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ORIGINAL ARTICLE

Peat: home to novel syntrophic species that feed acetate- and hydrogen-scavenging methanogens

Oliver Schmidt, Linda Hink, Marcus A Horn and Harold L Drake
Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany

Syntrophic bacteria drive the anaerobic degradation of certain fermentation products (e.g., butyrate, ethanol, propionate) to intermediary substrates (e.g., H_2, formate, acetate) that yield methane at the ecosystem level. However, little is known about the *in situ* activities and identities of these syntrophs in peatlands, ecosystems that produce significant quantities of methane. The consumption of butyrate, ethanol or propionate by anoxic peat slurries at 5 and 15 °C yielded methane and CO_2 as the sole accumulating products, indicating that the intermediates H_2, formate and acetate were scavenged effectively by syntrophic methanogenic consortia. 16S rRNA stable isotope probing identified novel species/strains of *Pelobacter* and *Syntrophomonas* that syntrophically oxidized ethanol and butyrate, respectively. Propionate was syntrophically oxidized by novel species of *Syntrophobacter* and *Smithella*, genera that use different propionate-oxidizing pathways. Taxa not known for a syntrophic metabolism may have been involved in the oxidation of butyrate (*Telmatospirillum*-related) and propionate (unclassified *Bacteroidetes* and unclassified *Fibrobacteres*). Gibbs free energies (ΔGs) for syntrophic oxidations of ethanol and butyrate were more favorable than ΔGs for syntrophic oxidation of propionate. As a result of the thermodynamic constraints, acetate transiently accumulated in ethanol and butyrate treatments but not in propionate treatments. Aceticlastic methanogens (*Methanosarcina*, *Methanosaeta*) appeared to outnumber hydrogenotrophic methanogens (*Methanocella*, *Methanoregula*), reinforcing the likely importance of aceticlastic methanogenesis to the overall production of methane. ΔGs for acetogenesis from H_2 to CO_2 approximated to -20 kJ mol^{-1} when acetate concentrations were low, indicating that acetogens may have contributed to the flow of carbon and reductant towards methane.
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Introduction

Plant-derived organic polymers (e.g., cellulose) are mineralized to carbon dioxide (CO_2) by fungi and bacteria under oxic conditions (Westermann, 1993). However, a complex network of interwoven degradation processes that are catalyzed by different metabolic guilds of microbes is required to completely mineralize plant-derived organic polymers in anoxic habitats (e.g., water-saturated peat) when CO_2 is the main terminal electron acceptor (Supplementary Figure S1) (Zehnder, 1978; McInerney and Bryant, 1981; Drake *et al.*, 2009). Although initial (i.e., the hydrolysis of polymers) and terminal (i.e., methanogenesis) anaerobic degradation steps have been extensively studied at the ecosystem level in diverse environments, the intermediary steps, that is, the production and subsequent transformation of fermentation products by primary and secondary (i.e., syntrophic) fermenters, have for the most part remained a 'black box' within the intermediary ecosystem metabolism of certain methane-emitting environments such as peatlands (Drake *et al.*, 2009).

Butyrate, ethanol and propionate are important intermediates in different peatlands (Metje and Frenzel, 2005, 2007; Hunger *et al.*, 2015; Schmidt *et al.*, 2015; Tveit *et al.*, 2015). The *in situ* conversion of these intermediates by a syntrophic methane-forming consortia is only thermodynamically favorable for the syntrophic fermenter if the methanogenic partner that cannot by itself use these substrates keeps the concentration of H_2 or formate low enough via interspecies transfer of H_2 or formate, respectively (Supplementary Table S1; Schink, 1997). Most studies on syntrophs have been conducted with a few model organisms isolated from anaerobic sludge and incubated as mono- or defined cocultures at moderate temperatures and near neutral pH (McInerney *et al.*, 2009; Schink and Stams, 2013). However, northern peatlands, methanogenic environments that store ~30% (i.e., 450 Gt) of the terrestrial carbon reserve as recalcitrant peat and produce 23–40% of the globally emitted methane (CH_4), are characterized by low soil temperatures and acidic pH (Fung *et al.*, 1991; Gorham, 1991; Hein *et al.*, 1997), and it is unclear if well-known model

Correspondence: HL Drake, Department of Ecological Microbiology, University of Bayreuth, Dr.-Hans-Frisch-Strasse 1-3, Bayreuth 95440, Germany.
E-mail: HLD@Uni-Bayreuth.De
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syntrophs or hitherto unknown syntrophic species are active under the low temperature, acidic conditions that predominate in northern peatlands. Thus, the objective of this study was to resolve the methanogenic syntrophic community of the extensively studied model peatland Schlöppnerbrunnen.

Materials and methods

Sampling site

The moderately acidic (pore water pH approximates to 4.5) methane-emitting fen Schlöppnerbrunnen is located in the Lehstenbach catchment of the Fichtelgebirge (translates as Spruce Mountains) in southeast Germany at 700 m above sea level (50°07′53′′N, 11°52′51′′E) (Paul *et al.*, 2006; Hamberger *et al.*, 2008). Soil samples of 10–30 cm depth were taken in close proximity to each other (0.5–10 m) in May 2013 (air temperature: 5 °C) with a soil corer (8 cm in diameter). Approximately 8 kg of fresh peat soil was collected. Soil samples were transferred into airtight sterile plastic bags and cooled on ice until processed in the lab within 5 h of sampling.

Preparation and incubation conditions of anoxic peat soil slurries (microcosms)

Freshly collected peat soil was homogenized by manually mixing all soil cores in one plastic bag. Two hundred grams of homogenized peat soil (88.6% moisture content) were diluted with 400 ml of fresh surface water (collected during sampling in the fen) in sterile 1 L infusion flasks (Müller & Krempel, Bülach, Switzerland) that were sealed with screw caps and rubber stoppers (Glasgerätebau Ochs, Bovenden, Germany) and flushed with 100% sterile dinitrogen. Flasks were shaken manually to homogenize the slurries and then incubated without shaking. A total number of 30 microcosms were prepared. Twenty microcosms were preincubated for 28 days at 15 °C (a temperature reached in the fen soil during summer) and 10 microcosms for 38 days at 5 °C (mean annual temperature at the fen site) vertically in the dark (Supplementary Figure S2). Preincubation was carried out to fully reduce alternative electron acceptors present in the fen (e.g., nitrate, ferric iron or sulfate), to deplete easily degradable endogenous carbon sources and thus to create stable methanogenic conditions (Drake *et al.*, 2009).

Main incubation: The preincubated microcosms were grouped into sets of five replicates and supplemented with low *in situ* relevant concentrations (300–750 μM) of either [^{12}C]ethanol (at 5 °C and 15 °C), sodium [^{12}C]butyrate (at 15 °C), sodium [^{12}C]propionate (at 15 °C) or anoxic water (unsupplemented controls; at 5 and 15 °C). Substrates were refed when they were consumed, and (transiently accumulating) acetate concentrations were similar to those in unsupplemented controls (Supplementary Figures S3–S8). After 88 days of incubation, one replicate each of the ethanol and butyrate treatment at 15 °C were refed with [^{13}C]ethanol and sodium [^{13}C]butyrate (Campro Scientific GmbH, Berlin, Germany), respectively. In total, 18 and 24 mM ^{13}C-carbon was added in the [^{13}C]ethanol and [^{13}C]butyrate replicate, respectively. No [^{13}C]substrate incubations were conducted for propionate treatments at 15 °C and ethanol treatments at 5 °C because of financial constraints. Samples of the headspace gas phase for gas chromatographic analysis and of the liquid phase for pH measurements and the analysis of dissolved organic compounds were taken using sterile syringes. Headspace gas phases were exchanged regularly with 100% sterile dinitrogen to prevent an accumulation of CO_2 and CH_4 to *in situ* irrelevant high concentrations. In treatments fed with either sodium propionate or sodium butyrate, the pH was regularly adjusted by adding 50–300 μl of a 2.5 M hydrogen chloride solution.

