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1 **A novel mesocosm set-up reveals strong methane emission**
2 **reduction in submerged peat moss *Sphagnum cuspidatum* by**
3 **tightly associated methanotrophs.**

4 **Running title:** Low CH₄ emission by *Sphagnum*-associated CH₄ oxidizers

5

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21 **Abstract**

22 Wetlands present the largest natural sources of methane (CH₄) and their potential CH₄ emissions
23 greatly vary due to the activity of CH₄-oxidizing bacteria associated with wetland plant species. In this
24 study, the association of CH₄-oxidizing bacteria with submerged *Sphagnum* peat mosses was studied,
25 followed by the development of a novel mesocosm set-up. This set-up enabled the precise control of
26 CH₄ input and allowed for monitoring the dissolved CH₄ in a *Sphagnum* moss layer while mimicking
27 natural conditions. Two mesocosm set-ups were used in parallel: one containing a *Sphagnum* moss
28 layer in peat water, and a control only containing peat water. Moss-associated CH₄ oxidizers in the
29 field could reduce net CH₄ emission up to 93%, and in the mesocosm set-up up to 31%. Furthermore,
30 CH₄ oxidation was only associated with *Sphagnum*, and did not occur in peat water. Especially
31 methanotrophs containing a soluble methane monooxygenase enzyme were significantly enriched
32 during the 32 day mesocosm incubations. Together these findings showed the new mesocosm setup
33 is very suited to study CH₄ cycling in submerged *Sphagnum* moss community under controlled
34 conditions. Furthermore, the tight association between *Sphagnum* peat mosses and methanotrophs
35 can significantly reduce CH₄ emissions in submerged peatlands.

36

37 **Keywords:** Methanotrophy, Peatland, *Sphagnum* moss, Methane cycle, mesocosm, climate change,
38 soluble methane monooxygenase

39 Introduction

40 Methane (CH₄) has a 25 times higher Global Warming Potential (GWP) than carbon dioxide (CO₂; on a
41 100 year time scale) and is the second most important greenhouse gas (GHG), contributing for about
42 16% to global warming [1, 2]. CH₄ in the atmosphere originates from both natural and anthropogenic
43 sources. Wetlands are the largest natural CH₄ source, emitting an estimated 167 Tg CH₄ yr⁻¹ into the
44 atmosphere [3], indicating an imbalance between CH₄ production and CH₄ consumption by
45 methanotrophs. Climate change has the potential to further stimulate the emission of CH₄ from
46 (especially arctic) wetlands [4]. Therefore, it is important to understand sources, sinks and microbial
47 transformations of CH₄ in wetland ecosystems.

48 CH₄ cycling in peat ecosystems is affected by peat degradation and subsequent restoration [5–7].
49 Restored (rewetted) sites appear to emit more CH₄, indicating that restored conditions stimulate
50 methanogenesis, and that methanotrophy cannot keep up. One well-known factor controlling CH₄
51 cycling in wetlands is the water-table [8, 9]. The CH₄ emission from rewetted peatlands remains low
52 when the water table remains well below the field surface. However, when the water-table rises, CH₄
53 emission strongly increases [10, 11]. As an example, the Mariagepeel peatland in The Netherlands has
54 been drained for many years, leading to severe drought. The peatland was rewetted again for
55 restoration purposes, which resulted in a strong decrease of CO₂ emissions that originated from the
56 aerobic oxidation of organic material, whereas the emission of the much stronger greenhouse gas CH₄
57 emission strongly increased [10]. The CH₄ emission in rewetted peatlands seems to be strongly
58 reduced by development of (aquatic) *Sphagnum* mosses, which harbor CH₄-oxidizing microorganisms
59 [6, 10, 12]. It is, however, challenging to study CH₄ dynamics in primary stages of peat development
60 (either restored/natural) without disturbing the site. Furthermore, also abiotic factors such as
61 temperature, water quality and light availability on site cannot be controlled as well as in the
62 laboratory, making experimental work and predictions about peat development and CH₄ cycling at
63 least cumbersome.

64 As mentioned above, CH₄ emissions are caused by an imbalance between CH₄ production and
65 consumption. The CH₄ emitted by peatlands is mainly produced by methanogenic Archaea [13]. In the
66 anaerobic, submerged peat layers that are devoid of electron acceptors other than CO₂, methanogens
67 produce CH₄ from a limited number of substrates and/or in syntrophic interaction with other
68 anaerobes that degrade organic carbon (C). However, not all of the CH₄ produced reaches the
69 atmosphere, due to methanotrophs that oxidize CH₄ to CO₂ [14, 15]. The oxidation of CH₄ is performed
70 both aerobically (e-acceptor: O₂) by CH₄-oxidizing bacteria (MOB), and anaerobically (AOM) by
71 Archaea and bacteria (e-acceptors: nitrite, nitrate, metal-oxides, humic acids, and sulfate [16]). Both
72 aerobic and anaerobic CH₄ oxidation contribute to the reduction of CH₄ emissions from peatlands [12,
73 17–19]. Within the MOB the enzyme methane monooxygenase (MMO) is responsible for the oxidation
74 of CH₄ to methanol. The majority of MOB have a copper containing, membrane bound form of MMO
75 (pMMO) [20]. In addition, a small fraction of the MOB also has a soluble form of MMO (iron containing
76 sMMO) [20]. The sMMO seems to be only expressed when copper limitation is experienced and has a
77 less restricted substrate specificity than pMMO [20]. Peatland methanotrophs typically possess both
78 pMMO and sMMO [12, 21–23], which can be targeted via the *pmoA* and *mmoX* genes encoding one
79 of the subunits, respectively. Some peatland and marine methanotrophs are unique in that they only
80 possess sMMO [23–27]. Also *mmoX* transcripts indicate that sMMO is an active enzyme in peatlands
81 [28], although its importance is not yet well understood.

82 Studies have shown that aerobic CH₄ oxidation is most prominent in submerged *Sphagnum* mosses in
83 a range of peatlands [12, 29, 30]. Furthermore, the association between methanotrophs and
84 *Sphagnum* was shown to be mutually beneficial Raghoebarsing et al. [31]. The methanotrophs convert
85 CH₄ into CO₂, thereby relieving part of the CO₂ limitation that *Sphagnum* mosses experience [32]
86 especially under submerged conditions [12, 31]. The aerobic MOB in return benefit from O₂ produced
87 and shelter provided by the moss [12].

