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

Xi Wen[1*], Viktoria Unger[2*], Gerald Jurasinski[2], Franziska Koebsch[2], Fabian Horn[1], Gregor Rehder[3], Torsten Sachs[4], Dominik Zak[5,6], Gunnar Lischeid[7,8], Klaus-Holger Knorr[9], Michael Böttcher[10], Matthias Winkel[1], and Susanne Liebner[1,11].

[1]Section 5.3 Geomicrobiology, GFZ German Research Centre for Geosciences, Helmholtz Centre Potsdam, Telegrafenberg, Potsdam, 14473, Germany
[2]Landscape Ecology and Site Evaluation, Faculty for Agricultural and Environmental Sciences, Rostock University, Rostock, 18059, Germany
[3]Department of Marine Chemistry, Leibniz Institute for Baltic Sea Research, Warnemünde, 18119, Germany
[4]Section 1.4 Remote Sensing, GFZ German Research Centre for Geosciences, Helmholtz Centre Potsdam, Telegrafenberg, Potsdam, 14473, Germany
[5]Department of Bioscience, Aarhus University, Silkeborg, 8600, Denmark
[6]Department of Chemical Analytics and Biogeochemistry, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, 12587, Germany
[7]Institute of Landscape Hydrology, Leibniz Center for Agricultural Landscape Research, Münchberg, 15374, Germany
[8]Institute of Earth and Environmental Science, University of Potsdam, Potsdam, 14476, Germany
[9]Institute of Landscape Ecology, University of Münster, Münster, 48149, Germany
[10]Geochemistry and Stable Isotope Biogeochemistry, Leibniz Institute for Baltic Sea Research, Warnemünde, 18119, Germany
[11]University of Potsdam, Institute of Biochemistry and Biology, Potsdam, 14469, Germany

Correspondence to: Viktoria Unger (viktoria.unger@uni-rostock.de), Franziska Koebsch (franziska.koebsch@uni-rostock.de)

*Shared first authorship – the two first authors contributed equally to preparation of this work

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

62 emission rates in association with environmental variables such as water level, plant community,
63 and aspects of site geochemistry (Abdalla et al. 2016). Site geochemistry indeed plays an important
64 role for methanogenic communities, as methanogenesis is suppressed in presence of
65 thermodynamically more favorable terminal electron acceptors (TEAs, Blodau 2011). Due to a
66 smaller pool of more favorable electron acceptors and high availability of carbon substrates,
67 organic-rich soils such as peat rapidly establish methanogenic conditions when anoxic (Segers
68 1998, Keller and Bridgham 2007, Knorr and Blodau 2009). Despite their decisive role as producers
69 (i.e. methanogens) and consumers (i.e. methanotrophs) of CH_4 (Conrad 1996), only a few studies
70 have combined a characterization of the CH_4-cycling microbial community, site geochemistry, and
71 observed patterns of CH_4 production. Existing studies have been conducted in oligotrophic and
72 mesotrophic boreal fens (e.g., Juottonen et al. 2005, Yrjälä et al. 2011, Juottonen et al. 2012),
73 alpine fens (e.g., Liebner et al. 2012, Urbanová et al. 2013, Cheema et al. 2015, Franchini et al.
74 2015), subarctic fens (Liebner et al. 2015), and incubation experiments (e.g., Jerman et al. 2009,
75 Knorr and Blodau 2009, Urbanová et al. 2011, Emsens et al. 2016). Several studies on CH_4-cycling
76 microbial communities have been conducted in minerotrophic temperate fens (e.g., Cadillo-Quiroz
77 et al. 2008, Liu et al. 2011, Sun et al. 2012, Zhou et al. 2017), but these sites were not subject to
78 drainage or rewetting. To our knowledge, only one study has directly compared *in situ* abundances
79 of methanogens and methanotrophs in drained versus rewetted fens (Juottonen et al. 2012). The
80 studied sites, however, were nutrient-poor fens with acidic conditions.
81 While studies of nutrient-poor and mesotrophic boreal fens have documented post-rewetting CH_4
82 emissions comparable to or lower than at pristine sites (Komulainen et al. 1998, Tuittila et al. 2000,
83 Juottonen et al 2012), studies of temperate nutrient-rich fens have reported post-flooding CH_4
84 emissions dramatically exceeding emissions in pristine fens (e.g., Augustin and Chojnicki 2008,
85 Hahn et al. 2015). These high emissions typically occur together with a significant dieback in
86 vegetation, a mobilization of nutrients and electron acceptors in the upper peat layer, and increased
87 availability of dissolved organic matter (Zak and Gelbrecht 2007, Hahn-Schöfl et al. 2011, Hahn

88 et al. 2015, Jurasinski et al. 2016). Vanselow-Algan et al. (2015) have shown that such high CH_4
89 fluxes may continue for decades following rewetting even in bogs. Because of their potential to
90 remain significant CH_4 sources on decadal timescales, there is an urgent need to characterize CH_4-
91 cycling microbial communities and geochemical conditions in rewetted minerotrophic fens.
92 Therefore, in this study, we examined microbial community composition and abundance in
93 relation to post-flooding geochemical conditions in two rewetted fens in northeastern Germany. In
94 both fens, CH_4 emissions increased dramatically after rewetting (Augustin and Chojnicki 2008,
95 Hahn-Schöfl et al. 2011, Hahn et al. 2015, Jurasinski et al. 2016). Average annual CH_4 emissions
96 have decreased in both fens since the initial peak (Franz et al. 2016, Jurasinski et al. 2016).
97 Nevertheless, fluxes remained higher than under pre-flooding conditions (ibid.), and higher than
98 in pristine fens (Urbanová et al. 2013, Minke et al 2016).
99 We expected patterns in microbial community composition would reflect the geochemical
100 conditions of the two sites and hypothesized a high abundance of methanogens relative to
101 methanotrophs in both fens. We also expected acetoclastic methanogens, which typically thrive in
102 nutrient-rich fens (Kelly et al. 1992, Galand 2005), to dominate the methanogenic community in
103 both fens.
104