Chemical analyses

Fresh peat soil was weighed, dried at 80 °C for 72 h and weighed again to determine the soil moisture content. An InLab R422 pH electrode (InLAB Semi-Micro; Mettler-Toledo, Gießen, Germany) was used to measure pH. Dissolved organic compounds were measured by high-performance liquid chromatography and the gases CH_4, CO_2 and H_2 by gas chromatography (Küsel and Drake, 1995). Amounts of gases in headspaces were calculated from the ideal gas law, taking into consideration temperature, actual pressure and volumes of gas phases in microcosms. Amounts of gases dissolved in liquid phases were calculated from standard solubility tables (Blachnik, 1998). pH-dependent amounts of bicarbonate were included in addition to CO_2 in the gas and liquid phases to calculate total amounts of CO_2. Gas concentrations (μM or mM) throughout the study represent the combined amounts of a gas in the gas and liquid phases divided by the volume of the liquid phase. One micromolar of CH_4 or H_2 approximated to 2 Pa and 1 μM of CO_2 approximated to 1 Pa.

Nucleic acid extraction

RNA was coextracted together with DNA from fresh peat soil (four extractions) and from microcosms (one extraction for each replicate) by bead-beating lysis, organic solvent extraction and precipitation (Griffiths *et al.*, 2000). DNA was removed from RNA/DNA coextracts using RNase-free DNase (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Separation of 'heavy' and 'light' RNA by density gradient centrifugation

RNA stable isotope probing was performed according to Whiteley *et al.* (2007). Six hundred nanograms of RNA, derived from microcosms supplemented

with [^{12}C]ethanol, [^{13}C]ethanol, [^{12}C]butyrate or [^{13}C] butyrate at the start and end timepoint of [^{13}C] substrate addition, was added to the gradient solution (buoyant density 1.79 g ml^{-1}) and filled into OptiSeal Tubes (Beckmann, Fullerton, CA, USA). Isopycnic centrifugation (1 30 000 *g* at 20 °C for 67 h; vertical rotor VTi 65.2; Beckmann) was performed to separate 'heavy', potentially ^{13}C-labeled RNA from 'lighter' ^{12}C-labeld RNA. Fractions of 450 µl each were collected. The density of fractions was determined by weighing at 25 °C (Supplementary Figure S9). RNA precipitation from fractions was performed as described (Degelmann *et al.*, 2009), and RNA concentrations were determined with Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Karlsruhe, Germany). RNA was stored at – 80 °C.

Reverse transcription-polymerase chain reaction
RNA was reversely transcribed into complementary DNA using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Polymerase chain reaction and cloning
Bacterial and archaeal 16S rRNA sequences were amplified using complementary DNA as published elsewhere (Schmidt *et al.*, 2015). Conditions for polymerase chain reaction were modified as follows: no precycles were run and annealing at 50 °C was reduced to 30S. Cloning of purified polymerase chain reaction products was performed as published before (Schmidt *et al.*, 2015). Sequencing was carried out by Macrogen (Seoul, South Korea).

Sequence analyses
Bacterial and archaeal 16S rRNA complementary DNA sequences (~880 bp in length) were analyzed with ARB (http://www.arb-home.de; version 2005; Ludwig *et al.*, 2004), aligned with the SINA Webaligner (http://www.arb-silva.de) and imported into a 16S rRNA gene-based database retrieved from the SILVA hompage (Pruesse *et al.*, 2007). Chimeric sequences were identified as published before (Schmidt *et al.*, 2015). Sequences were compared with those in public databases using BLASTn 2.2.27 (Zhang *et al.*, 2000). The DOTUR software (Schloss and Handelsman, 2005) was used to assign bacterial and archaeal 16S complementary DNA sequences within operational taxonomic units (OTUs) based on a similarity cutoff of 87.5% (family level) and 95% (genus level), respectively (Yarza *et al.*, 2008).

Thermodynamic calculations, recoveries and substrate to CH_4 and CO_2 ratios
ΔGs were calculated from standard Gibbs free energies of formation (G_f^0; Thauer *et al.*, 1977), standard reaction enthalpies of formation (H_f^0; Lange, 1967; Stumm and Morgan, 1981) and concentrations of products and reactants measured in anoxic microcosms using the Nernst and Van't Hoff equations (Conrad and Wetter, 1990). A concentration of 1 µM was assumed when a certain substance could not be detected but its concentration was needed for the calculation of the ΔG. Electron and carbon recoveries were calculated as follows: cumulative amounts of CH_4 and CO_2 formed in unsupplemented control microcosms were subtracted from the cumulative amounts of CH_4 and CO_2 (Supplementary Figure S10) formed in ethanol-, butyrate- or propionate-supplemented microcosms between the end of the preincubation and the end of the main incubation (resulting in net amounts of CH_4 and CO_2). Cumulative CO_2 amounts were corrected as indicated in Supplementary Figure S10. Amounts of electrons and carbon atoms from net amounts of CH_4 and CO_2 were divided by the total amount of electrons and carbon atoms supplemented as substrate (number of electrons/carbon atoms per molecule: CH_4, 8/1; CO_2, 0/1; ethanol, 12/2; butyrate, 20/4; propionate, 14/3).

Nucleotide sequence accession numbers
Sequences were submitted to the European Nucleotide Archive (accession numbers LK024545–LK026322).

Results and discussion

Production of methane by unsupplemented fresh peat soil
During the preincubation, CO_2 accumulated without delay in anoxic microcosms; in contrast, only minor amounts of acetate and propionate were formed, and methane production did not start before 10 and 20 days at 15 and 5 °C, respectively (Supplementary Figure S2). The production of CO_2 without an appreciable production of methane or fermentation products (such as acetate or propionate) during the preincubation period indicated that the mineralization of endogenous sources of carbon was linked to the consumption of residual electron acceptors other than CO_2, such as oxygen, nitrate, sulfate or ferric iron (Paul *et al.*, 2006; Reiche *et al.*, 2008; Drake *et al.*, 2009; Palmer *et al.*, 2010; Pester *et al.*, 2010).

After the preincubation, CO_2 and methane were the only detected end products that accumulated at both 15 and 5 °C in unsupplemented controls (Supplementary Figures S3 and S7). This result is in contrast to other studies where acetate, ethanol, butyrate or propionate were detected at mM concentrations in anoxic microcosms of unsupplemented peat at the end of anoxic incubation, especially at lower temperatures (Metje and Frenzel, 2005; Tveit *et al.*, 2015). The low steady-state concentrations of organic acids and alcohols observed in unsupplemented controls at 5 and 15 °C in this study indicate that the hydrolysis of organic matter rather than syntrophic methanogensis was rate limiting (Supplementary Figures S3 and S7).

