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1 **Distinct anaerobic bacterial consumers of cellobiose-derived carbon in boreal fens**

2 **differing in CH_4 vs. CO_2 production ratio**

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11

12 Running title: Anaerobic cellobiose consumers in boreal fens

13

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19 **Abstract**

20 Northern peatlands in general have high methane (CH_4) emissions, but individual peatlands show
21 considerable variation as CH_4 sources. Particularly in nutrient poor peatlands, CH_4 production can be
22 low and exceeded by carbon dioxide (CO_2) production from unresolved anaerobic processes. To clarify
23 the role anaerobic bacterial degraders play in this variation, we compared consumers of cellobiose-
24 derived carbon in two fens differing in nutrient status and the ratio of CH_4 and CO_2 produced. After
25 [^{13}C]cellobiose amendment, the mesotrophic fen produced equal amounts of CH_4 and CO_2. The
26 oligotrophic fen had lower CH_4 production but produced 3-59 times more CO_2 than CH_4. RNA stable
27 isotope probing revealed that in the mesotrophic fen with higher CH_4 production cellobiose-derived
28 carbon was mainly assimilated by various recognized fermenters of *Firmicutes* and by *Proteobacteria*.
29 The oligotrophic peat with excess CO_2 production revealed a wider variety of cellobiose-C consumers
30 including *Firmicutes* and *Proteobacteria* but additionally more unconventional degraders, such as
31 *Telmatobacter*-related *Acidobacteria* and subphylum 3 of *Verrucomicrobia*. Prominent and potentially
32 fermentative *Planctomycetes* and *Chloroflexi* did not appear to process cellobiose-C. Our results show
33 that anaerobic degradation resulting in different levels of CH_4 production can involve distinct sets of
34 bacterial degraders. By distinguishing cellobiose degraders from the total community, this study
35 contributes to defining anaerobic bacteria that process cellulose-derived carbon in peat. Several of the
36 identified degraders, particularly fermenters and potential Fe(III) or humic substance reducers in the
37 oligotrophic peat, represent promising candidates for resolving the origin of excess CO_2 production in
38 peatlands.

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39 **Importance**

40 Peatlands are major sources of the greenhouse gas methane (CH_4), yet in many peatlands CO_2
41 production from unresolved anaerobic processes exceeds CH_4 production. Anaerobic degradation
42 produces the precursors of CH_4 production but also represents competing processes. We show that
43 anaerobic degradation leading to high or low CH_4 production involved distinct sets of bacteria. Well-
44 known fermenters dominated in a peatland with high CH_4 production, while novel and unconventional
45 degraders could be identified in a site where CO_2 production greatly exceeds CH_4 production. Our
46 results help identify and assign functions to uncharacterized bacteria that promote or inhibit CH_4
47 production and reveal bacteria potentially producing the excess CO_2 in acidic peat. This study
48 contributes to understanding the microbiological basis for different levels of CH_4 emission from
49 peatlands.

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50 ## Introduction

51 Methane (CH_4) emission rates from peatlands, which together with other wetlands form the largest
52 natural source of CH_4 (1), vary considerably with water table level, temperature, vegetation, and
53 peatland type (2, 3). These factors have been shown to affect the activity and community composition
54 of CH_4-producing archaea and in some cases the pathway of CH_4 production (4-11). It is much less
55 clear how the environmental variation of peatland ecosystems influences the anaerobic bacterial
56 degraders, which produce the precursors of methanogenesis and on the other hand compete with
57 methanogens for substrates.

58 Peat is partially decomposed plant material, the main components being cellulose,
59 hemicellulose, and recalcitrant compounds such as lignin and humic substances (12). In anoxic peat
60 below the water level, decomposition of plant-derived polysaccharides requires a cooperation of
61 functional microbial groups: primary fermenters hydrolyzing polymers and fermenting the monomers
62 such as sugars, secondary fermenters turning the resulting organic acids into the methanogenic
63 substrates acetate and H_2+CO_2, and finally methanogens producing CH_4 (13). Polysaccharide
64 hydrolysis and thus the activity of non-methanogenic anaerobic bacteria has been proposed to be the
65 rate-limiting step for wetland CH_4 production (5, 14-16).

66 The complete anaerobic degradation of carbohydrates under methanogenic conditions,
67 when both acetate and H_2+CO_2 are precursors of methanogenesis, should theoretically produce equal
68 amounts of CH_4 and CO_2 (17). In contrast, a large excess of anaerobic CO_2 production is often
69 observed in peat, particularly in low pH and nutrient-poor peatlands (18-22). These sites in particular
70 but northern peatlands in general have low levels of Fe(III), nitrate, and sulfate, which is considered to
71 limit CO_2 production resulting from the degradation of organic matter with the reduction of these
72 inorganic electron acceptors (18, 19). Small sulfate pools in peat may have unexpectedly high turnover

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73 rates (23, 24), but currently the main proposed sources for the excess CO_2 in nutrient-poor peat are
74 fermentation or anaerobic respiration with phenolic and quinone-containing compounds of organic
75 residues, commonly referred to as humic substances, as electron acceptors (13, 19, 21, 25).
76 Identification of the anaerobic bacterial degraders active in peatlands with contrasting CH_4 vs. CO_2
77 production could provide insights into the processes producing the excess CO_2.

78 The current information on peat microbes involved in the intermediary anaerobic
79 processes, leading to methanogenesis or excess CO_2 production, is still restricted to relatively few
80 studies and sites. Work on a temperate spruce fen has identified cellulose, glucose, and xylose
81 fermenters, including *Bacteroidetes*, *Acidobacteria*, *Firmicutes*, *Spirochaeta*, *Actinobacteria*, and
82 *Proteobacteria* (26-30). Metagenomic studies in a *Sphagnum* bog and in Arctic peat have indicated
83 *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Deltaproteobacteria* as potential fermenters (14, 31,
84 32). A comparison of eutrophic to oligotrophic temperate peatlands found indications of functional
85 redundancy, with similar anaerobic processes being carried out by different communities (33). This
86 raises the question on whether and how the community composition of the anaerobic bacterial
87 degraders relates to the eventual CH_4 production of a peatland.

88 We set out to compare the active anaerobic bacterial degraders in two boreal peatlands
89 differing as CH_4 sources. These adjacent *Sphagnum* fens are rather similar in vegetation composition
90 and pH but differ in nutrient status and, more importantly, in the pathway of organic matter degradation
91 and the ratio of CH_4 and CO_2 produced (21). The mesotrophic fen has higher CH_4 production, higher
92 contribution of the acetoclastic pathway of CH_4 production and a distinct methanogen community from
93 the oligotrophic fen (8, 21, 34). We targeted consumers of cellobiose-derived carbon, representing
94 bacteria processing labile carbon from cellulose degradation, by incubating peat with [^{13}C]cellobiose
95 and following the label in the produced CH_4 and CO_2 and in RNA by stable isotope probing (SIP). Our

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96 aim was to 1) identify cellobiose consumers and fermenters in boreal *Sphagnum* fens and 2) compare
97 the active anaerobic degraders in two fens differing as CH_4 sources. By selecting outwardly similar but
98 functionally contrasting sites, we aimed to differentiate between degraders active under high CH_4
99 production and under lower CH_4 production when CO_2 is the main gaseous end product.

