more decomposed peat. In contrast, the largest rates of CH<sub>4</sub> production usually occur close to or just below the water table level, regardless whether that level is close to the surface of the peat, or deeper in the profile (Sundh et al. 1994; Clymo and Pearce 1995; Kettunen et al. 1999). The reason being that the soil immediately below the water table level provides the best combination of anoxic conditions and production of the metabolic substrates for methanogens (acetate, H<sub>2</sub>). Rates of CH<sub>4</sub> production in samples without added ethanol showed no relationship with water table level (Figure 2). However, with the addition of ethanol, rates of CH<sub>4</sub> production in soils from the continental bog showed a strong linear relationship ( $r^2 = 0.77$ ) with the largest rates in samples at the water table level and slower rates in deeper samples. Since peat soils from the internal lawn and permafrost mound showed no relationship with depth relative to the water table level, we conclude that permafrost itself and permafrost melting creates a patchy environment for methanogens.

This idiosyncrasy also was evident in the response of CH<sub>4</sub> production to added ethanol. Although added ethanol reduced the lag time before the onset of CH<sub>4</sub> production, samples from the continental bog and internal lawn still showed greater 10-fold range in rates of CH<sub>4</sub> production with added ethanol. Because the ethanol response occurred with a short lag time, we conclude that the patchiness in rates of CH<sub>4</sub> production reflects patchy distribution of methanogen populations in these peat soils. No response to added ethanol in peat soils from the permafrost mound indicates an unresponsive population. Ethanol is a good metabolic substrate to stimulate methanogenic activity. Although few methanogens appear to use ethanol directly (Zellner and Winter 1987), all methanogens can take advantage of the H<sub>2</sub> and acetate produced by anaerobic ethanol oxidation in peat soils (Coles and Yavitt 2002; Metje and Frenzel 2005).

The long-term incubation and recovery experiment provided insight into methanogen population activity. In each peatland type we selected type we selected one sample with a CH<sub>4</sub> production rate <10 nmol g<sup>-1</sup> and one sample with a CH<sub>4</sub> production rate >600 nmol g<sup>-1</sup>. After the long incubation, then warming to 25°C, both samples from the continental bog and internal lawn had large ethanol-induced rates of CH<sub>4</sub> production. This finding suggests that low rates in the 'fresh' samples might be transient. It is likely that the cold temperature treatment changed dominance among methanogen populations in the samples. In contrast, the very long lag time before the onset of any CH<sub>4</sub> production in samples from the permafrost mound again indicates the very low population sizes and unresponsive nature.

Despite long cold winters in the boreal zone, only anaerobic CO<sub>2</sub> production in peat soils from the internal lawn showed evidence of the largest rates at colder (10°C) than warmer tem-

peratures. Methanogens in all three sites had a temperature optima close to 35°C, which is typical of methanogenesis even in soils that experience prolonged cold temperatures (Zinder 1993). Indeed, only warm temperature induced large rates of CH<sub>4</sub> production in a majority of samples from the permafrost mound.

The predominance of sequences in the *Methanosarcinaceae* in peat soil from the internal lawn suggests the acetoclastic pathway for methanogenesis, which occurs only in members of the *Methanosarcinaceae* and *Methanosaetaceae* (Garcia et al. 2000). Moreover, the dominance of *Methanosarcinaceae* rather than *Methanosaetaceae* suggests that relatively large, millimolar concentrations of acetate fuel CH<sub>4</sub> production. *Methanosarcinaceae* have a high threshold for acetate and out compete members of the *Methanosaetaceae* at high acetate concentrations, whereas *Methanosaetaceae* dominate at low acetate concentrations (Westermann et al. 1989). Although acetate can cause toxicity problems in acidic pH soil (Russell 1992), acetoclastic methanogenesis and *Methanosarcinaceae* characterized a bog peatland with pH 4.8 soil in Siberia (Kotsyurbnko et al. 2004). Moreover, Wagner et al. (2005) found that adding 20 mM acetate to peat soil from a wet collapse scar in a more northern permafrost bog induced a large increase in CH<sub>4</sub> production. Several pure culture strains of *Methanosarcina* have been isolated that can tolerate pH as acidic as 4.3, but only in the presence of methanol and H<sub>2</sub> (Maestrojuan and Boone 1991). In contrast, *Methanosaetaceae* seem to account for acetoclastic methanogenesis in cool temperate bogs (Galand et al. 2005; Cadillo-Quiroz et al. 2006).