Average methane production rates were 2.9 μmol g^{-1} of $soil_{dw}$ per day at 15 °C and 0.89 μmol g^{-1} of $soil_{dw}$ per day at 5 °C, respectively. Similar rates have been reported for peat soil from the fen (3.3 μmol g^{-1} of $soil_{dw}$ per day at 15 °C and 0.17–0.54 μmol g^{-1} of $soil_{dw}$ per day at 5 °C; Schmidt *et al.*, 2015) and with subarctic peat soil (1.5 μmol g^{-1} of $soil_{dw}$ per day at 15 °C and 0.75 μmol g^{-1} of $soil_{dw}$ per day at 4 °C; Metje and Frenzel, 2007). CO_2:methane ratios at the end of the incubation were 2.0 and 2.4 at 15 and 5 °C, respectively. That the CO_2:methane ratios were >1 indicated that methanogenesis was not the sole terminal process (this conclusion assumes that CO_2 and methane were derived from carbon at the oxidation state of carbon in glucose). In this regard, the pool of internal inorganic terminal electron acceptors other than CO_2 (nitrate, sulfate and ferric iron) is relatively small and was depleted after 16 days of anaerobic incubation at 15 °C in this peat soil (Küsel *et al.*, 2008). Thus, these electron acceptors should have been depleted during the preincubation and alone should not account for the observed CO_2:methane ratios obtained for the anoxic incubation after the preincubation. Humic substances that are abundant in peat can also act as electron acceptors (Trckova *et al.*, 2005; Keller *et al.*, 2009; Lipson *et al.*, 2013). An anoxic sulfur cycle, where reduced sulfur compounds are reoxidized by redox-active humic substances, was proposed to account for high CO_2 production in long-term anoxic incubated peat mesocosms of the fen (Knorr and Blodau, 2009; Pester *et al.*, 2012). Such an anoxic sulfur cycle driven by humic substances may have contributed to CO_2:methane ratios of >1 in the microcosm experiments. Nevertheless, methanogenesis contributed to about half of the CO_2 produced during organic matter mineralization at 15 °C and only slightly less at 5 °C according to the CO_2:methane ratios. These results support the hypothesis that methanogenesis is one of several anaerobic processes that contribute to the overall mineralization of organic matter in this fen (Knorr *et al.*, 2009).

Oxidation of ethanol, butyrate and propionate

Ethanol, butyrate and propionate are common fermentation products (Zidwick *et al.*, 2013) and were produced in varying amounts during the fermentation of cellulose, glucose, xylose and *N*-acetyl-glucoseamine in anoxic microcosms of peat soil from the fen Schlöppnerbrunnen (Hamberger *et al.*, 2008; Wüst *et al.*, 2009; Schmidt *et al.*, 2015). In this study, preincubated anoxic microcosms were pulsed with low concentrations (300–750 μM) of ethanol, butyrate or propionate and incubated at 15 °C to identify processes that lead to the oxidation of these three fermentation products. The utilization of ethanol at 5 °C was also evaluated.

Ethanol was consumed rapidly and without delay, whereas butyrate and especially propionate were consumed more slowly (Figures 1 and 2). Subsequent pulses of substrates resulted in faster consumption of substrates (Supplementary Figures S4a–S6a and S8a). Acetate accumulated transiently and was subsequently consumed in ethanol and butyrate treatments (Supplementary Figures S4b, S5b and S8b). Hardly any transient accumulation of acetate was observed in propionate treatments where detected acetate concentrations never exceeded 40 μM, which is in the range of what was detected in unsupplemented controls (Supplementary Figures S3a and S6b). Isobutyrate transiently accumulated in butyrate treatments and was subsequently consumed parallel to butyrate consumption (Supplementary Figure S5a), indicating an isomerization of butyrate to isobutyrate as observed in syntrophic methanogenic cultures (Wu *et al.*, 1994). H_2 concentrations in headspace gas phases were relatively low and ranged between 3 Pa (ethanol treatments at 5 °C) and 17 Pa (ethanol treatments at 15 °C), indicating that either (a) H_2 was not an important intermediate or (b) H_2 scavaging was efficient in all treatments (Supplementary Figures S3d–S8d). Formate was below the detection limit of ~10 μM in any of the anoxic microcosms, indicating that formate, similar to hydrogen, was either (a) not formed or (b) effectively scavenged by formate-oxidizing methanogens or acetogens. Effective hydrogen and formate scavanging is supported by the finding that H_2 and formate were formed in glucose-, xylose- or *N*-acetylglucoseamine-supplemented microcosms and both stimulated acetogenesis and methanogenesis in hydrogen- or formate-supplemented microcosms of the fen (Hamberger *et al.*, 2008; Wüst *et al.*, 2009; Hunger *et al.*, 2011).

CO_2 and methane were the sole detected accumulating end products of ethanol, butyrate and propionate oxidation (Supplementary Figure S10). Observed substrate:methane:CO_2 ratios were close to theoretical ratios for complete substrate conversion to methane and CO_2 (Table 1). Electron recoveries of ~90% and carbon recoveries ranging from 75% to 104% also reflect the near stoichiometric conversion of substrates to methane and CO_2 (Table 1).

Thermodynamics of syntrophic processes, methanogenesis and acetogenesis

ΔGs between the first and second substrate pulses were calculated for syntrophic, methanogenic and acetogenic processes according to reactions in Supplementary Table S1. ΔGs for syntrophic ethanol and butyrate oxidation ranged from −31 to −5 and −20 to −3 kJ mol^{-1}, respectively (Figures 1c, f and 2c). Syntrophic oxidation of supplemented propionate (Figure 1i) or endogenously formed propionate (Figures 1l and 2f) was less exergonic and ranged from −17 to +10 kJ mol^{-1}. Propionate concentrations decreased (Figures 1g and j,) despite ΔGs of >-10 kJ mol^{-1}, which is near to the thermodynamic limit for the synthesis of ATP and thus growth

