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Research Article

RESEARCH ARTICLE

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

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One sentence summary: The authors assess functioning, diversity, and spatial organization of methanotrophic communities in drained and rewetted peat meadows with different water table management and agricultural practice.

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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 to neither major changes in abundances of methane-oxidizing bacteria (MOB) nor major changes in CH_4 consumption kinetics in drained agriculturally used peat meadows. Radiolabeling 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.

Keywords: restoration; ^{14}C labelling; atmospheric CH_4; spatial micro-distribution; peat land; pyrosequencing

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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 has 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 and 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 and Setter 2010) and the loss of wetland biodiversity (Quesnelle, Fahrig and Lindsay 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, Nol and Abraham 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 biofilter 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 oxidation kinetics deviating from those in wetlands (Bender and 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 and phenotypical characteristics and phylogeny, proteobacterial MOB have been classified into type Ia, type Ib and type II (Hanson and Hanson 1996; Bodrossy *et al.* 2003). Today, they are mainly grouped taxonomically into Gamma- and Alphaproteobacteria. 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 a 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, Lipski and Dunfield 2003). While the closest relative of USC-α is a 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 itself, 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 and CH_4 oxidation kinetics were compared in drained and rewetted peat soils, comprising both environments with high internal CH_4 supply and those with atmospheric levels of CH_4. Spatial organization of aerobic CH_4 consumption in a drained peat soil with and without short-term flooding was assessed by radio-imaging of intact soil cores incubated with labeled $^{14}CH_4$ at elevated (10 000 ppmv) or near-atmospheric (10 ppmv) concentrations.

MATERIALS AND METHODS

Study site and experimental design: long-term hydrological restoration

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

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

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

At both sites, soil samples were taken in October 2010, at the end of the growing season. To representatively cover within-site variability, five evenly distributed 2 × 2 m plots were chosen. In each plot, three soil cores (20 cm length, 3.8 cm diameter) were taken at random locations with a soil corer. Upon arrival in the laboratory, replicated cores were sliced and the 3–9 cm layer processed. We chose this layer because initial tests had shown that CH_4 oxidation was highest at this depth (unpublished data). Roots were removed from the soil, and the sample was mixed, freeze dried and stored at room temperature in the dark until further analyses. The main soil characteristics are summarized in Supplementary Table S1.

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

An additional pair of study sites was located at the former experimental dairy farm of the University of Wageningen in Zegveld, the Netherlands (52°8′25.9″ N, 4°50′19.7″ E). A former agricultural pasture was temporarily flooded for 12 weeks in a wetland bird-management initiative. The pasture had been flooded for the first time in February 2010 and for the second time in February 2011 and is still used for extensive agriculture (cattle and sheep). Next to this site, we selected a non-flooded drained reference site with similar characteristics and agricultural history. At each study site, we selected four plots of 1 m^2, which were marked with bamboo sticks for re-sampling over the season. In each plot, CH_4 emission was measured in duplicate points

before soil cores were collected for further analysis in the laboratory. In the following sampling campaigns two new points were selected within the 1 m^2 area and prepared in the same way as described above. In total, per sampling event and per site we obtained eight samples and CH_4 flux readings from two points at four plots. The soil was processed as described above. The main soil parameters, pH and moisture content based on dry weight are given in Supplementary Table S2.

CH_4 oxidation kinetics

Apparent enzyme kinetics, i.e. the apparent half-saturation constant ($K_{m(app)}$) and the maximum rate of CH_4 consumption (V_{max}), were determined from all soil samples the day following sample collection. Assays were performed by incubating 5 g of processed soil, suspended in 10 ml of MilliQ water (MILLI-Q Reagent Water System, Millipore, Billerica, MA, USA) in 120 ml serum flasks capped with a butyl rubber stopper (Sigma-Aldrich, St Louis, MO, USA). CH_4 was added to the headspace to achieve mixing ratios of approximately 50, 100, 500, 1000, 5000 and 10 000 ppmv, and 20 000 ppmv only for long-term hydrological restored sites. 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 $K_{m(app)}$ were derived from non-linear 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, 3 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 analysed for CH_4 concentration by GC-FID (Ultra GC gas chromatograph, Interscience, Breda, the Netherlands, 30 m × 0.32 mm 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 labeled with $^{14}CH_4$ under elevated (10 000 ppmv) CH_4 concentrations, 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 *et al.* (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 c 500 kBq $^{14}CH_4$. Labeling was carried out in gas-tight jars, for 7 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 leveled 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 the image processing toolbox of MATLAB (MathWorks, Natick, MA, USA).

MOB community composition

DNA was extracted from all soil samples by a modified method (see Henckel, Friedrich and Conrad 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 Biomedicals, Santa Clara, CA, USA) and bead-beated using a FastPrep-24 instrument (MP Biomedicals). Samples were then centrifuged (10 min, 20 800 × *g*). A 600 μl volume of the supernatant was 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 (400 μl supernatant). Before DNA precipitation we initially treated the supernatant with 7.5 M sodium acetate to remove humic substances.

DNA from the combined 1 ml of supernatant was purified and precipitated (Lueders, Manefield and Friedrich 2004). Finally, pelleted nucleic acids were suspended in 30 μl of elution buffer (Qiagen, Hilden, Germany) and stored at −20°C. DNA quality and quantity were determined using a Nano-Drop Spectrophotometer (Thermo Scientific, Madison, WI, USA).

