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

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Abstract

The restoration of peatlands is an important strategy to counteract subsidence and loss of biodiversity. However, responses of important microbial soil processes are poorly understood. We assessed functioning, diversity, and spatial organization of methanotrophic communities in drained and rewetted peat meadows with different water table management and agricultural practice. Results show that the methanotrophic diversity was similar between drained and rewetted sites with a remarkable dominance of the genus *Methylocystis*. Enzyme kinetics depicted no major differences, indicating flexibility in the methane (CH_4) concentrations that can be used by the methanotrophic community. Short-term flooding led to temporary elevated CH_4 emission but neither to major changes in abundances of MOB nor in CH_4 consumption kinetics in drained agriculturally used peat meadows. Radiolabelling and autoradiographic imaging of intact soil cores revealed a markedly different spatial arrangement of the CH_4 consuming zone in cores exposed to near-atmospheric and elevated CH_4. The observed spatial patterns of CH_4 consumption in drained peat meadows with and without short-term flooding highlighted the spatial complexity and responsiveness of the CH_4 consuming zone upon environmental change. The methanotrophic microbial community is not generally altered and harbors MOB that can cover a large range of CH_4 concentrations offered due to water-table fluctuations, effectively mitigating CH_4 emissions.

Introduction

Methane (CH_4) is an important greenhouse gas (GHG) that contributes significantly (17%) to the total anthropogenic radiative forcing of 2.83 W m^{-2} (Myhre, *et al.*, 2013). However, the annual growth rate of atmospheric CH_4 varied largely over the last 30 years (Kirschke, *et al.*, 2013). The causes for these anomalies are not fully understood but may be linked to changes in wetland CH_4 emissions such as: changes in wetland area, wetland management (e.g. fertilizer use in rice cultivation), and climatic effects affecting water table and temperature (Bousquet, *et al.*, 2006, Kai, *et al.*, 2011, Spahni, *et al.*, 2011, Kirschke, *et al.*, 2013, Turetsky, *et al.*, 2014).

In recent years, many peatlands were restored to natural wetlands to counteract subsidence of the peat soil (Verhoeven & Setter, 2010) and the loss of wetland biodiversity (Quesnelle, *et al.*, 2013). A primary restoration strategy is to raise the water table and adapt land use, i.e. reduce or abandon agriculture. While these strategies were successful in increasing the abundance of wetland birds species (Tozer, *et al.*, 2010), their consequences for GHG emissions are unclear. Rewetting likely reduces total C losses, but CH_4 emission may increase (Hendriks, *et al.*, 2007).

Next to anaerobic CH_4 oxidation, aerobic methane-oxidizing bacteria (MOB) play a key role in mitigating atmospheric CH_4 emissions. In environments such as wetlands, MOB act as a bio-filter mitigating the release of CH_4 from soil-internal sources to the atmosphere by approximately 40% (Frenzel, 2000). In environments such as dry forest and dry grassland, MOB with deviating oxidation kinetics than in wetlands (Bender & Conrad, 1992, Knief, *et al.*, 2006) contribute 6% to the global atmospheric CH_4 sink (Denman, *et al.*, 2007).

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

found within the family *Methylococcaceae*. Based on different physiological, biochemical, phenotypical characteristics and phylogeny, proteobacterial MOB have been classified into type Ia, type Ib and type II (Hanson & Hanson, 1996, Bodrossy, *et al.*, 2003). Today, they are mainly grouped taxonomically into Gamma- and Alphaproteobacteria, respectively. MOB oxidize CH_4 via methanol to carbon dioxide. The *pmoA* gene encodes a subunit of the particulate CH_4 monooxygenase (pMMO), a key enzyme catalyzing the first step in the aerobic CH_4 oxidation pathway of MOB. The *pmoA* gene is present in the majority of MOB and therefore has been widely used as phylogenetic and functional marker to target these organisms from the environment (McDonald, *et al.*, 2008).

MOB that are capable of oxidizing CH_4 at atmospheric concentration have not been cultivated yet. However, *pmoA* sequence analyses of soils showing atmospheric CH_4 uptake revealed distinct clusters, termed upland soil cluster (USC) α and γ (Holmes, *et al.*, 1999, Henckel, *et al.*, 2000, Knief, *et al.*, 2003). While the closest relative of USC α is member of the genus *Methylocapsa* (Ricke, *et al.*, 2005), USC γ is more closely related to the family Methylococcaceae (Knief, *et al.*, 2003). Next to these dominant groups other putative atmospheric MOB have been detected (Horz, *et al.*, 2005, reviewed in Kolb, 2009).