100

101 Materials and methods

102 Study site and sampling

103 We sampled an oligotrophic fen and a mesotrophic fen within the Lakkasuo mire complex in southern
104 Finland (61°47′N, 24°18′E) (35). The sampling sites are located within 500 meters from each other.
105 Both fens are minerotrophic (i.e., in contact with groundwater) with the mesotrophic fen being more
106 nutrient-rich than the oligotrophic fen (35). Vegetation at the oligotrophic site consists mainly of sedge
107 *Carex lasiocarpa* and mosses *Sphagnum papillosum* and *S. fallax*. The main species at the mesotrophic
108 site are sedge *C. lasiocarpa* and mosses *S. flexuosum* and *S. fallax*. Peat at the sampled depths consists
109 of partially degraded plant remains with no observable mineral soil. Water table depth at the time of
110 sampling in November 2011 was at 15±2 cm below peat surface at the oligotrophic site and at 4±2 cm
111 below surface at the mesotrophic site. Peat pH was 4.8±0.1 at the oligotrophic site and 5.1±0.1 at the
112 mesotrophic site.

113 Three replicate peat cores were collected from each site by cutting out a 10 cm × 10 cm
114 core with a knife to the depth of 30 cm from peat surface. Peat cores were stored overnight at +4 °C
115 and divided into two sections, 10-20 cm and 20-30 cm from peat surface. A portion of the sections was
116 frozen for RNA analysis of the initial peat. For initial measurements of CH_4 and CO_2 production
117 potential and for further incubation for the labeling experiment, 15 ml of peat was placed in 120-ml

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118 flasks with 30 ml of H_2O previously flushed with N_2 to remove oxygen. Each sample was bottled in
119 duplicate. Flushing with N_2 was repeated, and the flasks were closed with rubber septa and incubated in
120 dark at 15 °C. Methane and CO_2 production were followed by gas chromatography (36) with four
121 samplings of the headspace gas during 191 hours. Production rates include gaseous and dissolved gases
122 and are given per grams dry weight (gdw). Ratio of peat dry weight to peat wet weight was
123 0.099±0.007.

124

125 **Incubation and sampling for stable isotope analysis**

126 The incubation for the labeling experiment started with addition of uniformly labeled [^{13}C]cellobiose
127 (Omicron Biomedicals) to one of each pair of flasks in the initial measurements for the 20-30 cm layer,
128 when peat had been in the flasks for 10 days. Before the addition, the flasks were flushed with N_2 to
129 remove accumulated gases. The flasks received 0.5 ml of 18 mM cellobiose to a final concentration of
130 0.2 mM injected through the septum. The other flask of each pair received the same amount of
131 [^{12}C]cellobiose as a control for ^{13}C enrichment. The flasks were shaken by hand and incubated in dark
132 at 15 °C. The cellobiose addition was repeated 12 times during 25 days (every second or third day).
133 Altogether, each flask received 118 µmol of cellobiose and 1.42 mmol of ^{13}C, yielding 94 µmol ^{13}C (or
134 ^{12}C for controls) per g (fresh weight) of peat. During the pulse labeling, CH_4 and CO_2 production was
135 followed with three measurements per week. The flasks were sampled for analysis of ^{13}C-CH_4, ^{13}C-
136 CO_2 and peat at 7, 14, and 28 days. Headspace gas was sampled through the septum and injected into
137 N_2-flushed 12-ml vials (Labco). After gas sampling, the flasks were opened under N_2 flow and 2 × 1.5
138 ml of peat slurry was removed and frozen at -80 °C for RNA extraction for SIP.

139

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140 **Isotope analysis of CO_2 and CH_4**

141 The $^{13}C/^{12}C$ ratio of CO_2 and CH_4 was analyzed from the 12-ml vials described above. First, 1 ml was
142 injected into a second, pre-evacuated and N_2-flushed 12-ml vial, which was analyzed for $^{13}C/^{12}C$ ratio
143 in CO_2. The sample was injected into a Trace-GC with a Precon Interface connected with a continuous-
144 flow isotope-ratio mass spectrometer (IRMS; Thermo Finnigan DELTA XP^{Plus}, Bremen, Germany).
145 Separation of CO_2 from N_2O and other gases was performed with a Pora PLOT Q column (27.5 m
146 length; 0.32 mm i.d.; Varian) at 25 °C using He as carrier gas. Laboratory standards were prepared
147 using CO_2 gas diluted in N_2 and calibrated against the international IAEA CH-6 reference material
148 (International Atomic Energy Agency, Vienna, Austria) via elemental analysis IRMS (EA-IRMS). The
149 remaining gas in the original 12-ml vials was then analyzed for $^{13}C/^{12}C$ ratio of CH_4. The same protocol
150 was used as described above with the exception that an additional, manual cold trap was cooled by
151 liquid nitrogen in the Precon unit to purge the sample gas of O_2 and N_2 while trapping CH_4 in the loop.
152 Additionally, CH_4 was oxidized to CO_2 by reaction with nickel oxide at 1000°C. The precision (one
153 standard deviation) of standard gas for 10 consecutive measurements is ~0.1‰. Isotope ratios are
154 reported in terms of ^{13}C at% values, where at%^{13}C = $^{13}C/(^{12}C+^{13}C)*100$. Natural abundance of ^{13}C was
155 taken into account by analysis of [^{12}C]cellobiose-incubated samples as controls. ^{13}C at% values were
156 used to calculate the production of ^{13}C-CH_4 and ^{13}C-CO_2 based on the overall production of CH_4 and
157 CO_2 (Table S1).

158

159 **RNA extraction**

160 The method of RNA extraction was modified from previously published methods (37-39). Frozen peat
161 (0.5 g wet weight) was added to two 2-ml tubes containing 0.5 cm of quartz sand. Both tubes received

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162 350 µl of lysis buffer (2% cetyl trimethylammonium bromide [CTAB], 2% polyvinyl pyrrolidone
163 [PVP], 100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2.0 M NaCl, 0.5 g l^{-1} spermidine, 2% β-
164 mercaptoethanol) (40) and 350 µl of phenol (pH 8). Cell lysis was carried out in FastPrep (Qbiogene,
165 Illkirch, France) with setting 5.5 m s^{-1} for 30 s. After centrifugation at 20 800 g at 4 °C for 5 min, the
166 supernatants from both tubes were combined and 700 µl of phenol-chloroform-isoamylalcohol
167 (50:49:1) was added. Samples were mixed by inverting the tube repeatedly and centrifuged as above
168 for 3 min. Chloroform-isoamyl alcohol (700 µl, 24:1) was added to the supernatant. After mixing and
169 centrifugation, the supernatant was passed through a polyvinyl polypyrrolidone (PVPP) column by
170 centrifugation at 1300 g for 3 min. The flow-through was precipitated with 1 volume of 20%
171 polyethylene glycol in 2.5 M NaCl and 20 µg of glycogen (RNA grade, ThermoScientific) on ice for
172 1.5 h. After centrifugation at 20 800 g at 4 °C for 30 min, the pellet was washed with 70% ethanol and
173 dissolved in 50 µl of diethylpyrocarbonate (DEPC)-treated H_2O. Co-extracted DNA was removed by
174 treating RNA with DNase I (Thermo Scientific). Concentration of RNA was determined with Qubit
175 RNA HS Assay Kit and Qubit fluorometer (Invitrogen).