88 Molecular surveys showed that several CH₄-oxidizing bacteria are present in *Sphagnum* dominated
89 peatlands. *Alphaproteobacterial* methanotrophs typically dominate in 16S rRNA gene libraries over

90 the other methanotroph-containing (sub)phyla *Gammaproteobacteria* and *Verrucomicrobia*
91 (*Methylacidiphilaceae* [33–35]. Within the *Alphaproteobacteria* especially methanotrophs of the
92 family *Methylocystaceae* (*Methylocystis spp.*) and the acidophilic methanotrophs of the family
93 *Beijerinckiaceae* (*Methylocella*, *Methyloferula*, *Methylocapsa*) are often found and several of these
94 have been isolated from peatlands [24–26, 36, 37]. Using Fluorescence *in situ* Hybridization (FISH)
95 combined with confocal microscopy, *Alphaproteobacteria* have shown to be localized inside
96 *Sphagnum* mosses, in the dead hyaline cells [38]. Furthermore, *Verrucomicrobia* including the class
97 containing CH₄ oxidizers, *Methylacidiphilae*, can make up 10% of the total microbial community
98 associated with *Sphagnum*. However, the *Methylacidiphilae* found with *Sphagnum* mosses have not
99 yet been coupled to CH₄-oxidizing activity [34, 39, 40]. Their role in peatland C cycling has yet to be
100 confirmed [23, 41–43].

101 The goal of this study was to design and test a new mesocosm set-up where a submerged *Sphagnum*
102 community could be mimicked under fully controlled conditions. In this way, the irregularity and
103 variability often encountered in field studies could be excluded. The new set-up was used to study the
104 association between CH₄ oxidizers and a layer of submerged *Sphagnum* mosses. We hypothesized that
105 the submerged *Sphagnum* moss layer acts as a biofilter for CH₄, thereby reducing CH₄ emission to the
106 atmosphere. Furthermore, it is expected that the CH₄-oxidizing microorganisms are associated with
107 *Sphagnum*, rather than the peat water. Monitoring of the CH₄ flux throughout the mesocosm
108 incubation, as well as CH₄ batch assays and molecular analysis of 16S rRNA gene amplicons and qPCR
109 on 16S rRNA, *pmoA* and *mmoX* showed that during the 32 days of incubation aerobic methanotrophs
110 were highly active and enriched in the mesocosm.

111 **Materials & Methods**

112 *Sampling site and field measurements*

113 The sampling site was located in the Mariapeel (51°24'28.4"N, 5°55'8"E), a peat bog nature
114 conservation area in the south of the Netherlands. This site was visited for measurements and
115 sampling on 09/08/2017. Net diffusive gas fluxes of CO₂ and CH₄ were measured in the field using a
116 fast greenhouse gas analyzer with cavity ringdown spectroscopy (GGA-24EP; Los Gatos Research, USA)
117 connected to a Perspex chamber (15 cm in diameter). The chamber was put on top of the moss layer
118 for 10 min to measure fluxes of CO₂ and CH₄. In total 3 independent measurements were taken within
119 2 m distance from each other. After removal of the peat moss layer measurements were repeated,
120 after an equilibration period of 15 min. Submerged *Sphagnum cuspidatum* moss and water were
121 collected after the measurements.

122 Upon arrival in the laboratory, 1 set of mosses was used to determine field activity, and another part
123 was washed using sterile demineralized H₂O. One fraction of water was used to determine field
124 activity, the other fraction was filtered (2 – 5 nm, HF80S dialysis filter, Fresenius Medical Care,
125 Homburg, Germany). All samples were stored at 4 °C (1 week) until the start of the incubation.

126

127 *Mesocosm design*

128 The mesocosm consists of a glass cylinder with a diameter of 12 cm and a height of 54 cm, to which a
129 separate reservoir is connected (see Supplementary Figs. 1 and S1). The total reservoir volume is 0.5
130 L, the connector tube volume is 0.07 L and the total column volume is 6.11 L. The water level in the
131 mesocosms was maintained at 5.09 L, leaving a headspace of 1.02 L in the column. The column
132 headspace was closed throughout the day using a greased lid with sampling port. Several sampling
133 ports (in the reservoir, cylinder headspace and in the cylinder at 10, 20, 30, 35 and 40 cm height) allow
134 for sampling of either the gas or water phase. Throughout the mesocosm incubation all sampling ports
135 were closed off using boiled, red butyl rubber stoppers and capped using metal crimp caps.

136

137 *Mesocosm incubation*

138 The mesocosms were autoclaved prior to use. Two mesocosms were simultaneously incubated for
139 this experiment. A moss mesocosm, containing 100 *Sphagnum cuspidatum* plants (6 cm length, 120 g
140 fresh weight) in filtered peat water (5.09 L), and a control mesocosm which contained only filtered
141 peat water (5.09 L). Both mesocosms had an acclimatization period of 7 days prior to sampling.
142 The CH₄ was added via the reservoir headspace and dissolved into the water by stirring with a 2 cm
143 magnetic stir bar at 250 rpm. Throughout the week, lids were opened each morning for 1 h to allow
144 aeration, after which they were closed for the rest of the day. The CH₄ supply in the reservoir
145 headspace was replaced daily, directly after aeration, with a mixture of 50ml 99% CH₄ and 5 ml CO₂.
146 The mesocosm experiment was performed twice, each time for 32 days. Incubations were performed
147 at room temperature. The light regime consisted of 16 h daylight (150 μmol m⁻² s⁻¹ photosynthetically
148 active radiation at vegetation level) and 8 h of darkness. Light was supplied on top of the mesocosm
149 column, via 120 deep red/white LED lamps (Philips, Green-Power LED, Poland).

