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Predominance of methanogens over methanotrophs contributes to high methane emissions in rewetted fens

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Abstract. The rewetting of drained peatlands alters peat geochemistry and often leads to sustained elevated methane emission. Although this methane is produced entirely by microbial activity, the distribution and abundance of methane-cycling microbes in rewetted peatlands, especially in fens, is rarely described. In this study, we compare the community composition and abundance of methane-cycling microbes in relation to peat porewater geochemistry in two rewetted fens in northeastern Germany, a coastal brackish fen and a freshwater riparian fen, with known high methane fluxes. We utilized 16S rDNA high-throughput sequencing and quantitative polymerase

chain reaction on 16S rDNA, *mcrA*, and *pmoA* genes to determine microbial community composition and the abundance of total bacteria, methanogens, and methanotrophs. Electrical conductivity was more than three times higher in the coastal fen than in the riparian fen, averaging 5.3 and 1.5 mS cm^{-1}, respectively. Porewater concentrations of terminal electron acceptors varied within and among the fens. This was also reflected in similarly high intra- and inter-site variations of microbial community composition. Despite these differences in environmental conditions and electron acceptor availability, we found a low abundance of methanotrophs and a high abundance of methanogens, represented in particular by *Methanosaetaceae*, in both fens. This suggests that rapid re/establishment of methanogens and slow re/establishment of methanotrophs contributes to prolonged increased methane emissions following rewetting.

1 Introduction

Rewetting is a technique commonly employed to restore ecological and biogeochemical functioning of drained fens. However, while rewetting may reduce carbon dioxide (CO_2) emissions (Wilson et al. 2016), it often increases methane (CH_4) emissions in peatlands that remain mostly inundated following rewetting. The factors that contribute to the magnitude and duration of this increase are still uncertain (Joosten et al. 2015, Abdalla et al. 2016). On a 100-year time scale CH_4 has a global warming potential 28 times stronger than CO_2 (Myhre et al. 2013); thus, increased CH_4 emissions could potentially offset the benefit of decreased CO_2 emissions (Jurasinski et al. 2016). Although a recent increase in rewetting projects in Germany and other European nations has prompted a number of studies of methane cycling in rewetted peatlands (e.g., Jerman et al. 2009, Hahn-Schöfl et al. 2011, Urbanová et al. 2013, Hahn et al. 2015, Vanselow-Algan et al. 2015, Zak et al. 2015, Emsens et al. 2016), the post-rewetting distribution and abundance of methane-cycling microbes in rewetted fens has seldom been examined (but see Juottonen et al. 2012, Urbanová et al. 2013).

Peat CH_4 production and release is governed by a complex array of interrelated factors including climate, water level, plant community, nutrient status, site geochemistry, and the activity of microbes (i.e. bacteria and archaea) that use organic carbon as energy source (Segers 1998, Abdalla et al. 2016). To date, the vast majority of studies in rewetted fens have focused on quantifying CH_4

emission rates in association with environmental variables such as water level, plant community, and aspects of site geochemistry (Abdalla et al. 2016). Site geochemistry indeed plays an important role for methanogenic communities, as methanogenesis is suppressed in presence of thermodynamically more favorable terminal electron acceptors (TEAs, Blodau 2011). Due to a smaller pool of more favorable electron acceptors and high availability of carbon substrates, organic-rich soils such as peat rapidly establish methanogenic conditions when anoxic (Segers 1998, Keller and Bridgham 2007, Knorr and Blodau 2009). Despite their decisive role as producers (i.e. methanogens) and consumers (i.e. methanotrophs) of CH_4 (Conrad 1996), only a few studies have combined a characterization of the CH_4-cycling microbial community, site geochemistry, and observed patterns of CH_4 production. Existing studies have been conducted in oligotrophic and mesotrophic boreal fens (e.g., Juottonen et al. 2005, Yrjälä et al. 2011, Juottonen et al. 2012), alpine fens (e.g., Liebner et al. 2012, Urbanová et al. 2013, Cheema et al. 2015, Franchini et al. 2015), subarctic fens (Liebner et al. 2015), and incubation experiments (e.g., Jerman et al. 2009, Knorr and Blodau 2009, Urbanová et al. 2011, Emsens et al. 2016). Several studies on CH_4-cycling microbial communities have been conducted in minerotrophic temperate fens (e.g., Cadillo-Quiroz et al. 2008, Liu et al. 2011, Sun et al. 2012, Zhou et al. 2017), but these sites were not subject to drainage or rewetting. To our knowledge, only one study has directly compared *in situ* abundances of methanogens and methanotrophs in drained versus rewetted fens (Juottonen et al. 2012). The studied sites, however, were nutrient-poor fens with acidic conditions.

While studies of nutrient-poor and mesotrophic boreal fens have documented post-rewetting CH_4 emissions comparable to or lower than at pristine sites (Komulainen et al. 1998, Tuittila et al. 2000, Juottonen et al 2012), studies of temperate nutrient-rich fens have reported post-flooding CH_4 emissions dramatically exceeding emissions in pristine fens (e.g., Augustin and Chojnicki 2008, Hahn et al. 2015). These high emissions typically occur together with a significant dieback in vegetation, a mobilization of nutrients and electron acceptors in the upper peat layer, and increased availability of dissolved organic matter (Zak and Gelbrecht 2007, Hahn-Schöfl et al. 2011, Hahn

et al. 2015, Jurasinski et al. 2016). Vanselow-Algan et al. (2015) have shown that such high CH_4 fluxes may continue for decades following rewetting even in bogs. Because of their potential to remain significant CH_4 sources on decadal timescales, there is an urgent need to characterize CH_4-cycling microbial communities and geochemical conditions in rewetted minerotrophic fens. Therefore, in this study, we examined microbial community composition and abundance in relation to post-flooding geochemical conditions in two rewetted fens in northeastern Germany. In both fens, CH_4 emissions increased dramatically after rewetting (Augustin and Chojnicki 2008, Hahn-Schöfl et al. 2011, Hahn et al. 2015, Jurasinski et al. 2016). Average annual CH_4 emissions have decreased in both fens since the initial peak (Franz et al. 2016, Jurasinski et al. 2016). Nevertheless, fluxes remained higher than under pre-flooding conditions (ibid.), and higher than in pristine fens (Urbanová et al. 2013, Minke et al 2016).

We expected patterns in microbial community composition would reflect the geochemical conditions of the two sites and hypothesized a high abundance of methanogens relative to methanotrophs in both fens. We also expected acetoclastic methanogens, which typically thrive in nutrient-rich fens (Kelly et al. 1992, Galand 2005), to dominate the methanogenic community in both fens.