176

177 **Density gradient centrifugation of RNA**

178 Separation of ^{13}C-labeled RNA from ^{12}C-RNA was carried out by density gradient centrifugation in
179 cesium trifluoroacetate (CsTFA) gradients (41) for RNA from day 7 and day 14. Gradient medium was
180 prepared by mixing 4.8 ml of CsTFA (2.0 mg ml^{-1}, GE Healthcare), 3.4% formamide, and 938 µl of
181 gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA) for a final volume of 6 ml. Density of
182 CsTFA and the final gradient medium were checked before use by measuring the refractory index.
183 Gradient medium and RNA sample (500 ng RNA in a volume of 54.5 µl) were loaded into 6-ml
184 polyallomer crimp tubes (Sorvall) and centrifuged at 129 000 g for 65 h in a Discovery 100 centrifuge

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185 with a TV-1665 rotor (Sorvall). Each centrifugation included two control tubes without RNA or with
186 RNA from a cultivated strain for determining the density of fractions. After centrifugation, gradients
187 were fractionated into 14 fractions of 400 µl by pumping water to the top of the tube using a Gilson
188 Minipuls 3 (speed 3, ca. 0.5 ml min^{-1}) and collecting the fractions from the bottom. Densities of the
189 fractions from the control tubes were measured by a refractometer and by weighing the fractions.

190 RNA in the fractions was precipitated with isopropanol and 20 µg of glycogen at -20 °C
191 overnight, centrifuged at 20 800 g at 4 °C for 30 min, washed with 70% ethanol, and dissolved in 15 µl
192 of DEPC-H_2O. Concentration of RNA was determined with Qubit RNA HS Assay Kit and Qubit
193 fluorometer (Invitrogen). Fractions containing ^{13}C-labeled RNA and ^{12}C- RNA were identified by
194 comparing RNA concentrations in the density profiles of [^{13}C] and [^{12}C]cellobiose-incubated samples
195 (Fig. S1). Two to three fractions were pooled to form the final heavy (^{13}C-enriched) and light (^{12}C)
196 RNA fractions of each sample.

197

198 **Reverse transcription and 16S rRNA gene amplicon sequencing**

199 The heavy and light RNA fractions and RNA from initial peat (4 µl aliquots) were reverse transcribed
200 with Maxima H Minus reverse transcriptase (200 U, Thermo Scientific) and random hexamers (Thermo
201 Scientific) following the manufacturer's instructions.

202 Bacterial 16S rRNA gene PCR for reverse transcription products was carried out as a two-step PCR,
203 where the first step primers (341f and 805r) (42) contained adapters for introducing Illumina adapters
204 and dual barcodes in the second step. The first step PCR primers were thus adapter-341f (5'-
205 ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN**CCTACGGGNGGCWGCAG**-3';
206 adapter sequence followed by primer sequence in bold) and adapter-805r (5'

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207 AGACGTGTGCTCTTCCGATCT**GACTACHVGGGTATCTAATCC**-3'). The first step PCR was
208 carried out in duplicate in 20-µl reactions containing 1 × Q5 reaction buffer, 0.2 mM dNTPs, 0.5 µM
209 primers, 0.4 U Q5 high-fidelity DNA polymerase (New England Biolabs) and 1 µl reverse transcription
210 product (diluted if necessary) as template. Cycling conditions were 98 °C for 1 min, followed by 15-20
211 cycles of 98 °C for 10 s, 62 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 2 min. The
212 duplicate products were pooled and purified with Agencourt AMPure XP purification system
213 (Beckman Coulter). The second PCR step with barcoded primers (forward
214 AATGATACGGCGACCACCGAGATCTACAC-[index]-ACACTCTTTCCCTACACGACG; reverse
215 CAAGCAGAAGACGGCATACGAGAT-[index]-
216 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT), which bind to the first-step adapters and
217 incorporate Illumina adapters, was carried out using 1 µl of the first product (dilution 1:1-1:50
218 depending on concentration) as template with 0.1 µM of primers, annealing temperature of 66 °C, and
219 12-17 cycles. Products were purified as above and quantified with PicoGreen kit (Life Technologies).
220 Products were sequenced at the SciLifeLab SNP/ SEQ sequencing facility at Uppsala University,
221 Uppsala, Sweden on Illumina MiSeq (2×300 bp).
222

223 **Sequence analysis**

224 After raw amplicon sequencing data was demultiplexed into individual samples, sequence pairs were
225 assembled using an in-house bioinformatic pipeline (43). The pipeline further removed sequences with
226 missing primers and unassigned base pairs. The quality filtering removed on 17% of reads, leaving 2.16
227 million reads of 400-430 bp and on average 41379 reads per sample. The quality filtered assembled
228 reads were clustered into operational taxonomical units (OTUs) and filtered for chimeras using
229 UPARSE (1.5 % sequence dissimilarity cutoff) (44), which resulted in 9243 OTUs. Singletons were

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230 removed. Taxonomy was assigned using CREST (45) and the ribosomal sequence database SilvaMod
231 based on the release 106 of the SILVA non-redundant SSURef database (46). The sequence data were
232 sub-sampled to 13226 reads per sample, the number of reads in the sample with the fewest reads after
233 all the filtering steps, using the command sub.sample in mothur (v. 1.35.1)(47).
234

235 **Statistical analyses**

236 We used t-tests to compare CH_4 and CO_2 production rate of the original peat of the oligotrophic and
237 mesotrophic fen (n=3). Methane production was $\log_{10}$ transformed before testing. ^{13}C-CH_4 and ^{13}C-
238 CO_2 production rates between peat types during weeks 1, 2, and 3 (n=3) were tested with repeated
239 measures analysis of variance using function lme in R package nlme (48) with week as a random
240 factor. All analyses were carried out in R v.3.2.0 (49). P values were considered statistically significant
241 at $p < 0.05$.

242 Bacterial community in ^{13}C and ^{12}C fractions at days 7 and 14 and in initial peat of the
243 fens were visualized by non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis
244 distances of OTU data subsampled to 13226 reads per sample (n=3). Differences with peat type,
245 fraction, and time point were tested with permutational multivariate analysis of variance
246 (PERMANOVA, function adonis in R, Bray-Curtis distances). Analyses were carried out in R with
247 vegan package (v. 2.0) (50).

