**RESEARCH ARTICLE**

**Anaerobic trophic interactions of contrasting methane-emitting mire soils: processes versus taxa**

Sindy Hunger, Anita S. Gößner and Harold L. Drake*

Department of Ecological Microbiology, University of Bayreuth, 95440 Bayreuth, Germany

*Corresponding author: Department of Ecological Microbiology, University of Bayreuth, 95440 Bayreuth, Germany. Tel: (49) (0)921-555640; Fax: (49) (0)921-555793; E-mail: HLD@Uni-Bayreuth.De

One sentence summary: Wetlands such as mires are a primary global source of methane, and the study provides physiological and molecular insights on the methanogenic food webs of contrasting mires.

**ABSTRACT**

Natural wetlands such as mires contribute up to 33% to the global emission of methane. The emission of methane is driven by trophic interactions of anaerobes that collectively degrade biopolymers. The hypothesis of this study was that these interactions in contrasting methane-emitting mire soils are functionally similar but linked to dissimilar taxa. This hypothesis was addressed by evaluating anaerobic processes and microbial taxa of eutrophic, mesotrophic and oligotrophic mire soils. Glucose was fermented to various products (e.g. H₂, CO₂, butyrate, acetate). Acetoclastic methanogenesis occurred, and acetogenesis and methanogenesis transformed H₂-CO₂ to acetate and methane, respectively. Although product profiles, cultivable cell numbers and gene copy numbers [mcrA (encodes alpha-subunit of methyl-CoM reductase) and 16S rRNA genes] were similar for all mire soils, only approximately 15% of detected family-level bacteria and species-level methanogens were shared by all mire soils. Approximately, 40% of the detected family-level taxa of each mire soil have no cultured isolates. Acidic conditions appeared to restrict the number of dominant phylotypes. The results indicated (a) that microbial processes which drive methanogenesis are similar but facilitated by dissimilar microbial communities in contrasting mire soils and (b) that mire soils harbor a large number of taxa with no cultured isolates.

**Keywords:** acetogenesis; methanogenesis; fermentation; peatlands; wetlands

**INTRODUCTION**

Natural wetlands are the single most important source of methane and contribute up to 33% to the global annual emission of methane (Stocker et al. 2013). Wetlands are distinguished from other terrestrial ecosystems by having (a) a water table near the land surface, (b) unique soil conditions that are strongly influenced by the limited availability of molecular oxygen (O₂) and (c) a specialized biota that is characterized by plants and other organisms that are adapted to wet and reduced soils (Charman 2002; Rydin and Jeglum 2006). Mires are a type of wetland that includes fens (water and nutrients derived from precipitation and other sources) and bogs (precipitation as primary source of water and nutrients), and peat-forming mires are often called peatlands (Charman 2002). Only about 2.3% (i.e. 4 x 10⁶ km²) of the earth’s terrestrial surface is covered by peatlands, and most peatlands are located in the northern hemisphere (Gorham 1991; Immirzi, Maltby and Clymo 1992). Despite this relatively small area, peatlands of the northern hemisphere store about one third (i.e. 455 Pg carbon) of the total global pool of soil carbon and contribute considerably to the emission of methane (Gorham 1991; Stocker et al. 2013).

The emission of methane in water-saturated soils is linked to the microbe-mediated anaerobic degradation of plant-derived
polymers that yield intermediates that drive methanogenesis if carbon dioxide (CO₂) is the primary terminal electron acceptor available (Zehnder 1978; McNerny and Bryant 1981). At the level of the system, the network of intermediary processes of a methanogenic foodweb can be viewed as ‘intermediary ecosystem metabolism’ (Drake, Horn and Wüst 2009). Hydrolysis of plant-derived polymers such as lignocellulose yields sugars (e.g. glucose) that can be fermented to acetate and other fatty acids, alcohols, and molecular hydrogen (H₂) and CO₂ by primary fermenters of anoxic soils (Hamberger et al. 2008; Drake, Horn and Wüst 2009). Intermediates like acetate and H₂-CO₂ are potential substrates of methanogens and have been shown to be drivers of methane production in different peatland soils (McNerny and Bryant 1981; Abbanat et al. 1989; Westermann 1993; Bräuer, Yavitt and Zinder 2004; Wüst, Horn and Drake 2009b). H₂-CO₂ can also be utilized by acetogens, and methanogens and acetogens may under certain conditions compete for the same substrates in fen and bog soils (Bräuer, Yavitt and Zinder 2004; Deppe, McKnight and Blodau 2010; Hunger et al. 2011). However, most microbiological studies that have investigated the production of methane by such ecosystems have focused on either a single intermediary process linked to methane or a single wetland soil (e.g. Kotsyurbanen et al. 1996; Bräuer, Yavitt and Zinder 2004; Cadillo-Quiroz et al. 2006; Drake, Horn and Wüst 2009b; Lin et al. 2014a, b), and little is known about the potential differences and similarities of anaerobic processes and associated microbial communities that drive methanogenesis in contrasting methane-emitting mire soils.

The hypothesis of this study was that trophic interactions of anaerobes in contrasting methane-emitting mire soils are functionally similar but linked to dissimilar taxa. This hypothesis was addressed by (a) examining glucose-, acetate- and H₂-CO₂-driven anaerobic processes; (b) resolving the bacteria and methanogens potentially associated with these anaerobic processes; and (c) estimating cell and gene copy numbers of four contrasting eutrophic, mesotrophic and oligotrophic methane-emitting mire soils.

### MATERIALS AND METHODS

#### Sampling sites

Soil samples were taken in the summer or early autumn between June 2011 and August 2013 from four mires in Germany (Table 1). Soil samples that were used for the analyses of cultivable microorganisms and gene copy numbers were collected within 9 days from all mires. Soils were sampled from 5–30 cm depth and transported on ice in airtight sterile plastic bags for microcosm experiments and for analysis of soil parameters, or transported on dry ice and stored at −20°C for molecular analysis. Three soil samples that were obtained 4–100 m apart from each other were analyzed for each of the mires.