2 Methods

2.1 Study sites

The nature reserve “Heiligensee and Hütelmoor” (‘Hütelmoor’ in the following, approx. 540 ha, 54°12'36.66" N, 12°10'34.28" E), is a coastal, mainly minerotrophic fen complex in Mecklenburg-Vorpommern (NE Germany) that is separated from the Baltic Sea by a narrow (~100 m and less) dune dike (Fig. 1a and b). The climate is temperate in the transition zone between maritime and continental with an average annual temperature of 9.1 °C and an average annual precipitation of 645 mm (data derived from grid product of the German Weather Service, reference climate period:

1981–2010). Episodic flooding from storm events delivers sediment and brackish water to the site (Weisner and Schernewski 2013). The vegetation is a mixture of salt-tolerant macrophytes, with dominant to semi-dominant stands of *Phragmites australis*, *Bolboschoenus maritimus*, *Carex acutiformis*, and *Schoenoplectus tabernaemontani.* The dominating plants are interspersed with open water bodies that are colonized by *Ceratophyllum demersum* in summer (Koch et al. 2017). Intense draining and land amelioration practices began in the 1970s, which lowered the water level to 1.6 m below ground surface and caused aerobic decomposition and concomitant degradation of the peat (Voigtländer et al. 1996). The upper peat layer varies in depth between 0.6 and 3 m and is highly degraded, reaching up to H10 on the von Post humification scale (Hahn et al. 2015). Active draining ended in 1992, but dry conditions during summertime kept the water table well below ground surface (Schönfeld-Bockholt et al. 2005, Koebsch et al. 2013) until concerns of prolonged aerobic peat decomposition prompted the installation of a weir in 2009 at the outflow of the catchment (Weisner and Schernewski 2013). After installation of the weir, the site was fully flooded year-round with an average water level of 0.6 m, and annual average CH_4 flux increased ~186-fold from 0.0014 ± 0.0006 kg CH_4 m^{-2} a^{-1} to 0.26 ± 0.06 kg CH_4 m^{-2} a^{-1} (Hahn et al. 2015).

The study site polder Zarnekow ('Zarnekow' in the following, approx. 500 ha, 53°52'31.10" N, 12°53'19.60" E) is situated in the valley of the River Peene in Mecklenburg-Vorpommern (NE Germany, Fig. 1a and c). The climate is slightly more continental compared to the Hütelmoor, with a mean annual precipitation of 544 mm and a mean annual temperature of 8.7 °C (German Weather Service, meteorological station Teterow, 24 km southwest of the study site; reference period 1981–2010). The fen can be classified as a river valley mire system consisting of spring mires, wider percolation mires, and flood mires along the River Peene. Drainage and low-intensity agricultural use began in the eighteenth century when land-use changed to pastures and grassland. This was intensified by active pumping in the mid-1970s. Due to land subsidence of several decimeters, after rewetting (October 2004) water table depth increased to 0.1–0.5 m above peat surface. The upper horizon is highly decomposed (0–0.3 m), followed by moderately decomposed peat to a

depth of 1 m and a deep layer of slightly decomposed peat up to a maximum depth of 10 m. The open water bodies are densely colonized by *Ceratophyllum* spp. and *Typha latifolia* is the dominant emergent macrophyte (Steffenhagen et al. 2012). Following flooding, CH_4 flux rates increased to ~0.21 kg m^{-2} a^{-1} (Augustin and Chojnicki 2008). No pre-rewetting CH_4 flux data were available for the Zarnekow site but published CH_4 flux rates of representative drained fens from the same region have been shown to be negligible (Augustin et al. 1998).

2.2 Collection of peat cores and porewater samples

Peat and porewater samples were collected at four different locations in Hütelmoor (October 2014) and at five locations in Zarnekow (July 2015) and spanned a distance of 1,200 m and 250 m, respectively, to cover the whole lateral extension at each site (Fig. 1b and c). Peat cores were collected with a Perspex liner (ID: 60 mm, Hütelmoor) and a peat auger (Zarnekow). In order to minimize oxygen contamination, the outer layer of the peat core was omitted. Subsamples for molecular analysis were immediately packed in 50 ml sterile Falcon tubes and stored at -80 °C until further processing.

Pore waters in Hütelmoor were collected with a stainless-steel push-point sampler attached to a plastic syringe to recover the samples from 10 cm depth intervals. Samples were immediately filtered with 0.45 µm membrane disposable syringe filters. Pore waters in Zarnekow were sampled with permanently installed dialysis samplers consisting of slotted polypropylene (PP) pipes (length: 636 mm, ID: 34 mm) surrounded with 0.22 µm polyethersulfone membrane. The PP pipes were fixed at distinct peat depths (surface level, 20 and 40 cm depth) and connected with PP tubes (4x6 mm IDxAD). Water samples were drawn out from the dialysis sampler pipes with a syringe through the PP tube.

At both sites, electrical conductivity (EC), dissolved oxygen (DO) and pH were measured immediately after sampling (Sentix 41 pH probe and a TetraCon 325 conductivity measuring cell attached to a WTW multi 340i handheld; WTW, Weilheim). Headspace CH_4 concentrations of porewater samples were measured with an Agilent 7890A gas chromatograph (Agilent

Technologies, Germany) equipped with a flame ionization detector and a Carboxen PLOT Capillary Column or HP-Plot Q (Porapak-Q) column. The measured headspace CH_4 concentration was then converted into a dissolved CH_4 concentration using the temperature-corrected solubility coefficient (Wilhelm et al. 1977). Isotopic composition of dissolved CH_4 for Hütelmoor was analyzed using the gas chromatography-combustion-technique (GC-C) and the gas chromatography-high-temperature-conversion-technique (GC-HTC). The gas was directly injected in a Gas Chromatograph Agilent 7890A, methane was quantitatively converted to CO_2 and the $\delta^{13}C$ values were then measured with the isotope-ratio-mass-spectrometer MAT-253 (Thermo Finnigan, Germany). The $\delta^{13}C$ of dissolved methane in Zarnekow was analyzed using a laser-based isotope analyzer equipped with a small sample isotope module for analyses of discrete gas samples (cavity ring down spectroscopy CRDS; Picarro G2201-I, Santa Clara, CA, USA). Calibration was carried out before, during and after analyses using certified standards of known isotopic composition (obtained from Isometric Instruments, Victoria, BC, Canada, and from Westfalen AG, Münster, Germany). Reproducibility of results was typically +/- 1 ‰. In the presence of high concentrations of hydrogen sulfide interfering with laser-based isotope analysis, samples were treated with iron(III) sulfate to oxidize and/or precipitate sulfide. For both sites, sulfate and nitrate concentrations were analyzed by ion chromatography (IC, Thermo Fisher Scientific Dionex) using an Ion Pac AS-9-HC 4 column, partly after dilution of the sample. Dissolved metal concentrations were analyzed by ICP-OES (iCAP 6300 DUO, Thermo Fisher Scientific). Accuracy and precision were routinely checked with a certified CASS standard as previously described (Kowalski et al. 2012).

2.3 Gene amplification and phylogenetic analysis

Genomic DNA was extracted from 0.2–0.3 g of duplicates of peat soil per sample using an EurX Soil DNA Kit (Roboklon, Berlin, Germany). DNA concentrations were quantified with a Nanophotometer P360 (Implen GmbH, München, DE) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany). Polymerase chain reaction (PCR) amplification of bacterial and

archaeal 16S rRNA genes was performed using the primer combination of S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Herlemann et al. 2011) and S-D-Arch-0349-a-S-17/S-D-Arch-0786-a-A-20 (Takai and Horikoshi 2000), respectively. The PCR mix contained 1x PCR buffer (Tris•Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7) (QIAGEN, Hilden, Germany), 0.5 µM of each primer (Biomers, Ulm, Germany), 0.2 mM of each deoxynucleoside (Thermo Fisher Scientific, Darmstadt, Germany) and 0.025 U μl^{-1} hot start polymerase (QIAGEN, Hilden, Germany). PCR samples were kept at 95 °C for 5 min to denature the DNA, with amplification proceeding for 40 cycles at 95 °C for 1 min, 56 °C for 45 s and 72 °C for 90 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. PCR products were purified with a Hi Yield Gel/PCR DNA fragment extraction kit (Süd-Laborbedarf, Gauting, Germany). PCR products of three individual runs per sample were combined. PCR products of different samples were pooled in equimolar concentrations and compressed to a final volume of 10 µl with a concentration of 200 ng μl^{-1} in a vacuum centrifuge Concentrator Plus (Eppendorf, Hamburg, Germany).