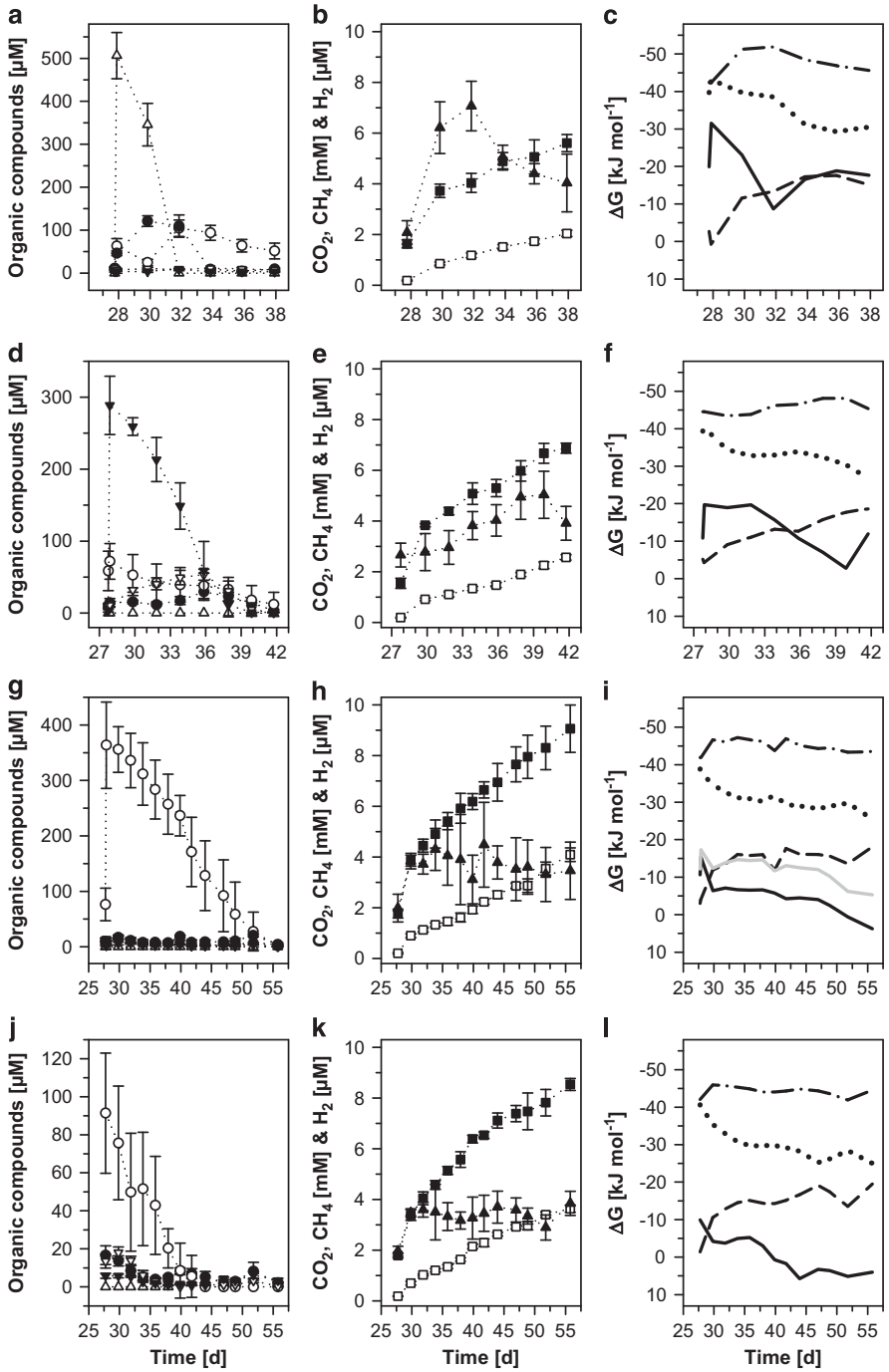


Figure 1 Concentrations of organic compounds (**a, d, g, j**), gases (**b, e, h, k**) and Gibbs free energies (**c, f, i, l**) of anoxic peat soil microcosms incubated at 15 °C. Shown is the time frame between the first and second substrate supplementations after the preincubation. See Supplementary Figure S2 for the preincubation and Supplementary Figures S3–S6 for the complete incubation after the preincubation. (**a–c**) Ethanol treatments; (**d–f**) butyrate treatments; (**g–i**) propionate treatments; (**j–l**) unsupplemented controls. Values are means of five replicates. Error bars indicate standard deviations. Symbols: ●, acetate; ○, propionate; ▼, butyrate; ∇, isobutyrate; Δ, ethanol; ▲, H_2; ■, CO_2; □, CH_4; solid black line, syntrophic oxidation of ethanol (**c**), butyrate (**f**), propionate according to reaction 6 in Supplementary Table S1 (**i** and **l**); solid gray line, syntrophic oxidation of propionate according to reaction 8 in Supplementary Table S1 (**i**); dashed line, acetogenesis; dotted line, aceticlastic methanogenesis; dashed-dotted line, hydrogenotrophic methanogenesis.

(Müller *et al*., 2010). Syntrophic propionate oxidation may have occurred in microzones where thermodynamic conditions were more exergonic compared with the bulk soil slurry. Such microzones could occur in microbial aggregates of syntrophic propionate oxidizers juxtaposed to hydrogenotrophic methanogens (Conrad *et al*., 1985). The hydrogenotrophic methanogens within the aggregate could maintain H_2 concentrations that yield thermodynamic conditions that sustain the growth of

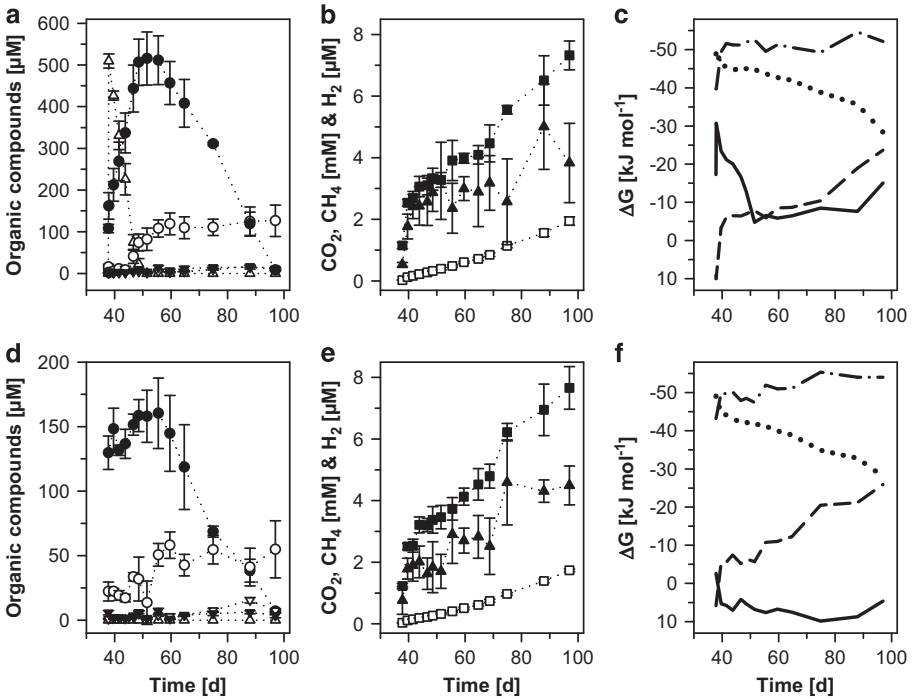


Figure 2 Concentrations of organic compounds (**a** and **d**), gases (**b** and **e**) and Gibbs free energies (**c** and **f**) of anoxic peat soil microcosms incubated at 5 °C. Shown is the time frame between the first and second substrate supplementations after the preincubation. See Supplementary Figure S2 for the preincubation and Supplementary Figures S7–S8 for the complete incubation after the preincubation. (**a–c**) Ethanol treatments; (**d–f**), unsupplemented controls. Values are means of five replicates. Error bars indicate standard deviations. Symbols: ●, acetate; ○, propionate; ▼, butyrate; ∇, isobutyrate; Δ, ethanol; ▲, H_2; ■, CO_2; □, CH_4; solid line, syntrophic oxidation of ethanol (**c**) and propionate according to reaction 6 in Supplementary Table S1 (**f**); dashed line, acetogenesis; dotted line, aceticlastic methanogenesis; dashed-dotted line, hydrogenotrophic methanogenesis.

Table 1 Substrate to CH_4 and CO_2 ratios and recoveries of anoxic peat soil microcosms[a]

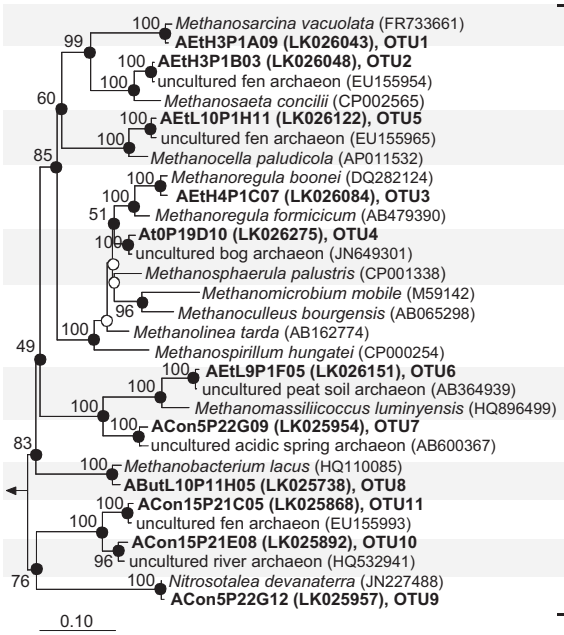
Treatment	*Incubation time frame (days)*	*Substrate: CH_4:CO_2 ratio*		*Electron recovery (%)*	*Carbon recovery (%)*
		Observed	*Theoretical*		
Ethanol (15 °C)	28–114	1:1.35:0.44	1:1.5:0.5[b]	90	89
Ethanol (5 °C)	38–218	1:1.31:0.20	1:1.5:0.5[b]	87	75
Butyrate (15 °C)	28–108	1:2.28:1.77	1:2.5:1.5[b]	91	101
Propionate (15 °C)	28–195	1:1.60:1.53	1:1.75:1.25[b]	92	104