150

151 *Mesocosm CH₄ fluxes*

152 After the acclimatization period the fate of CH₄ was followed through the mesocosm over time (0 - 32
153 days). To determine the concentration of CH₄ in the headspace or the concentration of dissolved CH₄
154 in water, gas and water samples were collected via the different sampling ports. A volume of 0.5 ml
155 gas or 0.5 ml water was taken and injected into a closed 5.9 ml Exetainer vial (Labco, Lampeter, UK).
156 The concentration of CH₄ in the headspaces of the reservoir and the column were determined by
157 taking samples directly after closing the column in the morning (0 h) and before opening the column
158 for aeration again (23 h). The concentration of dissolved CH₄ throughout the column was determined
159 once a week, by sampling water at 4 different time points during the day (0 h, 3 h, 7 h, 23 h after
160 closing the headspace).
161 The CH₄ concentration in the Exetainers was measured at least 4 h after sampling to allow for
162 equilibration between Exetainer headspace and liquid. The CH₄ concentration was measured using a

163 gas chromatograph with a flame-ionized detector and a Porapak Q column as described by De Jong et
164 al. [4].

165 Net CH₄ flux in the mesocosm was calculated as the change in CH₄ concentration in the headspace of
166 the mesocosm column for each day and divided by the surface area (0.01131 m²) of the mesocosm
167 column.

168

169 *Potential CH₄ oxidation rates*

170 The CH₄ oxidation rates were determined in triplicate in batch incubations prior to and after
171 mesocosm incubation. Prior to the mesocosm incubation, both unwashed and washed moss (3 g fresh
172 weight) as well as unfiltered and filtered porewater (12 ml) were placed into autoclaved 120 ml serum
173 vials and closed with boiled, red-butyl rubber stoppers and metal crimp-caps. Each batch flask
174 received 2 ml 99% CH₄. The CH₄ concentration in the headspace was followed in time as described for
175 mesocosm CH₄ fluxes.

176 At the end of the mesocosm experiment, potential CH₄ oxidation rates were determined for the
177 mosses from moss mesocosm and for porewater from both the moss and control mesocosm. Samples
178 were incubated as described above. Two sets of each 3 replicates were incubated, where one set was
179 used to determine CH₄ oxidation rates and the other set received the acetylene (6 ml 99.9% (C₂H₂)),
180 an inhibitor of the CH₄ monooxygenase enzyme, which was added after 10 h of incubation.

181 The concentrations of CH₄ were calculated using a calibration curve that was measured daily.
182 Ultimately, the CH₄ concentrations were plotted over time, from which CH₄ oxidation rates were
183 calculated from the slope of the linear part of the graph.

184

185 *Elemental analysis water*

186 Both unfiltered and filtered peat water was sampled and analyzed. The pH was measured and
187 elemental composition was determined using the auto analyzer and the ICP-OES as explained before
188 [34]. Dissolved CH₄ in field porewater was determined by injection of 1 ml porewater into a closed

189 Exetainer (5.9 ml), after 6 hours the headspace CH₄ concentration was measured as described above.

190 Data are shown in Supplementary Table S9.

191

192 *DNA extraction*

193 DNA extraction was performed by grinding 5 g of mosses (fresh weight) using pestle and mortar and

194 liquid nitrogen, after which DNA was extracted using the DNeasy Powersoil DNA extraction kit

195 following manufacturers protocol (Qiagen Benelux B.V., Venlo, Netherlands). DNA quality was

196 checked by gel electrophoresis (1% agarose gel in TBE buffer) and fluorometrically using the Qbit

197 dsDNA HS Assay Kit (Invitrogen, Thermo Fisher, Carlsbad, CA).

198

199 *Amplicon sequencing and analysis*

200 Barcoded Amplicon sequencing of the amplified V3-V4 region of the bacterial 16s rRNA gene (primers

201 Bact-341f and Bact 785r [44]) was done using Illumina Miseq, performed by BaseClear B.V. (Leiden,

202 the Netherlands). A total of 326 045 reads was obtained. The reads were quality filtered and analyzed

203 using Mothur (v1.36.1), following the Illumina Standard Operating Procedure (SOP, accessed on May

204 8th 2018, Kozich et al. 2013). Merged reads shorter than 400 bp were discarded, chimeras were

205 removed using the UCHIME algorithm [46] and the remaining sequences were clustered at 97%

206 identity. The resulting OTUs were classified based on the SILVA v132 16s rRNA gene non-redundant

207 database (SSURef_99_v132_SILVA). Next, non-target sequences (Chloroplasts, Mitochondria,

208 unknown, Archaea and Eukaryota) were removed from the dataset. See Supplementary Tables S1 and

209 S2 for full overview of read processing. The output was analyzed with R (version 3.4.0 by the R

210 Development Core Team [47]) and Rstudio v1.1.456 [48] using the packages Phyloseq [49] and vegan

211 [50]. Singletons were removed, and read libraries of all samples were rarefied by random subsampling

212 (seed: 12345) to 6 500 reads per sample (Rarefaction curves are depicted in Supplementary Fig. S2).

213 A PcoA plot (Supplementary Fig. S4) was created using Phyloseq, and based upon Bray-Curtis

214 dissimilarity matrix on rarefied data. All sequencing data can be accessed in GenBank NCBI BioProject
215 PRJNA517391.

216

217 *Quantitative PCR*

218 Copy numbers of the Bacterial 16S rRNA gene (for all primers see Table S3; Bact 341f - Bact 785r;
219 Klindworth et al. 2013), as well as functional genes *pmoA* (primers A189f-A682r; Holmes et al. 1995)
220 and *mmoX* (*mmoX1*-*mmoX2*; Miguez et al. 1997) were quantified using a qPCR approach. The qPCR
221 reaction mix consisted of PerfeCTA Quanta master mix (Quanta Bio, Beverly, MA) and 0.5 ng sample
222 DNA and 1µl of each primer (10 µM). In negative controls DNA was replaced by sterilized milli-Q water.
223 The qPCR reaction mix was loaded in triplicate into a 96-well optical PCR plates (Bio-Rad Laboratories
224 B.V., Veenendaal, The Netherlands), closed with an optical adhesive cover (Applied Biosystems, Foster
225 City, CA) and reactions were performed with a C1000 Touch thermal cycler equipped with a CFX96
226 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).
227 Standard curves were obtained via 10-fold dilution series of a PGEM T-easy plasmid (Promega,
228 Madison, WI) containing the target gene. The data was analyzed using Bio-Rad CFX Manager version
229 3.0 (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Triplicate analysis per samples were
230 averaged prior to statistical analysis.