#### Anoxic microcosms

Soil samples were homogenized, and 10 g of fresh weight soil was placed in sterile 250 ml-infusion flasks (Merck ABS, Dietikon, Switzerland) and was diluted with 35 ml-anoxic mineral solution. The pH of the mineral solution was adjusted according to the corresponding pH of the mire pore water. The mineral solution contained (in mg L⁻¹) mineral salts [KH₂PO₄ 10; NH₄Cl 4.6; MgCl₂·6H₂O 10; CaCl₂·2H₂O 10 (modified from Wüst, Horn and Drake 2009b)], trace metals [MnSO₄·H₂O 2.5; FeCl₃·4H₂O 0.7; CoCl₂·6H₂O 1; CaCl₂·2H₂O 1; ZnCl₂ 0.5; Al₂(SO₄)₃·12H₂O 0.2; H₂BO₃ 0.1; Na₂MoO₄·2H₂O 0.1; CuSO₄·5H₂O 0.1; Na₂WO₄·2H₂O 0.05; NiCl₂·2H₂O 0.2; H₂SeO₃ 0.5 (modified Balch et al. 1979)] and 10-ml vitamin solution (Balch et al. 1979). The infusion flasks were sealed with rubber stoppers and crimps, and flushed with sterile helium (100%). Anoxic solutions and substrates were prepared by using modified Hungate techniques (Hugnate 1969; Daniel and Drake 1993) and were sterilized by autoclaving or filter sterilization. Triplicate soil microcosms were supplemented with either 5 mM glucose, 0.3 mM acetate or 10 ml of H₂-CO₂ (4:1). Glucose and H₂-CO₂ were added once at the beginning of incubation, and acetate was added repeatedly every week. Triplicate unsupplemented soil microcosms served as controls.

### Table 1. Characteristics of contrasting mires in Germany.

<table>
<thead>
<tr>
<th>Type of mire</th>
<th>Mire 1</th>
<th>Mire 2</th>
<th>Mire 3</th>
<th>Mire 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of peat</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>pH</td>
<td>7.6 ± 0.4</td>
<td>4.3 ± 0.1</td>
<td>4.9 ± 0.9</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>81 ± 4</td>
<td>87 ± 6</td>
<td>93 ± 6</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Total C (g kg⁻¹)</td>
<td>259 ± 70</td>
<td>380 ± 31</td>
<td>459 ± 6</td>
<td>482 ± 13</td>
</tr>
<tr>
<td>Total N (g kg⁻¹)</td>
<td>20 ± 4</td>
<td>17 ± 4</td>
<td>10 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>13</td>
<td>22</td>
<td>46</td>
<td>53</td>
</tr>
<tr>
<td>NPOC (mg L⁻¹)</td>
<td>79 ± 32</td>
<td>115 ± 31</td>
<td>105 ± 36</td>
<td>171 ± 21</td>
</tr>
<tr>
<td>Cl⁻ (mg L⁻¹)</td>
<td>7.2 ± 4.7</td>
<td>21.1 ± 0.9</td>
<td>3.2 ± 1.9</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>NO₃⁻ (mg L⁻¹)</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>SO₄²⁻ (mg L⁻¹)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

| Main vegetation | Oxalis acetosella, Carex remota, Urtica dioica, Chaerophyllum hirsutum, Rumex conglomeratus, Myosotis palustris, Picea abies | Carex rostrata, Carex nigra, Carex canescens, Molinia caerulea, Eriophorum vaginatum, Juncus effusus, Sphagnum sp. | Juncus conglomeratus, Molinia caerulea, Calluna vulgaris, Sphagnum sp. | Carex pauciflora, Eriophorum vaginatum, Eriophorum angustifolium, Pinus rotundata, Sphagnum sp. |
| Location        | Oberpfalz | Erzgebirge | Oberpfalz | Erzgebirge |

*Of pure water.

*Of soil dry weight.

*Non-purgeable organic carbon (NPOC).
Soil microcosms were incubated at 15°C in the dark. Sterile syringes were used to sample gas and liquid phases. The loss of gas and liquid due to sampling (1 ml for each sampling) was equal in all treatments, and the amounts of substrates and products were mathematically corrected accordingly. Liquid samples were stored at −20°C for molecular and chemical analyses.

Analytical techniques

Dry weight of soil, pH, concentrations of organic acids and concentrations of gases were determined (Küsel and Drake 1995; Wüst, Horn and Drake 2009a; Hunger et al. 2011). Nitrate, sulfate, phosphate, chloride, total nitrogen, total carbon in solid samples and non-purgeable organic carbon (NPOC) were analyzed at the Center for Analytical Chemistry (Bayreuth Centre of Ecological and Environmental Research, University of Bayreuth, Bayreuth, Germany) (Hunger et al. 2011). Units of concentration are per gram soil fresh weight [g soil fw⁻¹].

Most probable number (MPN)

The MPN growth medium contained (in mg L⁻¹) yeast extract (0.05), tryptone (0.05), minerals (see above), trace elements (see above), vitamins (see above), and root or soil extract (50 ml L⁻¹) (modified from Balch et al. 1979; Wüst, Horn and Drake 2009b). Cycloheximide (0.5 g L⁻¹) was added to theoxic medium to inhibit the growth of euukaryotes. Root extracts from mires 2, 3 and 4 were prepared by grinding 300-g fresh weight roots with a mortar and pestle and liquid nitrogen. Deionized water was added to ground roots to a final volume of 1 L. There were very few roots in mire 1 and no root extract was obtained. Instead, 500-g deionized water was added to 500-g soil. Solutions with ground roots and soil were autoclaved, incubated for 1 week, and filtered, resulting in clear root and soil extracts. The pH of the solutions was adjusted to the pH of the corresponding mire pore water. Solutions for the 10-fold dilution series were prepared as described above but did not contain yeast extract or tryptone. Two oxic and two anoxic 10-fold dilution series were prepared from three soil samples from each mire soil. Microbes were dispersed in the first dilution of the 10-fold dilution series with help of a dispersion solution (150-g sodium pyrophosphate L⁻¹⁻¹) and a mechanical procedure (1 h at 15°C with 100 cycles min⁻¹ on an end-over-end shaker (Bast 1999). The 10-fold dilution series was used to inoculate wells in quadruplicates in 96-well plates. Incubated 96-well plates were incubated in the dark at 15°C under oxic or anoxic conditions for 18 weeks. Wells were scored positive for growth if the optical density (Milton Roy Spectronic 501, Bausch & Lomb Inc., Rochester, NY, USA) at 660 nm increased at least 0.01 OD units and growth was visible to the naked eye. MPN values were calculated with the software MPN Calculator (http://www2workout.com/mcurie/mpn/index.html). Cultivable cell numbers are per gram soil dry weight [g soil dw⁻¹].

