

1 **Compositional and functional stability of aerobic methane consuming**
2 **communities in drained and rewetted peat meadows**

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26 **Abstract**

27 The restoration of peatlands is an important strategy to counteract subsidence and loss of
28 biodiversity. However, responses of important microbial soil processes are poorly
29 understood. We assessed functioning, diversity, and spatial organization of methanotrophic
30 communities in drained and rewetted peat meadows with different water table management
31 and agricultural practice. Results show that the methanotrophic diversity was similar between
32 drained and rewetted sites with a remarkable dominance of the genus *Methylocystis*. Enzyme
33 kinetics depicted no major differences, indicating flexibility in the methane (CH₄)
34 concentrations that can be used by the methanotrophic community. Short-term flooding led to
35 temporary elevated CH₄ emission but neither to major changes in abundances of MOB nor in
36 CH₄ consumption kinetics in drained agriculturally used peat meadows. Radiolabelling and
37 autoradiographic imaging of intact soil cores revealed a markedly different spatial
38 arrangement of the CH₄ consuming zone in cores exposed to near-atmospheric and elevated
39 CH₄. The observed spatial patterns of CH₄ consumption in drained peat meadows with and
40 without short-term flooding highlighted the spatial complexity and responsiveness of the CH₄
41 consuming zone upon environmental change. The methanotrophic microbial community is
42 not generally altered and harbors MOB that can cover a large range of CH₄ concentrations
43 offered due to water-table fluctuations, effectively mitigating CH₄ emissions.

44

45 **Introduction**

46 Methane (CH₄) is an important greenhouse gas (GHG) that contributes significantly (17%) to
47 the total anthropogenic radiative forcing of 2.83 W m⁻² (Myhre, *et al.*, 2013). However, the
48 annual growth rate of atmospheric CH₄ varied largely over the last 30 years (Kirschke, *et al.*,
49 2013). The causes for these anomalies are not fully understood but may be linked to changes
50 in wetland CH₄ emissions such as: changes in wetland area, wetland management (e.g.
51 fertilizer use in rice cultivation), and climatic effects affecting water table and temperature
52 (Bousquet, *et al.*, 2006, Kai, *et al.*, 2011, Spahni, *et al.*, 2011, Kirschke, *et al.*, 2013,
53 Turetsky, *et al.*, 2014).

54 In recent years, many peatlands were restored to natural wetlands to counteract
55 subsidence of the peat soil (Verhoeven & Setter, 2010) and the loss of wetland biodiversity
56 (Quesnelle, *et al.*, 2013). A primary restoration strategy is to raise the water table and adapt
57 land use, i.e. reduce or abandon agriculture. While these strategies were successful in
58 increasing the abundance of wetland birds species (Tozer, *et al.*, 2010), their consequences
59 for GHG emissions are unclear. Rewetting likely reduces total C losses, but CH₄ emission
60 may increase (Hendriks, *et al.*, 2007).

61 Next to anaerobic CH₄ oxidation, aerobic methane-oxidizing bacteria (MOB) play a
62 key role in mitigating atmospheric CH₄ emissions. In environments such as wetlands, MOB
63 act as a bio-filter mitigating the release of CH₄ from soil-internal sources to the atmosphere
64 by approximately 40% (Frenzel, 2000). In environments such as dry forest and dry grassland,
65 MOB with deviating oxidation kinetics than in wetlands (Bender & Conrad, 1992, Knief, *et*
66 *al.*, 2006) contribute 6% to the global atmospheric CH₄ sink (Denman, *et al.*, 2007).

67 Aerobic MOB are found within the phylum of the Proteobacteria and
68 Verrucomicrobia. The methanotrophic Alphaproteobacteria include the families
69 *Methylocystaceae* and *Beijerinckiaceae*, while the methanotrophic Gammaproteobacteria are

70 found within the family *Methylococcaceae*. Based on different physiological, biochemical,
71 phenotypical characteristics and phylogeny, proteobacterial MOB have been classified into
72 type Ia, type Ib and type II (Hanson & Hanson, 1996, Bodrossy, *et al.*, 2003). Today, they are
73 mainly grouped taxonomically into Gamma- and Alphaproteobacteria, respectively. MOB
74 oxidize CH₄ via methanol to carbon dioxide. The *pmoA* gene encodes a subunit of the
75 particulate CH₄ monooxygenase (pMMO), a key enzyme catalyzing the first step in the
76 aerobic CH₄ oxidation pathway of MOB. The *pmoA* gene is present in the majority of MOB
77 and therefore has been widely used as phylogenetic and functional marker to target these
78 organisms from the environment (McDonald, *et al.*, 2008).

79 MOB that are capable of oxidizing CH₄ at atmospheric concentration have not been
80 cultivated yet. However, *pmoA* sequence analyses of soils showing atmospheric CH₄ uptake
81 revealed distinct clusters, termed upland soil cluster (USC) α and γ (Holmes, *et al.*, 1999,
82 Henckel, *et al.*, 2000, Knief, *et al.*, 2003). While the closest relative of USC α is member of
83 the genus *Methylocapsa* (Ricke, *et al.*, 2005), USC γ is more closely related to the family
84 Methylococcaceae (Knief, *et al.*, 2003). Next to these dominant groups other putative
85 atmospheric MOB have been detected (Horz, *et al.*, 2005, reviewed in Kolb, 2009).

86 Rewetting agriculturally used peat will affect methanotrophic communities because a
87 well aerated and dry environment with predominantly sub-atmospheric CH₄ concentrations is
88 converted back to a soil environment with high internal CH₄ supply. It currently is unclear
89 whether the original methanotrophic communities largely persist and or whether a new
90 microbial community with new dominant species establishes, with possible consequences for
91 CH₄ cycling. To investigate this we focused on MOB communities in Dutch peat meadows
92 differing in water table management, agricultural practice, and restoration time. MOB
93 community composition as well as CH₄ oxidation kinetics in drained and rewetted peat soils
94 was compared, comprising both environments with high internal CH₄ supply and atmospheric

95 levels of CH₄. Spatial organization of aerobic CH₄ consumption in a drained peat soil with
96 and without short-term flooding was assessed by radio-imaging of intact soil cores incubated
97 with labelled ¹⁴C- CH₄ at elevated (10.000ppmv) or near-atmospheric (10ppmv)
98 concentrations.