248 The OTUs enriched in the ^{13}C fractions compared to the ^{12}C fractions were identified with
249 R package edgeR (51). Data were not subsampled for these analyses (52). Filtering was applied to keep
250 only OTUs with more than five reads in at least four samples within each comparison of 12 samples,
251 i.e., within ^{13}C and ^{12}C fractions of two time points with three replicates. The filtered data was

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252 normalized by the relative log expression (RLE) method as implemented in standard edgeR protocols.
253 We used the general linear model (glm) approach to account for the two time points of 7 and 14 days.
254 Trended dispersion was estimated with the power method and the tagwise dispersion with df=5. In
255 testing for enriched ('differentially expressed') OTUs, we accepted OTUs with $p < 0.01$, false
256 discovery rate (FDR) < 0.05 and $\log_2$ fold change (logFC) > 2 as enriched in the ^{13}C fractions. To
257 exclude OTUs migrating to the ^{13}C fraction without actual ^{13}C incorporation, we tested for OTUs
258 enriched in the fractions corresponding to the density of the ^{13}C fraction in samples incubated with
259 [^{12}C]cellobiose. Based on this test, one OTU from the mesotrophic peat was removed from the set of
260 enriched OTUs. In further analyses, the read numbers of the enriched OTUs are from the subsampled
261 data. Sequences similar to representative sequences of enriched OTUs were searched using Blast.
262
263 **Accession number**
264 Raw amplicon sequences were deposited in the GenBank sequence read archive under accession
265 number SRP075161.
266

267 Results

268 **Methane and anaerobic CO_2 production**

269 Potential CH_4 production in initial measurements before cellobiose addition was higher in peat from the
270 20-30 cm layer than in the 10-20 cm layer (data not shown). The depth of 20-30 cm was therefore
271 chosen for the labeling experiment to represent the methanogenic peat layer. Before cellobiose
272 amendment, the mesotrophic fen showed higher CH_4 production (36 ± 18 nmol grams dry weight

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273 (gdw)$^{-1}$ h^{-1}, mean ± SD) than the oligotrophic fen (1.2 ± 0.8 nmol gdw^{-1} h^{-1}; t-test p = 0.003). The rates
274 of anaerobic CO_2 production did not significantly differ between mesotrophic (116 ± 72 nmol gdw^{-1} h^{-}
275 ^{1}) and oligotrophic peat (61 ± 10 nmol gdw^{-1} h^{-1}, t-test p = 0.32). Rates of CO_2 production of the
276 original unamended peat were on average 3 times higher than CH_4 production in the mesotrophic fen
277 and 50 times higher in the oligotrophic fen.

278 After the addition of [^{13}C]cellobiose, ^{13}C-enrichment of CH_4 demonstrated consumption
279 of cellobiose with methanogenesis as a terminal process (Fig. 1A). The ^{13}C-enrichment of CH_4
280 increased from day 7 to day 28, whereas the enrichment of CO_2 remained fairly stable over time. The
281 level of enrichment did not vary with peat type (Fig. 1A). The mesotrophic peat had consistently higher
282 ^{13}C-CH_4 production, i.e., CH_4 originating from labeled cellobiose, than the oligotrophic peat (p =
283 0.0001, Fig. 1B). Methane production was highest in the first week and then decreased in both peat
284 types. Anaerobic ^{13}C-CO_2 production did not differ with peat type (p = 0.84, Fig. 1B). In both peat
285 types, CO_2 production increased after the first week and was highest in the second week. Accordingly,
286 in the first week the ratio of ^{13}C-CO_2 and ^{13}C-CH_4 production was 1.4 ± 0.3 in the mesotrophic peat
287 and 5 ± 2 in the oligotrophic peat, but in the second week the ratio increased to 7 ± 5 in the
288 mesotrophic peat and 32 ± 24 in the oligotrophic peat. At weeks 3 and 4 the ratio decreased to 14 ± 6 in
289 the oligotrophic peat and 5 ± 2 in the mesotrophic peat, still being higher than in the first week.

290

291 **Bacterial communities**

292 The bacterial community of the original peat differed between the oligotrophic and mesotrophic fen
293 (Fig. 2; PERMANOVA R^2 = 0.48, p = 0.001). The most common bacterial phyla in the oligotrophic fen
294 were Parcubacteria (Candidate division OD1, 15.4 ± 2.2% of reads), Planctomycetes (13.2 ± 3.6%),

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295 and Acidobacteria (10.2 ± 2.0%) (Table S2). In the mesotrophic fen, the groups with the highest
296 relative abundance were Deltaproteobacteria (17.6 ± 5.9%), Chloroflexi (10.9 ± 4.6%), and
297 Acidobacteria (10.8 ± 4.2%).

298 After incubation with [^{13}C]cellobiose and separation of RNA into heavy (^{13}C-enriched)
299 and light (^{12}C) fractions, OTUs differed between the fractions (PERMANOVA $R^2 = 0.34$, $p = 0.001$)
300 and between the oligotrophic and mesotrophic peat (PERMANOVA $R^2 = 0.27$, $p = 0.001$) (Fig. 2).
301 Light fractions at day 7 differed from the original peat (PERMANOVA $R^2 = 0.17$, $p = 0.007$). No
302 change was observed between day 7 and day 14 in the overall community (PERMANOVA $R^2 = 0.01$, p
303 = 0.80) or in the mesotrophic peat despite the clear decrease in CH_4 production (PERMANOVA $R^2 =$
304 0.06, p = 0.35). Unlabeled controls that received [^{12}C]cellobiose, used to identify the density gradient
305 fractions containing ^{13}C-RNA and to exclude OTUs migrating to the heavy fraction without ^{13}C
306 incorporation, grouped with the light fractions (data not shown).

307 Looking at the fold changes of the OTU abundances in the heavy and light fractions
308 revealed that *Firmicutes* and *Betaproteobacteria* were strongly represented in the heavy fractions (Fig.
309 3, Table S3). Several groups common in the original peat were primarily detected in the light fractions
310 (*Planctomycetes*, *Deltaproteobacteria, Chloroflexi*) or were very rare in incubated peat
311 (*Parcubacteria*/Candidate division OD1). To identify the most likely cellobiose-C consumers, we
312 looked in more detail for those OTUs consistently more abundant in the heavy fractions compared to
313 the light fractions by using an approach developed for detecting differential gene expression. In the
314 mesotrophic peat, we identified 70 ^{13}C-enriched OTUs, 44 of which belonged to *Firmicutes* (Table 1,
315 Table S4, Table S5). The oligotrophic peat revealed higher number and higher taxonomic variety of
316 ^{13}C-enriched OTUs than the mesotrophic peat with 118 enriched OTUs and *Acidobacteria*,
317 *Verrucomicrobia*, *Firmicutes*, *Betaproteobacteria*, and *Alphaproteobacteria* as the largest groups.

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318 Eleven OTUs in the oligotrophic peat and six OTUs in the mesotrophic peat became more enriched
319 from day 7 to day 14 (Table 1, Table S5), further supporting their ^{13}C-labeling.

320 Firmicutes classified to the families *Clostridiaceae* and *Veillonellaceae* were identified as
321 prominent cellobiose-C consumers in both peat types. In unincubated peat, *Firmicutes* formed only 0.1-
322 1.0% of the total community, but by day 7 they had increased to 21-69% in the heavy fractions (Table
323 S3). *Clostridiaceae* in the oligotrophic peat, including the two most abundant ^{13}C-enriched OTUs
324 (Otu2, Otu4), had high sequence similarities to several acid-tolerant and sugar-fermenting *Clostridium*
325 species (Table S4, S5). In the mesotrophic peat, the two most abundant *Clostridium* OTUs (Otu1,
326 Otu3) showed similarities of 99-100% to uncultured clostridia from wetland soils (26, 28, 53, 54). Both
327 oligotrophic and mesotrophic peat showed *Veillonellaceae* OTUs similar to genera *Pelosinus*,
328 *Propionispira* and *Psychrosinus* (Table S4), but several additional *Veillonellaceae* OTUs from the
329 mesotrophic peat had no close matches to described species. Only three enriched *Firmicutes* OTUs
330 were unique to the oligotrophic peat, and they were similar to "*Psychrosinus fermentas*" (Otu110,
331 1663, 5263; Table S4, S5). The mesotrophic peat, on the other hand, showed 24 unique enriched
332 *Clostridiaceae* and *Veillonellaceae* OTUs and two additional families: *Lachnospiraceae* and
333 *Ruminococcaceae*. All four *Ruminococcaceae* OTUs (Otu40, 64, 145, 316) were 94-96% similar to
334 *Ethanoligenens harbinense* and 96-98% similar to a sequence from acidic peat (HG324862, 28). The
335 closest matches to *Lachnospiraceae* (Otu119) were gut microbes.