231

232 *Statistics*

233 The CH₄ flux in the field and in the mesocosm, CH₄ oxidation rates in batch and qPCR data were
234 analyzed using R version 3.4.0 by the R Development Core Team [47]. In order to allow for parametrical
235 statistical tests, Shapiro-Wilk's test was used on the residual (stats-package) to test the normality of
236 the data and Levene's test (car-package) was used to test for homogeneity of variance. If assumptions
237 of tests were not met, data was log-transformed (ln), which was the case for the field CH₄ flux data. A
238 paired T-test was used to test whether the net CH₄ flux in the field was affected by the presence of
239 moss (moss field / moss removed). Differences between material (moss/peatwater) in the potential

240 CH₄ oxidation activity prior to mesocosm incubation was tested using a non-parametric Kruskal Wallis
241 tests. Within each material (moss/peatwater) the effect of treatment (field / washing or filtering) was
242 tested using an independent T-test.
243 Differences between mesocosms (moss / control), material (moss / peat water) and inhibitor (yes/no)
244 in the potential CH₄ oxidation activity after mesocosm incubation, were tested using a 3-way Anova,
245 followed by a Tukey HSD post-hoc test. Differences in copy number between each moss sample (Moss
246 Field/Moss Washed/Moss incubated) within each target gene (*16S rRNA/mmoX/pmoA*) was analyzed
247 using a one-way Anova, followed by a Tukey HSD post-hoc test. Here, the data for *16S rRNA* gene and
248 *mmoX* gene were log-transformed (ln) prior to analysis.

249

250 **Results**

251 *Field CH₄ flux*

252 To estimate diffusive CH₄ emissions in the field, flux chamber measurements were carried out in plots
253 with submerged *Sphagnum* mosses before and after removal of the moss layer. The CH₄ emission in
254 the field situation with the submerged *Sphagnum* moss layer resulted in a net total of 4.1 ± 2.1 mmol
255 CH₄ m⁻² day⁻¹ (mean \pm SEM, n=3; Fig. 2). Removal of the *Sphagnum* moss layer significantly increased
256 the net CH₄ emission ($t_{(2)} = -6.1$, $p < 0.05$) to a total of 60 ± 32 mmol CH₄ m⁻² day⁻¹ (Fig. 2).

257

258 *Methane oxidation activity prior to mesocosm incubation*

259 The CH₄ oxidation rates associated with the *Sphagnum* moss and peat water were determined prior
260 to the incubation in the mesocosm, using batch assays (Fig. 3). *Sphagnum* mosses showed much higher
261 CH₄ oxidation rates (average rate mosses 143 ± 17 $\mu\text{mol g DW}^{-1} \text{ day}^{-1}$, Fig. 3) compared to peat water,
262 which had virtually no activity (0.05 ± 0.06 $\mu\text{mol g DW}^{-1} \text{ day}^{-1}$; $\chi^2 = 7.5$, $p < 0.01$, Supplementary Fig.
263 S5). Washing of the *Sphagnum* mosses was reduced the CH₄ oxidation rate 121 $\mu\text{mol g DW}^{-1} \text{ day}^{-1}$; $t_{(2)}$
264 $= 1.5$, $p > 0.05$).

265

266 *Mesocosm incubation*

267 Two parallel mesocosm incubations were performed, one including a *Sphagnum* layer and one
268 without. The net CH₄ flux in the mesocosm showed a similar pattern for both mesocosms until day 8
269 of the incubation (Fig. 4). After 8 days, the moss mesocosm headspace always showed a lower CH₄
270 concentration than the control mesocosm with only peat water. Furthermore, the emission from the
271 *Sphagnum* moss mesocosm gradually decreased over the 32 day of the incubation, which is a strong
272 indication of increasing CH₄ oxidation activity. The variation in Fig. 4 is partly due to the daily manual
273 refreshment of CH₄ and air. The experiment was repeated for a second time, and the replicate
274 incubation showed a similar pattern, with lower CH₄ emission with the presence of *Sphagnum* moss
275 layer (Supplementary Fig. S8 and Tables S7 and S8).

276

277 *Methane oxidation activity after mesocosm incubation*

278 After 32 days of incubation in the mesocosms, the CH₄ oxidation activity was determined in batch for
279 each element of both mesocosms (water and/or moss). The CH₄ oxidation activity was on average 189
280 μmol CH₄ g⁻¹ DW day⁻¹ (Table 1) in mosses. Even after mesocosm incubation the peat water showed
281 no CH₄ oxidation activity ($R^2 < 0.9$; see Table 1 and Figs. S6 and S7), indicating that the water is not a
282 favorable place for MOB. In the presence of acetylene, CH₄ oxidation associated with the mosses was
283 almost completely inhibited ($F_{(1,4)} = 981.3$, $p < 0.001$), indicating that the CH₄ oxidation rate is entirely
284 associated with methanotrophic microorganisms in or at the moss. Compared to the start of the
285 incubation, the CH₄ oxidation activity associated with mosses had increased by 155% (from 121 to 189
286 μmol g DW⁻¹ day⁻¹; Table 1 and Fig. 3).

287

288 *qPCR*

289 To quantify the microbial community, both qPCR and amplicon sequencing of 16S rRNA genes were
290 performed. Quantification of the bacteria (16S rRNA gene; Fig. 5) showed that bacterial copy numbers
291 differed between all stages ($F_{(2,6)} = 34.3$, $p < 0.001$). Substantial amounts (98%) of presumably epiphytes

292 were washed away (Tukey HSD $p < 0.001$). At the end of the incubations the copy numbers were back
293 to about 97% of the original value (Tukey HSD $p < 0.05$).

294 Quantification of methanotrophic microorganisms by *mmoX* gene and *pmoA* gene amplification
295 showed a similar trend (*mmoX* $F_{(2,6)} = 40.7$, $p < 0.001$; *pmoA* $F_{(2,6)} = 27.1$, $p < 0.001$; Fig. 5). The *pmoA*-
296 containing methanotrophs were overall less abundant than *mmoX*-containing methanotrophs (resp.
297 10^5 vs. 10^{10} copies). The washing step greatly reduced the abundance of the *mmoX*-containing
298 methanotrophs from 10^{10} to 10^2 copies (Tukey HSD $p < 0.001$), whereas *pmoA*-containing
299 methanotrophs were much less affected (remained around 10^5 copies; Tukey HSD $p > 0.05$). Upon
300 mesocosm incubation *mmoX* copies increased from 10^2 to 10^8 (Tukey HSD $p < 0.001$), while *pmoA*-
301 containing methanotrophs marginally increased from 10^5 to 10^6 copies (Tukey HSD $p < 0.01$).