105 **2 Methods**

106 **2.1 Study sites**

107 The nature reserve "Heiligensee and Hütelmoor" ('Hütelmoor' in the following, approx. 540 ha,
108 54°12'36.66" N, 12°10'34.28" E), is a coastal, mainly minerotrophic fen complex in Mecklenburg-
109 Vorpommern (NE Germany) that is separated from the Baltic Sea by a narrow (~100 m and less)
110 dune dike (Fig. 1a and b). The climate is temperate in the transition zone between maritime and
111 continental with an average annual temperature of 9.1 °C and an average annual precipitation of
112 645 mm (data derived from grid product of the German Weather Service, reference climate period:

113 1981–2010). Episodic flooding from storm events delivers sediment and brackish water to the site
114 (Weisner and Schernewski 2013). The vegetation is a mixture of salt-tolerant macrophytes, with
115 dominant to semi-dominant stands of *Phragmites australis*, *Bolboschoenus maritimus*, *Carex*
116 *acutiformis*, and *Schoenoplectus tabernaemontani.* The dominating plants are interspersed with
117 open water bodies that are colonized by *Ceratophyllum demersum* in summer (Koch et al. 2017).
118 Intense draining and land amelioration practices began in the 1970s, which lowered the water level
119 to 1.6 m below ground surface and caused aerobic decomposition and concomitant degradation of
120 the peat (Voigtländer et al. 1996). The upper peat layer varies in depth between 0.6 and 3 m and
121 is highly degraded, reaching up to H10 on the von Post humification scale (Hahn et al. 2015).
122 Active draining ended in 1992, but dry conditions during summertime kept the water table well
123 below ground surface (Schönfeld-Bockholt et al. 2005, Koebsch et al. 2013) until concerns of
124 prolonged aerobic peat decomposition prompted the installation of a weir in 2009 at the outflow
125 of the catchment (Weisner and Schernewski 2013). After installation of the weir, the site was fully
126 flooded year-round with an average water level of 0.6 m, and annual average CH_4 flux increased
127 ~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).
128 The study site polder Zarnekow (‘Zarnekow’ in the following, approx. 500 ha, 53°52'31.10" N,
129 12°53'19.60" E) is situated in the valley of the River Peene in Mecklenburg-Vorpommern (NE
130 Germany, Fig. 1a and c). The climate is slightly more continental compared to the Hütelmoor, with
131 a mean annual precipitation of 544 mm and a mean annual temperature of 8.7 °C (German Weather
132 Service, meteorological station Teterow, 24 km southwest of the study site; reference period 1981–
133 2010). The fen can be classified as a river valley mire system consisting of spring mires, wider
134 percolation mires, and flood mires along the River Peene. Drainage and low-intensity agricultural
135 use began in the eighteenth century when land-use changed to pastures and grassland. This was
136 intensified by active pumping in the mid-1970s. Due to land subsidence of several decimeters,
137 after rewetting (October 2004) water table depth increased to 0.1–0.5 m above peat surface. The
138 upper horizon is highly decomposed (0–0.3 m), followed by moderately decomposed peat to a

139 depth of 1 m and a deep layer of slightly decomposed peat up to a maximum depth of 10 m. The
140 open water bodies are densely colonized by *Ceratophyllum* spp. and *Typha latifolia* is the dominant
141 emergent macrophyte (Steffenhagen et al. 2012). Following flooding, CH_4 flux rates increased to
142 ~0.21 kg m^{-2} a^{-1} (Augustin and Chojnicki 2008). No pre-rewetting CH_4 flux data were available
143 for the Zarnekow site but published CH_4 flux rates of representative drained fens from the same
144 region have been shown to be negligible (Augustin et al. 1998).

145 **2.2 Collection of peat cores and porewater samples**

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

153 Pore waters in Hütelmoor were collected with a stainless-steel push-point sampler attached to a
154 plastic syringe to recover the samples from 10 cm depth intervals. Samples were immediately
155 filtered with 0.45 µm membrane disposable syringe filters. Pore waters in Zarnekow were sampled
156 with permanently installed dialysis samplers consisting of slotted polypropylene (PP) pipes
157 (length: 636 mm, ID: 34 mm) surrounded with 0.22 µm polyethersulfone membrane. The PP pipes
158 were fixed at distinct peat depths (surface level, 20 and 40 cm depth) and connected with PP tubes
159 (4x6 mm IDxAD). Water samples were drawn out from the dialysis sampler pipes with a syringe
160 through the PP tube.

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

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

186 **2.3 Gene amplification and phylogenetic analysis**

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

191 archaeal 16S rRNA genes was performed using the primer combination of S-D-Bact-0341-b-S-
192 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-
193 A-20 (Takai and Horikoshi 2000), respectively. The PCR mix contained 1x PCR buffer (Tris•Cl,
194 KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7) (QIAGEN, Hilden, Germany), 0.5 µM of each primer
195 (Biomers, Ulm, Germany), 0.2 mM of each deoxynucleoside (Thermo Fisher Scientific,
196 Darmstadt, Germany) and 0.025 U μl^{-1} hot start polymerase (QIAGEN, Hilden, Germany). PCR
197 samples were kept at 95 °C for 5 min to denature the DNA, with amplification proceeding for 40
198 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
199 was added to ensure complete amplification. PCR products were purified with a Hi Yield Gel/PCR
200 DNA fragment extraction kit (Süd-Laborbedarf, Gauting, Germany). PCR products of three
201 individual runs per sample were combined. PCR products of different samples were pooled in
202 equimolar concentrations and compressed to a final volume of 10 µl with a concentration of 200
203 ng μl^{-1} in a vacuum centrifuge Concentrator Plus (Eppendorf, Hamburg, Germany).
204 Illumina sequencing was performed by GATC Biotech AG using 300 bp paired-end mode and a
205 20% PhiX Control v3 library to counteract the effects of low-diversity sequence libraries. Raw
206 data was demultiplexed using an own script based on CutAdapt (Martin 2011). Ambiguous
207 nucleotides at sequence ends were trimmed and a 10% mismatch was allowed for primer
208 identification, whereas barcode sequences needed to be present without any mismatches and with
209 a minimum Phred-Score of Q25 for each nucleotide. After sorting, overlapping paired-end reads
210 were merged using PEAR [Q25, p 0.0001, v20] (Zhang et al. 2014). The orientation of the merged
211 sequences was standardized according to the barcode information obtained from demultiplexing.
212 Low-quality reads were removed using Trimmomatic [SE, LEADING Q25, TRAILING Q25,
213 SLIDINGWINDOW 5:25; MINLEN 200] (Bolger et al. 2014). Chimeric sequences were removed
214 using USEARCH 6.1 and the QIIME-script identify_chimeric_seqs.py (Caporaso et al. 2010). Pre-
215 processed sequences were taxonomically assigned to operational taxonomic units (OTUs) at a
216 nucleotide sequence identity of 97% using QIIME's pick_open_reference_otus.py script and the