336 The majority of the ^{13}C-enriched *Betaproteobacteria* OTUs in both peat types were
337 classified as *Neisseriales* and *Rhodocyclales*. Many of these OTUs (Table S4) showed high sequence
338 similarity to two wetland isolates: fermentative *Paludibacterium yongneupense* growing at lower pH
339 than related strains (55) and aerobic *Uliginosibacterium gangwonense* (56).

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340 Enriched *Alphaproteobacteria* OTUs belonged to *Rhodospirillales* and *Rhizobiales*. Both
341 the oligotrophic and the mesotrophic peat revealed several OTUs (Table S4) highly similar to
342 *Telmatospirillum siberiense*, an acidotolerant fermentative peat isolate (57). *Rhizobiales* OTUs were
343 mostly detected as ^{13}C-enriched in the oligotrophic peat, and most of them were similar to one of two
344 stalked fermentative strains able to use Fe(III) as an electron acceptor: *Rhizomicrobium electricum* (58;
345 Otu761, 463, 1222) and *Rhizomicrobium palustre* (59; Otu32).

346 Only two ^{13}C-enriched *Acidobacteria* OTUs, classified to *Holophagae*, were detected in
347 the mesotrophic peat (Otu63, Otu2366). In the oligotrophic peat, we identified 26 ^{13}C-enriched
348 *Acidobacteria* OTUs, and 12 of them showed 97-99% sequence similarity to cellulolytic *Telmatobacter*
349 *bradus* isolated from a bog with pH 4 and fermenting sugars including cellobiose (60) (Table S4). The
350 rest of the *Acidobacteria* OTUs belonging to classes *Acidobacteria* and *Holophagae* were highly
351 similar (99-100%) to sequences from acidic peat (26, 28, 33, 61, 62), alpine tundra soil (63), or paddy
352 soil (Table S5).

353 *Verrucomicrobia* were identified as consumers of cellobiose-derived carbon only in the
354 oligotrophic fen. Fourteen out of twenty OTUs were classified to subphylum 3 (OPB35 soil group)
355 (Table S5). These OTUs showed only ≤ 91% sequence similarity to the described members of
356 subphylum 3, which all are aerobic (64-66). In addition, OTUs identified as *Spartobacteria* (Otu470,
357 Otu1065) and *Opitutae* (Otu224, 266, 364, 613) were detected, both of which include a species
358 fermenting sugars and polysaccharides (67, 68). All *Verrucomicrobia* OTUs were most similar to
359 uncultured *Verrucomicrobia* from various soil, wetland and freshwater environments.

360 *Bacteroidetes*, previously identified as a major fermentative group in peat (28), were not
361 prominent among our ^{13}C-enriched OTUs. However, two relatively minor *Bacteroidetes* OTUs (Otu24,

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362 Otu198) in the mesotrophic peat identified as *Porphyromonadaceae* became more ^{13}C-enriched with

363 time (Table 1, Table S5).

364

365 **Discussion**

366 We compared consumers of cellobiose-derived carbon in two peat types with similar anaerobic CO_2

367 production but distinct rates of CH_4 production, suggesting differences in the processes of organic

368 matter degradation. The ratio of CO_2 to CH_4 production of close to 1 in the mesotrophic peat during the

369 first week of the incubation implies that methanogenesis was the dominant terminal process. The

370 considerably higher CO_2/CH_4 ratios in the oligotrophic peat throughout the incubation, ranging from 2

371 to 59, indicate that carbon was also directed to non-methanogenic processes. Our CO_2/CH_4 ratios of

372 both original and cellobiose-amended peat were higher than those of Galand et al. (21) for the same

373 sites but support the same pattern of lower contribution of methanogenesis as a terminal process in the

374 oligotrophic peat.

375 A clear separation of the bacterial community composition with peat type persisted with

376 cellobiose amendment, and the results showed distinct cellobiose-C consuming taxa with peat type and

377 a higher variety of taxa in the oligotrophic peat. These included *Acidobacteria* and *Verrucomicrobia*,

378 which were present in the mesotrophic peat at similar or higher relative abundances as in the

379 oligotrophic peat but were not as heavily labeled (Table S2, Table S3), suggesting they did not

380 assimilate cellobiose-derived carbon to the same extent as in the oligotrophic peat. *Verrucomicrobia*

381 have been associated with plant polymer degradation in soils including anoxic peat (32, 69) and in the

382 case of *Opitutaceae* with glucose fermentation in peat (33). Subphylum 3, the most abundant

383 verrucomicrobial group detected here as cellobiose ^{13}C-consumers, occurs in soil and peat (70-72) but

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384 contains no previously reported fermenters. According to recent metagenomic data, some members of
385 this group possess genes for polysaccharide hydrolysis (73). Our results show that subphylum 3
386 contains members able to assimilate cellobiose carbon under anoxic, oligotrophic conditions, extending
387 the physiologies for subphylum 3 and revealing potential novel peatland fermenters.

388 *Acidobacteria* are common in anoxic peat and more prominent with lower pH and
389 oligotrophy (4, 33, 34, 74-77). Accordingly, they were more prominent in processing cellobiose-C in
390 the oligotrophic peat than in the mesotrophic peat. *Acidobacteria* closely related to *Telmatobacter*
391 *bradus*, detected as ^{13}C-enriched exclusively in the oligotrophic peat, are emerging as important
392 anaerobic and aerobic degraders of cellulose-derived carbon in peatlands with pH < 5 (28, 78).
393 Additionally, other *Acidobacteriaceae* and *Holophagaceae* appeared to participate in anaerobic carbon
394 processing, but the roles of these taxa are more difficult to determine. Known *Holophagaceae* have
395 diverse physiologies, but members of this group also assimilated ^{13}C from cellulose in acidic peat (28).

396 Firmicutes assimilating ^{13}C were particularly prominent and varied in the mesotrophic
397 peat, and those found in the oligotrophic peat were mostly a subset of this larger variety. Nutrient status
398 has been previously shown to affect the distribution of *Clostridia* in a freshwater marsh between
399 eutrophic and oligotrophic soils (53). The two *Firmicutes* families detected as ^{13}C consumers only in
400 the mesotrophic peat, *Ruminococcacae* and *Lachnospiraceae*, are best known to inhabit digestive tracts
401 of mammals (79). Interestingly, the occurrence of *Ruminococcaceae* and *Lachnospiraceae* in sheep
402 rumen was associated with high CH_4 emission (80), fitting their detection in the mesotrophic peat with
403 higher CH_4 production. *Ruminococcaceae* have been indicated as fermenters in peat more acidic than
404 our sites (28, 33), suggesting that the lack of detection in the oligotrophic peat may not be related to the
405 slightly lower pH. *Lachnospiraceae* have been detected in degradation of rice straw (81) but not

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406 reported as peatland fermenters previously. Both *Lachnospiraceae* and *Ruminococcaceae* have,
407 however, been suggested to be well-suited for plant material degradation (82).