99

100 **Materials and Methods**

101 *Study site and experimental design: long-term hydrological restoration*

102 Our first study was carried out in the Horstermeer polder (52°14'00.0 N, 05°04'00.0 E)
103 and Stein (52°01'07.0 N, 04°46'00.0 E) in the Netherlands. These areas have previously been
104 described (Schrier-Uijl, *et al.*, 2010, Schrier-Uijl, *et al.*, 2014). At both locations, we selected
105 sites that were artificially drained and sites where the water table has been raised as a
106 restoration measure after drainage.

107 In Horstermeer, the rewetted site has been out of agricultural practice for more than
108 15 years. Hydrological isolation of the rewetted site raised ground water level close to the soil
109 surface and even above. The drained reference site has been out of intensive agricultural
110 practice for more than 7 years and today is mainly used for extensive pasturing while keeping
111 the water table artificially low.

112 In Stein, the rewetted site has been out of intensive agriculture for more than 20 years
113 but is used for extensive pasturing today. The drained reference site is still under intensive
114 agriculture and the water table is kept constant at low levels.

115 At both sites, soil samples were taken in October 2010, at the end of the growing
116 season. To representatively cover within-site variability, five evenly distributed 2 × 2 m plots
117 were chosen. In each plot, three soil cores (20 cm length, 3.8 cm diameter) were taken at
118 random locations with a soil corer. Upon arrival in the laboratory, replicated cores were
119 sliced and the 3-9cm layer processed. We chose this layer because initial tests had shown that

120 CH₄ oxidation was highest at this depth (unpublished data). Roots were removed from the
121 soil, the sample was mixed, freeze dried, and stored at room temperature in the dark until
122 further analyses. The main soil characteristics are summarized in Table S1.

123

124 *Study site and experimental design: drainage after short-term flooding*

125 An additional pair of study sites was located at the former experimental dairy farm of
126 the University of Wageningen in Zegveld, the Netherlands (52°8'25.9 N, 4°50'19.7 E). A
127 former agricultural pasture was temporarily flooded for 12 weeks in a wetland bird-
128 management initiative. The pasture has been flooded for the first time in February 2010 and
129 the second time in February 2011 and is still used for extensive agriculture (cattle and sheep).
130 Next to this site, we selected a non-flooded drained reference site with similar characteristics
131 and agricultural history. At each study site, we selected four plots of 1 m², which were
132 marked with bamboo sticks for re-sampling over the season. In each plot, CH₄ emission was
133 measured in duplicate points before soil cores were collected for further analysis in the
134 laboratory. In the following sampling campaigns two new points were selected within the 1
135 m² area and prepared in the same way as described above. In total, per sampling event and
136 per site we obtained 8 samples and CH₄ flux readings from two points at four plots. The soil
137 was processed as described above. The main soil parameters, pH and moisture content based
138 on dry weight are given in Table S2.

139

140 *CH₄ oxidation kinetics*

141 Apparent enzyme kinetics, i.e. the apparent half-saturation constant (K_{mapp}) and the
142 maximum rate of CH₄ consumption (V_{max}) were determined from all soil samples the day
143 following sample collection. Assays were performed by incubating 5 g of processed soil,
144 suspended in 10 ml of MilliQ water (MILLI-Q Reagent Water System, Millipore) in 120 ml

145 serum flasks capped with a butyl rubber stopper (Sigma Aldrich). CH₄ was added to the
146 headspace to achieve mixing ratios of approximately 50, 100, 500, 1000, 5000, 10.000 and
147 20.000 (only for long-term hydrological restored sites) ppmv. Soil slurries were incubated on
148 a rotary shaker (120 rpm) in the dark at room temperature. CH₄ consumption was monitored
149 by GC-FID analysis (HP 5890 Gas Chromatograph, Hewlett-Packard) over a period of 1-4
150 days including 5-12 measurements. Individual CH₄ oxidation rates for each concentration per
151 sample were calculated by linear regression. V_{max} and K_{mapp} were derived from nonlinear
152 regression using the nlstools package as implemented in the statistical software R (R
153 Development Core Team, 2013).

154

155 *CH₄ flux field measurements at the drained sites with and without short-term flooding*

156 CH₄ fluxes were monitored over the growing season in 2011. Measurements were taken in
157 May before the removal of water, three days after removal of water and in the beginning of
158 June, July and August. We used acrylic cylinders (40 cm length, 10 cm diameter) equipped
159 with two-way sampling ports through which headspace gas samples could be collected. These
160 samples were transferred into evacuated glass vials with a rubber stopper (Terumo, Belgium)
161 and analyzed for CH₄ concentration by GC-FID (Ultra GC gas chromatograph, Interscience,
162 Breda, the Netherlands, 30m x 0.32mm ID Rt-Q-Bond capillary column). CH₄ fluxes were
163 derived from the linear increase in CH₄ concentration in the headspace.

164

165 *¹⁴CH₄ labelling of cores; preparation of soil sections and imaging at the drained sites with 166 and without short-term flooding*

167 Both from the control as well as the temporarily flooded site in Zegveld, two pairs of cores
168 were taken in October 2011, at the end of the growing season. One soil core per pair was
169 labelled with ¹⁴C-CH₄ under elevated (10.000 ppmv) while the other was incubated under

170 near-atmospheric (10 ppmv) CH₄ concentrations. An additional soil core from the
171 temporarily flooded site was incubated at near-atmospheric conditions. The procedure was
172 performed as described in Stiehl-Braun and colleagues (2011). In brief, soil cores were
173 sampled by using polyethylene tubes (16 cm length, 5.7 cm diameter) and labeled with a total
174 activity of ca. 500 kBq ¹⁴CH₄. Labelling was carried out in gas-tight jars, for seven days. CH₄
175 headspace concentrations were monitored, and unlabeled CH₄ added to keep concentrations
176 close to the target values of 10 or 10.000 ppmv. To fix and solidify, the labeled soil cores
177 were freeze-dried and impregnated with epoxy resin, using a vacuum chamber, the fixed soil
178 cores were then cut horizontally into three sections of 5 cm height, and a vertical slice of
179 approximately 1 cm thickness cut from the center of each of these cylindrical sections, using
180 a diamond circular saw. These sections were mounted on 3 mm glass slides with epoxy resin,
181 and levelled with a diamond cup mill. Then, autoradiographs of the soil sections were
182 obtained by exposing phosphor imaging plates for 5 days. The imaging plates were scanned
183 by red-excited blue fluorescence scanning at a resolution of 50 µm. The images from the
184 three slides per core were then recomposed using MATLABs image processing toolbox.