Rewetting agriculturally used peat will affect methanotrophic communities because a well aerated and dry environment with predominantly sub-atmospheric CH_4 concentrations is converted back to a soil environment with high internal CH_4 supply. It currently is unclear whether the original methanotrophic communities largely persist and or whether a new microbial community with new dominant species establishes, with possible consequences for CH_4 cycling. To investigate this we focused on MOB communities in Dutch peat meadows differing in water table management, agricultural practice, and restoration time. MOB community composition as well as CH_4 oxidation kinetics in drained and rewetted peat soils was compared, comprising both environments with high internal CH_4 supply and atmospheric

95 levels of CH_4. Spatial organization of aerobic CH_4 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 ^{14}C- CH_4 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 CH_4 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 m^2, which were
132 marked with bamboo sticks for re-sampling over the season. In each plot, CH_4 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 m^2 area and prepared in the same way as described above. In total, per sampling event and
136 per site we obtained 8 samples and CH_4 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 *CH_4 oxidation kinetics*
141 Apparent enzyme kinetics, i.e. the apparent half-saturation constant (K_{mapp}) and the
142 maximum rate of CH_4 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

serum flasks capped with a butyl rubber stopper (Sigma Aldrich). CH_4 was added to the headspace to achieve mixing ratios of approximately 50, 100, 500, 1000, 5000, 10.000 and 20.000 (only for long-term hydrological restored sites) ppmv. Soil slurries were incubated on a rotary shaker (120 rpm) in the dark at room temperature. CH_4 consumption was monitored by GC-FID analysis (HP 5890 Gas Chromatograph, Hewlett-Packard) over a period of 1-4 days including 5-12 measurements. Individual CH_4 oxidation rates for each concentration per sample were calculated by linear regression. V_{max} and K_{mapp} were derived from nonlinear regression using the nlstools package as implemented in the statistical software R (R Development Core Team, 2013).

CH_4 flux field measurements at the drained sites with and without short-term flooding

CH_4 fluxes were monitored over the growing season in 2011. Measurements were taken in May before the removal of water, three days after removal of water and in the beginning of June, July and August. We used acrylic cylinders (40 cm length, 10 cm diameter) equipped with two-way sampling ports through which headspace gas samples could be collected. These samples were transferred into evacuated glass vials with a rubber stopper (Terumo, Belgium) and analyzed for CH_4 concentration by GC-FID (Ultra GC gas chromatograph, Interscience, Breda, the Netherlands, 30m x 0.32mm ID Rt-Q-Bond capillary column). CH_4 fluxes were derived from the linear increase in CH_4 concentration in the headspace.

$^{14}CH_4$ labelling of cores; preparation of soil sections and imaging at the drained sites with and without short-term flooding

Both from the control as well as the temporarily flooded site in Zegveld, two pairs of cores were taken in October 2011, at the end of the growing season. One soil core per pair was labelled with ^{14}C- CH_4 under elevated (10.000 ppmv) while the other was incubated under

near-atmospheric (10 ppmv) CH_4 concentrations. An additional soil core from the temporarily flooded site was incubated at near-atmospheric conditions. The procedure was performed as described in Stiehl-Braun and colleagues (2011). In brief, soil cores were sampled by using polyethylene tubes (16 cm length, 5.7 cm diameter) and labeled with a total activity of ca. 500 kBq $^{14}CH_4$. Labelling was carried out in gas-tight jars, for seven days. CH_4 headspace concentrations were monitored, and unlabeled CH_4 added to keep concentrations close to the target values of 10 or 10.000 ppmv. To fix and solidify, the labeled soil cores were freeze-dried and impregnated with epoxy resin, using a vacuum chamber, the fixed soil cores were then cut horizontally into three sections of 5 cm height, and a vertical slice of approximately 1 cm thickness cut from the center of each of these cylindrical sections, using a diamond circular saw. These sections were mounted on 3 mm glass slides with epoxy resin, and levelled with a diamond cup mill. Then, autoradiographs of the soil sections were obtained by exposing phosphor imaging plates for 5 days. The imaging plates were scanned by red-excited blue fluorescence scanning at a resolution of 50 µm. The images from the three slides per core were then recomposed using MATLABs image processing toolbox.