217 GreenGenes database 13.05 (McDonald et al. 2012) as reference. The taxonomic assignment of
218 representative sequences was further checked for correct taxonomical classification by
219 phylogenetic tree calculations in the ARB environment referenced against the SILVA database
220 (https://www.arb-silva.de) version 119 (Quast et al. 2013). The resulting OTU table was filtered
221 for singletons, OTUs assigned to chloroplasts or mitochondria, and for low-abundance OTUs
222 (below 0.2% within each sample). Archaeal and bacterial samples were processed separately while
223 only OTUs that were assigned to the respective domain were considered for further analysis. The
224 16S rRNA gene sequence data have been deposited at NCBI under the Bioproject PRJNA356778.
225 Hütelmoor sequence read archive accession numbers are SRR5118134-SRR5118155 for bacterial
226 and SRR5119428-SRR5119449 for archaeal sequences, respectively. Zarnekow accession
227 numbers are SRR6854018-SRR6854033 and SRR6854205-SRR6854220 for bacterial and
228 archaeal sequences, respectively.

229 **2.4 qPCR analysis**

230 Quantitative polymerase chain reaction (qPCR) for the determination of methanotrophic and
231 methanogenic functional gene copy numbers and overall bacterial 16S rRNA gene copy numbers
232 was performed via SybrGreen assays on a Bio-Rad CFX instrument (Bio-Rad, Munich, Germany)
233 with slight modifications after Liebner et al. (2015). The functional methanotrophic *pmoA* gene
234 was amplified with the primer combination A189F/Mb661 (Kolb et al. 2003) suitable for detecting
235 all aerobic methanotrophic Proteobacteria. Annealing was done at 55 °C after a 7-cycle-step
236 touchdown starting at 62 °C. The functional methanogenic *mcrA* gene was amplified with the
237 *mlas/mcrA*-rev primer pair (Steinberg and Regan 2009) with annealing at 57 °C. The bacterial 16S
238 rRNA gene was quantified with the primers Eub341F/Eub534R according to Degelmann et al.
239 (2010) with annealing at 58 °C. Different DNA template concentrations were tested prior to the
240 qPCR runs to determine optimal template concentration without inhibitions through co-extracts.
241 The 25 µl reactions contained 12.5 µl of iTaq universal Sybr Green supermix (Bio-Rad, Munich,
242 Germany), 0.25 µM concentrations of the primers, and 5 µl of DNA template. Data acquisition

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

249 **2.5 Data visualization and statistical analysis**

250 All data visualization and statistical analysis were done in R (R Core Team). The taxonomic
251 relative abundances across samples were visualized through bubble plots with the R package
252 ggplot2 (Wickham 2009). Differences in microbial community composition were visualized with
253 2-dimensional non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances. The
254 NMDS ordinations were constructed using R package vegan (Oksanen et al. 2017). An
255 environmental fit was performed on the ordinations to determine the measured geochemical
256 parameters that may influence community composition. The geochemical data were fitted to the
257 ordinations as vectors with a significance of $p < 0.05$. Depth profiles were constructed with the
258 porewater geochemical data, as well as with the microbial abundances, to elucidate depthwise
259 trends and assess whether differences in microbial community and abundances among the two fens
260 are related to differences in their respective geochemistry.
261

262 **3 Results**

263 **3.1 Community composition of bacteria and archaea**

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

269 Zarnekow. Within Proteobacteria, the alpha subdivision was the most dominant group, having
270 contributed 26.7% to all the libraries on average (Fig. 3). The family *Hyphomicrobiaceae*
271 dominated the Alphaproteobacteria, and was distributed evenly across samples, but missing in the
272 surface and bottom peat layers in Hütelmoor core (HC) 2. In addition, methanotrophs were clearly
273 in low abundance across all samples. Of the few methanotrophs that were detected, type II
274 methanotrophs (mainly *Methylocystaceae*) outcompeted type I methanotrophs (mainly
275 *Methylococcaceae*) in the community, while members of the genus *Methylocella* were absent (Fig.
276 3).

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

292 **3.2 Environmental characteristics and site geochemistry**

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

295 than three times higher in Hütelmoor than in Zarnekow, averaging 5.3 and 1.5 mS cm^{-1},
296 respectively. Mean pH was approximately neutral (6.5 to 7) in the upper peat profile and
297 comparable in both fens until a depth of about 30 cm where pH was ~6 in the Hütelmoor.
298 Concentrations of the TEAs nitrate and sulfate were lower in Zarnekow and near zero in the pore
299 water at all depths, while nitrate and sulfate were abundant in the upper and lower peat profile in
300 Hütelmoor at ~1.5 to 3.0 mM and ~4 to 20 mM, respectively (Fig. 5). Iron concentrations were
301 higher in the Hütelmoor pore water, while manganese concentrations were higher in Zarnekow
302 pore water. Dissolved oxygen concentrations in the upper peat profile (i.e. 0 to 25 cm depths) were
303 much higher in Hütelmoor than in Zarnekow (Fig. 5). Here DO concentrations averaged ~0.250
304 mM until a depth of 15 cm at which they dropped sharply, reaching concentrations slightly below
305 0.050 mM at 25 cm. In Zarnekow, DO concentrations did not exceed 0.1 mM and varied little with
306 depth. Regarding geochemical conditions, HC 1 was distinct from all other Hütelmoor cores and
307 more similar to Zarnekow cores. In HC 1 – the core taken nearest to potential freshwater sources
308 (Fig. 1b) – pore water EC and DO concentrations were lower while pH was slightly higher than
309 all other Hütelmoor cores. Moreover, this was the only Hütelmoor core where nitrate
310 concentrations were undetectable (Fig. 5). Dissolved CH_4 concentrations were high, varied within
311 and among fens and were slightly higher in Zarnekow pore water. Stable isotope ratios of $\partial^{13}C$-
312 CH_4 (Fig. 5) in the upper peat (approx. −59‰) suggest a predominance of acetoclastic
313 methanogenesis, with a shift to hydrogenotrophic methanogenesis around −65‰ in the lower peat
314 profile. Also, shifts toward less negative $\partial^{13}C$-CH_4 values in the upper peat layer, as in HC 1 and
315 HC 2, could indicate partial oxidation of CH_4 occurred (Chasar et al. 2000).