185

186 *MOB community composition*

187 DNA was extracted from all soil samples by a modified method (see Henckel *et al.*, 1999 for
188 details). In brief, approximately 0.2 g of freeze-dried soil was suspended in 750 µl 120 mM
189 sodium phosphate buffer and 250 µl sodium dodecyl sulphate solution in bead-beating vials
190 (Lysing Matrix E, MP BIO, USA) and bead-beated using a FastPrep-24 Instrument (MP BIO,
191 USA). Samples were then centrifuged (10 min., 20.800 x g). 600 µl of the supernatant were
192 collected. The soil pellet was re-extracted with 600 µl sodium phosphate buffer and 200 µl
193 sodium dodecyl sulphate solution followed by another bead beating and centrifugation step

194 (400 µl supernatant). Before DNA precipitation we initially treated the supernatant with 7.5
195 molar sodium acetate to remove humic substances.
196 DNA from the combined 1 ml of supernatant was purified and precipitated (Lueders, *et al.*,
197 2004). Finally, pelleted nucleic acids were suspended in 30 µl of elution buffer (Qiagen,
198 Germany) and stored at -20 °C. DNA quality and quantity was determined using a Nano-
199 Drop Spectrophotometer (Thermo Scientific, USA).

200 We first performed Sanger sequencing of the *pmoA* gene from clone libraries
201 generated using the pGEM-T Easy Vector Systems (Promega, USA). Amplicons were made
202 using the forward primer A189f and either the reverse primer mb661r or A682r (table S3) as
203 described before (Henneberger, *et al.*, 2012). In brief, 0.5 ng of template DNA were added to
204 a total reaction volume of 25 µl of 1 x MasterAmpF PCR premix (Epicentre, USA) and 0.5 U
205 of Taq polymerase (Invitrogen, USA). PCR was performed with 5 min initial denaturation at
206 94 °C, followed by a temperature gradient which consisted of 94 °C for 60 s, 11 cycles
207 starting at 62 °C and decrease by 1 °C per cycle, and 60 s final extension at 72 °C.
208 Subsequently 24 cycles followed with annealing at 52 °C for 60 s and a 10-min final
209 extension at 72 °C.

210 For an in depth analysis of community composition, samples from the long-term
211 hydrological restored and drained sites were subjected to NGS (454 pyrosequencing)
212 amplicon sequencing (GS FLX, titanium chemistry; Roche). The barcoded amplicons were
213 obtained in a two-step PCR approach using the primer pair A189f and A650r (table S3). In a
214 first step, *pmoA* genes were amplified in 35 cycles and PCR conditions as described above. In
215 a second step, 1 µl of a 25 fold dilution of PCR product from first step was amplified using
216 the same primers in 25 cycles with an annealing temperature of 52°C. Three reactions were
217 carried out for each sample, pooled, purified via gel extraction and sent for analyses to GATC
218 Biotech, Konstanz, Germany.

219 Sequence reads larger than 400 bp were analyzed as described in (Lüke & Frenzel,
220 2011). In brief, nucleotide sequences were translated into amino acid sequences and reads
221 containing frame shifts were removed from further analysis. Phylogeny was inferred using
222 the Neighbor Joining algorithm with Jukes Cantor correction. Processing and analysis was
223 done using the ARB software (Ludwig, *et al.*, 2004). Nucleotide sequences found in this
224 study were deposited at the EMBL European Nucleotide Archive (ENA) under the study
225 accession number XXXXXXXXX.

226

227 *MOB abundances*

228 We followed total abundances of different MOB groups over the growing season at the short-
229 term flooded and drained reference using quantitative PCR (qPCR). Therefore all four
230 samples were pooled for each site and time point. Type Ia, Ib and type II subgroups were
231 quantified using *pmoA-specific* qPCR assays described by Kolb et al. (2003). Prior to qPCR
232 template was checked for PCR inhibition by template dilution. The three assays were
233 performed in duplicate as described by Pan et al. (2010). In brief, 12.5µl 2 x SensiFAST
234 SYBR No-ROX Kit (Bioline, Germany), 2 µl of diluted DNA template (1 ng per µl) and 0.8
235 mM of each primer were mixed to a total volume of 25µl. The qPCR conditions were as
236 follows: 15 min initial denaturation at 95°C, followed by 45 cycles of denaturation at 95°C
237 for 20 s, annealing at 64°C for 20 s, and extension at 72°C for 45 s. DNA melting curves were
238 analyzed at temperatures ranging from 70 to 99°C and fluorescence was recorded at 84°C. All
239 assays were performed with a Rotor-Gene 6000 thermal cycling system (Corbett Research,
240 Australia). Samples were added to aliquots of the master mixture using the QIAgility liquid
241 handling system (Qiagen, Netherlands). To quantify total copy number of each individual
242 assay the Rotor-Gene Q Series Software (Qiagen, Netherlands) was used.

243 In addition, we tested all samples at the drained sites with and without short-term
244 flooding and at the long-term hydrological restored sites for the putative atmospheric MOB
245 upland soil cluster (USC) α . The primers A189f-Forest675r targeting specifically USC α
246 *pmoA* genes were used (Kolb *et al.*, 2003). We followed the protocol as described in Barbosa
247 and colleagues (2015). These assays were performed with the CFX96 qPCR cycler (Biorad,
248 Germany). To quantify total copy number of each individual assay the Bio-Rad CFX
249 Manager Series (version 3.0.1224.1015) software was used.