[a]Concentrations of unsupplemented control microcosms were substracted from that of supplemented microcosms to calculate ratios and recoveries (see Materials and methods section).
[b]Complete oxidation of substrate to CH_4 and CO_2 according to reactions 15, 14 and 13 in Supplementary Table S1, respectively.

syntrophs and methanogens (Krylova and Conrad, 1998). In this regard, H_2 concentrations of 1 Pa would yield a ΔG of $\sim -15\,kJ\,mol^{-1}$ for the syntrophs and $-25\,kJ\,mol^{-1}$ for hydrogenotrophic methanogens (with 10 µM acetate, 10 µM propionate, 3 kPa CO_2, 1.5 kPa CH_4, 15 °C and pH 5.3; Supplementary Figure S11).

ΔGs for aceticlastic methanogenesis were $\leqslant -25\,kJ\,mol^{-1}$ despite acetate concentrations as low as 4 µM (Figures 1 and 2). These exergonic ΔGs indicate that aceticlastic methanogens may have sustained low acetate concentrations, which, along with low H_2 concentrations, are thermodynamically favorable for syntrophs (Dong *et al.*, 1994; Metje and Frenzel, 2007).

Hydrogenotrophic methanogenesis was always far more exergonic compared with acetogenesis from H_2 to CO_2 (Figures 1 and 2). However, ΔGs were still exergonic enough ($-20\,kJ\,mol^{-1}$ at 15 °C and $-25\,kJ\,mol^{-1}$ at 5 °C) to sustain the growth of acetogens on H_2–CO_2 when transiently accumulated acetate was consumed (e.g., by aceticlastic methanogens). Psychotolerant acetogens are able to use H_2 at concentrations as low as 4 Pa (Conrad and Wetter, 1990), and thermodynamic calculations suggested that acetogens may have been metabolically active at H_2 concentrations as low as 2 Pa under the experimental conditions used in this study (Supplementary Figure S12). Thus, acetogens might have contributed to the consumption of hydrogen.



	Rel. abundance[%]								
		15°C						5°C	
	F	E		B		P	C	E	C
		H	L	H	L				
Methanosarcina vacuolata (FR733661) / AEtH3P1A09 (LK026043), OTU1	51	83	63	95	80	77	35	85	68
AEtH3P1B03 (LK026048), OTU2 / uncultured fen archaeon (EU155954) / Methanosaeta concilii (CP002565)	34	7	15	2	4	8	51	8	16
AEtL10P1H11 (LK026122), OTU5 / uncultured fen archaeon (EU155965) / Methanocella paludicola (AP011532)	7	3	8	0	5	5	3	5	8
Methanoregula boonei (DQ282124) / AEtH4P1C07 (LK026084), OTU3 / Methanoregula formicicum (AB479390)	2	6	8	3	11	8	3	0	2
At0P19D10 (LK026275), OTU4 / uncultured bog archaeon (JN649301) / Methanosphaerula palustris (CP001338)	3	0	2	0	0	0	0	0	0
Methanomicrobium mobile (M59142) / Methanoculleus bourgensis (AB065298) / Methanolinea tarda (AB162774) / Methanospirillum hungatei (CP000254)									
AEtL9P1F05 (LK026151), OTU6 / uncultured peat soil archaeon (AB364939) / Methanomassiliicoccus luminyensis (HQ896499)	0	0	3	0	0	0	2	0	2
ACon5P22G09 (LK025954), OTU7 / uncultured acidic spring archaeon (AB600367)	0	0	0	0	0	0	0	0	2
Methanobacterium lacus (HQ110085) / AButL10P11H05 (LK025738), OTU8	0	1	0	0	1	0	0	0	0
ACon15P21C05 (LK025868), OTU11 / uncultured fen archaeon (EU155993)	0	0	0	0	0	2	5	0	2
ACon15P21E08 (LK025892), OTU10 / uncultured river archaeon (HQ532941)	0	0	2	0	0	2	2	2	0
Nitrosotalea devanaterra (JN227488) / ACon5P22G12 (LK025957), OTU9	3	0	2	0	0	0	0	0	2

Figure 3 Phylogenetic tree of archaeal 16S rRNA complementary DNA sequences retrieved from fresh peat and anoxic peat soil microcosms (bold), and reference sequences. The phylogenetic tree was calculated using the maximum parsimony method. Empty circles at nodes indicate congruent nodes in either the neighbor-joining or maximum likelihood tree. Congruent nodes in all three trees are indicated with filled circles and have bootstrap values (1000 resamplings) from the maximum parsimony tree. The bar indicates 0.1 change per nucleotide. *Escherichia coli* (AF233451) was used as outgroup. Sequence descriptor code: A, *Archaea*; Et, But, or Con, sequences obtained at the end of the incubation with ethanol, butyrate or unsupplemented control, respectively; H3 or H4, derived from 'heavy' fraction 3 or 4, respectively; L10, derived from 'light' fraction 10; 5 or 15, incubated at 5 or 15 °C, respectively; t0, fresh soil; the last 5–6 characters represent the clone identifier (e.g., P15F01 is from plate 15 position F01). Abbreviations: F, fresh peat; E, B or P, ethanol-, butyrate- or propionate-supplemented treatments, respectively; C, unsupplemented controls; H or L, derived from 'heavy' or 'light' fractions, respectively (Supplementary Figure S9).

H_2-driven acetogenesis would result in a high proportion of aceticlastic compared with hydrogenotrophic methanogenesis (Conrad, 1999), a possibility that is consistent with the high number of 16S rRNA complementary DNA sequences affiliated with aceticlastic methanogens in fresh peat as well as in the anoxic microcosms (Figure 3).

Bacterial taxa potentially linked to syntrophic processes

A total of 1129 bacterial 16S rRNA complementary DNA sequences were obtained from fresh peat or anoxic microcosms (Supplementary Table S2). Family-level coverages for the different clone libraries ranged from 84% to 89%, indicating that most of the family-level diversity present in the different samples was detected (Supplementary Table S2). Relative abundancies of 16S rRNA complementary DNA sequences from 'heavy' and 'light' fractions of [^{13}C]ethanol and [^{13}C]butyrate treatments were compared to identify ethanol- and butyrate-oxidizing syntrophs, respectively. Rarefaction analysis indicated that the diversity of bacterial family-level OTUs was higher if total RNA extracts were used to generate 16S rRNA complementary DNA sequences compared with 'heavy' or 'light' fractions of RNA (Supplementary Figure S13), indicating that populations with different RNA buoyant densities were successfully separated. It is therefore probable that labeled (i.e., 'heavy') RNA of taxa that assimilated ^{13}C-labeled carbon was separated from unlabeled (i.e., 'light') RNA of taxa that assimilated carbon derived from endogenous carbon sources. Syntrophic taxa that responded to the supplementation of butyrate, ethanol or propionate are discussed below. Taxa abundant in fresh peat or unsupplemented controls that could not be directly linked with syntrophic processes is presented in Supplementary Text S1.