Nucleic acid extraction and cloning

Nucleic acids were obtained from soil samples and from samples that were taken from each replicate of the microcosms on day 21 of the incubation. Nucleic acids were extracted by bead-beating lysis, organic solvent extraction and precipitation (Griffiths et al. 2000). Approximately, 120-mg glass beads (0.1 mm in diameter), 110-mg glass beads (0.5 mm in diameter), 30-mg glass beads (1 mm in diameter) and 2 glass beads (3 mm in diameter) were used for each extraction. DNA was amplified according to published protocols with the following primer sets (Lueders et al. 2001; Hunger et al. 2011): (a) 27f and 907r for bacterial 16S rRNA genes (Lane 1991), and (b) mcrAf and mcrAr for mcrA (Springer et al. 1995). Chemicals of PCR assays and final concentrations of reagents were as described (Hunger et al. 2011). PCR products for cloning were ligated into pGEM-T (Promega, Mannheim, Germany) or CloneJET vector plasmids (Thermo Fisher Scientific, MA, USA). Competent cells of Escherichia coli JM109 (Promega; protocol as per manufacturer's instructions) were transformed with ligated vector plasmids. Clones were randomly picked. A total of 42 clones per gene and per treatment or soil were analyzed (i.e. 1920 clones in total). Correct inserts were determined by M13 or pJET1.2 PCR (primer set M13f/M13r or pJET1.2f/pJET1.2r) according to published (Mensing 1983) or manufacturer’s protocols, respectively. Sequencing was done by Macrogen (Amsterdam, Netherlands).

Identification of phylotypes

Analysis and assignment of mcrA and bacterial 16S rRNA genes to species and families was performed with ARB (SILVA version from August 2012) and Mega as described (Ludwig et al. 2004; Hunger et al. 2011; Tamura et al. 2011). Chimeric sequences were eliminated from the analysis. Representative sequences from each family-level 16S rRNA gene and species-level mcrA phylotype were analyzed in 2014 with the Basic Local Alignment Search Tool (BLAST) to obtain sequences from the next related cultivated organisms. 16S rRNA gene sequence similarities of 87.5% and mcrA-encoded amino acid sequence similarities of 85.7% were used to determine family- and species-level phylotypes (OTUs), respectively (Yarza et al. 2008; Hunger et al. 2011). Coverage was calculated by published protocol (Schloss, Larget and Handelsman 2004).

Phylogenic trees of mcrA genes were calculated with nucleotide and amino acid sequences with the following algorithms: neighbor-joining (PAM or Olsen correction), maximum likelihood (Dayhoff correction) and maximum parsimony. The mcrA trees used a 100% similarity filter with 133 valid amino acid positions between 96 and 227 of mcrA of Methanocella paludicola SANAE or a 100% similarity filter with 397 nucleotide positions between 288 and 681 of mcrA of M. paludicola SANAE.

Classification of affiliated organisms was done according to the homepage of ‘List of prokaryotic names with standing in nomenclature’ (LPSN, http://www.bacterio.net). For example, mcrA amino acid sequence of clone A12C6 (LN716351) was 91% similar to the sequence of Methanoregula formica (determined by ARB similarity matrix and BLAST). The classification of M. formica to the family Methanoregulaceae was done according to LPSN.

Quantitative PCR (qPCR)

qPCR, melting curves and quality control were based on published protocols (Schellenberger, Drake and Kolb 2011; Depkat-Jakob et al. 2012). Three soil samples per mire were obtained for nucleic acid extractions. Each soil sample was extracted three times, and each extraction was used for the triplicate qPCR assay for determining gene copy numbers. For bacterial 16S rRNA genes, the primers EuB341f and EuB534r were utilized with 30 cycles of amplification (Muyzer, De Waal and Uitterlinden 1993). For mcrA genes, the primers mcrAf and mcrAr were utilized with 50 cycles of amplification (Springer et al. 1995). Each qPCR assay was prepared with SensiMix SYBR & Fluorescein Kit (Bioline,
 Luckenwalde, Germany). Final concentrations of PCR reagents were 1.25 μM (mcrA) or 0.75 μM (16S rRNA genes) of each primer, 0.25 mg bovine serum albumin ml⁻¹ (mcrA), and 6 mM (mcrA) or 3 mM (16S rRNA genes) MgCl₂.

Calculations of Gibb’s free energy

The changes of the Gibb’s free energies (ΔG) were calculated as described (Thauer, Jungermann and Decker 1977; Hunger et al. 2011). The following equations were used to calculate ΔG values for methanogenesis and acetogenesis: 4 H₂ + CO₂ → CH₄ + 2 H₂O (ΔG° = -130.8 kJ mol⁻¹); 4 H₂ + 2 CO₂ → CH₃COO⁻ + H⁺ + 2 H₂O (ΔG° varied from -99.7 to -118.4 kJ mol⁻¹). Values are given in kJ mol⁻¹ methane for methanogenesis and kJ mol⁻¹ acetate for acetogenesis.

Statistical analysis

Statistical tests (i.e. ANOVA, and Principal Component Analysis) were performed with R version 3.1.2 (http://www.rstudio.org/).

Accession numbers

The sequences obtained in this study are available from EMBL nucleotide sequence database under the following accession numbers: (a) bacterial 16S rRNA genes under LN715239–6107 and (b) mcrA genes under LN716108–7036.