302

303 *Microbial community (16S rRNA gene)*

304 The microbial community associated with the mosses was studied by 16S rRNA gene sequencing of
305 the V3-V4 region. Comparison of the moss microbial community in the field, after washing and after
306 incubation in the mesocosm shows a gradual change in microbial community. However, the main
307 classes remained present throughout the incubation. Furthermore, mesocosm incubation increased
308 diversity of the microbial community (Shannon and Chao 1 index, Table S4).

309 Looking at microbial community composition depicted as relative abundances in Fig. 6A, the
310 *Proteobacteria* were the overall dominant phylum. Relative abundance of *Proteobacteria* was not
311 affected by washing, but decreased during incubation in our mesocosm set-up. For the
312 *Verrucomicrobiae* the relative abundance was lower after washing and increased after incubation.
313 Especially the relative abundance of *Pedosphaerales* and *Opitutales* increased upon incubation
314 (Supplementary Table S5). When focusing on the methanotrophic community, the relative abundance
315 of Verrucomicrobial *Methylacidiphilales* increased by incubation (Fig. 6B). Other methanotrophic
316 species whose relative abundance increased upon incubation are *Methylomonas* spp. and
317 *Methylocystis* spp. (Fig. 6B). Only acidophilic *Methylocystis* isolates, *M. bryophila* and *M. heyeri* [53,

318 54], are known to contain both sMMO and pMMO, whereas neutrophilic *Methylocystis* and
319 *Methylocella* species isolated so far only contain pMMO.

320

321 **Discussion**

322 *Mesocosm approach*

323 Studying and sampling the *Sphagnum* microbiome in the field is challenging, because the microbial
324 community associated with the moss is influenced by many biotic and abiotic factors that are not
325 controlled for. After many field campaigns we set out to circumvent these challenges and fluctuations.
326 Therefore, we designed a novel mesocosm set up to mimic submerged *Sphagnum* moss ecosystem
327 and operated it under controlled laboratory conditions to shed light on the association between
328 aerobic CH₄ oxidizers and a submerged *Sphagnum cuspidatum* community. We hypothesized that the
329 submerged *Sphagnum* moss layer acts as a biofilter for CH₄ and expected that the CH₄-oxidizing
330 community was mainly associated with *Sphagnum* moss. Indeed, in this controlled mesocosm set-up,
331 we were able to mimic a significant reduction (31%) in CH₄ emissions as was also observed in the field
332 (Figs. 4 and S8). This CH₄ removal was only associated with the mosses and not found in the peat water.

333

334 The novel mesocosm set-up allowed for enrichment of both methanotrophic activity and their
335 abundance. Potential CH₄ oxidation batch assays revealed a significant increase in methanotrophic
336 activity after mesocosm incubation (from 121 ± 4 to 189 ± 6 μmol CH₄ g⁻¹ DW day⁻¹, resp. Fig. 3 & Table
337 1). Similarly, qPCR of functional methanotrophic genes (*mmoX* and *pmoA*), indicated that significant
338 numbers of CH₄-oxidizing bacteria were present in and on the moss and that their numbers increased
339 over the course of the incubation.

340

341 *Peat mosses strongly facilitate CH₄-oxidizing activity*

342 Washing of the moss and filtering of the peat water had little effect on CH₄ oxidation activity and
343 community composition, which underlines the tight association between CH₄ oxidizers and *Sphagnum*

344 mosses. Yet, qPCR revealed that bacterial copy numbers decreased by washing of the moss. The
345 number of sMMO-containing methanotrophs decreased most significantly during washing, indicating
346 that these methanotrophs might only be loosely attached epiphytes on the *Sphagnum* mosses.
347 However, they showed the highest increase (10^2 to 10^8 copies/g FW) upon mesocosm incubation,
348 equaling growth of up to 20 generations in 32 days. The transcription of *mmoX* gene and activity of
349 sMMO-containing methanotrophs has previously been reported in peatlands [55–57]. The increase in
350 sMMO copy number during incubation suggests that sMMO-containing methanotrophs are
351 environmentally relevant in acidic peatland ecosystems, especially in submerged conditions, but their
352 importance and contribution needs further study. Surprisingly, the pMMO-containing methanotrophs
353 were initially less abundant than sMMO-containing methanotrophs, but seemed more tightly
354 associated to the moss as washing had no effect on the copy numbers. There was hardly any increase
355 in abundance upon incubation. Lack of copper might explain why pMMO containing methanotrophs
356 did not thrive in the mesocosm incubation [20]. Ultimately, the enrichment of sMMO-containing
357 methanotrophs upon mesocosm incubation shows that this set-up can be used to further study the
358 functioning of sMMO methanotrophs in *Sphagnum* mosses as their ecology is far less understood than
359 that of canonical pMMO containing methanotrophs.

360

361 *Microbial community composition*

362 The *Sphagnum*-associated microbial community in all samples of this study showed high similarity to
363 previous *Sphagnum*-associated 16S rRNA gene libraries [34, 38, 39]. Similar dominant community
364 members were found in this study, with dominant phyla being the *Proteobacteria* (*Alpha*- and
365 *Gammaproteobacteria*), *Cyanobacteria* (*Oxyphotobacteria*) and *Acidobacteria* and a relatively high
366 abundance of *Verrucomicrobia*. Upon mesocosm incubation the microbial diversity increased,
367 potentially due to the limited amount of nutrients present compared to field conditions. The relative
368 abundance of *Verrucomicrobia* and *Planctomycetes* increased, whereas the relative abundance of the

369 *Proteobacteria* decreased. Which processes control the changes in the moss-associated microbial
370 community is topic for further study.

371

372 *Strong natural CH₄ filter*

373 The reduction of CH₄ emission by the *Sphagnum*-methanotroph interaction in the studied mesocosm
374 set-up is large (31%), compared to other high CH₄ producing moss-dominated ecosystems. In other
375 ecosystems CH₄ oxidation also mitigates CH₄ emission. For example, in the arctic tundra [28] 5% of the
376 total CH₄ emission is mitigated, whereas in hollows in *Sphagnum*-dominated peatland [58] measured
377 CH₄ production and oxidation rates and calculated that nearly 99% of the CH₄ emission was mitigated
378 by CH₄-oxidizing microorganisms. For free-floating wetland plants, it was shown that up to 70% of the
379 CH₄ emission may be oxidized by the combination of decreased flux rates and high CH₄-oxidizing
380 activity [59].