316 **3.3 Environmental drivers of microbial community composition**

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

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

328 **3.4 Total microbial and functional gene abundances**

329 Quantitative PCR results show that in both fens, *mcrA* abundance is up to two orders of magnitude
330 greater than *pmoA* abundance (Fig. 7, Tables 1 and 2). Gene copy numbers of *mcrA* are overall
331 higher and spatially more stable in Zarnekow than in Hütelmoor. Total microbial abundance
332 declined with depth more strongly in Hütelmoor than in Zarnekow (Fig. 7). There was a
333 pronounced decrease in microbial abundances at 20 cm depth in the Hütelmoor. For example, 16S
334 rRNA gene and *pmoA* gene copy numbers in deeper samples (below 20 cm depth) are one order
335 of magnitude lower than in upper samples on average, while the *mcrA* gene abundance are
336 approximately two orders of magnitude lower. Hütelmoor samples also exhibited larger
337 heterogeneity in terms of abundances than Zarnekow samples.
338

339 **4 Discussion**

340 **4.1 Fen geochemistry and relations to microbial community composition**

341 The rewetting of drained fens promotes elevated CH_4 production and emission, which can
342 potentially offset carbon sink benefits. Very few studies have attempted to link microbial
343 community dynamics and site geochemistry with observed patterns in CH_4 production and/or
344 emission in rewetted fens while such data are crucial for predicting long-term changes to CH_4
345 cycling (Galand et al. 2002, Yrjälä et al. 2011, Juottonen et al. 2012). In this study, we show that
346 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

373 methanogenesis using nitrate as an electron acceptor (Haroon et al. 2013). However, ANME-2D
374 has also been implicated in the iron-mediated anaerobic oxidation of methane (Ettwig et al. 2016),
375 and the HC 1 site showed slightly higher total iron concentrations. The relevance of ANME-2D as
376 CH_4 oxidizers in terrestrial habitats is still not clear. Rewetting converts the fens into widely
377 anaerobic conditions, thus providing conditions suitable for the establishment of anaerobic
378 oxidation of methane, but this has yet to be demonstrated in fens. The patchy occurrence and
379 locally high abundance of ANME-2D both in Hütelmoor and in Zarnekow suggests an ecological
380 relevance of this group. Shifts toward a less negative $\delta^{13}C$-CH_4 signature in the upper peat profile,
381 especially in HC 1 where ANME-2D was abundant, may indicate partial oxidation occurred, but
382 we could only speculate whether or not they are actively involved in CH_4 oxidation.
383 Although TEA input may be higher in the Hütelmoor, here, methanogenic conditions also
384 predominate. This finding contrasts the measured oxygen concentrations in the upper peat profile,
385 however seasonal analysis of oxygen concentrations in both sites suggests highly fluctuating
386 oxygen regimes both spatially and temporary (data not shown). Such non-uniform distribution of
387 redox processes has already been described elsewhere, in particular for methanogenesis (Hoehler
388 et al. 2001, Knorr et al. 2009). It is possible that oxygen levels in both fens are highly dynamic
389 allowing for both aerobic and anaerobic carbon turnover processes. Further, oxygen may not
390 necessarily be available within aggregates in which anaerobic pathways predominate. Anaerobic
391 conditions are also reflected by the extensive and stable occurrence of the strictly anaerobic
392 syntrophs (e.g., *Syntrophobacteraceae*, *Syntrophaceae*) in most samples, even in the top
393 centimeters. This suggests that syntrophic degradation of organic material is taking place in the
394 uppermost layer and the fermented substances are easily available for methanogens. Recent studies
395 from wetlands also show that methanogenesis can occur in aerobic layers, driven mainly by
396 Methanosaeta (Narrowe et al. 2017, Wagner 2017), which were detected in a high abundance in
397 this study (Fig. 4). As geochemistry and microbial community composition differ among the sites
398 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

425 dieback has been observed along with strong changes in surface peat geochemistry (Hahn-Schöfl
426 et al. 2011, Hahn et al. 2015). The anoxic conditions at the peat surface caused by inundation may
427 have disturbed existing methanotrophic niches, and further, hindered the establishment of new
428 ones, as oxygen availability is the most important factor governing the activity of most
429 methanotrophs (Le Mer and Roger 2001, Hernandez et al. 2015).
430 Comparable studies have so far been conducted in nutrient-poor or mesotrophic fens where post-
431 rewetting CH_4 emissions, though higher than pre-rewetting, did not exceed those of similar pristine
432 sites (e.g., Yrjälä et al. 2011, Juottonen et al. 2005, Juottonen et al. 2012). Nevertheless, there is
433 mounting evidence linking CH_4-cycling microbe abundances to CH_4 dynamics in rewetted fens.
434 Juottonen et al. (2012), for example, compared *pmoA* gene abundances in three natural and three
435 rewetted fens and found them to be lower in rewetted sites. The same study also measured a lower
436 abundance of *mcrA* genes in rewetted sites, which was attributed to a lack of available labile carbon
437 compounds. In peatlands, and especially fens, litter and root exudates from vascular plants can
438 stimulate CH_4 emissions (Megonigal et al. 2005, Bridgham et al. 2013, Agethen and Knorr 2018),
439 and excess labile substrate has been proposed as one reason for dramatic increases in CH_4
440 emissions in rewetted fens (Hahn-Schöfl et al. 2011). Future studies should compare pre- and post-
441 rewetting microbial abundances along with changes in CH_4 emissions, plant communities, and
442 peat geochemistry to better assess the effect rewetting has on the CH_4-cycling microbial
443 community.
444