RESULTS

Abiotic characteristics of mires

The four mire soils varied significantly in their pH (P < 0.001), water content (P < 0.002), concentration of total carbon (P < 0.001), concentration of total nitrogen (P < 0.002), concentration of PO₄³⁻ (P < 0.04) and had different vegetations (Table 1). Concentrations of Cl⁻, NO₃⁻ and SO₄²⁻ were similar (P > 0.06) in the different mire soils, and NPOC increased with decreasing pore water pH. The concentration of total carbon increased and the concentration of total nitrogen decreased with increasing water content. Based on the ratio of carbon to nitrogen and pH (Joosten 2001), the soil of mire 1 was eutrophic pH-neutral, the soil of mire 2 was mesotrophic acid and the soils of mires 3 and 4 were oligotrophic acidic.

Product profiles of anoxic un-supplemented microcosms

CO₂ and methane accumulated and traces of acetate, lactate and formate were detected in all un-supplemented microcosms (Fig. 1, data for lactate and formate not shown), suggesting that methanogenesis, fermentation and/or anaerobic respiration occurred at the expense of endogenous substrates.

Effect of supplemental glucose on product profiles

Glucose consumption stimulated fermentation and methanogenesis in all anoxic microcosms (Fig. 1). Theoretical recoveries of glucose-derived carbon and reductant in microcosms from all mires indicated that 21–29% of the carbon was recovered in CO₂, 21–40% of the reductant was recovered in butyrate and 16–24% of the reductant was recovered in acetate (Table S1, Supporting Information). Up to 13 and 15% of reductant from glucose were theoretically recovered in ethanol and H₂, respectively (Table S1, Supporting Information). Ethanol was produced during the degradation of glucose in microcosms from mires 1, 2 and 4, but not in microcosms from mire 3 (Fig. 1). Accumulated H₂ was consumed after glucose was completely degraded (Fig. 1). Acetate accumulated during the utilization of H₂ and CO₂ in microcosms from mire 1 (Fig. 1A and B). The consumption of CO₂ and the accumulation of acetate were not observed after H₂ was essentially consumed (Fig. 1A and B), indicating that acetogenesis was an ongoing process until H₂ was depleted. Propionate was a minor product of the degradation of glucose in microcosms from mires 1–3 but more abundant in microcosms of mire 4 (Fig. 1, Table S1, Supporting Information). Formate was detected periodically in trace amounts in all microcosms, suggesting that varying amounts of formate might have been formed and utilized during the different incubations.

Effect of supplemental H₂-CO₂ on product profiles

Supplemental H₂-CO₂ stimulated the production of acetate and methane in all anoxic microcosms (Fig. 2, Table S2, Supporting Information). Theoretical recoveries of glucose-derived carbon and reductant in microcosms from all mires indicated that 21–29% of the carbon was recovered in CO₂, 21–40% of the reductant was recovered in butyrate and 16–24% of the reductant was recovered in acetate (Table S1, Supporting Information). Ethanol was produced during the degradation of glucose in microcosms from mires 1, 2 and 4, but not in microcosms from mire 3 (Fig. 1). Accumulated H₂ was consumed after glucose was completely degraded (Fig. 1). Acetate accumulated during the utilization of H₂ and CO₂ in microcosms from mire 1 (Fig. 1A and B). The consumption of CO₂ and the accumulation of acetate were not observed after H₂ was essentially consumed (Fig. 1A and B), indicating that acetogenesis was an ongoing process until H₂ was depleted. Propionate was a minor product of the degradation of glucose in microcosms from mires 1–3 but more abundant in microcosms of mire 4 (Fig. 1, Table S1, Supporting Information). Formate was detected periodically in trace amounts in all microcosms, suggesting that varying amounts of formate might have been formed and utilized during the different incubations.
The consumption of CO₂ and H₂ was concomitant with the formation of acetate (Fig. 2), an activity indicative H₂-dependent acetogenesis. Acetate was consumed as soon as H₂ was depleted (Fig. 2B and F). Methane accumulated as long as H₂ and/or acetate were present (Fig. 2), which is indicative of hydrogenotrophic and acetoclastic methanogenesis, respectively. The apparent production of methane from H₂-CO₂ occurred before the apparent H₂-CO₂-dependent production of acetate in microcosms from mires 1 and 3 (Fig. 3). The theoretical recovery of reductant above 100% in microcosms of mires 1 and 2 (Table S3, Supporting Information) indicated that supplemental acetate enhanced the utilization of endogenous substrates.

Bioenergetics

The estimated Gibb’s free energies of H₂-dependent methanogenesis (i.e. −32 to −100 kJ mol⁻¹) and acetogenesis (i.e. −21 to −108 kJ mol⁻¹) indicated that those processes were thermodynamically feasible under the experimental conditions (Fig. 4). The estimated Gibb’s free energies of acetogenesis were 8–14 kJ mol⁻¹ more negative than of methanogenesis in microcosms of mire 1 (Fig. 4). In contrast, the estimated Gibb’s free energies of methanogenesis were 19–43, 23–32 and 23–30 kJ mol⁻¹ more negative than of acetogenesis in microcosms of mires 2, 3 and 4, respectively (Fig. 4).
Figure 5. Phylogenic maximum-likelihood tree of (a) representative species level amino acid sequences encoded by mcrA retrieved from contrasting mire soils and from microcosms at the end of the 21 day incubation, and (b) reference sequences. Filled dots at nodes indicate the confirmation of tree topology by six calculations with the same data set whereby nucleic acid and corresponding amino acid sequences were used with maximum-likelihood, neighbor-joining and maximum parsimony algorithms. Empty dots at nodes indicate the confirmation of tree topology by five out of the six calculations. Methanopyrus kandleri (AE009439) was used as outgroup. Bar indicates a 0.1 estimated change per amino acid. Accession numbers are given in parentheses. Legend: A, mire soil; B, unsupplemented microcosms; C, glucose-supplemented microcosms; D, acetate-supplemented microcosms; E, H₂-CO₂-supplemented microcosms; -, not detected. Coverage and number of sequences of gene libraries: mire 1, 99%, 232 sequences; mire 2, >99%, 226 sequences; mire 3, >99%, 238 sequences; mire 4, >99%, 235 sequences.