381 Yet, the CH₄ activity in the mesocosm set-up it is lower than the reduction found in the field. This is
382 likely to be caused by the peat moss density, which was much higher in the field, where the moss layer
383 was more than 50 cm deep. Although the stabilization of the net CH₄ flux in both mesocosms occurred
384 relatively quickly (8 days) and considerable CH₄ mitigation was measured after 32 days of incubation,
385 we believe that the CH₄ mitigation by the moss associated methanotrophs in the mesocosm will most
386 probably increase even further by prolonging the incubation time and increased amount of *Sphagnum*
387 mosses. Additionally, the mesocosm set-up could be improved by replacing the manual addition of
388 CH₄ and air of the mesocosm with a continuous supply system. In a continuous bioreactor set-up, the
389 system is even more stable, and variation is further reduced. The high reduction in CH₄ emission in
390 submerged *Sphagnum* emphasizes that the methanotrophs associated with *Sphagnum* are important
391 in CH₄ cycling in peatlands [12, 28–30], as they strongly regulate CH₄ emission from *Sphagnum*
392 dominated peatlands.

393

394 *Implications for degraded peatlands*

395 The large organic matter stocks in peatlands are a great potential source for CO₂ when these peatlands
396 are drained. Restoration measures aimed at preventing further oxidation and degradation of these
397 drained peatlands, often involve hydrological measures (rewetting), resulting in inundation of large
398 surface areas. After rewetting, anaerobic degradation of organic matter will result in high CH₄
399 production rates. As shown above, methanotrophs are tightly associated to *Sphagnum* mosses.
400 Presence of this consortium in restored peatlands can thereby strongly mitigate CH₄ emissions. Since
401 the presence and abundance of *Sphagnum* in peatlands is affected by peatland degradation as well
402 [60, 61], care should be taken to restore and facilitate *Sphagnum* mosses in restored peatlands.

403

404 *Conclusion*

405 *Sphagnum* mosses have many key roles in peat ecosystems [62], and this study shows that their
406 microbiome and specifically the methanotrophs associated with *Sphagnum* are crucial to keep CH₄
407 emissions from *Sphagnum*-dominated peatlands low. Peatland restoration practices involving
408 rewetting, should therefore aim to stimulate *Sphagnum* growth simultaneously, in order to keep CH₄
409 emissions at bay. The presented mesocosm set-up can be used to further study the effect of various
410 climate change relevant factors (such as temperature, pH, fertilization) on CH₄ cycling in submerged
411 *Sphagnum* moss ecosystems. Studying the influence of climate change on the *Sphagnum*-
412 methanotroph interaction and CH₄ balance is crucial to get a better understanding of the potential
413 positive feedback loop that reside in peatlands.

414

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418 Faculty of Science at Radboud University, especially Paul van der Ven and Sebastian Krosse are
419 thanked for measuring elemental composition of the water samples and determining stable isotope
420 contents.

421 **Availability of data and materials**

422 All sequencing data has been deposited in the NCBI SRA database, project number PRJNA517391.

423

424 **Competing interests**

425 The authors declare no competing financial interests.

426

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- 592

593 **Table & Figure legends**

594 **Table 1** Potential CH₄ oxidation rate in batch, after mesocosm incubation. Moss and peat water
595 samples from each mesocosm were incubated in batch, with or without acetylene. Different italic
596 letters indicate statistical differences between PMO rates, tested by 3-way Anova.

597

598 **Fig. 1** Schematic set up of mesocosm incubation with in **A.** control mesocosms containing only filtered
599 peat water (blue) and in **B.** the moss mesocosms, containing sphagnum moss layer (green) in filtered
600 peat water.

601

602 **Fig. 2** Net CH₄ flux (mmol CH₄ m⁻² day⁻¹) measured in the field with *Sphagnum* moss layer present
603 (green, n=3) and after moss removal (blue, n=3). Error bars indicate the standard error of the mean.

604

605 **Fig. 3** Potential CH₄ oxidation rate in batch, associated with field *Sphagnum* mosses (light green, μmol
606 CH₄ g⁻¹ DW day⁻¹) or washed *Sphagnum* mosses (darker colors) and rates in peat water unfiltered or
607 filtered. Error bars indicate the standard error of the mean (n=3).

608

609 **Fig. 4** Net CH₄ flux (mmol CH₄ m⁻² day⁻¹) from the mesocosms with *Sphagnum* moss (green) and the
610 control mesocosm with only peat water (blue) measured in the headspace over time (days). Each dot
611 represents the mean of 2 technical replicates.

612

613 **Fig. 5** Copy numbers of bacteria 16S rRNA, *pmoA* and *mmoX* genes obtained via qPCR. Error bars
614 indicate the standard error of the mean (n=3).

615

616 **Fig. 6 A** Phylogenetic classification of the bacterial community based on 16S rRNA gene amplification
617 and sequencing. Taxonomic groups with a relative abundance <1% are depicted as "Other". In **B**

618 specific relative abundances (RA in %) of methanotrophic bacteria in the bacterial 16s rRNA
619 community profile are shown.

620 **Tables**

621 **Table 1** Potential CH₄ oxidation rate in batch, after mesocosm incubation. Moss and peat water samples from
 622 each mesocosm were incubated in batch, with or without acetylene. Different italic letters indicate statistical
 623 differences between PMO rates, tested by 3-way Anova.