OTU35a was the most abundant bacterial OTU in 'heavy' fractions of the [^{13}C]ethanol-supplemented microcosm at 15 °C but was only a minor OTU in 'light' fractions (Figure 4 and Supplementary Table S3). No other OTU was considerably enriched in 'heavy' compared with 'light' fractions. Thus, OTU35a appeared to represent an important taxon associated with the consumption of ethanol. OTU35a

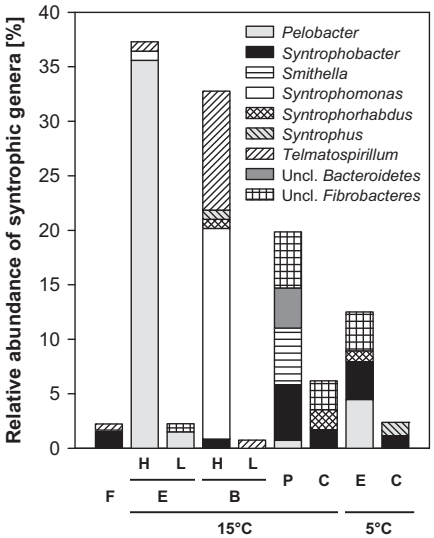


Figure 4 Relative abundances of known and potential novel syntrophic genera detected in fresh peat and anoxic peat soil microcosms (see Supplementary Table S3 for a complete list of all detected bacterial OTUs). Taxa correspond to the following OTUs: *Pelobacter*, 35a; *Syntrophobacter*, 37a; *Smithella*, 38b; *Syntrophomonas*, 79a; *Syntrophorhabdus*, 39; *Syntrophus*, 38a; *Telmatospirillum*, 26a; unclassified *Bacteroidetes*, 47; unclassified *Fibrobacteres*, 53. Abbreviations: F, fresh peat; E, B, or P, ethanol, butyrate or propionate treatments, respectively; C, unsupplemented controls; H or L, derived from 'heavy' or 'light' fractions, respectively.

was also detected in ethanol-supplemented microcosms but not in unsupplemented controls at 5 °C (Figure 4 and Supplementary Table S3). OTU35a was related to *Pelobacter propionicus* (Figure 5), which is known to convert ethanol to propionate (3 ethanol+2 bicarbonate → 2 propionate+1 acetate+1 proton+3 water; Schink *et al.*, 1987) but not syntrophically to acetate and H_2 (reaction 11 in Supplementary Table S1) as do *Pelobacter acetylenicus* and *Pelobacter carbinolicus* (Schink, 1984; Seitz *et al.*, 1990). However, only small concentrations of propionate were detected in ethanol-supplemented microcosms, whereas transient acetate accumulations were repeatedly observed at 15 and 5 °C after the addition of ethanol (Supplementary Figures S4b and S8b). These findings indicate that the fen harbors *P. propionicus*-affiliated bacteria that syntrophically oxidize ethanol to acetate and H_2 at *in situ* temperatures. Ethanol oxidation to propionate and acetate with Arctic peat was attributed to members of the *Actinobacteria* (Tveit *et al.*, 2015). However, none of the OTUs assigned to *Actinobacteria* responded to ethanol in this study (Supplementary Table S3).

OTU79a was the most abundant OTU in 'heavy' fractions and was not detected in 'light' fractions of the [^{13}C]butyrate-supplemented microcosm. OTU79a was related to *Syntrophomonas zehnderi* (Figure 5), which is a syntrophic butyrate oxidizer (Sousa *et al.*, 2007). Thus, *Syntrophomonas* might be an important genus contributing to syntrophic butyrate oxidation in the fen.

Two OTUs (OTU37a and OTU38b) that were affiliated with known syntrophic propionate oxidizers were more abundant in propionate-supplemented microcosms compared with fresh peat or unsupplemented controls (Figure 4). OTU37a was related to *Syntrophobacter wolinii* (Figure 5). Known *Syntrophobacter* species syntrophically oxidize propionate to acetate, hydrogen and CO_2 according to reaction 6 in Supplementary Table S1. OTU37a was also detected in ethanol treatments at 5 °C as well as unsupplemented controls at 15 and 5 °C, suggesting that OTU37a might have been associated with the consumption of the transiently formed propionate (Figure 4 and Supplementary Figures S3). Furthermore, OTU37a was the only OTU in fresh peat that was affiliated with any known syntroph (Figure 4). This finding as well as the finding that propionate and acetate were dominant products of cellulose fermentation (Schmidt *et al.*, 2015) suggests that syntrophic oxidation of propionate is important during the anaerobic mineralization of plant-derived organic matter in this fen. OTU38b was related to *Smithella propionica* (Figure 5) and was only detected in propionate treatments (Figure 4). *S. propionica* uses a propionate-degrading pathway that yields high amounts of acetate, very little hydrogen and no CO_2 (reaction 8 in Supplementary Table S1; De Bok et al., (2001). Thus, *Smithella* is more dependent on low acetate rather than low H_2 concentrations (Supplementary Figures S11 and S12), as was reflected by the more negative ΔGs in propionate treatments for *Smithella* compared with *Syntrophobacter* (Figure 1i). Syntrophs with different strategies for the degradation of propionate might prevent the accumulation of propionate and resulting acidification during periods of elevated H_2 or acetate in the fen. Almost no 16S rRNA sequences were affiliated to *Peptococcaceae*, an important propionate-oxidizing taxon in microcosms of swamp soil and Arctic peat (Chauhan and Ogram, 2006; Tveit *et al.*, 2015). The occurrence of different propionate oxidizers in contrasting wetlands is indicative of the functional redundancy of taxa associated with methanogenic foodwebs (Hunger *et al.*, 2015).

Sequence similarities within OTUs that were enriched by substrate addition and could be affiliated to known syntrophic genera (OTU35a, 37a, 79a and 38b) ranged between 95% and 99%. Thus, these OTUs represent a population of closely related but not identical species (Figure 5).

Active archaeal taxa in anoxic microcosms

A total of 649 archaeal 16S rRNA complementary DNA sequences derived from fresh peat or anoxic microcosms were obtained (Supplementary Table S2). Genus-level (95% similarity cutoff) coverages for the different clone libraries ranged from 94% to