250

251 *Statistics*

252 Possible differences in fluxes and kinetics between long-term hydrological restored sites,
253 short-term flooded, and drained sites were evaluated using a two-sided Student's t-test as
254 implemented in the statistical software R (R Development Core Team, 2013).

255

256 **Results**

257 *MOB community composition in long-term hydrological restored and drained peat meadows*

258 We performed Sanger sequencing of *pmoA* gene clone libraries and used the two widely
259 applied reverse primer mb661r and A682r, together still having the highest coverage of
260 aerobic MOB diversity. Members of the genus *Methylocystis* (type II MOB) were the
261 dominant MOB present (Figure 1, Figure S1), independent of agricultural history and
262 management. The reverse primer A682r also covers ammonia monooxygenase genes. At both
263 sites at Stein and for the drained reference site at Horstermeer next to a dominance of type II
264 MOB a large fraction of sequences belonging to the ammonium-oxidizing genus *Nitrosospira*
265 were detected (Figure 1, Figure S1).

266 Since drainage of peat soil can turn this environment with high internal CH₄ supply to a well
267 aerated and dry environment with predominantly sub-atmospheric CH₄ concentrations we

268 looked for the occurrence of putative atmospheric CH₄ oxidizers. We applied the *pmoA*
269 reverse primer A650r to increase the coverage of these putative high affinity MOB in a
270 pyrosequencing approach. In all four sites a large proportion of the putative atmospheric CH₄
271 oxidizers USC α and related were detected but also a small fraction of other putative
272 atmospheric CH₄ oxidizers such as TUSC (Figure 1). The qPCR assay specifically targeting
273 USC α *pmoA* genes revealed a low abundance of those organisms at the studied environments
274 (Figure 2). Similarly to the results of *pmoA* gene clone libraries the 454 pyro-sequencing
275 revealed a large proportion of *pmoA* sequences belonging to the genus *Methylocystis*, except
276 for the drained reference site at Stein (Figure 1).

277

278 *MOB community composition in drained peat meadows with and without short-term flooding*

279 The short-term effect of flooding on the methanotrophic community were quantitatively
280 followed for different sub-populations of MOB over a growing season in drained peat soils
281 with and without a short-term flooding (Figure 3). The qPCR data of the three major group of
282 MOB revealed temporal variability with a peak of all three sub-populations in samples taken
283 in June (Figure 3). Overall, the abundance of type II was a 1000 fold higher compared to type
284 Ia and Ib MOB (Figure 3). The qPCR assay specifically targeting USC α *pmoA* genes showed
285 that USC α were below the detection limit over the whole growing season (Figure 2).

286 We performed an additional Sanger sequencing of *pmoA* gene clone libraries using
287 only the reverse primer mb661r (Figure 1) to get a snapshot of the community composition at
288 the end of the experimental observations in the field. Similarly to clone libraries from long-
289 term hydrological restored and drained sites, the majority of the sequences were assigned to
290 type II MOB of the genus *Methylocystis* (Figure 1). Next to the dominance of type II MOB,
291 sequences were found belonging to type Ib MOB affiliated to the genus *Methylocaldum* at the

292 drained site (Figure 1). In addition, we detected type Ia MOB affiliated to the genus
293 *Methylosarcina* at the short-term flooded site.

294

295 *CH₄ oxidation kinetics*

296 CH₄ oxidation kinetics (i.e. K_{mapp} , V_{max}) were measured once at the long-term hydrological
297 restored and drained sites at Horstermeer and Stein and were followed over the growing
298 season at the drained sites with and without short-term flooding in Zegveld. Overall, V_{max} was
299 comparable between all sites, but K_{mapp} was lower at Horstermeer and Stein (Table 1 & 2).
300 Kinetics measured over the growing season in the short-term flooded soil in Zegveld showed
301 very high variability and were not significantly different based on Student's t-tests. Similarly,
302 calculated specific affinities showed no significant differences but displayed a large
303 variability (Table 1 & 2).

304 Short-term flooding increased CH₄ emissions significantly compared to a drained
305 reference site (Table 3). Once the short-term flooded site was re-drained CH₄ emissions
306 decreased continuously and approximated values measured at the reference site
307 approximately 4 weeks after drainage (Table 3).

308

309 *Spatial distribution of CH₄ assimilation in drained peat meadows with and without short-* 310 *term flooding*

311 Autoradiographic imaging of ¹⁴C-labelled soil cores at the same site incubated under elevated
312 (10.000 ppmv) and under near-atmospheric (10 ppmv) CH₄ concentrations showed that CH₄
313 assimilation was not homogenously distributed but displayed distinct spatial patterns
314 depending on CH₄ supply (Fig. 4). A visual exploration of the autoradiographs displayed a
315 very distinct and actively incorporating community restricted to the top soil under elevated
316 CH₄ concentrations while under near-atmospheric conditions CH₄ assimilation spread over

317 virtually the entire soil profile. These patterns were consistent both for the short-term flooded
318 site and the drained reference site (Figure 4). However, vertical profiles at the short-term
319 flooded site under near-atmospheric conditions displayed pronounced CH₄ uptake more
320 evenly distributed over the whole soil core, reflecting more the soil structure like pore or root
321 channels (Figure 4).

322

323 **Discussion**

324 *MOB community composition in long-term hydrological restored and drained peat meadows*

325 In this study, we compared MOB communities in Dutch peat meadows differing in water
326 table management, agricultural practice, and time since restoration. A key finding is the
327 remarkable dominance of type II MOB of the genus *Methylocystis* in both long-term
328 hydrological restored and drained sites (Figure 1, Figure S1). As shown by stable isotope
329 probing and metagenomic analyses *Methylocystis* is known to be the active and the
330 predominant MOB in acidic peatlands (Chen, *et al.*, 2008, Chen, *et al.*, 2008). Similarly, it
331 has been observed that all *pmoA* sequences along a water table drawdown gradient of a fen
332 ecosystem were assigned to the genus *Methylocystis* (Yrjala, *et al.*, 2011). In another study,
333 Juottonen and colleagues (2012) demonstrated a prevalence of *Methylocystis* species based
334 on DGGE analyses in 10-12 years restored and forestry-drained peatlands. Hence, the genus
335 *Methylocystis* seems to be commonly inhabiting peat soils. In this study it remains a
336 dominant MOB even after drastic land use changes. This finding is somewhat opposed to the
337 general observation from previous meta-analysis which demonstrated that overall microbial
338 community composition and functioning to be very sensitive to environmental
339 perturbation/alteration (Griffiths & Philippot, 2012, Shade, *et al.*, 2012).