445 **5 Conclusion**

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

451 lower CH_4 emission rates, we found that pristine wetlands generally have a higher abundance of
452 methanotrophs than measured in the fens in this study, suggesting the inundation and associated
453 anoxia caused by flooding disturbs methanotrophic niches and may negatively affect the ability of
454 methanotrophic communities to establish. The abundances of methane producers and consumers
455 are thus suggested as important drivers for continued elevated CH_4 emissions following the
456 rewetting of drained fens. Our results suggest that in the context of CH_4 cycling, rewetting drained
457 peatlands by flooding may be problematic if post-rewetting conditions hinder methanotroph
458 establishment. Management decisions regarding rewetting processes should consider that
459 disturbances to methanotrophic niches is possible if rewetting leads to long-term inundation of the
460 peat surface.
461
462 **Competing interests**
463 The authors declare that they have no conflict of interest.
464
465 **6 Acknowledgements**
466 This study was conducted within the framework of the Research Training Group 'Baltic
467 TRANSCOAST' funded by the DFG (Deutsche Forschungsgemeinschaft) under grant number
468 GRK 2000. This is Baltic TRANSCOAST publication no. GRK2000/000X. The financial support
469 to Xi Wen (Grant No. 201408620031 to X.W.) provided by the China Scholarship Council (CSC)
470 is gratefully acknowledged. This study was supported by the Helmholtz Gemeinschaft (HGF) by
471 funding the Helmholtz Young Investigators Group of S.L. (VH-NG-919) and T.S. (Grant VH-NG-
472 821), a Helmholtz Postdoc Programme grant to F.K. (Grant PD-129), and further supported by the
473 Terrestrial Environmental Observatories (TERENO) Network. The Leibniz Institute for Baltic Sea
474 Research (IOW) is also acknowledged for funding the lab work in this study. The European Social
475 Fund (ESF) and the Ministry of Education, Science and Culture of Mecklenburg-Western
476 Pomerania funded this work within the scope of the project WETSCAPES (ESF/14-BM-A55-

0030/16). Dr. Matthias Gehre, head of the Laboratory of Stable Isotopes at the Helmholtz Centre for Environmental Research, is acknowledged for measuring carbon isotopes of methane from Hütelmoor samples. Anke Saborowski and Anne Köhler are also acknowledged for support in the laboratory.

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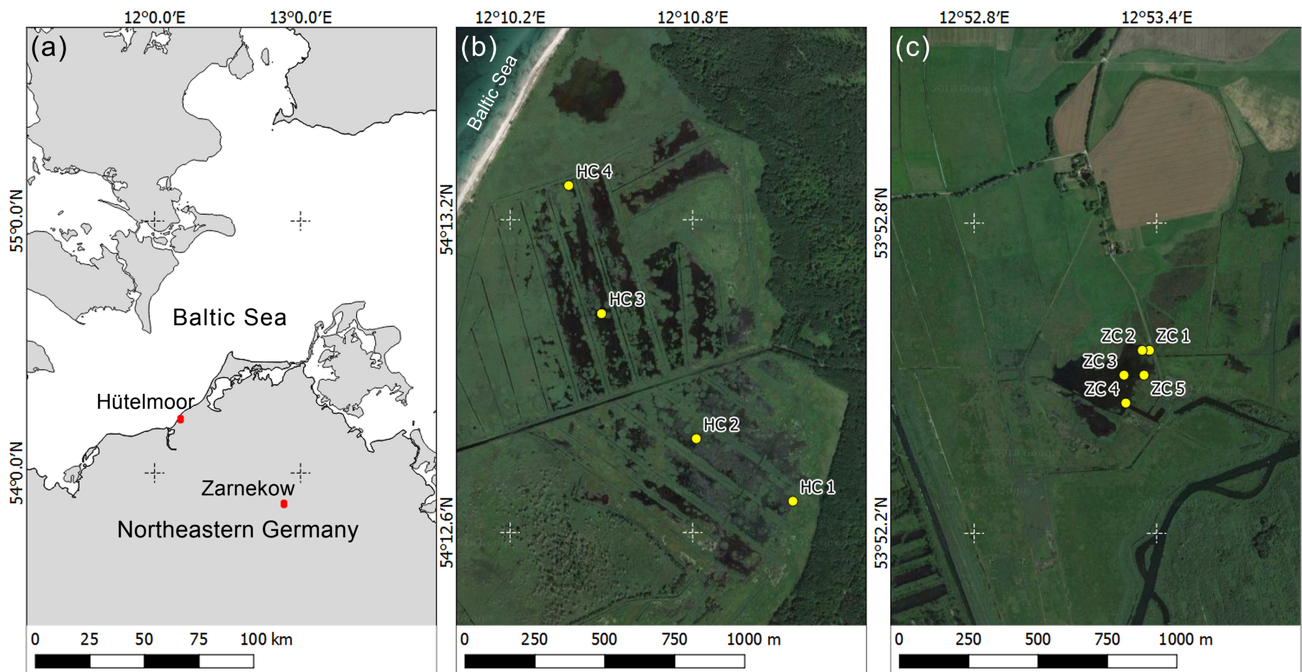