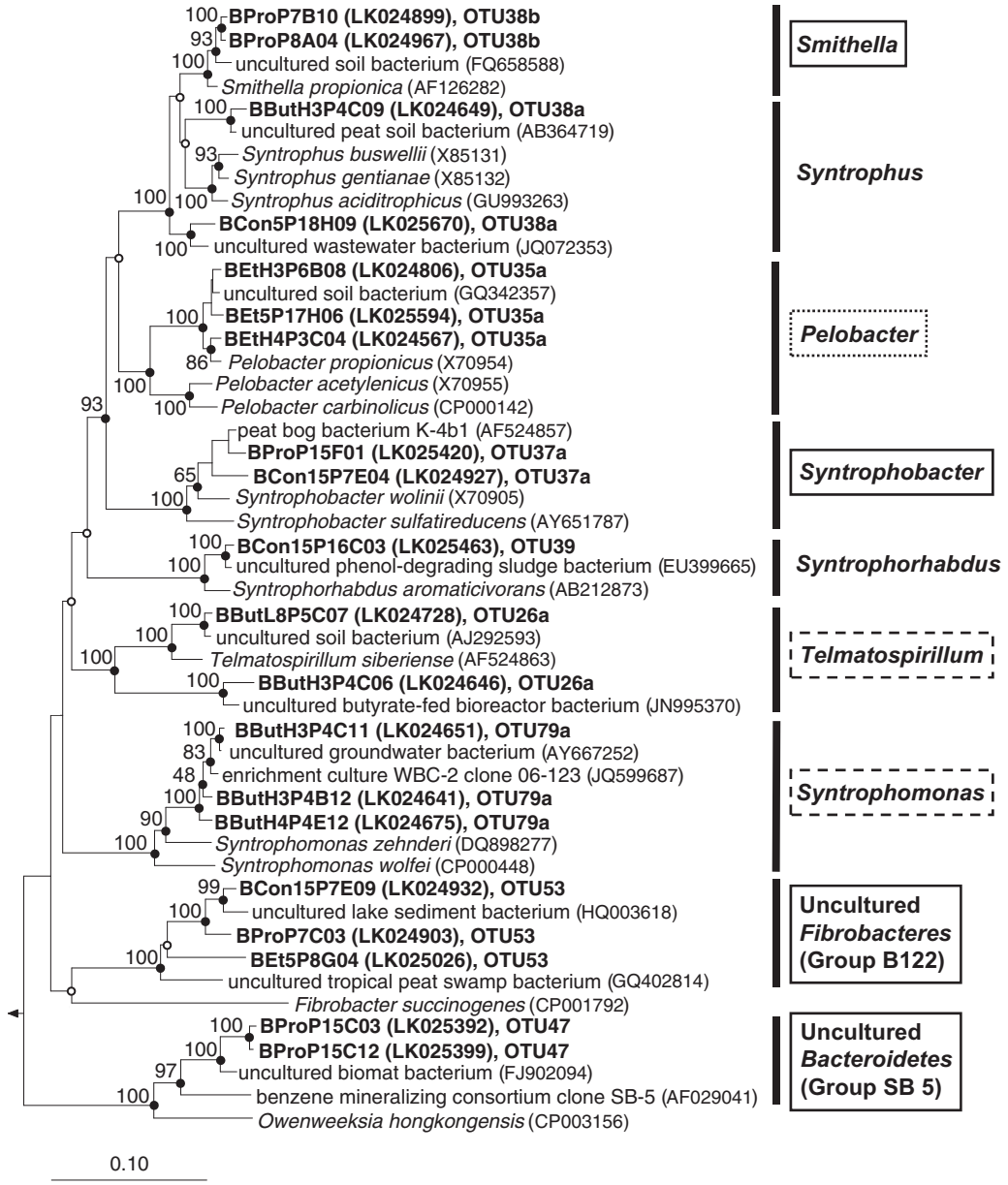


Figure 5 16S rRNA-based phylogenetic tree of bacterial OTUs (bold text) that responded to the supplementation of ethanol (dotted boxes), butyrate (dashed boxes) or propionate (solid boxes). OTUs that affiliated to genera known for syntrophic degradation of aromatic compounds are included in the tree. The phylogenetic tree was calculated using the neighbor-joining method. Empty circles at nodes indicate congruent nodes in either the maximum parsimony or maximum likelihood tree. Congruent nodes in all three trees are indicated with filled circles and have bootstrap values (1000 resamplings) from the neighbor-joining tree. The bar indicates 0.1 change per nucleotide. *Methanosarcina mazei* (AE008384) was used as outgroup. Sequence descriptor code: B, *Bacteria*; Et, But, Pro, or Con, sequences obtained at the end of the incubation with ethanol, butyrate, propionate or unsupplemented control, respectively; H3 or H4, derived from 'heavy' fraction 3 or 4, respectively; 5 or 15, incubated at 5 or 15 °C, respectively; the last 5–6 characters represent the clone identifier (e.g., P15F01 is from plate 15 position F01).

100%, which indicates sufficient sampling for the detection of most of the archaeal genera present in the different samplings (Supplementary Table S2). Rarefaction analysis indicated that the diversity of archaeal genus-level OTUs differed between the clone libraries, but no clear trends were apparent (Supplementary Figure S13).

All archaeal clone libraries were dominated by the aceticlastic methanogens *Methanosarcina* and *Methanosaeta*, and the sum of the relative abundancies of both genera ranged between 84% and 97% in fresh peat (Supplementary Text S1) and anoxic microcosms. It is therefore likely that aceticlastic methanogenesis was an important source of methane. Aceticlastic methanogensis was also the dominant methanogenic pathway in other peat soils (Metje and Frenzel, 2007; Tveit *et al.*, 2015), whereas hydrogenotrophic methanogenesis contributed to most of the methane production in other studies with peat soil (Horn *et al.*, 2003;

Metje and Frenzel, 2005). Within the aceticlastic methanogens, *Methanosarcina* was more abundant than *Methanosaeta* in fresh peat and in almost all microcosms. The relative abundance of *Methanosaeta* was higher compared with that of *Methanosarcina* only in unsupplemented controls at 15 °C. Different relative abundancies of *Methanosarcina* and *Methanosaeta* in the different treatments might be due to the different acetate requirements for both genera. Reported threshold concentrations for acetate were lower for *Methanosaeta* (<10 μM) compared with that for *Methanosarcina* (>100 μM) because these taxa have different mechanisms for the activation of acetate (Jetten *et al.*, 1992). On the other hand, *Methanosarcina* generates more ATP per mol acetate and tends to outgrow *Methanosaeta* in the presence of higher acetate concentrations (Jetten *et al.*, 1992). After the preincubation, acetate concentrations only occasionally exceeded 10 μM in unsupplemented controls at 15 °C (Supplementary Figure S3a), whereas acetate concentrations of >100 μM were repeatedly measured in most other treatments (Supplementary Figures S4b, S5b, S7a and S8b). Thus, *Methanosaeta* might have outcompeted *Methanosarcina* under the more 'acetate-starved' conditions in the unsupplemented control at 15 °C, whereas *Methanosarcina* may have dominated under 'acetate-rich' conditions in the other treatments.

However, the scenario above does not explain why *Methanosarcina* also dominated in propionate treatments at 15 °C in which acetate concentrations ranged mostly between 10 and 30 μM (Supplementary Figure S6b), which has not been reported to be sufficient for the growth of *Methanosarcina*. However, thermodynamic calculations indicated that ΔGs for acetate concentrations in the range of 1–10 μM acetate were exergonic enough for *Methanosarcina* to grow under the experimental conditions used (Supplementary Figure S11d). In addition, one could speculate that syntrophs with a propionate oxidation pathway similar to *Syntrophobacter* were juxtaposed to hydrogenotrophic methanogens (e.g., *Methanoregula* or *Methanocella*) and *Methanosarcina* (Figure 6). Hydrogenotrophic methanogens could sustain very low H_2 concentrations (1 Pa at 15 °C or 0.4 Pa at 5 °C) that could allow syntrophs to produce acetate concentrations high enough for *Methanosarcina* (50 μM) (Supplementary Figures S11 and S12).

Alternatively, syntrophs with a propionate oxidation pathway similar to *Smithella* could be associated with *Methanosarcina* as the hydrogenotrophic methanogen and *Methanosaeta* as aceticlastic methanogen (Figure 6). In this scenario, *Methanosaeta* would decrease local acetate concentrations to ~0.1 μM, allowing syntrophs to sustain local H_2 levels high enough for *Methanosarcina* (170 Pa at 15 °C and 90 Pa at 5 °C) (Supplementary Figures S11 and S12).

Species of *Methanosarcina* are metabolically versatile and can grow on methanol (+/− H_2) or methylamines in addition to acetate or H_2–CO_2 (Maestrojuán and Boone, 1991; Gunnigle *et al.*, 2013). The ability to use different methanogenic substrates would be advantageous for *Methanosarcina* under the substrate limited conditions of peat. Methanol is produced during the degradation of organic matter (Schink and Zeikus, 1980), and methanol stimulated methanogenesis in anoxic microcosms of peat from the investigated fen (Wüst *et al.*, 2009). Methylamines might be formed from glycine, sarcosine and betaine fermentation (Tveit *et al.*, 2015). It is likely that *Methanosarcina* had to compete for methanol with other more specialized methanol using methanogens with lower thresholds for methanol (and H_2), a competition that occurs in the hindgut of cockroaches (Sprenger *et al.*, 2007). In this regard, 16S rRNA complementary DNA sequences that were affiliated with *Methanomassiliicoccus luminyensis* (a methanogen that is restricted to growth on methanol plus H_2; Dridi *et al.*, 2012) were detected in some treatments.