340 A possible explanation for the strong resilience of the genus *Methylocystis* can be
341 found in their metabolic flexibility. Dunfield and colleagues (1999) demonstrated

342 consumption of atmospheric CH₄ by *Methylocystis* LR1 after long-term cultivation under low
343 CH₄ availability, later *Methylocystis* SC2 was found which harbors two isoenzymes of the
344 particulate methane monooxygenase, one oxidizing CH₄ at mixing ratios > 600 ppmV and a
345 second one oxidizing CH₄ at mixing ratios of < 100 ppmV (Baani & Liesack, 2008). In
346 addition, some *Methylocystis* species have been shown to be facultative MOB able to grow
347 on acetate and ethanol (Belova, *et al.*, 2011, Im, *et al.*, 2011). It has been suggested that this
348 trait enables them to survive in environments with fluctuating or limited CH₄ supply by using
349 the reducing power obtained from acetate oxidation to keep pMMO functioning at
350 atmospheric CH₄ concentrations.

351 A second finding from our study is the detection of putative atmospheric MOB (USC
352 α) in drained peat meadows and in long-term hydrological restored sites. However, a qPCR
353 assay targeting specifically USC α revealed low signals suggesting a minor role of this group
354 of organisms. In addition the clade MHP has been suggested to be involved in the oxidation
355 of atmospheric CH₄ in acidic upland meadows (discussed in Chen, *et al.*, 2008).

356

357 *MOB community composition in drained peat meadows with and without short-term flooding*

358 Next to the long-term hydrological restored and drained sites we also looked at short-term
359 effects of rewetting/flooding of drained peat soils. Monitoring the abundance of different sub-
360 groups of MOB by qPCR showed that the abundance of type II was a 1000-fold higher
361 compared to type Ia and Ib MOB. In line with Sanger sequencing results it suggests that also
362 in these peat soils type II and *Methylocystis* still persisted after land-use change. The genus
363 *Methylosarcina* can be very responsive within flooding gradients and therefore a short-term
364 flooding pulse may have favored the occurrence of the organisms (Bodelier, *et al.*, 2012). The
365 occurrence of the genus *Methylocaldum* at the drained reference site is most likely the result
366 of fertilization effects. During the growing season the reference site was fertilized with

367 manure and *Methylocaldum* has been clearly shown to be stimulated by nitrogen fertilization
368 (Noll, *et al.*, 2008).

369

370 *CH₄ oxidation kinetics*

371 The apparent half-saturation constant (K_{mapp}) can be used as indicator of the concentration
372 range of CH₄ at which the targeted MOB can be active. In CH₄-rich environments, CH₄ is
373 oxidized with a low apparent affinity ($K_{mapp} > 1 \mu\text{M}$), while in dry, well aerated environments
374 CH₄ is oxidized with a high apparent affinity (K_{mapp} ranges from 0.03–0.05 μM) (Bender &
375 Conrad, 1992). In addition, intermediate kinetic values have been observed for several
376 hydromorphic soils with a K_{mapp} higher than in most upland soils ($> 0.1 \mu\text{M}$) but lower than in
377 wetlands (Knief, *et al.*, 2006).

378 The CH₄ oxidation kinetics measured in rewetted and drained sites depicted large
379 dynamics. The K_{mapp} of rewetted and drained sites were in the range of many cultivated type I
380 and type II MOB, including *Methylocystis* species (2.2 – 10.3 μM) (Knief & Dunfield, 2005).
381 Observed kinetics value showed no indication for high affinity CH₄ oxidation. However, in
382 another study we demonstrated that metabolic traits such as CH₄ oxidation kinetics are not
383 phylogenetically conserved (Krause, *et al.*, 2014) which likely prevents the use of the
384 observed kinetics as a proxy for activity of specific MOB in field samples.

385

386 *CH₄ fluxes in drained peat meadows with and without short-term flooding*

387 Our results showed that short-term flooding (12-week stable water table) in an agriculturally
388 used peat land temporary increased CH₄ emissions. During and immediately after the
389 flooding period field fluxes were between 45 to 347 $\text{mg m}^{-2} \text{day}^{-1}$ which is in a similar range
390 as measured in previous studies for long-term water-table managed sites in Stein (528 mg m^{-2}
391 day^{-1}) and Horstermeer (331.2 $\text{mg m}^{-2} \text{day}^{-1}$) (Hendriks, *et al.*, 2007, Schrier-Uijl, *et al.*,

392 2010). In addition, observed values were comparable to natural wetlands with measured
393 fluxes between 72 to 1184 mg m⁻² day⁻¹ (Juottonen, *et al.*, 2012).

394

395 *Spatial distribution of CH₄ assimilation in drained peat meadows with and without short-*
396 *term flooding*

397 Restoring agriculturally used peat land back to natural wetlands turned a potential sink into a
398 source of CH₄. To further investigate the response of indigenous MOB to different CH₄
399 concentrations, we varied CH₄ availability and incubated soil cores under elevated (10.000
400 ppmV) or near-atmospheric (10 ppmV) CH₄ conditions.

401 Under elevated CH₄ concentrations, most ¹⁴CH₄ was assimilated in the top soil layers. This
402 suggests the presence of a community with the capacity to consume the offered CH₄
403 completely at these concentrations occurring usually in soil with an internal CH₄ sources e.g.
404 when flooded. The MOB present act as an efficient “filter” that reduces CH₄ emissions.