MOB community composition

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

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

MOB abundances

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

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

Statistics

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

Results

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

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

Since drainage of peat soil can turn this environment with high internal CH_4 supply to a well aerated and dry environment with predominantly sub-atmospheric CH_4 concentrations we

looked for the occurrence of putative atmospheric CH_4 oxidizers. We applied the *pmoA* reverse primer A650r to increase the coverage of these putative high affinity MOB in a pyrosequencing approach. In all four sites a large proportion of the putative atmospheric CH_4 oxidizers USC α and related were detected but also a small fraction of other putative atmospheric CH_4 oxidizers such as TUSC (Figure 1). The qPCR assay specifically targeting USC α *pmoA* genes revealed a low abundance of those organisms at the studied environments (Figure 2). Similarly to the results of *pmoA* gene clone libraries the 454 pyro-sequencing revealed a large proportion of *pmoA* sequences belonging to the genus *Methylocystis*, except for the drained reference site at Stein (Figure 1).

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

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

We performed an additional Sanger sequencing of *pmoA* gene clone libraries using only the reverse primer mb661r (Figure 1) to get a snapshot of the community composition at the end of the experimental observations in the field. Similarly to clone libraries from long-term hydrological restored and drained sites, the majority of the sequences were assigned to type II MOB of the genus *Methylocystis* (Figure 1). Next to the dominance of type II MOB, 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 *CH_4 oxidation kinetics*
296 CH_4 oxidation kinetics (i.e. $K_{m\text{app}}$, 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 $K_{m\text{app}}$ 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 CH_4 emissions significantly compared to a drained
305 reference site (Table 3). Once the short-term flooded site was re-drained CH_4 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 CH_4 assimilation in drained peat meadows with and without short-*
310 *term flooding*
311 Autoradiographic imaging of ^{14}C-labelled soil cores at the same site incubated under elevated
312 (10.000 ppmv) and under near-atmospheric (10 ppmv) CH_4 concentrations showed that CH_4
313 assimilation was not homogenously distributed but displayed distinct spatial patterns
314 depending on CH_4 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 CH_4 concentrations while under near-atmospheric conditions CH_4 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 CH_4 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 CH_4 by *Methylocystis* LR1 after long-term cultivation under low
343 CH_4 availability, later *Methylocystis* SC2 was found which harbors two isoenzymes of the
344 particulate methane monooxygenase, one oxidizing CH_4 at mixing ratios > 600 ppmV and a
345 second one oxidizing CH_4 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 CH_4 supply by using
349 the reducing power obtained from acetate oxidation to keep pMMO functioning at
350 atmospheric CH_4 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 CH_4 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

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

CH_4 oxidation kinetics

The apparent half-saturation constant (K_{mapp}) can be used as indicator of the concentration range of CH_4 at which the targeted MOB can be active. In CH_4-rich environments, CH_4 is oxidized with a low apparent affinity (K_{mapp} > 1 μM), while in dry, well aerated environments CH_4 is oxidized with a high apparent affinity (K_{mapp} ranges from 0.03–0.05 μM) (Bender & Conrad, 1992). In addition, intermediate kinetic values have been observed for several hydromorphic soils with a K_{mapp} higher than in most upland soils (> 0.1 μM) but lower than in wetlands (Knief, *et al.*, 2006).

The CH_4 oxidation kinetics measured in rewetted and drained sites depicted large dynamics. The K_{mapp} of rewetted and drained sites were in the range of many cultivated type I and type II MOB, including *Methylocystis* species (2.2 – 10.3 μM) (Knief & Dunfield, 2005). Observed kinetics value showed no indication for high affinity CH_4 oxidation. However, in another study we demonstrated that metabolic traits such as CH_4 oxidation kinetics are not phylogenetically conserved (Krause, *et al.*, 2014) which likely prevents the use of the observed kinetics as a proxy for activity of specific MOB in field samples.