Illumina sequencing was performed by GATC Biotech AG using 300 bp paired-end mode and a 20% PhiX Control v3 library to counteract the effects of low-diversity sequence libraries. Raw data was demultiplexed using an own script based on CutAdapt (Martin 2011). Ambiguous nucleotides at sequence ends were trimmed and a 10% mismatch was allowed for primer identification, whereas barcode sequences needed to be present without any mismatches and with a minimum Phred-Score of Q25 for each nucleotide. After sorting, overlapping paired-end reads were merged using PEAR [Q25, p 0.0001, v20] (Zhang et al. 2014). The orientation of the merged sequences was standardized according to the barcode information obtained from demultiplexing. Low-quality reads were removed using Trimmomatic [SE, LEADING Q25, TRAILING Q25, SLIDINGWINDOW 5:25; MINLEN 200] (Bolger et al. 2014). Chimeric sequences were removed using USEARCH 6.1 and the QIIME-script identify_chimeric_seqs.py (Caporaso et al. 2010). Pre-processed sequences were taxonomically assigned to operational taxonomic units (OTUs) at a nucleotide sequence identity of 97% using QIIME's pick_open_reference_otus.py script and the

GreenGenes database 13.05 (McDonald et al. 2012) as reference. The taxonomic assignment of representative sequences was further checked for correct taxonomical classification by phylogenetic tree calculations in the ARB environment referenced against the SILVA database (https://www.arb-silva.de) version 119 (Quast et al. 2013). The resulting OTU table was filtered for singletons, OTUs assigned to chloroplasts or mitochondria, and for low-abundance OTUs (below 0.2% within each sample). Archaeal and bacterial samples were processed separately while only OTUs that were assigned to the respective domain were considered for further analysis. The 16S rRNA gene sequence data have been deposited at NCBI under the Bioproject PRJNA356778. Hütelmoor sequence read archive accession numbers are SRR5118134-SRR5118155 for bacterial and SRR5119428-SRR5119449 for archaeal sequences, respectively. Zarnekow accession numbers are SRR6854018-SRR6854033 and SRR6854205-SRR6854220 for bacterial and archaeal sequences, respectively.

2.4 qPCR analysis

Quantitative polymerase chain reaction (qPCR) for the determination of methanotrophic and methanogenic functional gene copy numbers and overall bacterial 16S rRNA gene copy numbers was performed via SybrGreen assays on a Bio-Rad CFX instrument (Bio-Rad, Munich, Germany) with slight modifications after Liebner et al. (2015). The functional methanotrophic *pmoA* gene was amplified with the primer combination A189F/Mb661 (Kolb et al. 2003) suitable for detecting all aerobic methanotrophic Proteobacteria. Annealing was done at 55 °C after a 7-cycle-step touchdown starting at 62 °C. The functional methanogenic *mcrA* gene was amplified with the *mlas/mcrA*-rev primer pair (Steinberg and Regan 2009) with annealing at 57 °C. The bacterial 16S rRNA gene was quantified with the primers Eub341F/Eub534R according to Degelmann et al. (2010) with annealing at 58 °C. Different DNA template concentrations were tested prior to the qPCR runs to determine optimal template concentration without inhibitions through co-extracts. The 25 µl reactions contained 12.5 µl of iTaq universal Sybr Green supermix (Bio-Rad, Munich, Germany), 0.25 µM concentrations of the primers, and 5 µl of DNA template. Data acquisition

was always done at 80 °C to avoid quantification of primer dimers. The specificity of each run was verified through melt-curve analysis and gel electrophoresis. Only runs with efficiencies between 82 and 105% were used for further analysis. Measurements were performed in triplicates. We determined the ratio of methanogens to methanotrophs based on gene abundances of *mcrA* and *pmoA*. The marker gene for the soluble monooxygenase, *mmoX*, was neglected due to the absence of *Methylocella* in the sequencing data (Fig. 3).

2.5 Data visualization and statistical analysis

All data visualization and statistical analysis were done in R (R Core Team). The taxonomic relative abundances across samples were visualized through bubble plots with the R package ggplot2 (Wickham 2009). Differences in microbial community composition were visualized with 2-dimensional non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances. The NMDS ordinations were constructed using R package vegan (Oksanen et al. 2017). An environmental fit was performed on the ordinations to determine the measured geochemical parameters that may influence community composition. The geochemical data were fitted to the ordinations as vectors with a significance of $p < 0.05$. Depth profiles were constructed with the porewater geochemical data, as well as with the microbial abundances, to elucidate depthwise trends and assess whether differences in microbial community and abundances among the two fens are related to differences in their respective geochemistry.

3 Results

3.1 Community composition of bacteria and archaea

Bacterial sequences could be affiliated into a total of 30 bacterial phyla (Fig. 2). Among them, Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Nitrospirae and Bacteroidetes were present in all samples. With mean relative abundance of 48%, Proteobacteria was the most abundant phylum. Some taxa (e.g., Verrucomicrobia, Atribacteria (OP9), and AD3) were present only in Hütelmoor. Variation in community composition was larger in Hütelmoor samples than in

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Zarnekow. Within Proteobacteria, the alpha subdivision was the most dominant group, having contributed 26.7% to all the libraries on average (Fig. 3). The family *Hyphomicrobiaceae* dominated the Alphaproteobacteria, and was distributed evenly across samples, but missing in the surface and bottom peat layers in Hütelmoor core (HC) 2. In addition, methanotrophs were clearly in low abundance across all samples. Of the few methanotrophs that were detected, type II methanotrophs (mainly *Methylocystaceae*) outcompeted type I methanotrophs (mainly *Methylococcaceae*) in the community, while members of the genus *Methylocella* were absent (Fig. 3).

Within the archaeal community, Bathyarchaeota were mostly dominating over Euryarchaeota (Fig. 4). The MCG group (mainly the order of pGrfC26) in Bathyarchaeota prevailed across all samples but was especially abundant in HC 2 samples. In addition to Bathyarchaeota, methanogenic archaea were important, and on average contributed 30.6% to the whole archaeal community. Among the methanogens, acetoclastic methanogens were more abundant in most of the samples and *Methanosaetaceae* (24.8%) were the major component. They were present in most samples and much more dominant than *Methanosarcinaceae* (2.0%). Hydrogenotrophic methanogens, such as *Methanomassiliicoccaceae* (1.6%), *Methanoregulaceae* (1.2%) and *Methanocellaceae* (0.6%), albeit low in abundance, were detected in many samples. Hütelmoor samples displayed greater variability in archaeal community composition compared to Zarnekow samples. The putative anaerobic methanotrophs of the ANME-2D (Raghoebarsing et al. 2006) clade occurred in patchy abundance with dominance in single spots of both sites. In HC 1 they represented a mean relative abundance of 40.9% of total archaeal reads but were almost absent in all other Hütelmoor cores. In Zarnekow core (ZC) 3, ANME-2D represented up to approximately 30% of all archaea but were otherwise low in abundance.