405 Under near-atmospheric CH₄ concentrations, ¹⁴CH₄ assimilation was found over the entire
406 soil profile, reflecting a community which can assimilate atmospheric CH₄ but with a
407 capacity below the diffusion of CH₄ into the soil. This community presumably is active when
408 the soil water table is lower. Putative atmospheric MOB (e.g. USC α) are likely not the
409 responsible organisms here because a USC α specific qPCR assay did not give any signals.

410 We think in line with previous studies (Kolb & Horn, 2012) that *Methylocystis* species found
411 at these sites may be capable of oxidizing CH₄ over a large range clearly distinguishing this
412 genus from other cultivated MOB.

413

414 *Critical remarks and Conclusions*

415 We applied a comprehensive set of tools to compare diversity and activity of aerobic MOB in
416 drained and rewetted peat meadows with different water table management and different

417 agricultural practice but we may have missed some groups of MOB. Using our approach we
418 cannot detect methanotrophic Verrucomicrobia and MOB containing only a soluble CH₄
419 monooxygenases (sMMO). To best of our knowledge Verrucomicrobia have only been
420 detected in wetlands with a pH between 1.8 and 5.0 (Sharp, *et al.*, 2014) but our sampling
421 sites were less acidic (Table S1, S2) which suggest that they do not play a major role here.
422 From the sMMO containing MOB we additionally tested for the widely occurring genus
423 *Methylocella* (Rahman, *et al.*, 2011) but they were not detected by PCR using *Methylocella*
424 specific primers (data not shown). Next to aerobic MOB, recent studies have shown that
425 anaerobic CH₄ oxidation coupled to denitrification is more widely distributed and may play a
426 significant role as additional CH₄ sink in environments such as wetlands (Hu, *et al.*, 2014)
427 and lake sediments (Deutzmann, *et al.*, 2014).

428 Nevertheless, our findings conclusively show that rewetting and short-term flooding
429 of agriculturally used peat meadows are comprised of a fundamentally similar
430 methanotrophic community. The present MOB community members appear to process CH₄
431 at a wide range of concentrations that naturally occur due to water-table changes and
432 associated changes in O₂ availability and methanogenesis. The strong dominance of type II
433 aerobic MOB (*Methylocystis* sp.) suggests a very pronounced flexibility and persistence of
434 this group under land use change.

435

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443

444 **Conflict of interest**

445 The authors declare no conflict of interest.

446

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Table 1. Apparent enzyme kinetics and specific affinities from CH₄ oxidation of the methanotrophic community at long-term hydrological restored peat meadows in October 2010 (n=5; ± standard deviations). K_{mapp} is expressed in μM, V_{max} in μmol h⁻¹ g⁻¹ dry weight of soil and specific affinities in L h⁻¹ g⁻¹ dry weight of soil.

Site		Stein	Horstermeer
Apparent enzyme kinetics			
Drained site	V_{max}	0.11 ± 0.10	0.30 ± 0.20
	K_{mapp}	3.79 ± 1.94	4.94 ± 4.43
Restored site	V_{max}	0.32 ± 0.20	0.45 ± 0.29*
	K_{mapp}	7.72 ± 3.90	9.05 ± 5.30*
Specific affinities			
Drained site		0.03 ± 0.02	0.10 ± 0.09
Restored site		0.04 ± 0.02	0.05 ± 0.01*

647 * (n=3; ± standard deviations)
648

649 **Table 2.** Apparent enzyme kinetics and specific affinities from CH₄ oxidation of the
 650 methanotrophic community at drained after short-term flooding and drained peat meadows
 651 over the growing season in 2011 (n=4; ± standard deviations). K_{mapp} is expressed in μM, V_{max}
 652 in μmol h⁻¹ g⁻¹ dry weight of soil and specific affinities in L h⁻¹ g⁻¹ dry weight of soil.

Site		May 9	May 22	June 7	July 5	August 9
Apparent enzyme kinetics						
Drained site	V_{max}	n.d.	0.34 ± 0.17	0.18 ± 0.14	0.49 ± 0.42	0.34 ± 0.16
	K_{mapp}	n.d.	63.60 ± 71.16	35.01 ± 23.98	40.34 ± 34.14	21.97 ± 7.00
Short-term flooded site	V_{max}	n.d.	0.61 ± 0.24	0.36 ± 0.23	0.44 ± 0.42	0.47 ± 0.26
	K_{mapp}	n.d.	35.51 ± 35.95	35.40 ± 6.69	51.61 ± 43.13	12.99 ± 2.74
Specific affinities						
Drained site			0.010 ±	0.005 ±		
		n.d.	0.009	0.001	0.012 ± 0.002	0.013 ± 0.006
Short-term flooded site			0.051 ±	0.010 ±		
		n.d.	0.073	0.005	0.009 ± 0.001	0.031 ± 0.006

653

654 **Table 3.** CH₄ emission (in mg per m² per day) over the growing season in 2011 at Zegveld

Site	May 9	May 22	June 7	July 5	August 9
Drained site	16.39 ± 28.54	13.64 ± 11.29	61.06 ± 66.12	10.02 ± 14.23	45.51 ± 2.92
Short-term flooded site	195.98 ± 150.71	172.49 ± 86.94	129.50 ± 100.39	35.21 ± 51.99	74.68 ± 68.55