408 Our ^{13}C-enriched *Firmicutes* OTUs closely matched sequences from other peatlands and
409 acid-tolerant wetland isolates. These included *Clostridiaceae*, which are well-known saccharolytic and
410 cellulolytic fermenters and have been suggested to represent important fermenters in acidic peat (26,
411 28, 33, 52). Another major group of *Firmicutes* were *Veillonellaceae*, which have Gram-negative cell
412 walls and have so far been rarely detected in peat or soil habitats, although they have been actively
413 involved in rice straw degradation (81) and detected as minor peat taxa (33, 83). *Veillonellaceae* may
414 play a role as propionate-producing fermenters in peat: Several of our OTUs were similar to
415 propionate-producing genera *Pelosinus*, *Psychrosinus*, and *Propionispira* (Table S4), and
416 *Propionispira*-related glucose fermenters were previously detected in acidic peat under propionate-
417 producing conditions (26). *Veillonellaceae* also include homoacetogens producing acetate from H_2 and
418 CO_2, but none of our OTUs were similar to known acetogenic strains.

419 The large amount of ^{13}C-CO_2 detected throughout the incubation in the oligotrophic peat
420 could be the result of incomplete degradation or degraders using humic substances (HS) or inorganic
421 compounds as electron acceptors. Incomplete degradation, where electron donors and acceptors are
422 organic substances and which results in build-up of fermentation products instead of CH_4 production, is
423 one of the main proposed sources for the excess CO_2 production in peat (13, 21). The various
424 potentially fermentative groups that assimilated ^{13}C support incomplete degradation as a relevant
425 source. The role of HS reduction (84) is more difficult to evaluate, as HS reducers are poorly known
426 and include Fe(III)-reducing, sulfate reducing and fermentative bacteria (85-88). The labeled taxa in
427 the oligotrophic peat included *Holophagaceae* and *Anaeromyxobacter* (*Deltaproteobacteria*) with
428 members able to reduce HS analogue anthraquinone-2,6-disulfonate (AQDS; 89). None of the labeled

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429 taxa resembled known sulfate reducers but instead contained several potential Fe(III) reducers:
430 *Rhizomicrobium*, *Clostridia* related to *C. saccharobutylicum*, and *Acidobacteria* of groups 1 and 3 (90-
431 92). Fermentative Fe(III) reducers have been shown to occur in an acidic fen (93). The putative Fe(III)
432 reducers could also be speculated to reduce HS, although most have not been tested for it, and a group
433 3 acidobacterium *Paludibaculum fermentas* does not reduce AQDS (94). Whether the groups discussed
434 above are involved in HS or Fe(III) reduction and resposible for the excess CO_2 production should be
435 addressed in future studies. Other ^{13}C-CO_2 sources that cannot be ruled out are ^{13}C-CO_2 production by
436 microbes not assimilating ^{13}C, production by organisms other than bacteria such as non-methanogenic
437 archaea, or anaerobic CH_4 oxidation (95). Despite attempts, anaerobic CH_4 oxidation has not been
438 conclusively verified in this site (K. Peltoniemi, personal communication).

439 In the mesotrophic peat, CH_4 production decreased and CO_2 production increased
440 drastically after the first week, but we observed no shift in the bacterial community. The additional ^{13}C-
441 CO_2 was therefore possibly produced by *Firmicutes* and *Proteobacteria* that assimilated ^{13}C under
442 methanogenic conditions and remained active under lower CH_4 production. The decrease in CH_4
443 production may be due to inhibition of methanogenic activity by accumulation of fermentation
444 products such as organic acids, as has been observed in other peat incubations (28, 96). The incubated
445 mesotrophic peat revealead notable amounts of deltaproteobacterial Fe(III) and sulfate reducers, such
446 as *Geobacteraceae* (2-4% vs. < 1% in oligotrophic peat), *Syntrophaceae* (3-5% vs. 1-3%),
447 *Syntrophobacteraceae* (0.9-1.3% vs. 0.4-0.7%), and *Desulfobacteraceae* (0.1-0.5% vs. < 0.1%). These
448 taxa were, however, not enriched in the ^{13}C-fractions and thus were most likely not producing the
449 additional ^{13}C-CO_2 from [^{13}C]cellobiose, but they may be contributing to CO_2 production overall from
450 endogenous substrates.

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451 To avoid known sources of bias in SIP experiments, we added the ^{13}C-substrate as
452 repeated smaller pulses instead of a large single pulse and used RNA-SIP instead of DNA-SIP to allow
453 shorter incubation times. To exclude OTUs enriched in the heavy fraction for reasons other than ^{13}C-
454 labeling such as high GC percentage, we sequenced the fractions corresponding to the heavy fractions
455 in the unlabeled samples and removed such OTUs. Another source of bias, cross-feeding, should have
456 been reduced as the incubation flasks were flushed during the weekly sampling. Despite removal of the
457 gases, cross-feeding could still take place via organic acids. Therefore the detected labeled taxa may
458 include both primary fermenters, using cellobiose and glucose, and secondary fermenters consuming
459 the products of primary fermenters such as organic acids. Because cellobiose-C consumers were
460 identified based on enrichment in the heavy fractions compared to the light fractions, our analysis
461 focuses on the most strongly ^{13}C-labeled taxa. Therefore the analysis would miss OTUs assimilating
462 both ^{13}C-carbon and endogenous unlabeled carbon, thus appearing in the heavy fractions but in
463 addition strongly or predominantly in the light fractions.

464 Several potentially fermentative groups abundant in the original peat were not labeled
465 with ^{13}C from cellobiose. *Planctomycetes*, largely related to *Isosphaera*, became the most abundant
466 group in oligotrophic peat with cellobiose amendment, but only one rare OTU was identified as ^{13}C-
467 enriched. *Planctomycetes* have been described as aerobic, rarely fermentative carbohydrate degraders
468 in soil and peat (73, 97, 98) and proposed to be glucose fermenters in peat (33). Our results match those
469 of Schmidt et al. (28), where *Planctomycetes* were not labeled from ^{13}C-cellulose. Similarly,
470 *Chloroflexi* classified as *Anaerolineae* and *Caldilineae*, which consist of filamentous anaerobes that
471 ferment sugars and were enriched with glucose in peat (33, 99), were mostly detected in the light
472 fractions of the mesotrophic peat. Strong occurrence in the light fractions suggests that *Planctomycetes*
473 and *Chloroflexi* were active but largely not assimilating cellobiose-derived carbon. *Parcubacteria*

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474 (Candidate division OD1), which have been proposed to be fermentative (100, 101), were abundant in
475 the original oligotrophic peat but were rare and unlabeled after the incubation. Members of this group
476 were suggested to be symbiotic (102), which could explain why they thrive in natural but not in
477 incubated peat.