3.2 Environmental characteristics and site geochemistry

The two rewetted fens varied substantially in their environmental characteristics (e.g., proximity to the sea) and porewater geochemistry (Fig. 5, Tables 1 and 2). Electrical conductivity was more

than three times higher in Hütelmoor than in Zarnekow, averaging 5.3 and 1.5 mS cm^{-1}, respectively. Mean pH was approximately neutral (6.5 to 7) in the upper peat profile and comparable in both fens until a depth of about 30 cm where pH was ~6 in the Hütelmoor. Concentrations of the TEAs nitrate and sulfate were lower in Zarnekow and near zero in the pore water at all depths, while nitrate and sulfate were abundant in the upper and lower peat profile in Hütelmoor at ~1.5 to 3.0 mM and ~4 to 20 mM, respectively (Fig. 5). Iron concentrations were higher in the Hütelmoor pore water, while manganese concentrations were higher in Zarnekow pore water. Dissolved oxygen concentrations in the upper peat profile (i.e. 0 to 25 cm depths) were much higher in Hütelmoor than in Zarnekow (Fig. 5). Here DO concentrations averaged ~0.250 mM until a depth of 15 cm at which they dropped sharply, reaching concentrations slightly below 0.050 mM at 25 cm. In Zarnekow, DO concentrations did not exceed 0.1 mM and varied little with depth. Regarding geochemical conditions, HC 1 was distinct from all other Hütelmoor cores and more similar to Zarnekow cores. In HC 1 – the core taken nearest to potential freshwater sources (Fig. 1b) – pore water EC and DO concentrations were lower while pH was slightly higher than all other Hütelmoor cores. Moreover, this was the only Hütelmoor core where nitrate concentrations were undetectable (Fig. 5). Dissolved CH_4 concentrations were high, varied within and among fens and were slightly higher in Zarnekow pore water. Stable isotope ratios of $\partial^{13}C$-CH_4 (Fig. 5) in the upper peat (approx. −59‰) suggest a predominance of acetoclastic methanogenesis, with a shift to hydrogenotrophic methanogenesis around −65‰ in the lower peat profile. Also, shifts toward less negative $\partial^{13}C$-CH_4 values in the upper peat layer, as in HC 1 and HC 2, could indicate partial oxidation of CH_4 occurred (Chasar et al. 2000).

3.3 Environmental drivers of microbial community composition

Bacterial and archaeal population at both peatland sites showed distinct clustering (Fig. 6) with similarly high intra- and inter-site variations but greater overall variation in community composition in the Hütelmoor. Community composition varied much more strongly in HC 2 than in any other core (grey dashed-line polygon in Fig. 6). Bacterial communities in HC 1 were more

similar to communities in all Zarnekow cores than in other Hütelmoor cores (Fig. 6a). The archaeal community in HC 1 was more similar to Zarnekow cores as well (Fig. 6b). Overall, the influence of depth on microbial community was evident, especially in the Hütelmoor where the differences were more pronounced. Environmental fit vectors suggest pH, oxygen and alternative TEA availability as important factors influencing microbial community composition. The EC vector suggests the importance of brackish conditions in shaping microbial communities in the Hütelmoor (Fig. 6a - c).

3.4 Total microbial and functional gene abundances

Quantitative PCR results show that in both fens, *mcrA* abundance is up to two orders of magnitude greater than *pmoA* abundance (Fig. 7, Tables 1 and 2). Gene copy numbers of *mcrA* are overall higher and spatially more stable in Zarnekow than in Hütelmoor. Total microbial abundance declined with depth more strongly in Hütelmoor than in Zarnekow (Fig. 7). There was a pronounced decrease in microbial abundances at 20 cm depth in the Hütelmoor. For example, 16S rRNA gene and *pmoA* gene copy numbers in deeper samples (below 20 cm depth) are one order of magnitude lower than in upper samples on average, while the *mcrA* gene abundance are approximately two orders of magnitude lower. Hütelmoor samples also exhibited larger heterogeneity in terms of abundances than Zarnekow samples.

4 Discussion

4.1 Fen geochemistry and relations to microbial community composition

The rewetting of drained fens promotes elevated CH_4 production and emission, which can potentially offset carbon sink benefits. Very few studies have attempted to link microbial community dynamics and site geochemistry with observed patterns in CH_4 production and/or emission in rewetted fens while such data are crucial for predicting long-term changes to CH_4 cycling (Galand et al. 2002, Yrjälä et al. 2011, Juottonen et al. 2012). In this study, we show that CH_4-cycling microbial community composition is related to patterns in site geochemistry in two

347 rewetted fens with high CH_4 emissions, high methanogen abundances, and low methanotroph
348 abundances. Our results suggest that high methanogen abundances concurrent with low
349 methanotroph abundances contribute to increased CH_4 production and the resulting high emissions
350 in rewetted peatlands with readily available substrate. Thus, we present microbial evidence for
351 sustained elevated CH_4 emissions in mostly inundated rewetted temperate fens.
352 The environmental conditions and associated geochemistry of the two rewetted fens were largely
353 different. Depth profiles of porewater geochemical parameters show the fens differed in EC
354 throughout the entire peat profile, while pH and concentrations of alternative TEAs differed at
355 certain depths. In general, concentrations of TEAs oxygen, sulfate, nitrate, and iron were higher
356 in the Hütelmoor. In Zarnekow, geochemical conditions varied little across the fen and along the
357 peat depth profiles (Fig. 5). As expected, the geochemical heterogeneity was reflected in microbial
358 community structure in both sites, suggesting the importance of environmental characteristics and
359 associated geochemical conditions as drivers of microbial community composition (Figs. 2, 3, 4,
360 6). The NMDS ordinations (Fig. 6) show significant variation in archaeal and bacterial community
361 composition in the coastal brackish fen, and much less variation in the freshwater riparian fen.
362 Environmental fit vectors (Fig. 6) suggest that salinity (indicated by the EC vector), pH, oxygen
363 and alternative TEA availability are the most important measured factors influencing microbial
364 communities in the two fens. Patterns in microbial community composition have previously been
365 linked to salinity (e.g., Chambers et al. 2016), pH (e.g., Yrjälä et al. 2011), and TEA availability
366 in peatlands (e.g., He et al. 2015).
367 Comparing the geochemical depth profiles (Fig. 5) with the relative abundance of bacteria and
368 archaea (Figs. 3 and 4) provides a more complete picture of the relationships between microbial
369 communities and site geochemistry, particularly with respect to TEA utilization. While the
370 porewater depth profiles suggest there is little nitrate available for microbial use in HC 1, the
371 relative abundance plot for Archaea showed that this core was dominated by ANME-2D. ANME-
372 2D were recently discovered to be anaerobic methanotrophs that oxidize CH_4 performing reverse

methanogenesis using nitrate as an electron acceptor (Haroon et al. 2013). However, ANME-2D has also been implicated in the iron-mediated anaerobic oxidation of methane (Ettwig et al. 2016), and the HC 1 site showed slightly higher total iron concentrations. The relevance of ANME-2D as CH_4 oxidizers in terrestrial habitats is still not clear. Rewetting converts the fens into widely anaerobic conditions, thus providing conditions suitable for the establishment of anaerobic oxidation of methane, but this has yet to be demonstrated in fens. The patchy occurrence and locally high abundance of ANME-2D both in Hütelmoor and in Zarnekow suggests an ecological relevance of this group. Shifts toward a less negative $\delta^{13}C$-CH_4 signature in the upper peat profile, especially in HC 1 where ANME-2D was abundant, may indicate partial oxidation occurred, but we could only speculate whether or not they are actively involved in CH_4 oxidation.