841

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
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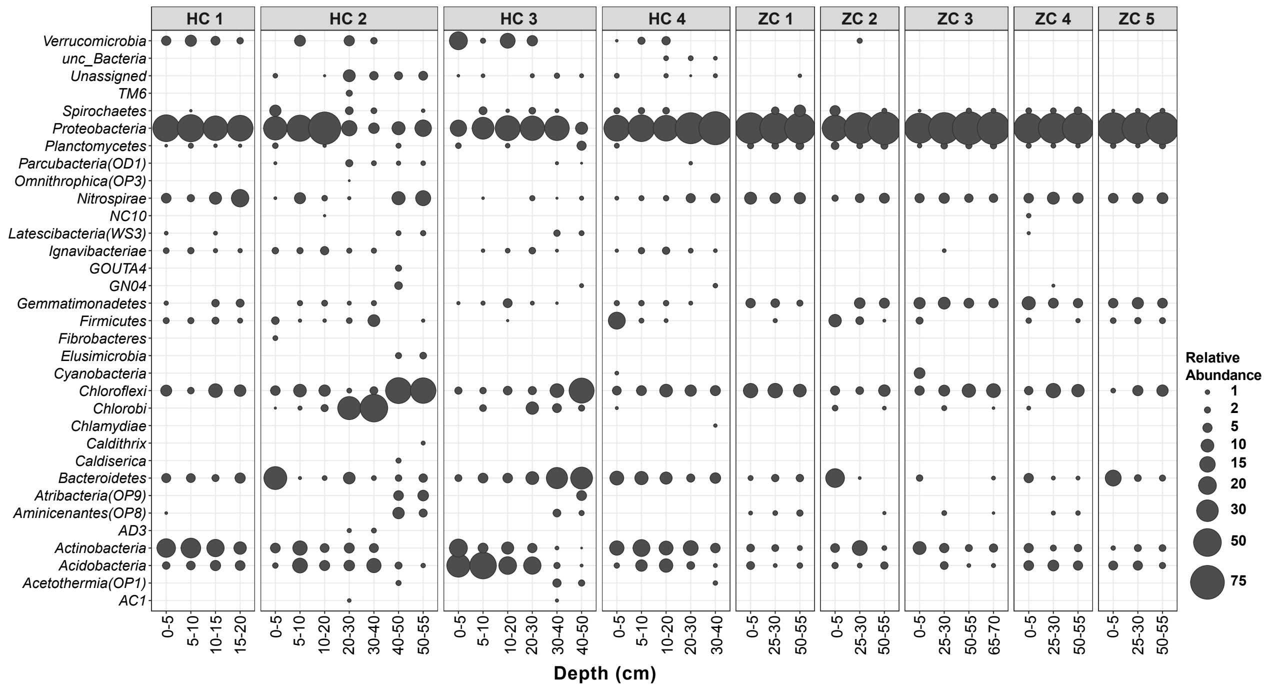
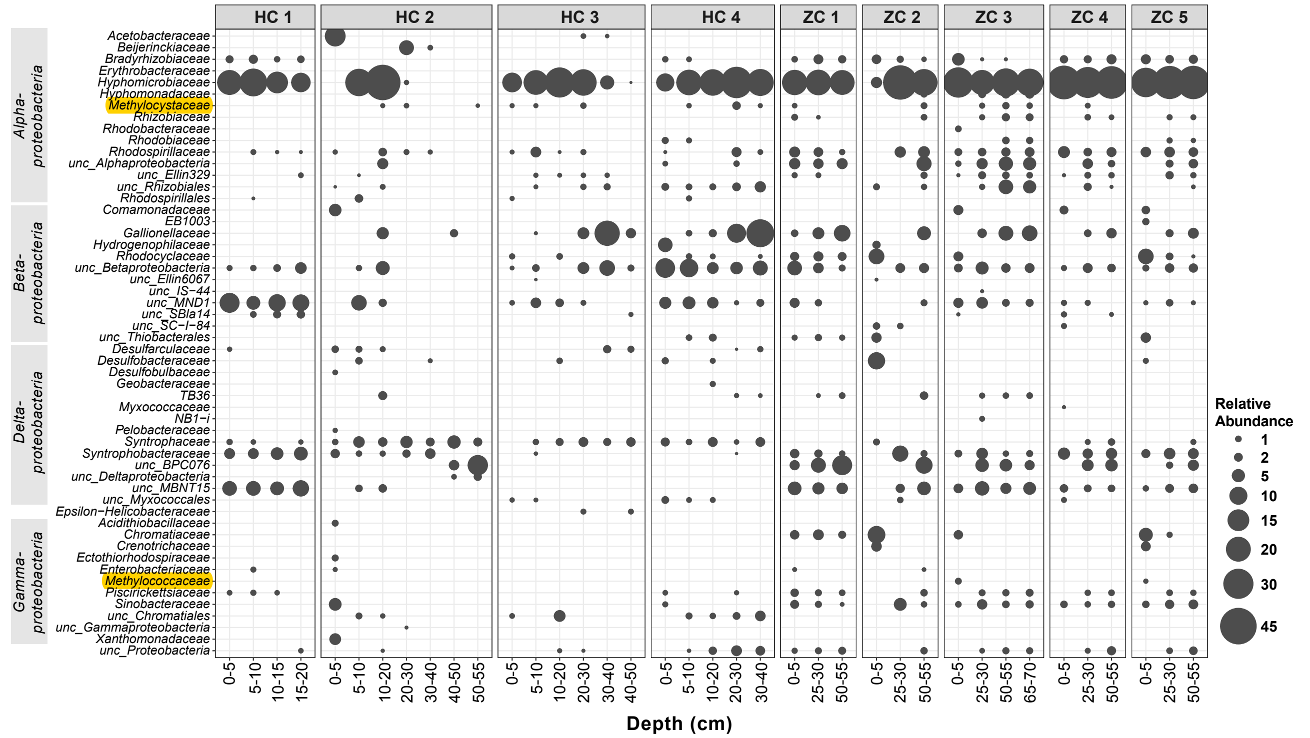


Figure 2: Relative abundances of different bacterial lineages in the study sites. Along the horizontal axis samples are arranged according to site and depth. The rank order along the vertical axis is shown for the phylum level.



849
850 **Figure 3:** Relative abundances of Proteobacteria phyla in the study sites. Along the horizontal axis samples are arranged according
851 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
852 possible the next higher assignable taxonomic level was used.
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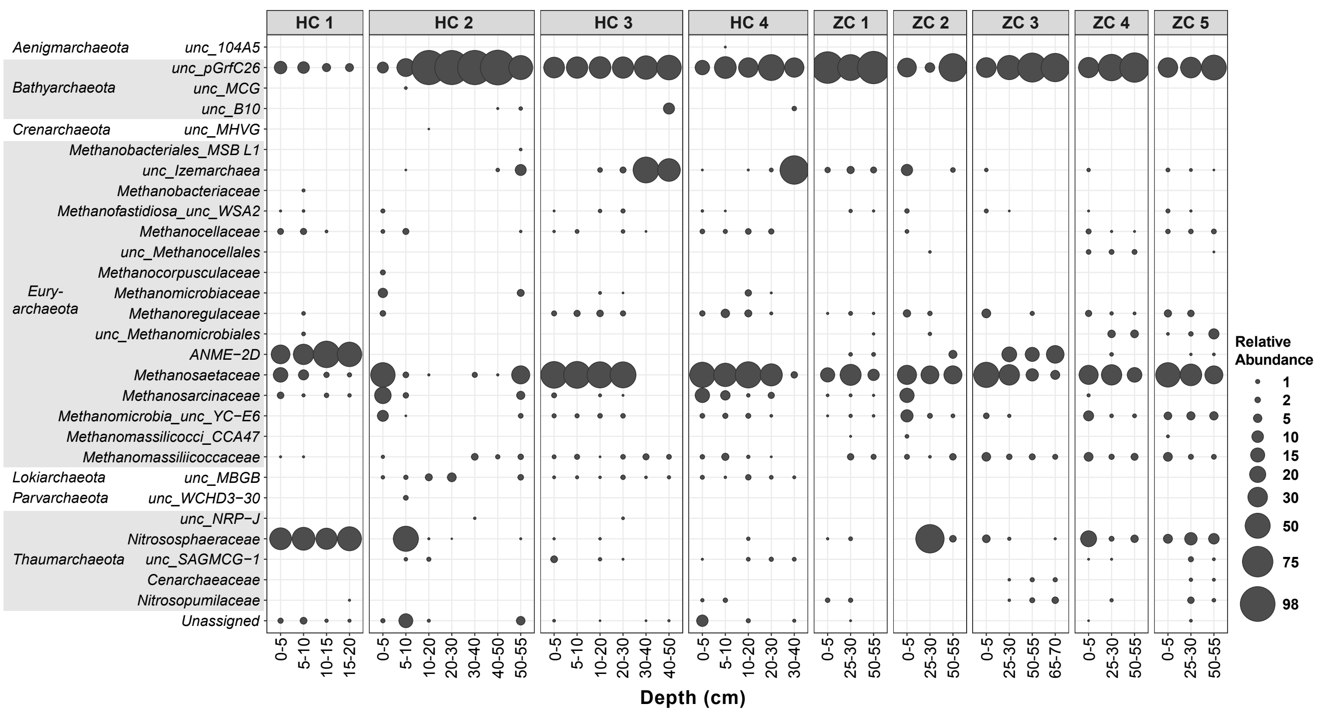