CH_4 fluxes in drained peat meadows with and without short-term flooding

Our results showed that short-term flooding (12-week stable water table) in an agriculturally used peat land temporary increased CH_4 emissions. During and immediately after the flooding period field fluxes were between 45 to 347 mg m^{-2} day^{-1} which is in a similar range as measured in previous studies for long-term water-table managed sites in Stein (528 mg m^{-2} day^{-1}) and Horstermeer (331.2 mg m^{-2} 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 CH_4 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 CH_4. To further investigate the response of indigenous MOB to different CH_4
399 concentrations, we varied CH_4 availability and incubated soil cores under elevated (10.000
400 ppmV) or near-atmospheric (10 ppmV) CH_4 conditions.
401 Under elevated CH_4 concentrations, most $^{14}CH_4$ was assimilated in the top soil layers. This
402 suggests the presence of a community with the capacity to consume the offered CH_4
403 completely at these concentrations occurring usually in soil with an internal CH_4 sources e.g.
404 when flooded. The MOB present act as an efficient "filter" that reduces CH_4 emissions.
405 Under near-atmospheric CH_4 concentrations, $^{14}CH_4$ assimilation was found over the entire
406 soil profile, reflecting a community which can assimilate atmospheric CH_4 but with a
407 capacity below the diffusion of CH_4 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 CH_4 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 CH_4
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 CH_4 oxidation coupled to denitrification is more widely distributed and may play a
426 significant role as additional CH_4 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 CH_4
431 at a wide range of concentrations that naturally occur due to water-table changes and
432 associated changes in O_2 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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Conflict of interest

The authors declare no conflict of interest.

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642
643 **Table 1.** Apparent enzyme kinetics and specific affinities from CH_4 oxidation of the
644 methanotrophic community at long-term hydrological restored peat meadows in October
645 2010 (n=5; ± standard deviations). K_{mapp} is expressed in µM, V_{max} in µmol h^{-1} g^{-1} dry weight
646 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 CH_4 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		n.d.	0.010 ± 0.009	0.005 ± 0.001	0.012 ± 0.002	0.013 ± 0.006
Short-term flooded site		n.d.	0.051 ± 0.073	0.010 ± 0.005	0.009 ± 0.001	0.031 ± 0.006

653

654 **Table 3.** CH_4 emission (in mg per m^2 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