Although TEA input may be higher in the Hütelmoor, here, methanogenic conditions also predominate. This finding contrasts the measured oxygen concentrations in the upper peat profile, however seasonal analysis of oxygen concentrations in both sites suggests highly fluctuating oxygen regimes both spatially and temporary (data not shown). Such non-uniform distribution of redox processes has already been described elsewhere, in particular for methanogenesis (Hoehler et al. 2001, Knorr et al. 2009). It is possible that oxygen levels in both fens are highly dynamic allowing for both aerobic and anaerobic carbon turnover processes. Further, oxygen may not necessarily be available within aggregates in which anaerobic pathways predominate. Anaerobic conditions are also reflected by the extensive and stable occurrence of the strictly anaerobic syntrophs (e.g., *Syntrophobacteraceae*, *Syntrophaceae*) in most samples, even in the top centimeters. This suggests that syntrophic degradation of organic material is taking place in the uppermost layer and the fermented substances are easily available for methanogens. Recent studies from wetlands also show that methanogenesis can occur in aerobic layers, driven mainly by Methanosaeta (Narrowe et al. 2017, Wagner 2017), which were detected in a high abundance in this study (Fig. 4). As geochemistry and microbial community composition differ among the sites in this study, it is thus notable that a similarly high abundance of methanogens, and low abundance

399 of methanotrophs was detected in both fens. The dominance of methanogens implies that readily
400 available substrates and favorable geochemical conditions promote high anaerobic carbon turnover
401 despite seasonally fluctuating oxygen concentrations in the upper peat layer.

402 **4.2 Microbial evidence for high CH_4 emissions**

403 Methanogens (mainly *Methanosaetaceae*) dominated nearly all of the various niches detected in
404 this study, while methanotrophs were highly under-represented in both sites (Figs. 3 and 4).
405 Functional and ribosomal gene copy numbers not only show a high ratio of methanogen to
406 methanotroph abundance (Fig. 7) irrespective of site and time of sampling, but also a small
407 contribution of methanotrophs to total bacterial population in both sites. Methanotrophs constitute
408 only ~0.06% of the total bacterial population in the Hütelmoor and ~0.05% at Zarnekow. It should
409 be noted that in this study we measured only gene abundances and not transcript abundances, so
410 that the pool both of active methanogens and methanotrophs was likely smaller than the numbers
411 presented here (Freitag and Prosser 2009, Freitag et al. 2010, Cheema et al. 2015, Franchini et al.
412 2015). Also, as we were unable to obtain microbial samples from before rewetting, a direct
413 comparison of microbial abundances was not possible. Compared to pristine fens, however, we
414 detected a relatively low abundance of methanotrophs. Liebner et al. (2015), for example, found
415 methanotrophs represented 0.5% of the total bacterial community in a pristine, subarctic
416 transitional bog/fen palsa, while *mcrA* and *pmoA* abundances were nearly identical. In a pristine
417 Swiss alpine fen, Liebner et al. (2012) found methanotrophs generally outnumbered methanogens
418 by an order of magnitude. Cheema et al. (2015) and Franchini et al. (2015) reported *mcrA*
419 abundances higher than *pmoA* abundances by only one order of magnitude in a separate Swiss
420 alpine fen. In the rewetted fens in our study, *mcrA* gene abundance was up to two orders of
421 magnitude higher than *pmoA* abundance (Fig. 7). As most methanotrophs live along the oxic-
422 anoxic boundary of the peat surface and plant roots therein (Le Mer and Roger 2001), the low
423 methanotroph abundances in both fens could be explained by disturbances to this boundary zone
424 and associated geochemical pathways following inundation. In rewetted fens, a massive plant

dieback has been observed along with strong changes in surface peat geochemistry (Hahn-Schöfl et al. 2011, Hahn et al. 2015). The anoxic conditions at the peat surface caused by inundation may have disturbed existing methanotrophic niches, and further, hindered the establishment of new ones, as oxygen availability is the most important factor governing the activity of most methanotrophs (Le Mer and Roger 2001, Hernandez et al. 2015).

Comparable studies have so far been conducted in nutrient-poor or mesotrophic fens where post-rewetting CH_4 emissions, though higher than pre-rewetting, did not exceed those of similar pristine sites (e.g., Yrjälä et al. 2011, Juottonen et al. 2005, Juottonen et al. 2012). Nevertheless, there is mounting evidence linking CH_4-cycling microbe abundances to CH_4 dynamics in rewetted fens. Juottonen et al. (2012), for example, compared *pmoA* gene abundances in three natural and three rewetted fens and found them to be lower in rewetted sites. The same study also measured a lower abundance of *mcrA* genes in rewetted sites, which was attributed to a lack of available labile carbon compounds. In peatlands, and especially fens, litter and root exudates from vascular plants can stimulate CH_4 emissions (Megonigal et al. 2005, Bridgham et al. 2013, Agethen and Knorr 2018), and excess labile substrate has been proposed as one reason for dramatic increases in CH_4 emissions in rewetted fens (Hahn-Schöfl et al. 2011). Future studies should compare pre- and post-rewetting microbial abundances along with changes in CH_4 emissions, plant communities, and peat geochemistry to better assess the effect rewetting has on the CH_4-cycling microbial community.

5 Conclusion

Despite a recent increase in the number of rewetting projects in Northern Europe, few studies have characterized CH_4-cycling microbes in restored peatlands, especially fens. In this study, we show that rewetted fens differing in geochemical conditions and microbial community composition have a similarly low abundance of methanotrophs, a high abundance of methanogens, and an established anaerobic carbon cycling microbial community. Comparing these data to pristine wetlands with

lower CH_4 emission rates, we found that pristine wetlands generally have a higher abundance of methanotrophs than measured in the fens in this study, suggesting the inundation and associated anoxia caused by flooding disturbs methanotrophic niches and may negatively affect the ability of methanotrophic communities to establish. The abundances of methane producers and consumers are thus suggested as important drivers for continued elevated CH_4 emissions following the rewetting of drained fens. Our results suggest that in the context of CH_4 cycling, rewetting drained peatlands by flooding may be problematic if post-rewetting conditions hinder methanotroph establishment. Management decisions regarding rewetting processes should consider that disturbances to methanotrophic niches is possible if rewetting leads to long-term inundation of the peat surface.

Competing interests

The authors declare that they have no conflict of interest.