855
856 **Figure 4:** Relative abundances of different archaeal lineages in the study sites. Along the horizontal axis samples are arranged
857 according to site and depth. The rank order along the vertical axis is shown for the family level. If an assignment to the family level
858 was not possible, the next higher assignable taxonomic level was used.
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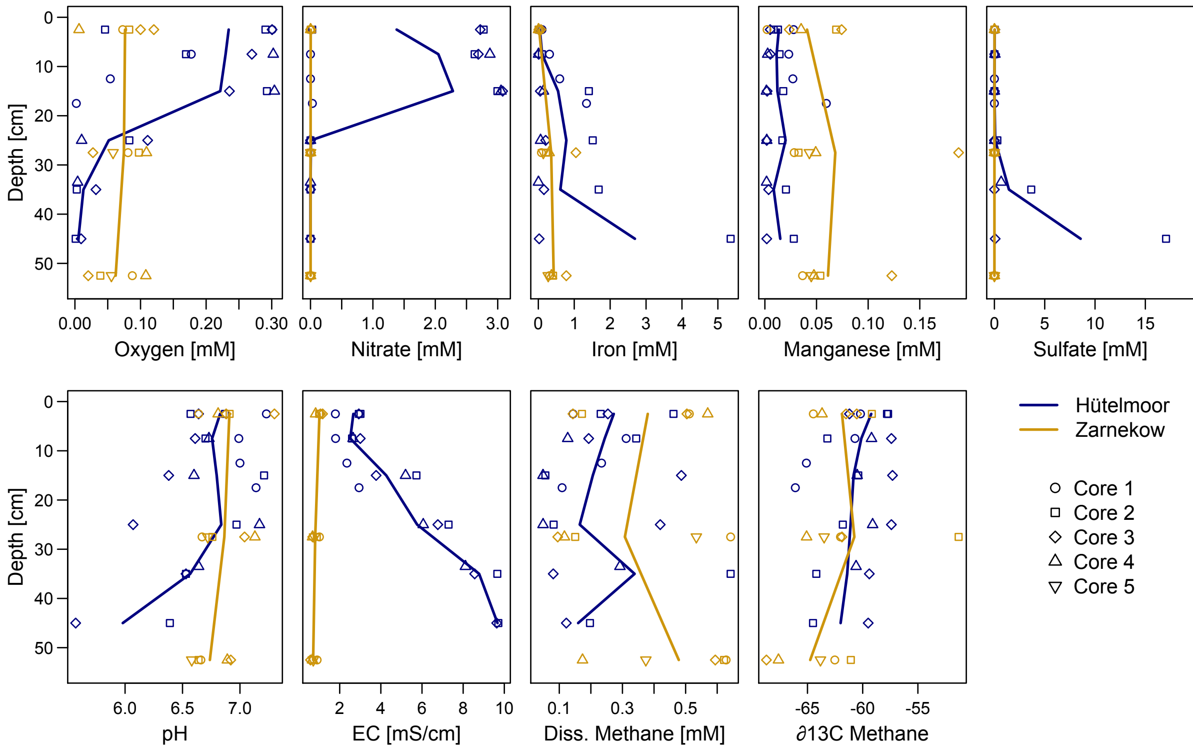

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863 **Figure 5:** Depth profiles of porewater geochemistry (see x-axis labels for considered variables) in both study sites. Lines connect
864 the respective means.
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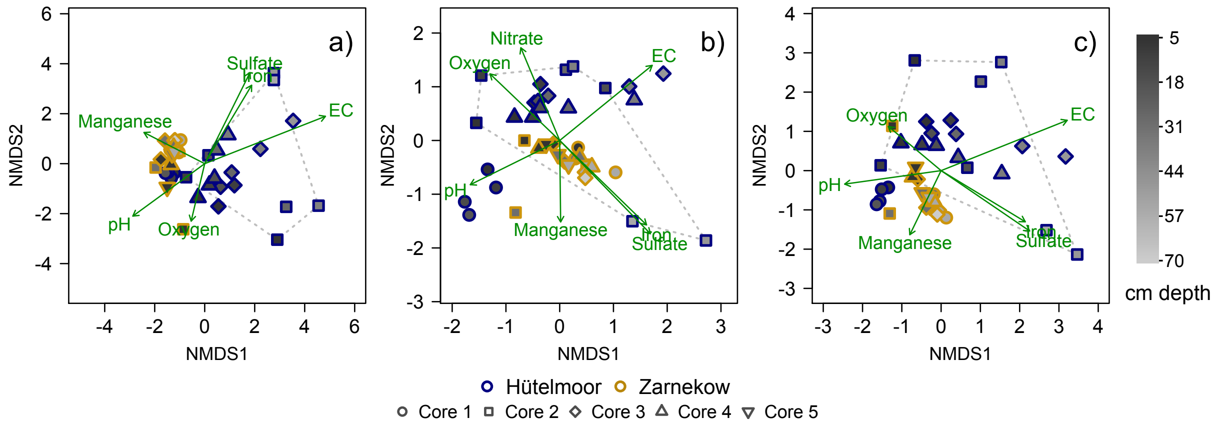