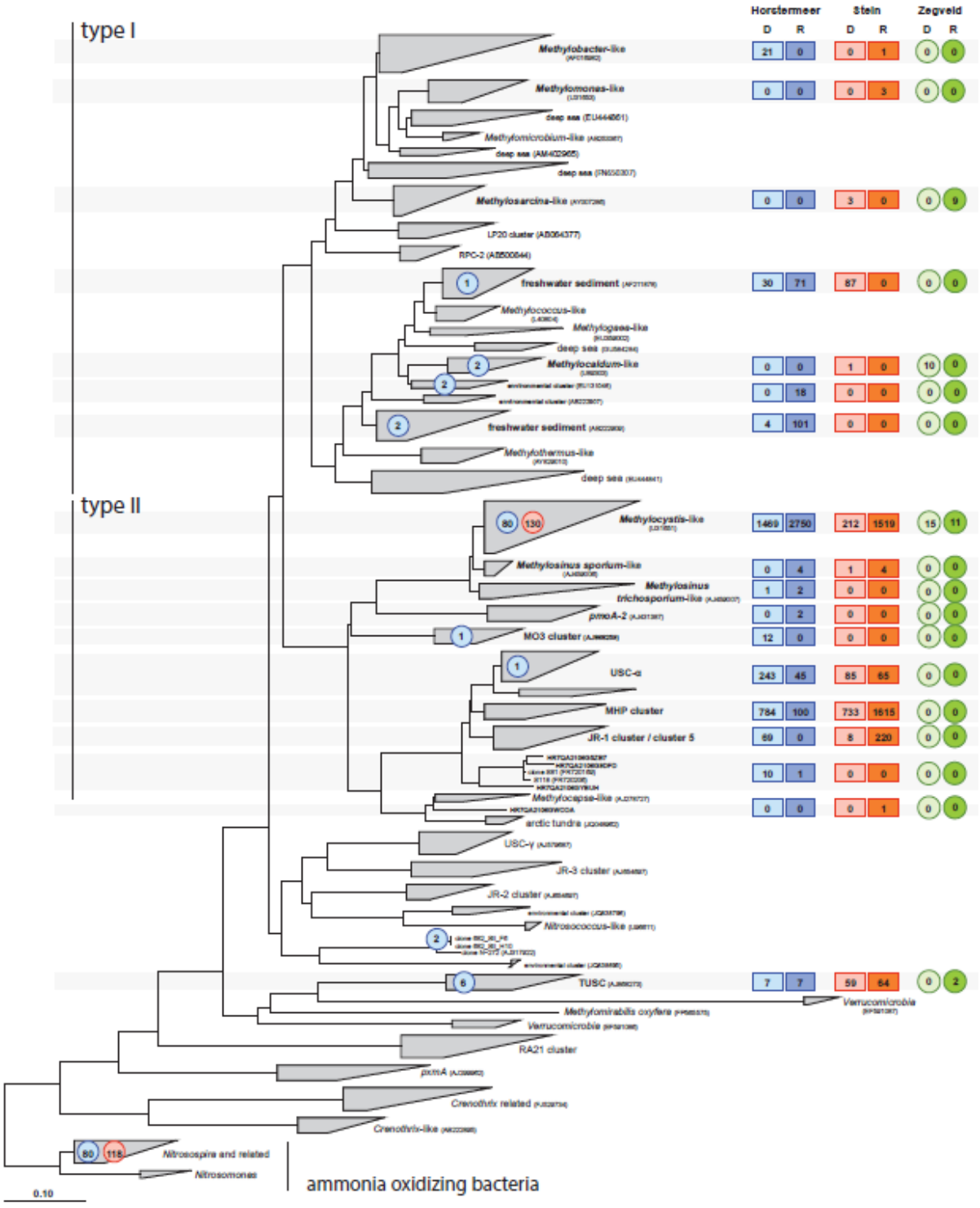
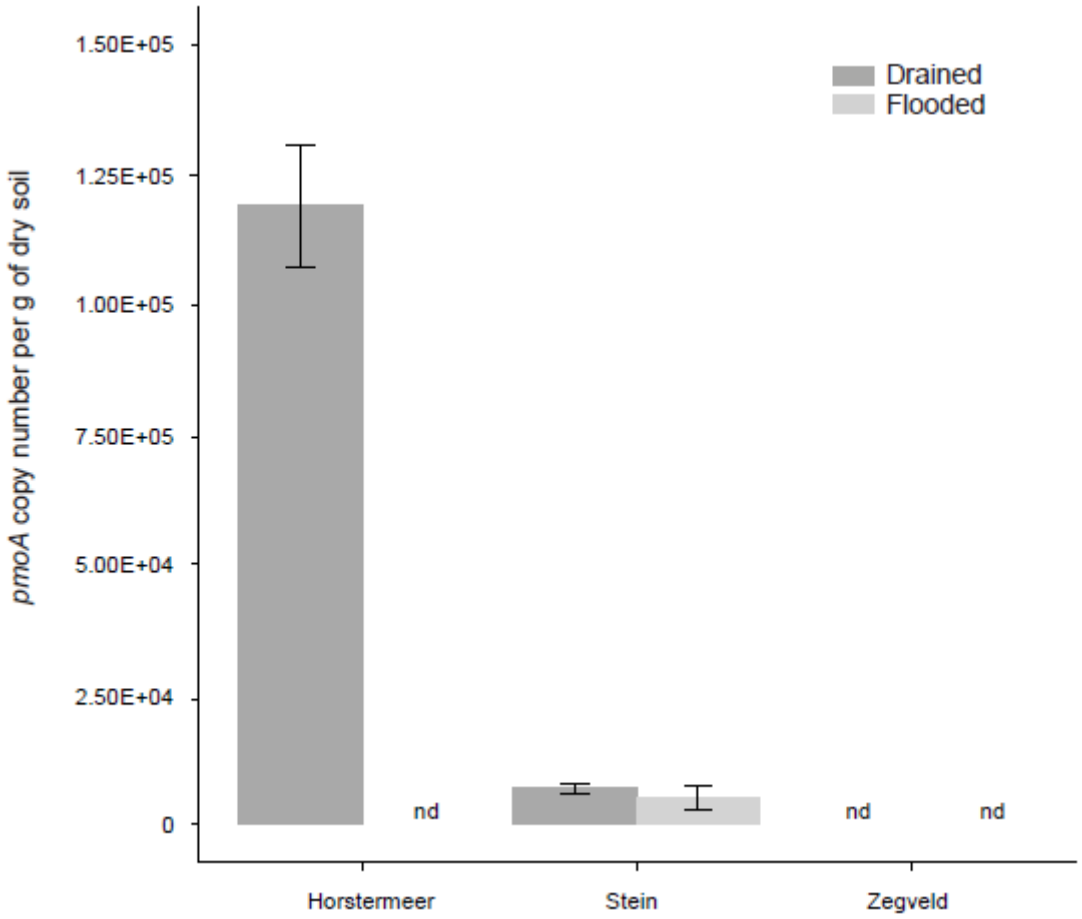