Diversity of mcrA gene phylotypes

A total of 10 family- and 20 species-level mcrA phylotypes were detected in microcosms and soils, including Methanobacteriaceae, Methanoccaceae, Methanoregulaceae, Methanosarcinaceae and five family-level phylotypes without any cultured isolates (Fig. 5). Some species-level phylotypes had a relative abundance of over 24% in a mire soil and decreased in abundance during incubation of the unsupplemented control [e.g. OTU 1 (Methanoregulaceae) and OTU 14 (Methanosarcinaceae) in microcosms of mire 2, and OTU 4 (Methanoregulaceae) in microcosms of mire 3] (Fig. 5). Other species-level phylotypes increased in relative abundance after supplementation of substrate [e.g. OTU 1 in microcosms of mire 2 after supplementation of H₂-CO₂, OTU 2 (Methanoregulaceae) in microcosms of mire 2 after supplementation of H₂-CO₂ and OTU 14 in microcosms of mires 1, 3 and 4 after supplementation of either glucose or acetate] (Fig. 5). Species-level phylotypes with a relative abundance of 24% or more had a total relative abundance of 29, 59, 50 and 76% in soils of mire 1 (pH 7.6), mire 2 (pH 4.3), mire 3 (pH 4.9) and
mire 4 (pH 3.9), respectively (Fig. 5), suggesting that acidic conditions restricted the relative number of dominant methanogenic phylotypes.

### Diversity of bacterial 16S rRNA gene phylotypes

A total of 13 phyla and 86 family-level phylotypes without any cultured isolates were detected in microcosms and soils (Tables 2 and S4, Supporting Information). Acidobacteria and Proteobacteria were the most abundant phyla in mire soils, and the relative abundance of Acidobacteria (especially Acidobacteriaceae, Tables 3 and S4, Supporting Information) increased with decreasing mire pore water pH (Table 2; Fig. S1, Supporting Information). Some family-level phylotypes within the Acidobacteria that had a relative abundance in the mire soil of over 10% decreased in relative abundance during the incubation period (e.g. phylotype 3 in mire 1, phylotype 5 in mire 2 and phylotype 1 in mire 4) (Table 3). The relative abundance of certain family-level phylotypes increased more greatly after supplementation of substrate than in the unsupplemented controls (e.g. Clostridiaceae in microcosms of mire 2 and 4 with supplemental glucose, Veillonellaceae in microcosms of mire 2 and 4 with supplemental H₂CO₂, Planctomycetaceae in microcosms of mire 2 and 3 with supplemental glucose and/or acetate, Hyphomicrobiaceae in microcosms of mire 1 with supplemental glucose and acetate, Methylocystaceae and Acetobacteriaceae in microcosms of mire 4 with supplemental acetate and family-level phylotype 28 in microcosms of mire 1 with supplemental H₂CO₂ (Table 3).

Family-level phylotypes with a relative abundance of 10% or more had a total relative abundance of 10, 38, 41 and 83% in soils of mire 1 (pH 7.6), mire 2 (pH 4.3), mire 3 (pH 4.9) and mire 4 (pH 3.9), respectively (Fig. 5), suggesting that acidic conditions restricted the relative number of dominant bacterial phylotypes.

### Differences and similarities of microbial communities

Major differences were observed between the microbial communities of the contrasting mires (Fig. 6). Only the following three species-level mcrA phylotypes (i.e. 15% of the 20 detected) were common to all mires: OTUs 2, 14 and 16 that were closely related to M. boonei, Methanosarcina vacuolata and Methanosaeta concilii, respectively (Fig. 5, Fig. 6). Furthermore, only the following 13 family-level 16S rRNA gene phylotypes (i.e. 15% of the 86 detected) were common to all mires: Acidobacteriaceae, Acidimicrobiaceae, Acidobacteriaceae, Beijerinckiaceae, Bradyrhizobiaceae, Chitinophagaceae, Clostridiaceae, Methylocystaceae, Planctomycetaceae, Thermomonosporaceae, a family-level phylotype affiliated with Conexibacteraceae, Palutilbacteraceae and Solirubrobacteraceae, and family-level phylotypes 1 and 41 (Table S4, Supporting Information; Fig. 6). Whereas Actinobacteria, Chloroflexi and Verrucomicrobia displayed a relative abundance of 10% or higher in certain mire soils, the relative abundance of these taxa were lower in other mire soils (e.g. the relative abundance of Actinobacteria in soil of mire 3 was 19% but was only 2% in soils of mire 2 and 4) (Table 2). A greater number of 16S rRNA family-level phylotypes without any cultured isolates were detected in eutrophic mire soil 1 (26) and mesotrophic mire soil 2 (19) than in the oligotrophic mire soils 3 (10) and 4 (11) (Table S4, Supporting Information). However, the relative percentage of family-level phylotypes without any cultured isolates was similar in all mire soils [i.e. mire 1, 45%; mire 2, 39%; mire 3, 34%; mire 4, 37% (includes both mcrA and 16S rRNA gene phylotypes)]. The majority of the species-level mcrA and family-level 16S rRNA gene phylotypes that were detected in microcosms or soils of mires 3 and 4 were also detected in microcosms or soils of mires 1 and 2 (Fig. 6).

### Gene copy numbers and cultivable cell numbers in mire soils

Copy numbers of 16S rRNA genes and mcrA did not vary significantly (F > 0.35) between mire soils, indicating that the
Table 3. Most abundant family-level phylotypes (i.e. phylotypes having a relative abundance of 10% or higher) and relative abundances of bacterial 16S rRNA gene sequences from mire soils and from microcosms at the end of the 21 day incubation.