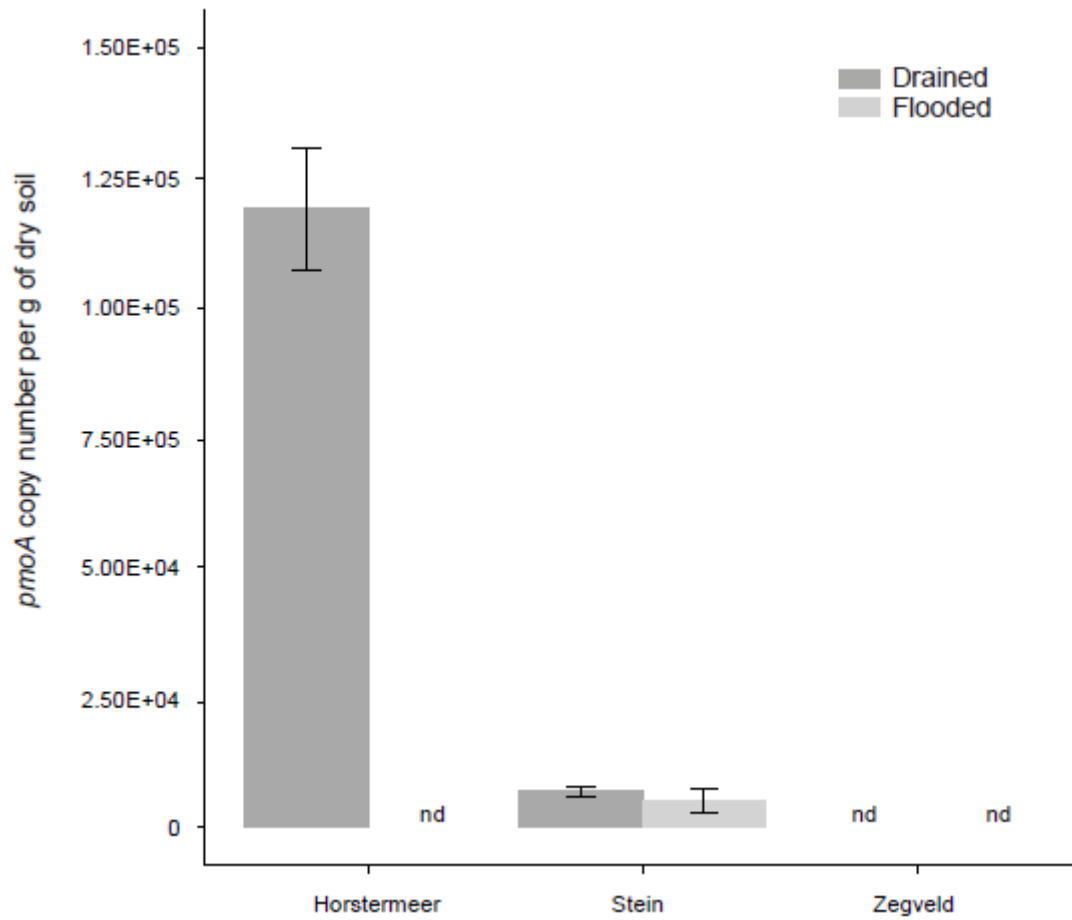
655 sites (n=8, standard deviation). Values in bold are significantly different at $P < 0.05$.

656

657

664 pyrosequencing, colored circles represent total number of clones retrieved from Sanger
665 sequencing. Sequences were obtained by PCR using different reverse primers (mb661R,
666 A682R). Sanger sequencing results for the long-term hydrological restored sites and drained
667 reference site were combined and mapped onto the tree (blue/red circles). Individual
668 proportions for each of the reverse primers (mb661R, A682R) are displayed in figure S1.
669 Lineages lacking isolates are named according to representative clones or to the environment
670 in which they were predominantly or initially found (RPC, rice paddy cluster; USC, upland
671 soil cluster; MHP, Moor House Peat; JR, Jasper Ridge; TUSC, tropical upland soil cluster).
672 The scale bar represents 0.1 changes per amino acid position.

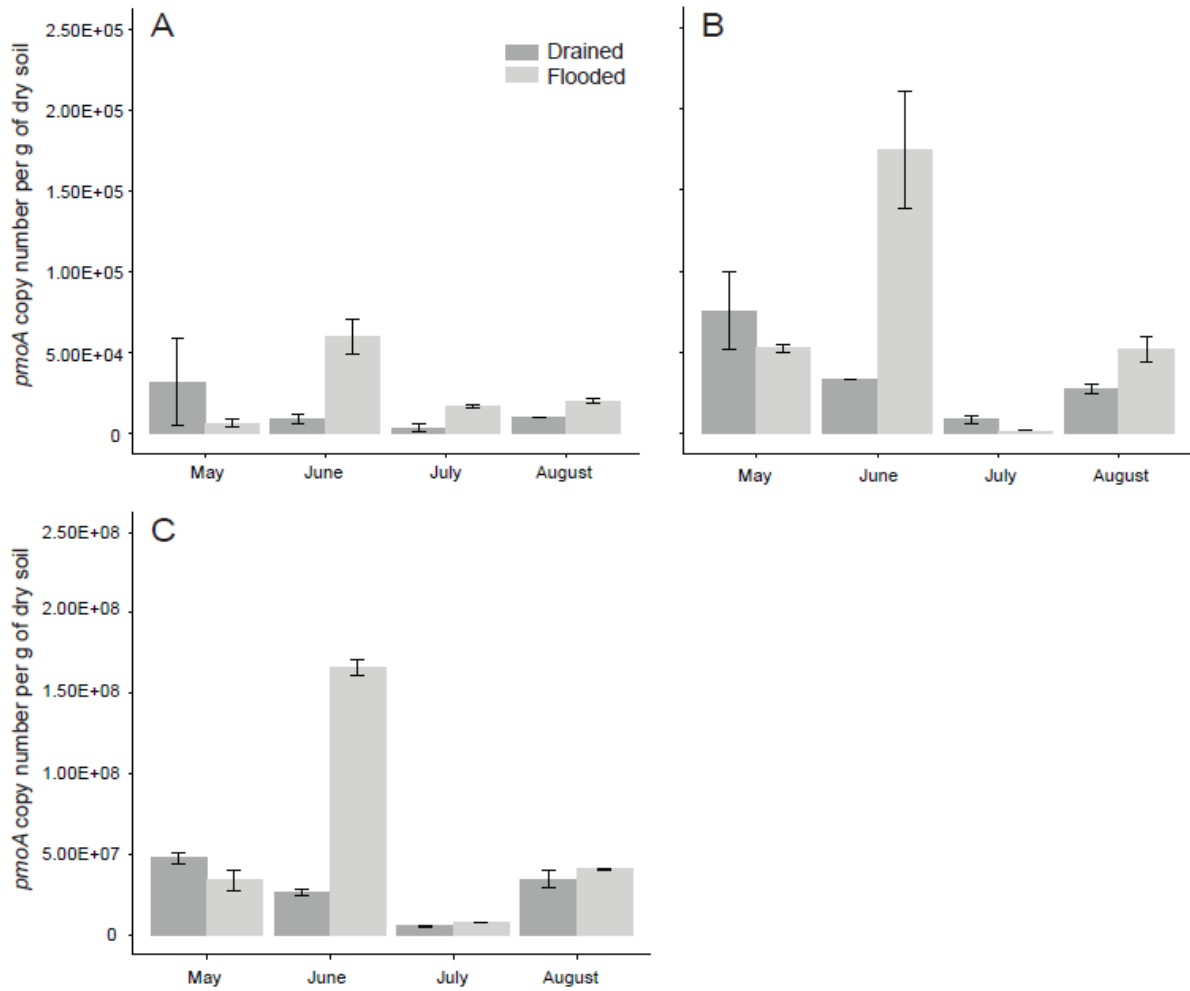
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675 **Figure 2.** *pmoA* gene copy numbers of the USC α MOB subgroup at different study sites
 676 (error bar = standard deviation). Dark grey shaded bars represent the drained reference site
 677 and light grey shaded bars the restored or short-term flooded site (in case of Zegveld). nd
 678 indicates that the target gene was below the detection limit of the qPCR assay. The limit of
 679 quantification is 5 target gene copies per reaction. Note for Zegveld samples that only the last
 680 time point is shown because results were below detection limit in all samples.