Figure 1. Neighbor-joining tree of *pmoA* sequences obtained by Sanger and 454-pyrosequencing in long-term hydrological restored sites and drained reference site (Horstermeer and Stein) as well as by Sanger sequencing from clone libraries at drained sites with and without short-term flooding (Zegveld); D = drained reference site, R =restored site. Colored boxes next to *pmoA* lineages represent total number of reads from 454-

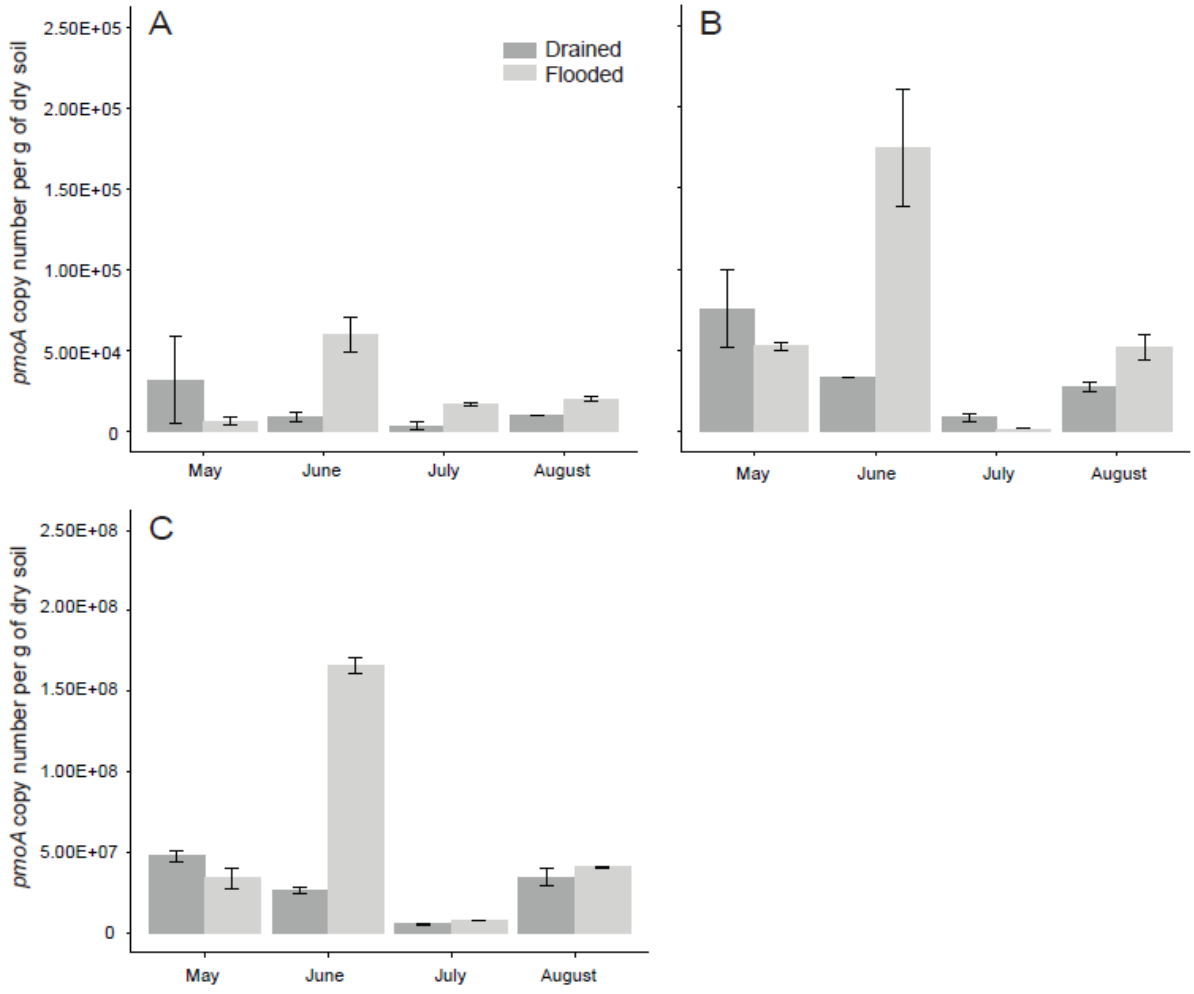
664 pyrosequencing, colored circles represent total number of clones retrieved from Sanger
665 sequencing. Sequences were obtained by PCR using different reverse primers (mb661R,
666 A682R). Sanger sequencing results for the long-term hydrological restored sites and drained
667 reference site were combined 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.
673