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868 **Figure 6:** NMDS plots showing (a) bacterial, (b) archaeal, and (c) microbial (bacterial plus archaeal) community composition
869 across the nine peat cores and their respective depth sections. The point positions represent distinct microbial 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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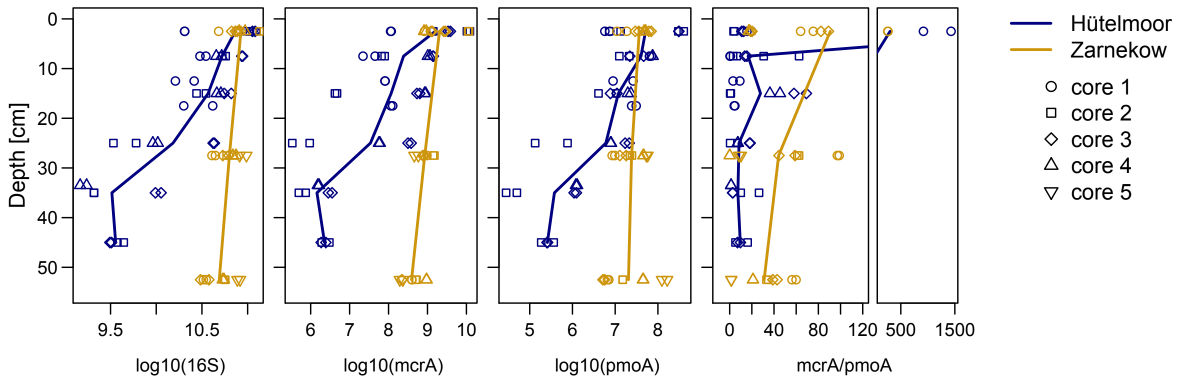

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879 **Figure 7:** Depth distribution of qPCR abundances for total microbial (16S), methanogen (*mcrA*), methanotroph (*pmoA*), and ratio
880 of *mcrA* to *pmoA* gene copy numbers in both sites. Microbial abundances were designated as numbers of gene copies per gram of
881 dry peat soil and are shown against sampling depth using log-transformed values. Solid lines indicate mean abundances. Note that
882 the plot at the right was split into two plots to capture very high *mcrA*/*pmoA* ratios in the upper peat layer.
883

Table 1: Environmental conditions, geochemical conditions, and microbial abundances in peat cores from the Hütelmoor, a coastal 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 ($\partial^{13}C–CH_4$), 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	$\partial^{13}C–CH_4$	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	2.04×10^{10}	1.15×10^{08}	6.60×10^{06}	17.7
5–10	7.0	1.80	-60.7	0.31	0.18	nd	0.31	0.02	0.01	3.25×10^{10}	3.36×10^{07}	6.68×10^{07}	0.51
10–15	7.0	2.35	-65.1	0.23	0.05	nd	0.60	0.03	nd	2.11×10^{10}	8.12×10^{07}	1.76×10^{07}	6.12
15–20	7.1	2.94	-66.1	0.11	nd	0.03	1.34	0.06	nd	3.08×10^{10}	1.21×10^{08}	2.76×10^{07}	4.41
HC 2, 0–5	6.9	3.01	-57.8	0.46	0.05	0.03	0.03	0.01	nd	1.10×10^{11}	1.13×10^{10}	1.03×10^{07}	1,170
5–10	6.7	2.60	-63.2	0.34	0.17	2.63	0.10	0.01	0.01	5.51×10^{10}	7.27×10^{07}	1.69×10^{07}	4.73
10–20	7.2	5.73	-60.4	0.06	0.29	3.00	1.41	0.02	nd	3.13×10^{10}	4.47×10^{06}	7.32×10^{06}	0.74
20–30	7.0	7.29	-61.8	0.08	0.08	nd	1.51	0.02	0.29	4.71×10^{09}	6.41×10^{05}	4.50×10^{05}	3.75
30–40	6.5	9.66	-64.2	0.64	nd	nd	1.68	0.02	3.66	2.09×10^{09}	6.21×10^{05}	3.90×10^{04}	18.3
40–50	6.4	9.71	-64.5	0.20	nd	nd	5.35	0.03	17.1	4.09×10^{09}	2.47×10^{06}	2.75×10^{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	1.10×10^{11}	1.34×10^{09}	3.51×10^{08}	3.86
5–10	6.6	3.00	-57.4	0.19	0.27	2.69	0.01	0.01	0.03	8.72×10^{10}	1.40×10^{09}	3.42×10^{07}	46.6
10–20	6.4	3.77	-57.3	0.49	0.24	3.08	0.05	nd	nd	6.08×10^{10}	5.86×10^{08}	9.35×10^{06}	63.6
20–30	6.1	6.77	-57.4	0.42	0.11	nd	0.20	nd	nd	4.26×10^{10}	3.48×10^{08}	1.92×10^{07}	18.2
30–40	6.5	8.56	-59.4	0.08	0.03	nd	0.16	nd	nd	1.05×10^{10}	3.20×10^{06}	1.17×10^{06}	2.74
40–50	5.6	9.36	-59.5	0.12	0.01	nd	0.02	nd	0.08	3.18×10^{09}	2.16×10^{06}	2.58×10^{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	1.17×10^{11}	3.63×10^{09}	3.09×10^{08}	11.7
5–10	6.7	2.65	-59.2	0.13	0.30	2.87	0.01	nd	0.05	4.87×10^{10}	1.09×10^{09}	7.51×10^{07}	14.5
10–20	6.6	5.20	-60.5	0.05	0.30	3.05	0.14	nd	nd	4.85×10^{10}	8.71×10^{08}	2.15×10^{07}	40.8
20–30	7.2	6.06	-59.1	0.05	0.01	nd	0.06	nd	0.02	9.78×10^{09}	5.82×10^{07}	7.91×10^{06}	7.36
30–40	6.6	8.11	-60.6	0.29	nd	nd	0.09	nd	0.67	1.60×10^{09}	1.58×10^{06}	1.25×10^{06}	1.27

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 ($\partial^{13}C–CH_4$), 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**	**$\partial^{13}C–CH_4$**	**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 $peat^{-1}$			
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