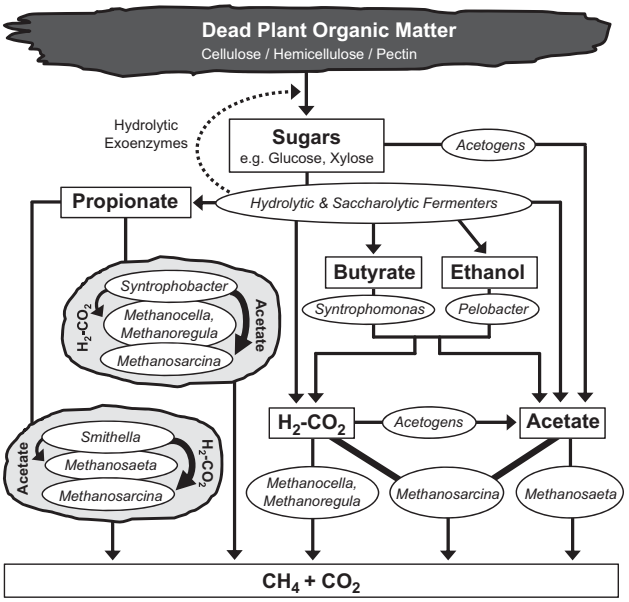


Figure 6 Hypothetical model of syntrophic processes during the complete mineralization of dead plant organic matter in the fen under methanogenic conditions. Thick lines and arrows indicate that *Methanosarcina* has relatively high thresholds for acetate and H_2. Propionate degraders might be juxtaposed to H_2- and acetate-consuming methanogens in a matrix that optimizes interspecies transfer of H_2 and acetate. Propionate oxidation by *Syntrophobacter* is particularly dependent on low H_2 concentrations (maintained by *Methanoregula* or *Methanocella*) and local acetate concentrations could be high enough for *Methanosarcina*. Propionate oxidation by *Smithella* allows for elevated local H_2 concentrations (high enough for *Methanosarcina*) if acetate concentrations are low (maintained by *Methanosaeta*). See Supplementary Figures S11 and S12 for thermodynamic calculations of pathways potentially involved in syntrophic propionate oxidation. Ethanol and butyrate degraders might also be located in close proximity to methanogens. However, calculated ΔGs were exergonic enough to enable a planktonic lifestyle for syntrophic butyrate and ethanol degraders. Note: Formate could be produced by syntrophs and fermenters in addition to H_2–CO_2.

Conclusions, limitations and future perspectives

A hypothetical model highlighting syntrophic processes that are crucial for the intermediary ecosystem metabolism in the investigated fen was constructed based on the process and phylogenic data collected in this study (Figure 6). Degradation of propionate, butyrate and ethanol was found to be associated with hitherto uncultured species/strains of the syntrophic genera *Syntrophobacter* (95% identity to cultured relatives), *Smithella* (97%), *Syntrophomonas* (95%) and *Pelobacter* (98%).

Genera not known for a syntrophic metabolism may also contribute to the degradation of propionate, butyrate and ethanol. In this respect, OTU26a was enriched in 'heavy' compared with 'light' fractions in [^{13}C]butyrate treatments (Figure 4). OTU 26a, which was related to *Telmatospirillum siberiense* (up to 95% identity), was highly similar to a clone sequence retrieved from a butyrate-fed anaerobic digestor (JN995370; Figure 5). This suggests a potential contribution of OTU26a to the degradation of butyrate. However, stable isotope probing is based on assimilation rather than dissimilation, and crossfeeding on [^{13}C]butyrate-derived acetate or ^{13}C-labeled dead biomass is not unlikely (Lueders *et al.*, 2004; Chauhan and Ogram, 2006). OTU53 and OTU47 (affiliated to the *Fibrobacteres* and *Bacteroidetes*, respectively; Figure 5) had increased abundancies in propionate treatments (Figure 4) and might thus represent unrecognized propionate oxidizers. In this regard, the *Bacteroidetes* were identified as potential propionate oxidizers in Arctic peat soil (Tveit *et al.*, 2015). Pure cultures of these taxa will be required to determine their syntrophic abilities.

Some sulfate-reducing bacteria are capable of a syntrophic lifestyle when sulfate is not available (Pester *et al.*, 2012). The detected sulfate reducers (e.g., *Desulfomonile* (OTU38d) and *Desulfovibrio* (OTU40a)) might have therefore contributed to the syntrophic degradation of ethanol and fatty acids and might do so *in situ* in the absence of sulfate. Experiments under alternating sulfate-reducing (i.e., with supplemental sulfate) and syntrophic (i.e., by adding a hydrogen-scavenging methanogen) conditions could more closely evaluate the syntrophic capabilities of the fen sulfate reducers.

Of the dominant syntrophic genera in propionate, butyrate and ethanol treatments, only *Syntrophobacter* (1.7% relative abundance) was detected in fresh peat, and a more extensive sequencing would be required to detect rare syntrophs. Thus, the proportion of syntrophs in the bacterial community of fresh peat was low. However, a low number of syntrophs can be sufficient for an effective conversion of fermentation products to methanogenic substrates as observed in anaerobic digestors in which the relative abundance of syntrophs was $<1\%$ during steady-state operation (Vanwonterghem *et al.*, 2014).

OTU38a and OTU39 were detected in several treatments and were related to *Syntrophus aciditrophicus* (92% identity) and *Syntrophorhabdus aromaticivorans* (95% identity), respectively. *Syntrophus* and *Syntrophorhabdus* are able to syntrophically oxidize aromatic compounds such as benzoate under methanogenic conditions (McInerney *et al.*, 2007; Qui *et al.*, 2008). Although the detection of 16S rRNA complementary DNA sequences related to syntrophs that oxidize aromatic compounds indicate that certain fen syntrophs may degrade aromatic compounds derived from lingocellulose or sphagnum biomass, further investigations are needed to characterize such syntrophs.

H_2 and formate, both potential products of syntrophic degradation, never accumulated, indicating that they may have been effectively scavenged by methanogens (e.g., *Methanocella* and *Methanoregula*) or acetogens (Figure 6). *Methanosarcina* may also contribute to hydrogenotrophic methanogensis *in situ* if local H_2 concentrations are high enough. Acetate, the other product of syntrophic degradation, is also produced by fermenters and acetogens and was probably the major methanogenic substrate. This is supported by the fact that aceticlastic methanogens outnumbered hydrogenotrophic methanogens to ~8 to 1 (Figure 3).

The collective results indicate that (i) propionate, butyrate and ethanol were degraded efficiently by hitherto uncultured species/strains of known syntrophic genera at 15 and 5 °C, (ii) hydrogenotrophic methanogens and acetogens may have competed for H_2 and (iii) acetate is the major methanogenic substrate under the experimental conditions. Horizontal, vertical and temporal differences of abiotic conditions as well as substrate and nutrient availability will theoretically affect the community composition and activity of syntrophs and methanogens in peatlands. It can therefore be postulated that both the capacity for syntrophic degradation as well as the predominance of a particular methanogenic pathway will vary spatially and temporally in the peatland.

Conflict of Interest

The authors declare no conflict of interest.

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