674

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



682

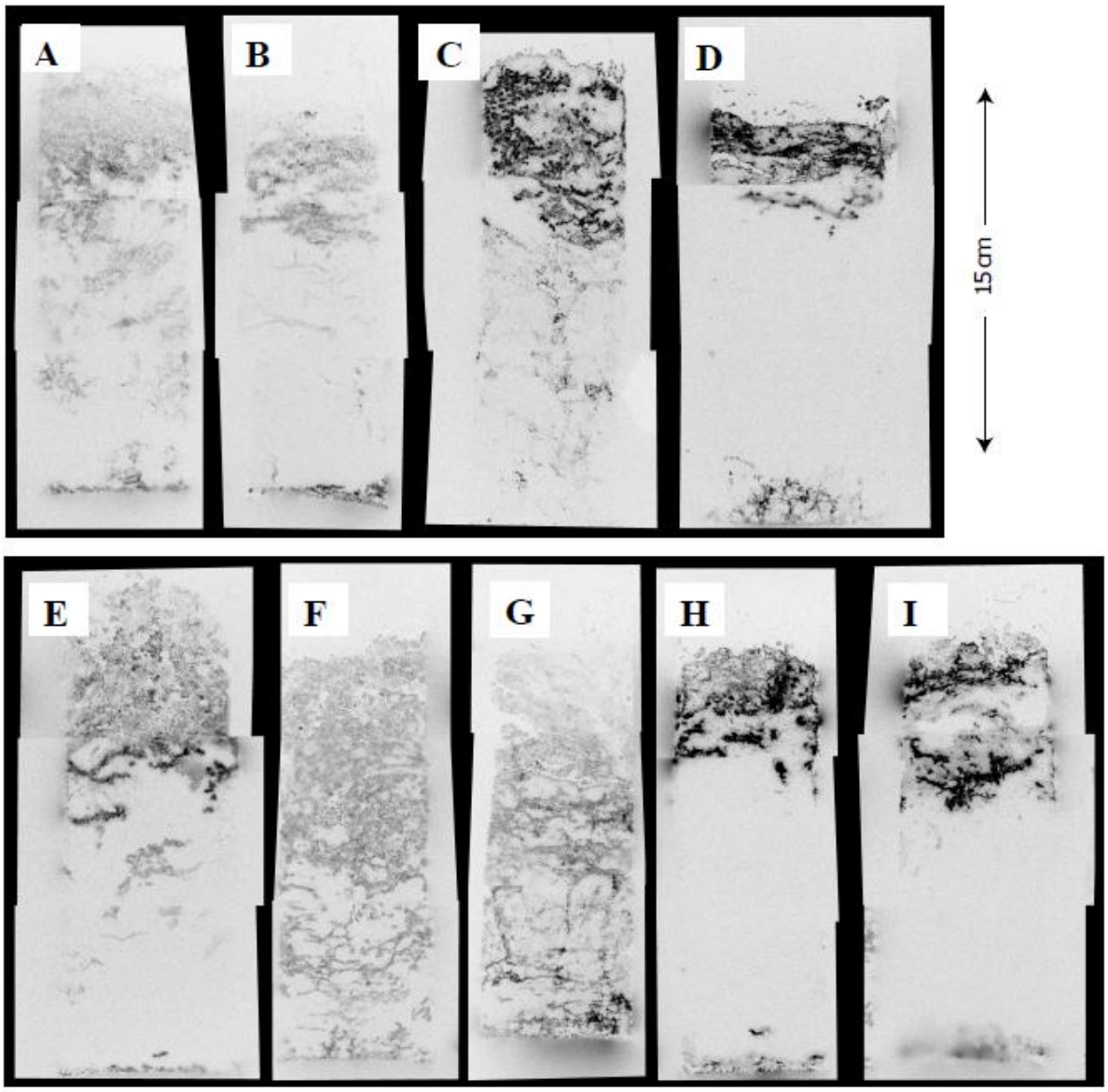
683 **Figure 3.** *pmoA* gene copy numbers of type Ia (A), type Ib (B) and type II (C) MOB

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 $^{14}CH_4$-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