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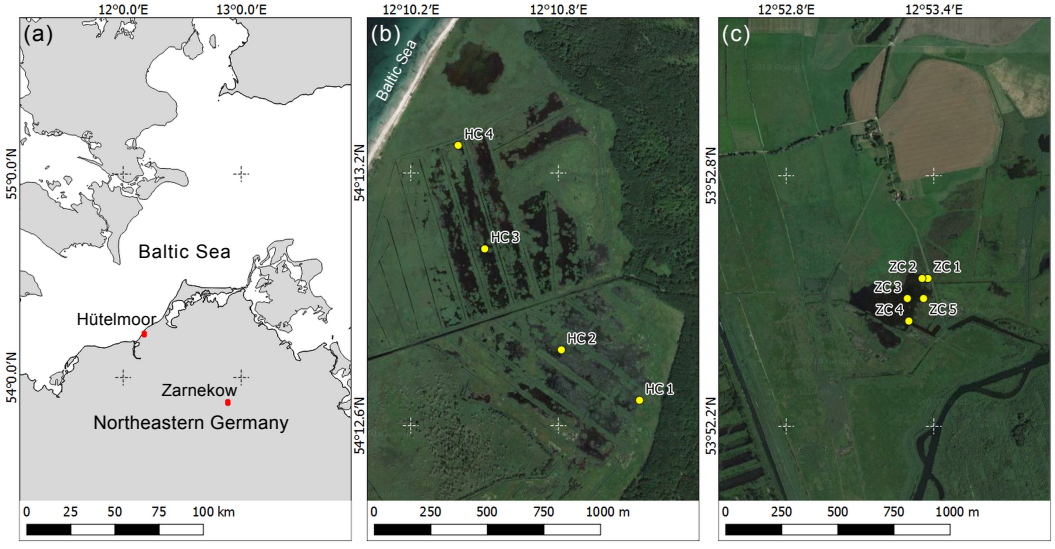

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842 **Figure 1:** Location of study sites in northeastern Germany (a) and sampling locations within sites (b) Hütelmoor and (c) Zarnekow.
843 Maps b) and c) are drawn to the same scale. Image source: (a) QGIS, (b) and (c) Google Earth via QGIS OpenLayer Plugin. Imagery
844 date: August 9, 2015.

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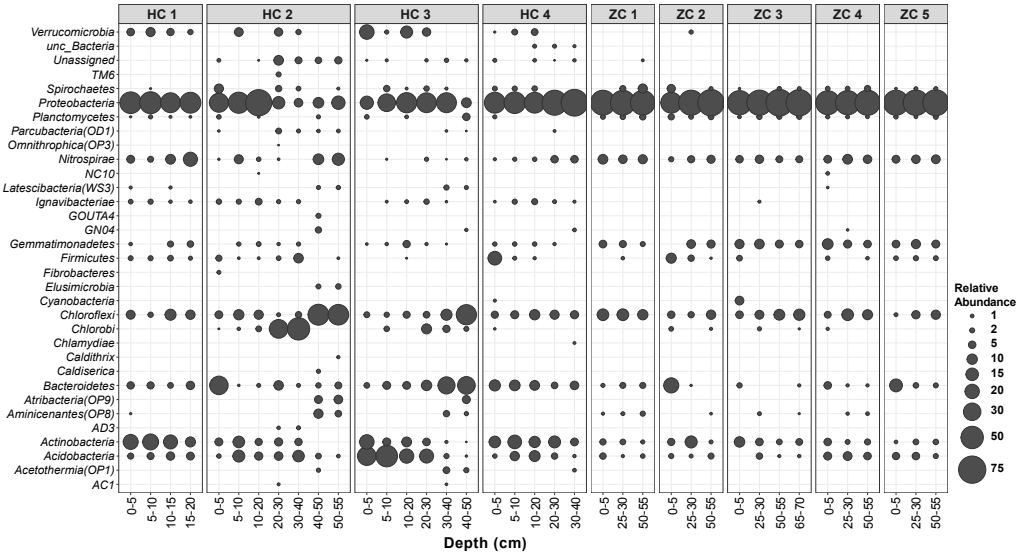

Figure 2: Relative abundances of different bacterial lineages in the study sites. Along the horizontal axis samples are arranged according to site and depth. The rank order along the vertical axis is shown for the phylum level.

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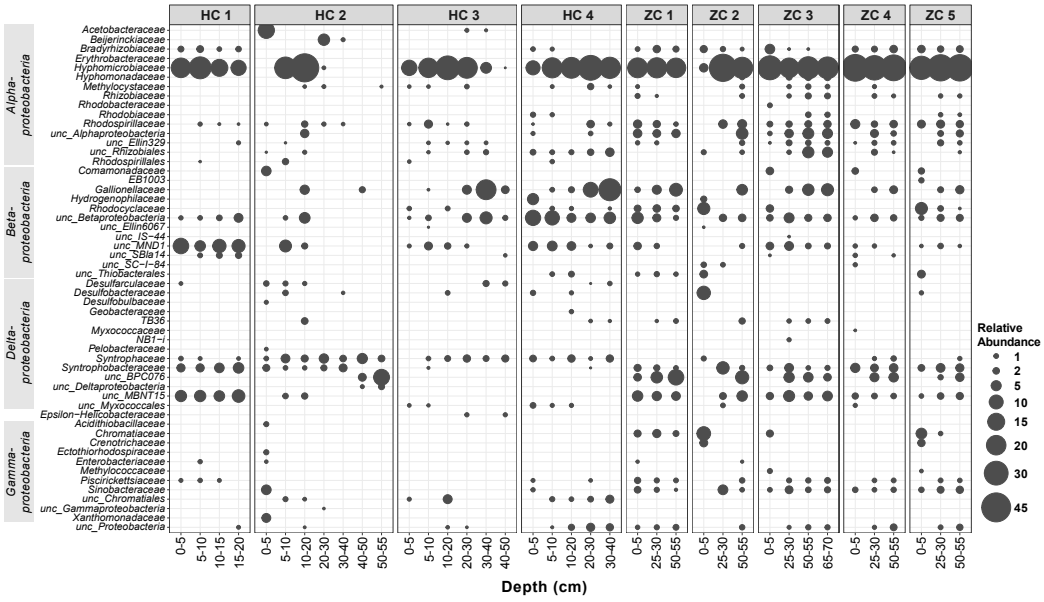

Figure 3: Relative abundances of Proteobacteria phyla in the study sites. Along the horizontal axis samples are arranged according to site and depth. The rank order along the vertical axis is shown for the family level. If an assignment to the family level was not possible the next higher assignable taxonomic level was used.

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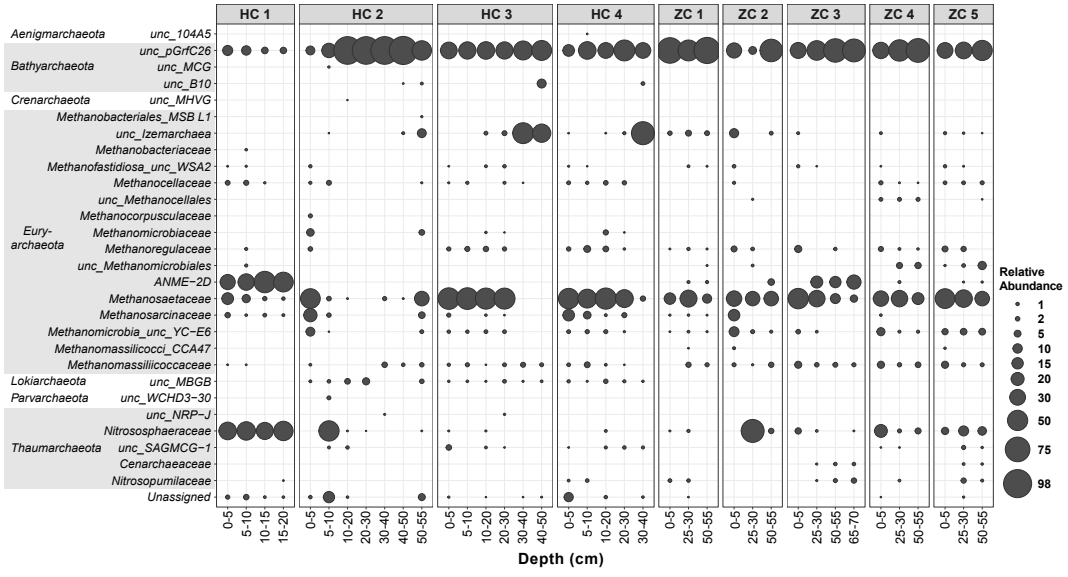

Figure 4: Relative abundances of different archaeal lineages in the study sites. Along the horizontal axis samples are arranged according to site and depth. The rank order along the vertical axis is shown for the family level. If an assignment to the family level was not possible, the next higher assignable taxonomic level was used.