478 To conclude, we showed that anaerobic degradation in peat, exemplified by cellobiose-C
479 consumers, can involve a clearly distinct set of bacteria depending on the amount of CH_4 produced.
480 With higher CH_4 production in the mesotrophic peat, cellobiose-derived carbon was mainly processed
481 by well-known fermenters within *Firmicutes* and by *Proteobacteria*. When CO_2 production by far
482 exceeded CH_4 production in the oligotrophic peat, more unconventional degraders or fermenters such
483 as *Telmatobacter*-related *Acidobacteria* and subphylum 3 of *Verrucomicrobia* were prevalent. The
484 groups we detected as ^{13}C-labeled in the oligotrophic peat, particularly those with known potential for
485 Fe(III) or HS reduction, such as *Acidobacteria*, *Holophagaceae*, *Deltaproteobacteria*, *Rhizobiales*, and
486 *Clostridiaceae*, should be further addressed in future studies to resolve the processes and electron
487 acceptors behind the anaerobic CO_2 production in acidic peatlands. By differentiating anaerobic
488 bacteria active in cellobiose processing from the rest of the community, this study contributes to
489 assigning functions to uncharacterized bacteria in peat and to understanding the microbiological basis
490 of differing levels of CH_4 production in peatlands.

491

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499

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Table 1. Numbers of bacterial 16S rRNA gene operational taxonomic units (OTUs, relative abundance categories of >2.5%, 0.25-2.5%, and 0.025-0.25% of reads) identified as enriched in the ^{13}C fractions of ^{13}C-cellobiose labelled peat from oligotrophic and mesotrophic fen.

Classification of OTUs		oligotrophic[a]			mesotrophic[a]			shared
		>2.5%	>0.25%	>0.025%	>2.5%	>0.25%	>0.025%	
Firmicutes	*Clostridiaceae*	3	2^{1}	3^{1}	8	7^{1}	2	
	Lachnospiraceae					1		
	Ruminococcaceae					3	1	13
	Veillonellaceae		7^{3}	2^{2}	1	9^{2}	7	
	unknown family					2	2	
Betaproteobacteria	*Burkholderiales*		1					
	Neisseriales	3^{1}	1	4	2		2	5
	Rhodocyclales	3	2	2	2	1	3	
	Myxococcales			1				
Alphaproteobacteria	*Rhizobiales*	1	3^{1}	3			1	5
	Rhodospirillales		4	3	1	3	3	
Deltaproteobacteria	*Anaeromyxobacter*			1				0
Gammaproteobacteria	*Tolumonas*		1		2			1
Acidobacteria	*Acidobacteriaceae*	1	13	4				
	Holophagaceae		3^{1}	3		1	1^{1}	2
	group 3		2					
Verrucomicrobia	subphylum 3		3	11				
	Spartobacteria		1	1				0
	Opitutae		2	2				
Spirochaetes	*Spirochaetales*		5	5			1	0
Bacteroidetes	*Bacteroidales*			2		1^{1}	1^{1}	
	Sphingobacteriales						1	0
	other/unknown		2	3				
Candidate division BRC1			1					0
Actinobacteria	*Coriobacteriia*		1^{1}					0
Armatimonadetes	group 1			2				0
Planctomycetes	*Phycisphaerae*			1				0
Chlorobi	*Ignavibacteria*			1				0
Melainabacteria							1	0

[a]The superscripts indicate the number of OTUs that became more ^{13}C-enriched from day 7 to day 14.

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781 **Figure legends**

782 **Fig. 1**. A) ^{13}C-enrichment of CH_4 and CO_2 and B) ^{13}C-CH_4 and ^{13}C-CO_2 production rates during
783 labeling experiment with [^{13}C]cellobiose in oligotrophic and mesotrophic peat at three time points
784 (n=3, mean± SD).

785

786 **Fig. 2.** Non-metric multidimensional scaling (NMDS) plot of bacterial community based on 16S rRNA
787 gene sequencing in original peat and in the heavy (^{13}C) and light (^{12}C) stable isotope fractions of
788 oligotrophic and mesotrophic peat. Stress = 0.084.

789

790 **Fig. 3.** Log_2 fold changes of read abundances showing the taxonomic distribution of bacterial
791 operational taxonomic units (OTUs, 150 most abundant) in heavy (^{13}C) and light (^{12}C) fractions at A)
792 day 7 and B) day 14. Reads more abundant in mesotrophic peat, x axis < 0; in oligotrophic peat, x axis
793 > 0; in ^{13}C fractions, y axis > 0; in ^{12}C fractions, y axis < 0. Solid grey line separates OTUs at least 2-
794 fold more abundant than in the other peat type or fraction. Fold changes were calculated from means of
795 three replicate incubations using data subsampled to 13226 reads for each sample. Symbol size is based
796 on the read number of an OTU over all the samples at each time point.

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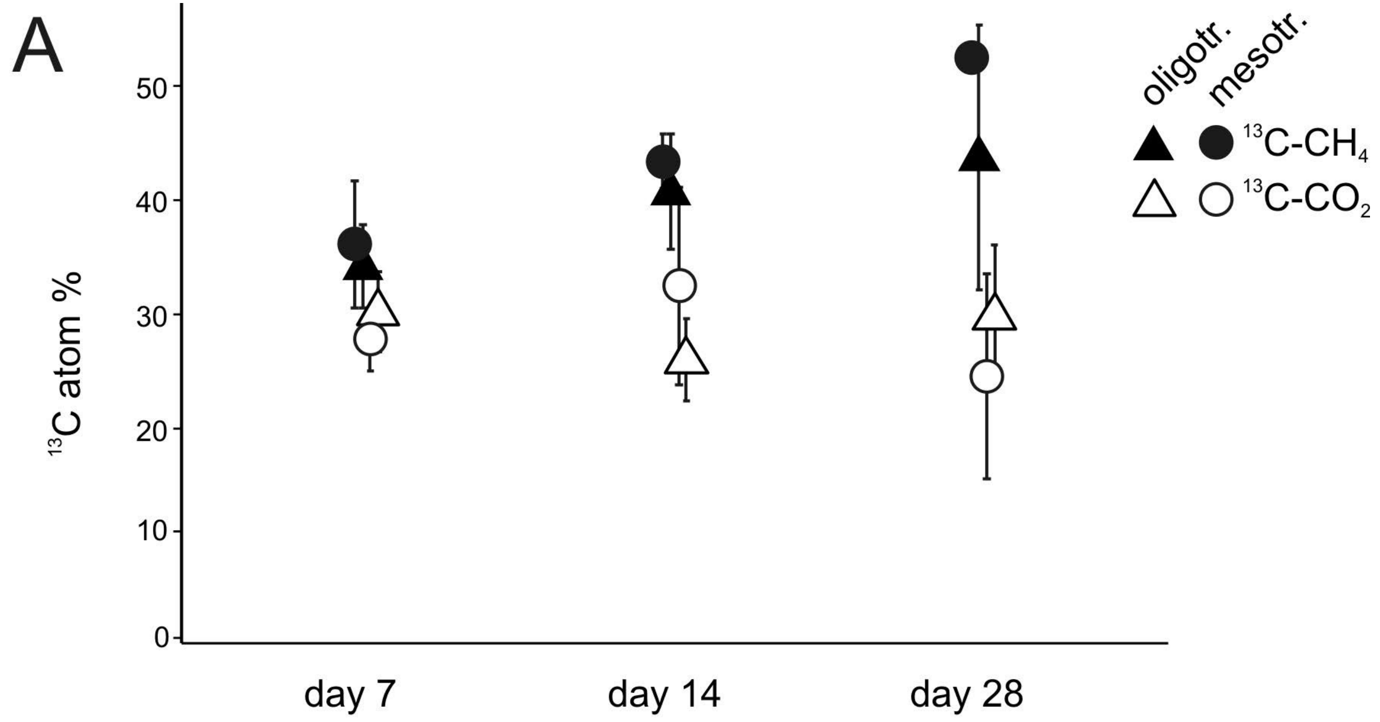