681



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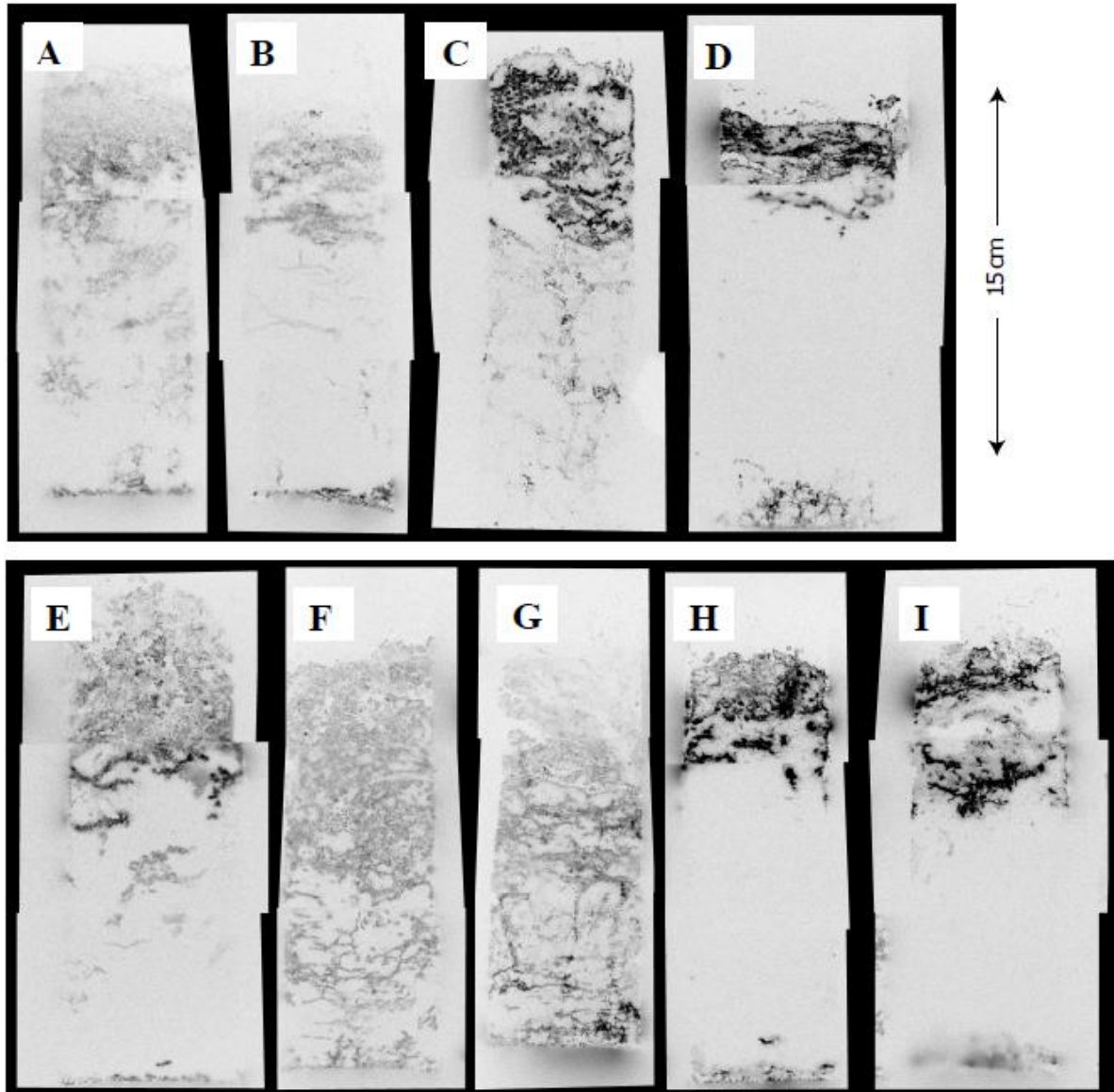
683 **Figure 3.** *pmoA* gene copy numbers of type Ia (A), type Ib (B) and type II (C) MOB

684 subgroups over the growing season at drained sites with and without short-term flooding (n =

685 2, error bar = standard deviation). Dark grey shaded bars represent the drained reference site

686 and light grey shaded bars the short-term flooded site.

687



688

689 **Figure 4.** Autoradiographs of $^{14}\text{CH}_4$ -labelled soil core sections from the drained reference
690 site (A-D) and short-term flooded (E-I). Soil cores were labeled with 10 ppmv (A-B, E-G) or
691 with 10000 ppmv of ^{14}C -labelled CH_4 (C-D, H-I). 15 cm cores were used. For each core the
692 three resulting sections of 5 cm height are displayed. For the short-term flooded site three
693 replicates were taken, all others were in duplicates.

694