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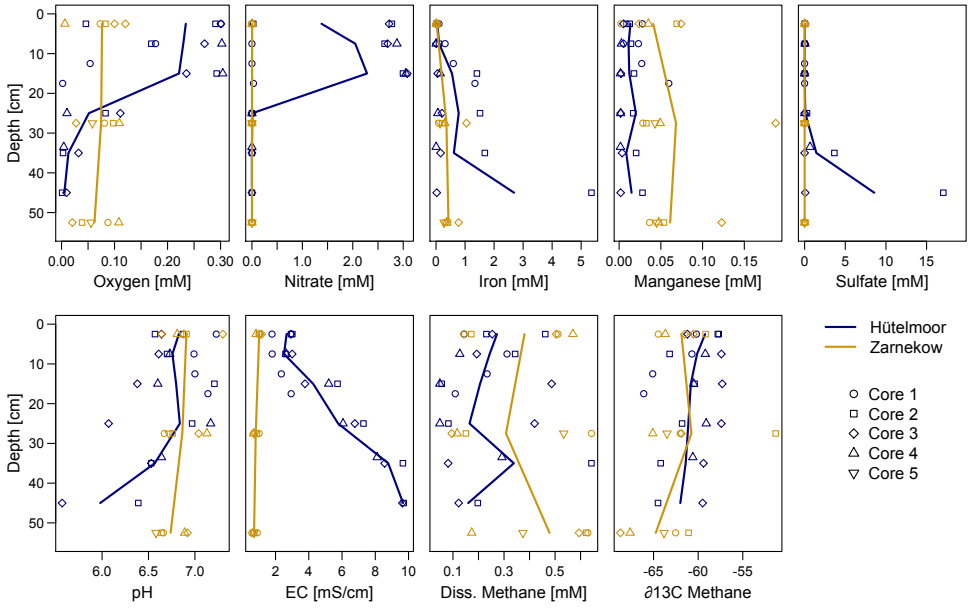

Figure 5: Depth profiles of porewater geochemistry (see x-axis labels for considered variables) in both study sites. Lines connect the respective means.

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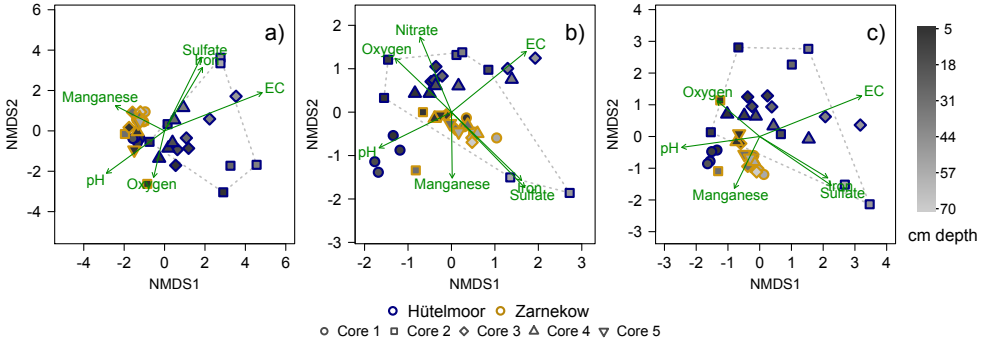

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868 **Figure 6:** NMDS plots showing (a) bacterial, (b) archaeal, and (c) microbial (bacterial plus archaeal) community composition
869 across the nine peat cores and their respective depth sections. The point positions represent distinct microbial communities, with
870 the border colors of the symbols referring to the study sites and their shapes representing the core number. The shading indicates
871 sample depth, with darker shades representing shallower depths, and lighter shades representing deeper depths. The dashed grey
872 polygon highlights the large variation in microbial community composition in HC 2. Environmental fit vectors with a significance
873 of $p < 0.05$ are shown in green.
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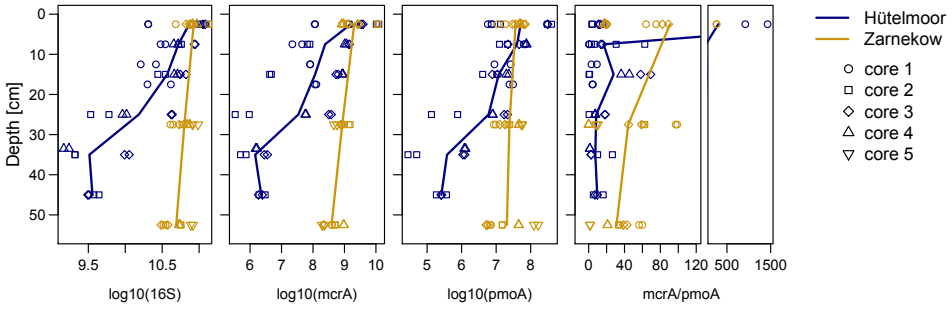

Figure 7: Depth distribution of qPCR abundances for total microbial (16S), methanogen (*mcrA*), methanotroph (*pmoA*), and ratio of *mcrA* to *pmoA* gene copy numbers in both sites. Microbial abundances were designated as numbers of gene copies per gram of dry peat soil and are shown against sampling depth using log-transformed values. Solid lines indicate mean abundances. Note that the plot at the right was split into two plots to capture very high *mcrA*/*pmoA* ratios in the upper peat layer.

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884 **Table 1:** Environmental conditions, geochemical conditions, and microbial abundances in peat cores from the Hütelmoor, a coastal minerotrophic fen
885 in northeastern Germany. Environmental conditions are described by pH and EC (electrical conductivity). Geochemical parameters shown are dissolved
886 methane (CH_4) concentrations, the isotopic signature of methane-bound carbon ($\partial^{13}C–CH_4$), and concentrations of terminal electron acceptors which
887 are denoted with their respective chemical abbreviations. Microbial abundances here represent the mean value of averaged subsamples for each depth
888 section (n=2). nd = not detected.
889
890