<table>
<thead>
<tr>
<th>Taxonomic level (phylum, family)</th>
<th>Relative abundance of 16S rRNA gene sequences (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mire 1</td>
</tr>
<tr>
<td>Acidobacteria</td>
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<tr>
<td>Acidobacteriaceae</td>
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<tr>
<td>Family-level phylotype 1(^b)</td>
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<tr>
<td>Family-level phylotype 3(^b)</td>
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<tr>
<td>Family-level phylotype 5(^b)</td>
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<tr>
<td>Actinobacteria</td>
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<tr>
<td>Acidimicrobiaceae</td>
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<tr>
<td>Family-level phylotype 13(^b)</td>
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<tr>
<td>Firmicutes</td>
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<tr>
<td>Clostridaceae</td>
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<tr>
<td>Family-level phylotype 28(^b)</td>
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<tr>
<td>Planctobacteria</td>
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<tr>
<td>Planctomycetaceae</td>
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<tr>
<td>Hyphomicrobiaceae</td>
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<td>Methyloptaceae</td>
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<tr>
<td>Acetobacteriaceae</td>
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<tr>
<td>Family-level phylotype 22(^b)</td>
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</table>

\(^a\) -, not detected; A, mire soil; B, unsupplemented microcosms; C, glucose-supplemented microcosms; D, acetate-supplemented microcosms; E, H\(_2\)-CO\(_2\)-supplemented microcosms. Coverage of gene libraries: mire 1, 93%; mire 2, 92%; mire 3, 93%; mire 4, 94%.

\(^b\) Sequences were considered to be family-level phylotypes without any cultured isolates if the 16S rRNA gene sequence was less than 87.5% similar to the sequence of the closest related cultured species (Yashiro et al. 2011).
abundance of bacteria and methanogens were relatively uniform in the contrasting mire soils (Fig. 7A). Gene copy numbers of 16S rRNA genes were significantly greater (P < 0.04) than gene copy numbers of mcrA (i.e. 0.1–28.8 × 10^9 16S rRNA genes g[^soil\text{dw}]^-1 vs 0.5–111.6 × 10^9 mcrA g[^soil\text{dw}]^-1) (Fig. 7A) in each mire soil. The average ratios of the gene copy numbers of 16S rRNA genes to mcrA of the contrasting mires approximated 55, 40, 40 and 75 for soils of mire 1, 2, 3 and 4, respectively. Although the cultivable numbers of microbes capable of aerobic growth appeared in some cases to be somewhat greater than the cultivable number of microbes capable of anaerobic growth (Fig. 7B), the quantities of microorganisms capable of aerobic and anaerobic growth were not significantly different in the contrasting mire soils (P > 0.15).

**DISCUSSION**

It is well accepted that methanogenesis in mire soils is driven by the network of intermediary processes that link the initial hydrolysis of plant-derived polymers to the terminal production of methane; however, information on the trophic interactions of methanogens and these microbes that catalyze system-level intermediary events is very fragmented, and hypothetical models of potential processes that such soils have in common are for the most part conceptualized rather than resolved (Zehnder et al. 1978; Mcinerney and Bryant 1981; Drake, Horn and Wüst 2009). Although product profiles, cultivable cell numbers and gene copy numbers were similar for the contrasting mire soils, only approximately 15% of detected family-level bacteria and species-level methanogens were shared by all mire soils. Furthermore, 40% of the detected family-level taxa of each mire soil have no cultured isolates, and some of the detected phyla have been rarely reported for such soils (e.g. Armatimonadetes, Chlorobi, Nitrospirae) (Juottonen et al. 2005; Dedysh et al. 2006; Kraigher et al. 2006; Drake, Horn and Wüst 2009; Dedysh 2011; Serkebaeva et al. 2013; Lin et al. 2014a,b; Schmidt et al. 2015).

**Fermentation and associated Bacteria**

CO_2, butyrate and acetate were the main products during the fermentation of glucose (Fig. 1), a fermentation product profile similar to those observed with monosaccharide-supplemented Tundra wetland soil and rice straw-supplemented paddy soil (Kotsyurbenko et al. 1996; CO_2 not determined; Glissman and Conrad 2000, CO_2 not determined). 16S rRNA gene sequences affiliated with Acidobacteriaceae, Clostridiaceae, Planctomycetaceae and Veillonellaceae increased in their abundance after supplementation of glucose (Table 3). Acidobacteriaceae accounted for 25–41% of the bacterial community in soils of all acid mires (Table 3). Acidobacteriaceae-affiliated sequences were most closely related to Telmatobacter. Telmatobacter is adapted to moderately acidic pH, grows under microaerophilic and anoxic conditions, and ferments sugars (e.g. glucose, xylose) and polysaccharides (e.g. cellulose, cellobiose) to acetate, H_2 and traces of other compounds (Pankratov et al. 2012). Clostridiaceae produce butyrate or acetate as major fermentation products together with other organic acids, alcohols, H_2 and CO_2, and can utilize a wide range of sugars and proteinaceous substrates (Wiegel 2009). Members of Schlesneria (Planctomycetaceae) have been isolated from peat bogs and were capable of fermenting carbohydrates (Kulichevskaya et al. 2007). Veillonellaceae ferment sugars mostly to acetate, propionate, CO_2 and H_2 (Rainey 2009b). Members of other families that were affiliated with detected 16S rRNA gene sequences are known to ferment glucose or other sugars e.g. Anaerolineaceae, Methylophilaceae (Pleomorphomonas) and Otitutaceae (Table S5) (Xie 2005; Yamada et al. 2006; Cho et al. 2011; Madhaiyan et al. 2013). Based on the known properties of the detected taxa, fermentative taxa accounted for 24, 47, 36 and 63% in the soils of mires 1, 2, 3 and 4, respectively (Table S5, Supporting Information, Fig. 8). These findings suggest that Acidobacteriaceae, Clostridiaceae, Planctomycetaceae and Veillonellaceae are representative of fermentative taxa in mire soils.
Methanogenesis and associated Archaea