624

Material	Mesocosm	Treatment	Potential methane oxidation rate ($\mu\text{mol CH}_4 \text{ g}^{-1} \text{ DW day}^{-1}$)	SEM	R ²	n
Moss	Moss		189 <i>a</i>	6	0.98	3
Moss	Moss	+ acetylene	2.0 <i>b</i>	2	0.30	3
Material	Mesocosm	Treatment	Potential methane oxidation rate ($\mu\text{mol CH}_4 \text{ ml}^{-1} \text{ day}^{-1}$)	SEM	R ²	n
Water	Moss		0.02 <i>a</i>	0.02	0.17	3
Water	Moss	+ acetylene	0.03 <i>a</i>	0.01	0.51	3
Water	Peatwater only		0.09 <i>b</i>	0.01	0.86	3
Water	Peatwater only	+ acetylene	0.05 <i>b</i>	0.01	0.67	3

625

626 **Figures**

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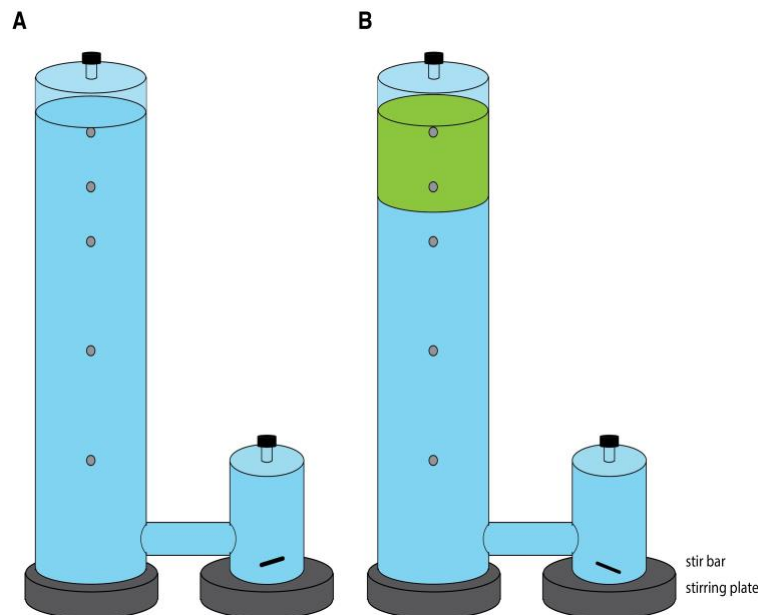
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Fig. 1 Schematic set up of mesocosm incubation with in **A.** control mesocosms containing only filtered peat water (blue) and in **B.** the moss mesocosms, containing sphagnum moss layer (green) in filtered peat water.

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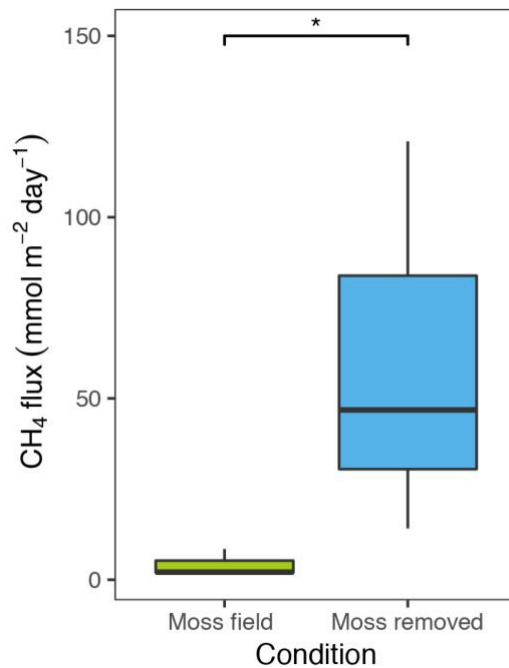
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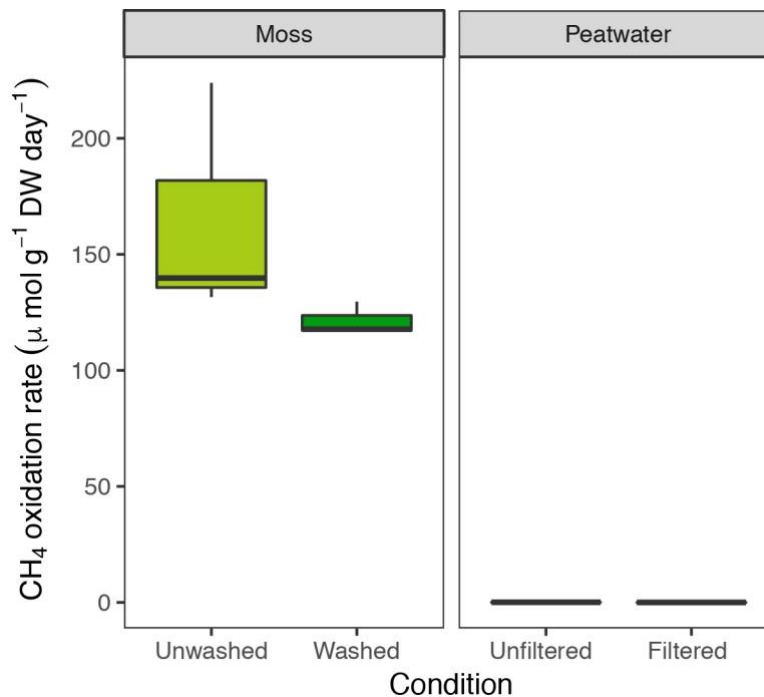
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Fig. 2 Net CH₄ flux (mmol CH₄ m⁻² day⁻¹) measured in the field with *Sphagnum* moss layer present (green, n=3) and after moss removal (blue, n=3). Error bars indicate the standard error of the mean.

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Fig. 3 Potential CH₄ oxidation rate in batch, associated with field *Sphagnum* mosses (light green, µmol CH₄ g⁻¹ DW day⁻¹) or washed *Sphagnum* mosses (darker colors) and rates in peat water unfiltered or filtered. Error bars indicate the standard error of the mean (n=3).

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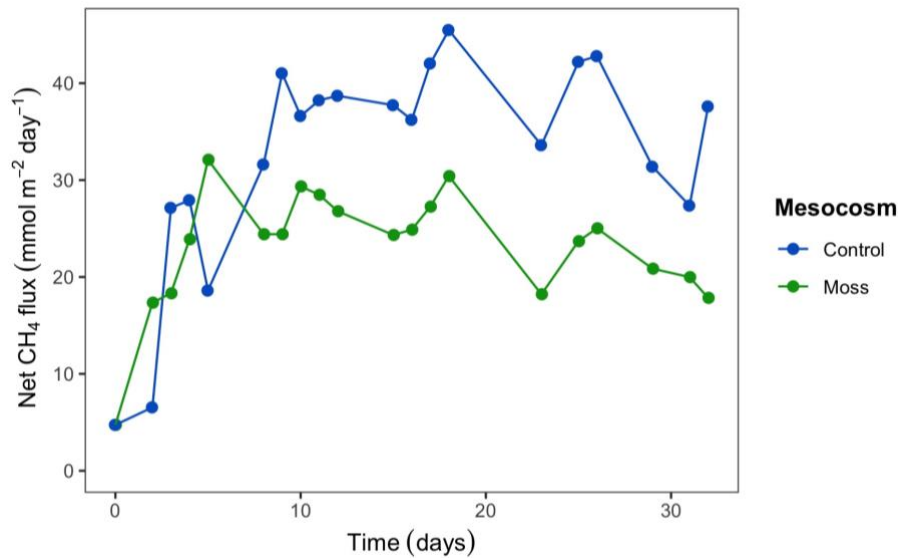
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Fig. 4 Net CH₄ flux (mmol CH₄ m⁻² day⁻¹) from the mesocosms with *Sphagnum* moss (green) and the control mesocosm with only peat water (blue) measured in the headspace over time (days). Each dot represents the mean of 2 technical replicates.