The prevalence of sequences in the *Methanobacteriales* and Rice Cluster I suggest methanogenesis from CO<sub>2</sub> reduction in the continental bog. Members of RC-I, although not yet cultivated, have been selectively enriched with H<sub>2</sub>-CO<sub>2</sub> (Lueders et al. 2001; Sizova et al. 2003) suggesting they utilize the CO<sub>2</sub> reduction pathway. Sizova et al. (2003) also established an enrichment culture derived from a peat soil that showed methanogenesis from CO<sub>2</sub>-reduction at pH 4.9 in which the dominant member was in the *Methanobacteriales*. Hoj et al. (2005) found members of *Methanobacteriales* in a cold, high Arctic wetland, but with pH 6.0 soils, which contrasts our sites that have acidic pH soil.

The combination of anaerobic ethanol oxidation and *Methanobacteriales* is especially notable. Metje and Frenzel (2005) found a *Methanobacteriales* sequence very closely affiliated with the type strain of *Methanobacterium bryantii*, which was isolated from a syntrophic ethanol-oxidizing coculture of "*Methanobacterium omelianskii*" (Bryant et al. 1967). The only known methanogen that can use ethanol directly is the thermophilic marine isolate *Methanogenium organophilum* in the *Methanomicrobiales* (Widdel et al. 1988). Coles and Yavitt (2002) found that ethanol added to peat soil from forested peatland fueled greater stoichiometric yield of CH<sub>4</sub> than that predicted from *M. organophilum*, suggesting either a new pathway for direct ethanol oxidation to CH<sub>4</sub> by a methanogen, or a priming effect of the added ethanol on carbon flow.

In both the internal lawn and the continental bog, the largest diversity of methanogens occurred in samples close to the water table level. The deepest sample that we examined in the internal lawn had a member of the *Methanosaetaceae*, whereas the deepest sample from the continental bog had a member of the *Methanosarcinaceae*. It is unusual to find methanogens using the acetoclastic pathway so deep in the peat soil, since this usually relies on an active bacterial assemblage to produce enough acetate. However, finding a gene sequence does not confirm activity in situ, and these sequences might be remnants of methanogen populations in the past.

We did not detect methanogens in four different samples from the permafrost mound. Other studies have found microorganisms within permafrost itself (Gilichinsky and Wagener 1995; Rivkina et al. 1998). Our results imply very small population sizes and/or inefficient DNA extraction. It would be interesting to determine whether methanogens in frozen soil layers of permafrost mounds have similar microbial composition to internal lawn peat, which would suggest that internal lawns assume resident methanogens and do not inherit populations from the continental bogs, despite the much larger extent of the bogs and close proximity to the internal lawns. However, the results presented here argue that internal lawns do not behave as islands colonized by methanogens from surrounding continental bogs. It is notable that during the course of ecological succession the internal lawns begin to look like continental bogs, as bog-like plant species, specifically *Sphagnum* mosses, grow over the fen-like plants, resulting in a fen-to-bog transition (cf., Hughes and Barber 2003). Analyses of methanogen populations in other fen-to-bog transitions, albeit without permafrost melting, reveal fairly distinct methanogen populations in the bog versus the fen (Cadillo-Quiroz et al. 2006; Merila et al. 2006).

In summary, average rates of CH<sub>4</sub> production were greater in the internal lawn (400 nmol g<sup>-1</sup> day<sup>-1</sup>) than in the continental bog (150 nmol g<sup>-1</sup> day<sup>-1</sup>), with much slower rates in the permafrost mound (10 nmol g<sup>-1</sup> day<sup>-1</sup>; although, one sample had 900 nmol g<sup>-1</sup> day<sup>-1</sup>). This site difference allows us to generalize that melting permafrost leads to the initiation and/or enhancement of CH<sub>4</sub> production. Although the emission of CH<sub>4</sub> into the atmosphere depends on a more complex set of processes, including CH<sub>4</sub> oxidation and transport or diffusion through the peat soil, our findings appear consistent with Turetsky et al. (2002) who reported up to 30-fold increases in CH<sub>4</sub> emissions in melted permafrost Canadian peatlands. We expand on the findings of Turetsky et al. (2002) by illustrating that CH<sub>4</sub> production is particularly patchy in permafrost and in melted permafrost soils. The mechanistic basis for patchiness still needs more work, which is essential for predictive models.

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