Methanogenesis in microcosms of all mire soils was stimulated by fermentation-derived intermediates and supplemental acetate and H₂-CO₂ (Figs 1–3), and mcrA sequences that were affiliated with acetoclastic and hydrogenotrophic methanogens were detected in all mire soils but with variable relative abundance (Fig. 5). Methanoseta and Methanosarcina dissimilirate acetate (Garrity and Holt 2001). Detected Methanosetaeae-affiliated mcrA sequences were more abundant in the near neutral pH soil of mire 1 than in the other more acidic mire soils 2–4 (Fig. 5, Fig. S1, Supporting Information), suggesting that Methanosaetaceae is not well adapted to acidic conditions. This possibility is reinforced by earlier observations on the occurrence of Methanosaetaceae in Finnish peatlands (Putkinen et al. 2009). Methanosarcina species also grow on H₂-CO₂ and may only use acetate if H₂ is depleted (Garrity and Holt 2001). Small amounts of H₂ were produced during the degradation of acetate in soil microcosms of mires 1–3 (Fig. 3), an observation that has been made with pure cultures of Methanosarcina (Garrity and Holt 2001). Acetate stimulated the production of methane (Fig. 3), and mcrA sequences affiliated to methanogens 2–4 (Fig. 5, Fig. S1, Supporting Information).
obligate acetoclastic methanogens were detected (Fig. 5), suggesting that acetate was dissimilated via acetoclastic methanogenesis. However, recoveries of reductant indicated that the dissimilation of endogenous substrates was enhanced by supplemental acetate, i.e. that acetate might have had a priming effect on microbes capable of utilizing endogenous substrates (Fontaine et al. 2004). In this regard, many mcrA sequences that were affiliated with hydrogenotrophic methanogens were detected in microcosms after the supplementation of acetate, implying that hydrogenotrophic methanogens might have been stimulated by acetate. Methanocella and Methanoregula produce methane from H$_2$-CO$_2$ and cannot dissiplate acetate but require acetate for assimilation (Sakai et al. 2008; Bräuer et al. 2011). Although Methanocellaceae was not detected in soils of the oligotrophic acidic mires 3 and 4 (Fig. 5), Methanocellales has been detected in another oligotrophic acidic mire soil at a depth of 25 cm or deeper (Lin et al. 2014b), suggesting that Methanocellaceae may occur in oligotrophic acidic mire soils but may not always be important to methanogenesis. Some species of Methanoregula, Methanocella and Methanobacterium can utilize formate (Garrity and Holt 2001; Sakai et al. 2008; Yashiro et al. 2011). Although methanol was not evaluated, species of Methanosarcina are able to utilize methanol, suggesting that methanol-dependent methanogenesis is a capacity of certain mire soil methanogens.

Most of the detected cultured methanogens grow over a pH range of 5–9 but prefer pH neutral conditions (Garrity and Holt 2001). An exception is M. boonei that has a more acidic pH range (i.e. pH 4.5–5.5) (Bräuer et al. 2011). The properties of M. boonei are consistent with the observation that the relative abundance of Methanoregula-affiliated sequences increased in mire soils with decreasing pore water pH (Fig. 5). Methanogens that can dissimilate H$_2$-CO$_2$ accounted for 56–87% of the detected abundance of methanogens in mire soils, whereas methanogens that can dissipilate acetate accounted for only 22–39% of the detected abundance of methanogens (Fig. 5), highlighting the potential contrasting importance of hydrogenotrophic and acetoclastic methanogenesis in the investigated mires.

**Acetogenesis and potentially associated Bacteria**

Acetogenesis competed with methanogenesis for H$_2$-CO$_2$ in microcosms of all mire soils (Fig. 2). Bog soil methanogens and acetogens can compete for H$_2$-CO$_2$ until approximately 4 mM acetate is produced, which is likely due to the acetate-dependent impairment of methanogenesis (Bräuer, Yavitt and Zinder 2004). Although the estimated Gibbs’ free energy of methanogenesis (Fig. 4) and the production of methane prior to the apparent hydrogenotrophic acetogenesis (Fig. 3) indicated that methanogens could outcompete acetogens for H$_2$-CO$_2$ under the conditions to which the acidic mire soils were experimentally subjected, 16S rRNA gene sequences affiliated with taxa that contain acetogens were detected in microcosms of all mire soils (e.g. Clostridium, Holophagaceae, Peptococcaceae, Ruminococcaceae, Spirochaetaceae and Veillonellaceae) (Bernalier et al. 1996; Drake, Küsel and Matthes 2006; Ezaki 2009; Rainey 2009a,b; Wiegel 2009; Paster 2011; Thrash and Coates 2011). In addition, 16S rRNA gene sequences affiliated with the acetogens Clostridium carboxidivorans (98% sequence similarity), C. drakei (97% sequence similarity) and C. magnus (96% sequence similarity) increased in abundance in the glucose treatment. These acetogens are capable of growth on sugars, alcohols and organic acids, and C. carboxidivorans and C. drakei are also capable of growth on H$_2$-CO$_2$, CO and amino acids (Drake, Gößner and Daniel 2008; Wiegel 2009). Veillonellaceae-affiliated 16S rRNA gene sequences increased in abundance in the H$_2$-CO$_2$ treatment (Table 3), and some of those sequences were most closely related to sequences from the monophyletic acetogenic genera Acetotena (91% sequence similarity) and Sporomusa (89% sequence similarity). Sporomusa utilizes H$_2$-CO$_2$, organic acids and alcohols whereas Acetotena utilizes H$_2$-CO$_2$ and sugars (Rainey 2009b). Acetogens that belong to the other aforementioned families are capable of utilizing diverse substrates including H$_2$-CO$_2$, organic acids, alcohols, aromatic compounds, amino acids and sugars (Bernalier et al. 1996; Drake, Gößner and Daniel 2008; Ezaki 2009; Paster 2011; Thrash and Coates 2011). The broad substrate spectrum of acetogens indicates that acetogens not only compete with methanogens for H$_2$-CO$_2$ but also compete with other microbes for a wide range of substrates in mire soils (Fig. 8) (Drake et al. 1997; Drake, Küsel and Matthies 2006).