Core, depth	pH	EC	$\partial^{13}C–CH_4$	Dissolved CH_4	O_2	NO_3^-	Fe	Mn	SO_4^{2-}	16S	*mcrA*	*pmoA*	*mcrA*/*pmoA*
cm		mS cm^{-1}		mM						gene copies g dry $peat^{-1}$			
HC 1, 0–5	7.2	1.79	-60.2	0.14	0.30	nd	0.10	0.03	0.03	$2.04x10^{10}$	$1.15x10^{08}$	$6.60x10^{06}$	17.7
5–10	7.0	1.80	-60.7	0.31	0.18	nd	0.31	0.02	0.01	$3.25\ x10^{10}$	$3.36x10^{07}$	$6.68x10^{07}$	0.51
10–15	7.0	2.35	-65.1	0.23	0.05	nd	0.60	0.03	nd	$2.11x10^{10}$	$8.12x10^{07}$	$1.76x10^{07}$	6.12
15–20	7.1	2.94	-66.1	0.11	nd	0.03	1.34	0.06	nd	$3.08x10^{10}$	$1.21x10^{08}$	$2.76x10^{07}$	4.41
HC 2, 0–5	6.9	3.01	-57.8	0.46	0.05	0.03	0.03	0.01	nd	$1.10x10^{11}$	$1.13x10^{10}$	$1.03x10^{07}$	1,170
5–10	6.7	2.60	-63.2	0.34	0.17	2.63	0.10	0.01	0.01	$5.51x10^{10}$	$7.27x10^{07}$	$1.69x10^{07}$	4.73
10–20	7.2	5.73	-60.4	0.06	0.29	3.00	1.41	0.02	nd	$3.13x10^{10}$	$4.47x10^{06}$	$7.32x10^{06}$	0.74
20–30	7.0	7.29	-61.8	0.08	0.08	nd	1.51	0.02	0.29	$4.71x10^{09}$	$6.41x10^{05}$	$4.50x10^{05}$	3.75
30–40	6.5	9.66	-64.2	0.64	nd	nd	1.68	0.02	3.66	$2.09x10^{09}$	$6.21x10^{05}$	$3.90x10^{04}$	18.3
40–50	6.4	9.71	-64.5	0.20	nd	nd	5.35	0.03	17.1	$4.09x10^{09}$	$2.47x10^{06}$	$2.75x10^{05}$	10.7
HC 3, 0–5	6.6	2.93	-57.7	0.23	0.29	2.77	0.11	0.01	0.04	$1.10x10^{11}$	$1.34x10^{09}$	$3.51x10^{08}$	3.86
5–10	6.6	3.00	-57.4	0.19	0.27	2.69	0.01	0.01	0.03	$8.72x10^{10}$	$1.40x10^{09}$	$3.42x10^{07}$	46.6
10–20	6.4	3.77	-57.3	0.49	0.24	3.08	0.05	nd	nd	$6.08x10^{10}$	$5.86x10^{08}$	$9.35x10^{06}$	63.6
20–30	6.1	6.77	-57.4	0.42	0.11	nd	0.20	nd	nd	$4.26x10^{10}$	$3.48x10^{08}$	$1.92x10^{07}$	18.2
30–40	6.5	8.56	-59.4	0.08	0.03	nd	0.16	nd	nd	$1.05x10^{10}$	$3.20x10^{06}$	$1.17x10^{06}$	2.74
40–50	5.6	9.36	-59.5	0.12	0.01	nd	0.02	nd	0.08	$3.18x10^{09}$	$2.16x10^{06}$	$2.58x10^{05}$	8.39
HC 4, 0–5	6.6	2.93	-61.2	0.25	0.30	2.72	0.02	0.01	0.04	$1.17x10^{11}$	$3.63x10^{09}$	$3.09x10^{08}$	11.7
5–10	6.7	2.65	-59.2	0.13	0.30	2.87	0.01	nd	0.05	$4.87x10^{10}$	$1.09x10^{09}$	$7.51x10^{07}$	14.5
10–20	6.6	5.20	-60.5	0.05	0.30	3.05	0.14	nd	nd	$4.85x10^{10}$	$8.71x10^{08}$	$2.15x10^{07}$	40.8
20–30	7.2	6.06	-59.1	0.05	0.01	nd	0.06	nd	0.02	$9.78x10^{09}$	$5.82x10^{07}$	$7.91x10^{06}$	7.36
30–40	6.6	8.11	-60.6	0.29	nd	nd	0.09	nd	0.67	$1.60x10^{09}$	$1.58x10^{06}$	$1.25x10^{06}$	1.27

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Table 2: Environmental conditions, geochemical conditions, and microbial abundances in peat cores from Zarnekow, a freshwater minerotrophic fen in northeastern Germany. Environmental conditions are described by pH and EC (electrical conductivity). Geochemical parameters shown are dissolved methane (CH_4) concentrations, the isotopic signature of methane-bound carbon ($\delta^{13}C–CH_4$), and concentrations of terminal electron acceptors which are denoted with their respective chemical abbreviations. Microbial abundances here represent the mean value of averaged subsamples for each depth section (n=2). nd = not detected.

Core, depth	pH	EC	$\delta^{13}C–CH_4$	Dissolved CH_4	O_2	NO_3^-	Fe	Mn	SO_4^{2-}	16S	*mcrA*	*pmoA*	*mcrA*/*pmoA*
cm		mS cm^{-1}		mM						gene copies g dry $peat^{-1}$			
ZC 1, 0–5	6.64	1.03	-64.5	0.51	0.07	0.001	0.007	0.002	0.002	$6.33x10^{10}$	$1.02x10^{09}$	$1.49x10^{07}$	69.7
25–30	6.67	1.14	-62.0	0.64	0.08	0.001	0.087	0.028	0.003	$4.25x10^{10}$	$8.96x10^{08}$	$9.14x10^{06}$	98.0
50–55	6.66	1.31	-62.5	0.63	0.09	0.005	0.310	0.037	0.002	$3.40x10^{10}$	$3.97x10^{08}$	$6.85x10^{06}$	58.1
ZC 2, 0–5	6.91	1.00	-59.2	0.17	0.08	0.004	0.012	0.069	0.007	$1.43x10^{11}$	$1.14x10^{10}$	$4.35x10^{07}$	261
25–30	6.76	1.29	-51.3	0.15	0.10	0.001	0.215	0.033	0.013	$6.44x10^{10}$	$1.45x10^{09}$	$2.34x10^{07}$	61.8
50–55	6.64	1.52	-61.1	0.62	0.04	nd	0.410	0.054	0.003	$5.64x10^{10}$	$5.10x10^{08}$	$1.50x10^{07}$	34.0
ZC 3, 0–5	6.88	1.17	-60.5	0.50	0.10	0.001	0.073	0.074	0.032	$7.86x10^{10}$	$2.78x10^{09}$	$3.26x10^{07}$	85.7
25–30	7.04	3.39	-61.9	0.10	0.03	0.002	1.046	0.188	0.003	$5.79x10^{10}$	$7.81x10^{08}$	$1.55x10^{07}$	51.8
50–55	6.92	3.82	-68.7	0.59	0.02	nd	0.779	0.123	0.003	$3.41x10^{10}$	$2.21x10^{08}$	$5.41x10^{06}$	40.9
ZC 4, 0–5	7.3	1.06	-61.5	0.14	0.12	0.010	0.013	0.024	0.035	$7.19x10^{10}$	$1.28x10^{09}$	$6.53x10^{07}$	19.6
25–30	7.13	1.58	-65.1	0.12	0.11	0.002	0.301	0.049	0.002	$7.19x10^{10}$	nd	$4.60x10^{07}$	-
50–55	6.89	1.51	-67.6	0.17	0.11	0.002	0.366	0.048	0.002	$5.42x10^{10}$	$9.47x10^{08}$	$4.50x10^{07}$	21.0
ZC 5, 0–5	6.81	0.83	-63.7	0.57	0.01	0.002	0.005	0.035	0.005	$8.73x10^{10}$	$8.73x10^{08}$	$4.97x10^{07}$	17.6
25–30	6.72	0.86	-63.5	0.53	0.06	0.002	0.139	0.043	0.001	$8.94x10^{10}$	$5.21x10^{08}$	$5.57x10^{07}$	93.4
50–55	6.58	1.00	-63.8	0.37	0.06	0.002	0.275	0.045	0.002	$8.00x10^{10}$	$2.14x10^{08}$	$1.44x10^{08}$	14.9