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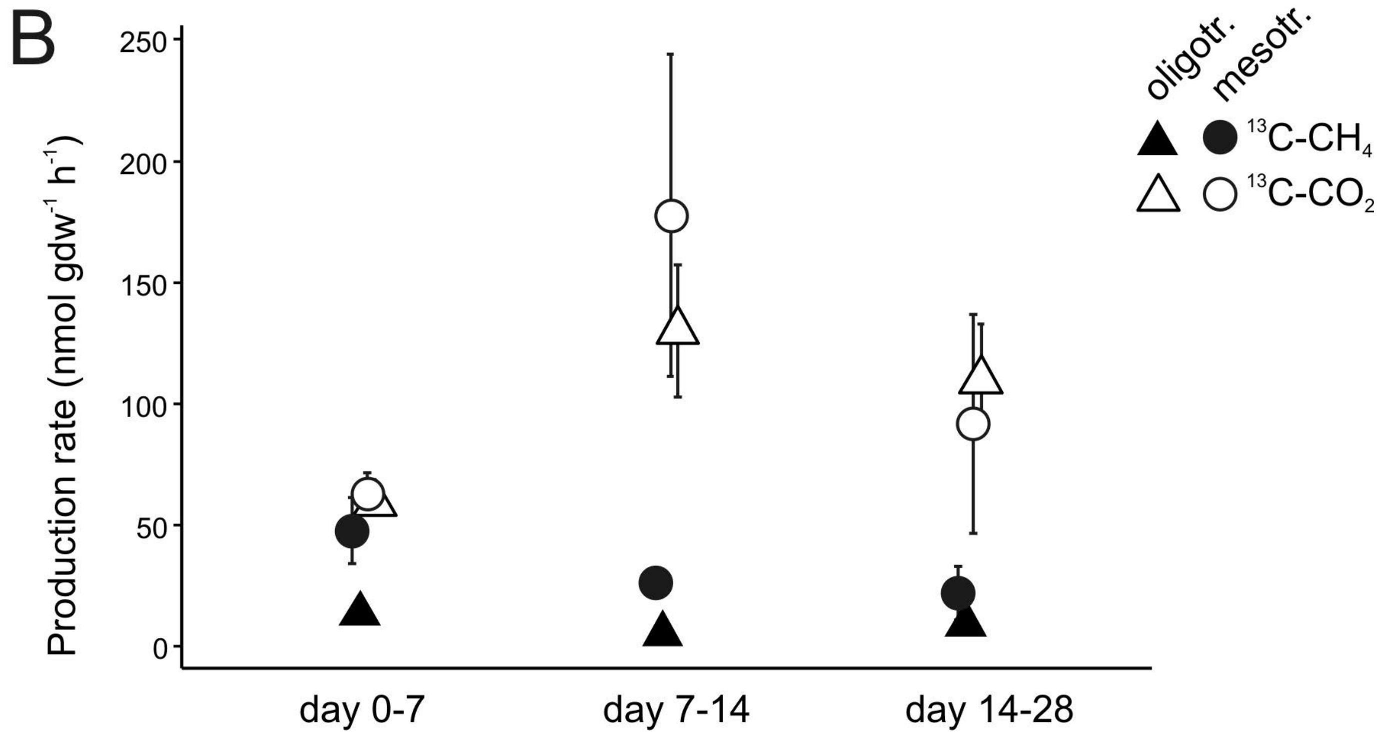

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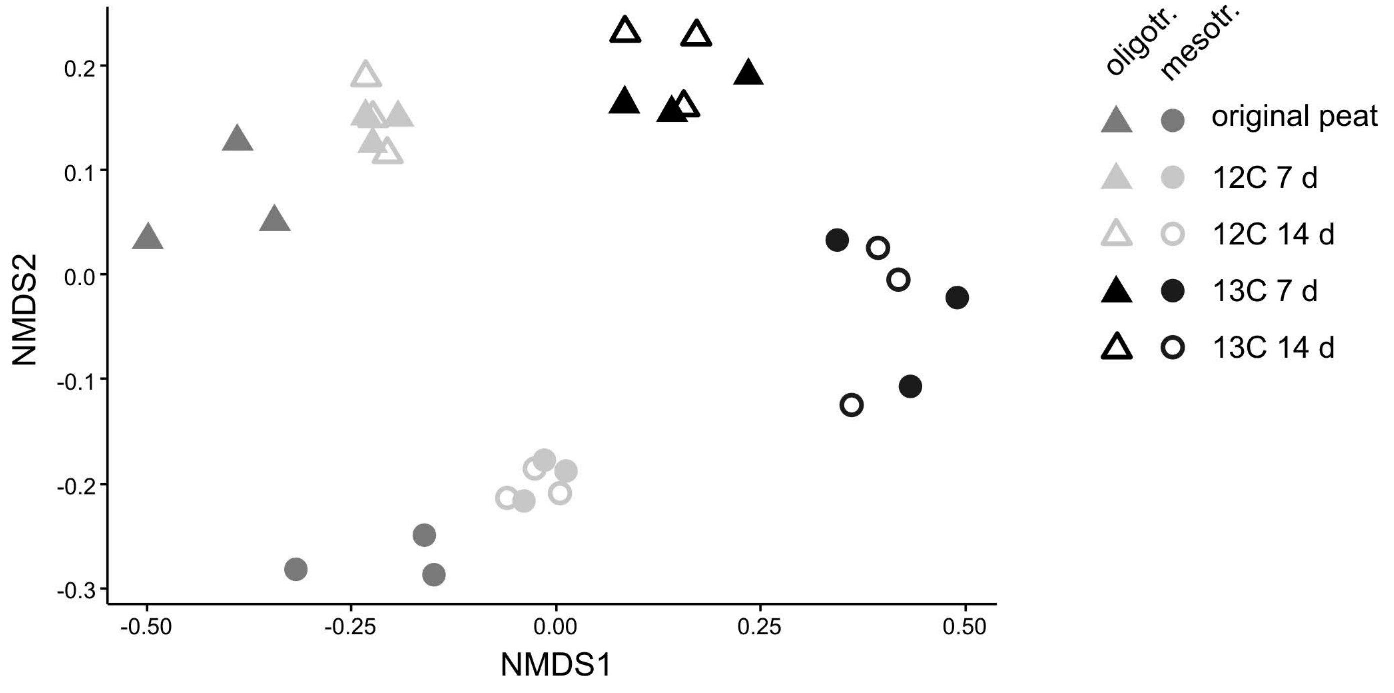

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