**Metabolic diversity of additional taxa**

16S rRNA gene sequences that were affiliated with taxa capable of anaerobic respiration were detected in all mires [e.g. Acidimicrobiaceae, Desulfotheromicrobiaceae and Holophagaceae (Geothrix); Table S5, Supporting Information] (Kuever, Rainey and Widdel 2005; Itoh et al. 2011; Thrash and Coates 2011). Collectively, the detected taxa can reduce sulfate, nitrate, Mn(IV), iron(III), citrate or humic acids, and may utilize organic acids, multi-carbon compounds or H$_2$ as sources of reductant (Kuever, Rainey and Widdel 2005; Thrash and Coates 2011). Some 16S rRNA gene sequences were affiliated with facultative aerobes that can reduce nitrate under anoxic conditions [e.g. Hyphomicrobiaceae (Hyphomicrobium, Rhodoplanes) and Methylocystaceae (Pleomorphomonas)] (Garrity, Bell and Liburn 2005a; Xie 2005; Okamura, Kanbe and Hiroishi 2009; Madhaiyan et al. 2013). Acidimicrobiaceae-affiliated sequences were closely related to Aciditetrirrimas ferrireducens, an acidophilic facultative aerobe that can grow on sugars under oxic conditions and reduce iron(III) autotrophically with H$_2$ under anoxic conditions (Itoh et al. 2011).

Some of the detected 16S rRNA gene sequences were affiliated with aerobic taxa such as Acidobacteriaceae, Acidobacteriaceae, Hypomicrobiaceae, Methylocystaceae and Rhodospirillaceae (Table S5, Supporting Information) (Garrity, Bell and Liburn 2005a; Thrash and Coates 2011). Members of these taxa can utilize polymers, sugars, organic acids, amino acids or alcohols (Garrity, Bell and Liburn 2005a; Sizova et al. 2007). Some members of these taxa can also (a) fix atmospheric nitrogen [e.g. Acetobacteraceae, Methylocystaceae], (b) grow under low pH conditions [e.g. Acetobacteraceae, Acidobacteriaceae], (c) grow via methylotrophy [e.g. Hyphomicrobiaceae, Methylocystaceae] and (d) grow photoheterotrophically [e.g. Acetobacteraceae, Rhodospirillaceae] (Garrity, Bell and Liburn 2005a; Sizova et al. 2007). Family 1-affiliated sequences were most closely related to the aerobic taxa Candidatus Solibacter usitatus and Bryobacter aggregatus. Candidatus S. usitatus may be able to utilize polymers, sugars, amino acids and alcohols, whereas B. aggregatus utilizes polysaccharides and sugars (Ward et al. 2009; Kulichevskaya et al. 2010), suggesting that members of family 1 might hydrolyze polymers and oxidize sugars in mire soils.

The availability of O$_2$ can fluctuate in mire soils due to varying water tables, and taxa with facultative metabolic potentials have theoretical advantages over O$_2$-sensitive obligate anaerobes such as methanogens during such fluctuations in O$_2$ availability. In this regard, (a) detected bacterial 16S rRNA gene sequences were affiliated with taxa that are thought to be obligate aerobes (e.g. Caulobacteraceae and Thermomonosporaceae;
Table S5, Supporting Information) (Garrity, Bell and Lilburn 2005a, Goodfellow and Trujillo 2012) and (b) tolerance to O₂ and/or the ability to grow under bothoxic and anoxic conditions are properties of many of the detected taxa (e.g. Acidimicrobiaceae and Acidobacteriaceae; Table S5, Supporting Information) (Itoh et al. 2011; Pankratov et al. 2012).

Some of the detected 16S rRNA gene sequences were affiliated with syntrophic fermenters (e.g. Syntrophorhabdaceae, Syntrophaceae, Syntrophobacteraceae). Members of those taxa ferment aromatic compounds or fatty acids in syntrophic association with a H₂-utilizing partner (Kuever, Rainey and Widdel 2005; Qiu et al. 2008). Oxalobacteraceae-affiliated sequences were most closely related to Oxalobacter, species of which can grow anaerobi-cally on oxalate and produce formate and CO₂ (Garrity, Bell and Lilburn 2005b). Some 16S rRNA gene sequences were affiliated with taxa that grow within eukaryotes (e.g. Xiphinema totabacteraceae, Cystiellaceae) (Vandekerckhove et al. 2000; Garrity, Bell and Lilburn 2005c), suggesting that certain eukaryotes in mire soils might harbor endosymbiotic bacteria. One 16S rRNA gene sequence was most closely related to Micavibrio (Bell and Lilburn), a taxon that preys preferentially on bacteria of the genera Pseudomonas and Xanthomonas (Kuever, Rainey and Widdel 2005). These collective properties illustrate the broad metabolic diversity of bacterial communities in mire soils.

Conclusions, limitations and future perspectives

The intent of the present study was to obtain insight into the methanogenic food webs of four contrasting methane-emitting mire soils (Fig. 8). The comparative processes and taxa reflect some of the overlapping trophic interactions as well as physiological and phylogenetic distinctions among the detected family-level bacterial taxa and species-level methanogens associated with these trophic interactions (Fig. 8). Although it can be postulated that times of sampling may have contributed to some of the differences observed in the detected taxa, the gene copy numbers of Archaea and Bacteria in mire soils may not differ significantly from one season to the next (Lin et al. 2014b).

This study provided system-level information on taxa that have been rarely and in some cases never evaluated for trophic interactions. Indeed, a large number of the detected family-level taxa of each mire soil were without any cultured isolates, reinforcing the fact that mires contain a very significant number of uncultured bacterial taxa that await characterization. A more extensive sequencing of the microbial communities will be required to gain a more complete understanding of how bacterial and archaeal species-level diversities differ in contrasting mire soils. Likewise, transcriptomic or proteomic analyses would provide insight on which taxa respond to a particular condition or perturbation thereof. In this regard, the functional redundancy (Miki et al. 2014) of the microbial communities is in particular reflected in the large number of the detected fermentative taxa that are not identical in each of the mires but nonetheless catalyze similar processes (Fig. 8).

Although it can be anticipated that pH is a main determinant to the microbial activities of contrasting mires (e.g. acidic conditions appeared to restrict the relative number of dominant phylotypes), other factors such as substrate availability and redox state of the soil will theoretically also have differential impact on microbial species. Current efforts are focused on determining which in situ parameters are primarily responsible for the engagement of different taxa that facilitate the flow of carbon to methane in contrasting mire soils.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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