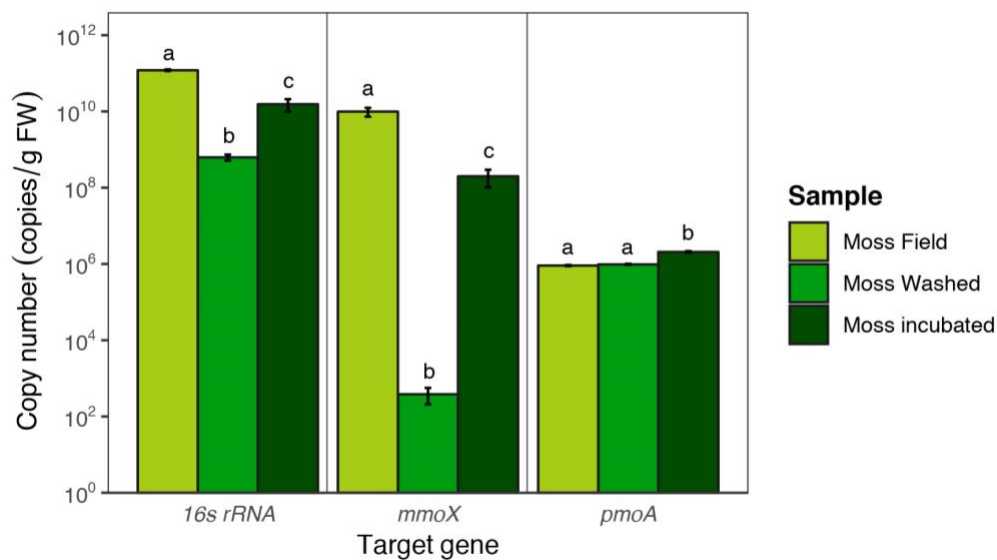


Fig. 5 Copy numbers of bacteria 16S rRNA, *pmoA* and *mmoX* genes obtained via qPCR. Error bars indicate the standard error of the mean.

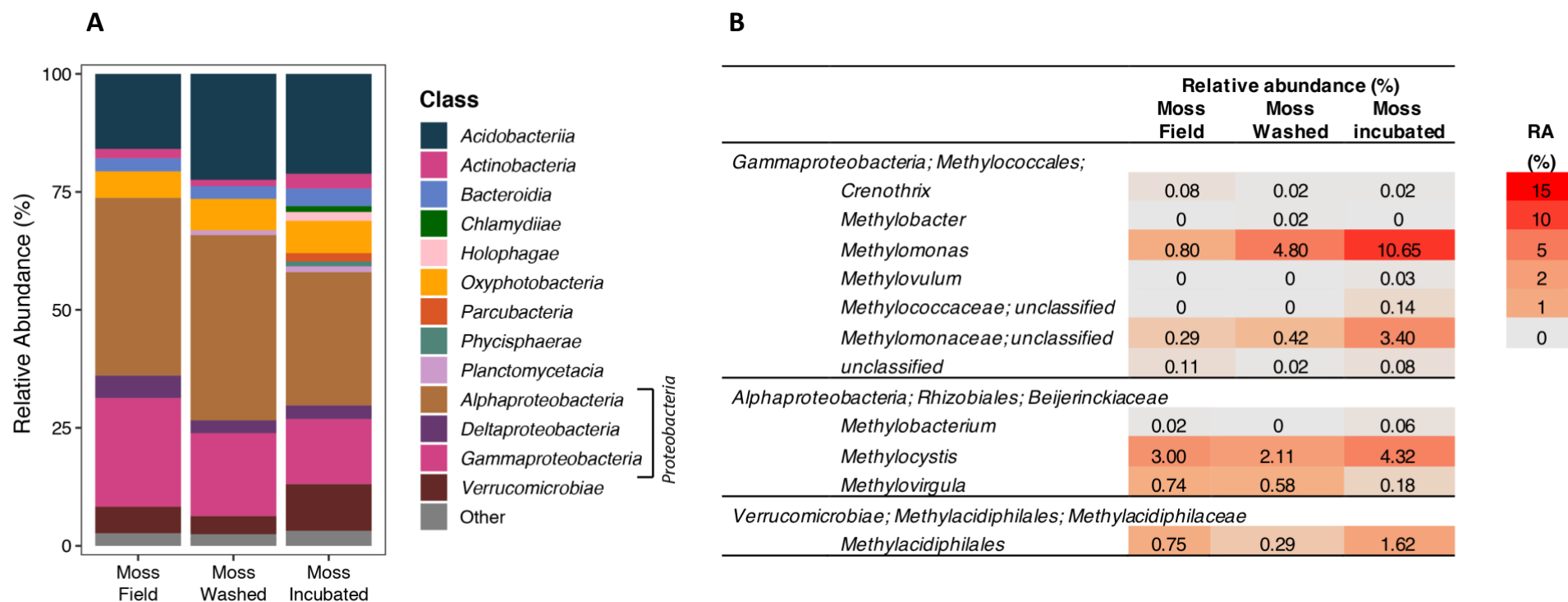


Fig. 6 A Phylogenetic classification of the bacterial community based on 16S rRNA gene amplification and sequencing. Taxonomic groups with a relative abundance <1% are depicted as "Other". In **B** specific relative abundances (RA in %) of methanotrophic bacteria in the bacterial 16s rRNA community profile are shown.