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

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

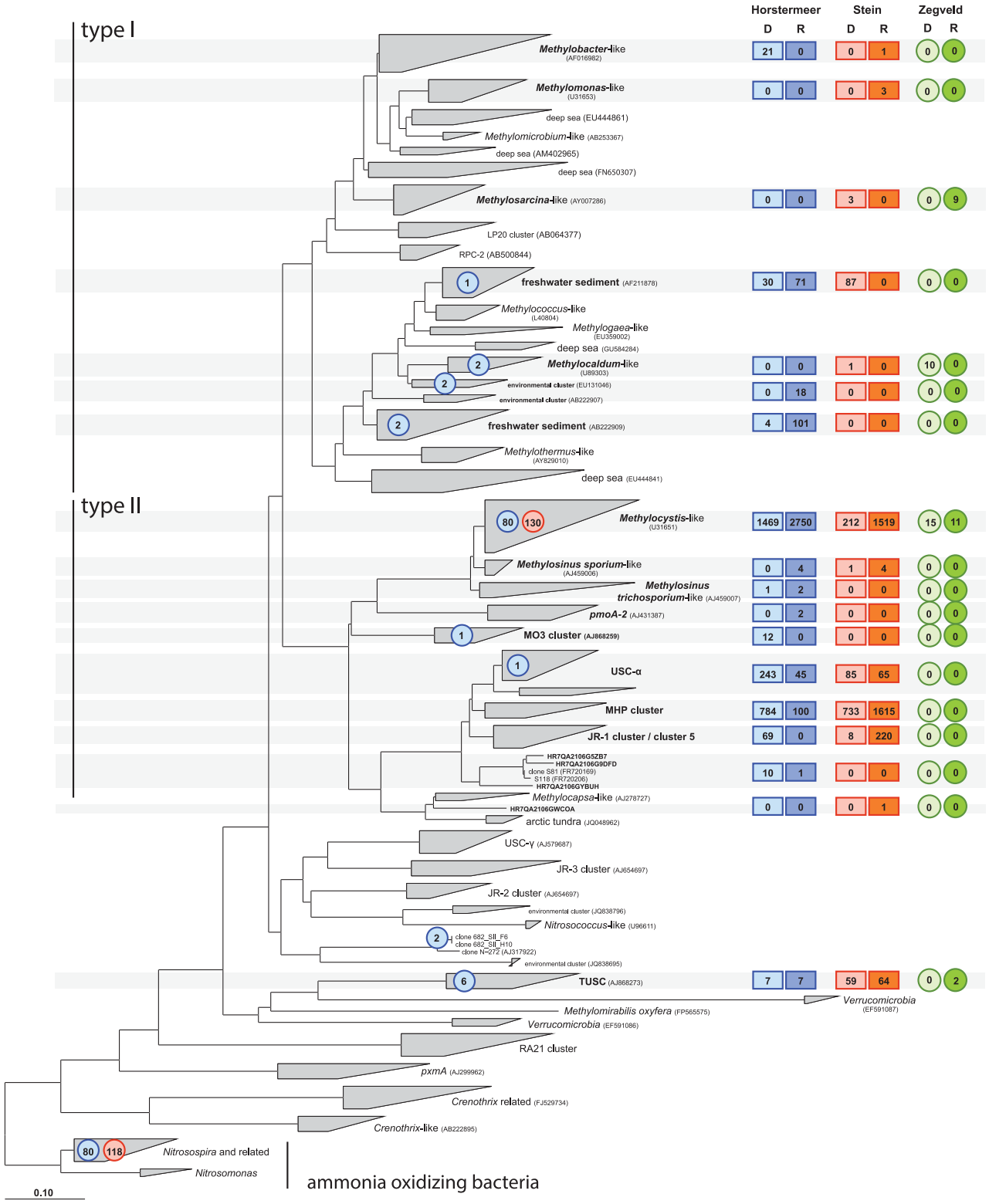

Figure 1. Neighbor-joining tree of *pmoA* sequences obtained by Sanger sequencing and 454-pyrosequencing in long-term hydrological restored sites and drained reference site (Horstermeer and Stein) as well as by Sanger sequencing from clone libraries at drained sites with and without short-term flooding (Zegveld); D, drained reference site; R, restored site. Colored boxes next to *pmoA* lineages represent total number of reads from 454-pyrosequencing, and colored circles represent total number of clones retrieved from Sanger sequencing. Sequences were obtained by PCR using different reverse primers (mb661R, A682R). Sanger sequencing results for the long-term hydrological restored sites and drained reference site were combined and mapped onto the tree (blue/red circles). Individual proportions for each of the reverse primers (mb661R, A682R) are displayed in Supplementary Fig. S1. Lineages lacking isolates are named according to representative clones or to the environment in which they were predominantly or initially found (JR, Jasper Ridge; MHP, Moor House Peat; RPC, rice paddy cluster; TUSC, tropical upland soil cluster; USC, upland soil cluster). The scale bar represents 0.1 changes per amino acid position.

Sequence reads larger than 400 bp were analysed as described in (Lüke and 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 PRJEB11175.

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. Types Ia, Ib and II subgroups were quantified using *pmo*A-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, 12.5 μl 2 × SensiFAST SYBR No-ROX Kit (Bioline, Luckenwalde, 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 25 μl. 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 analysed 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, Mortlake, NSW, Australia). Samples were added to aliquots of the master mixture using the QIAgility liquid handling system (Qiagen). To quantify total copy number of each individual assay the Rotor-Gene Q Series Software (Qiagen) 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 *pmo*A genes were used (Kolb *et al.* 2003). We followed the protocol as described in Lima, Muniz and Dumont (2014). These assays were performed with the CFX96 qPCR cycler (Bio-Rad, München, 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 sites 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 *pmo*A gene clone libraries and used the two widely applied reverse primers, mb661r and A682r, which together still have the highest coverage of aerobic MOB diversity. Members of the genus *Methylocystis* (type II MOB) were the dominant MOB present (Fig. 1 and Supplementary Fig. 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 (Fig. 1 and Supplementary Fig. S1).

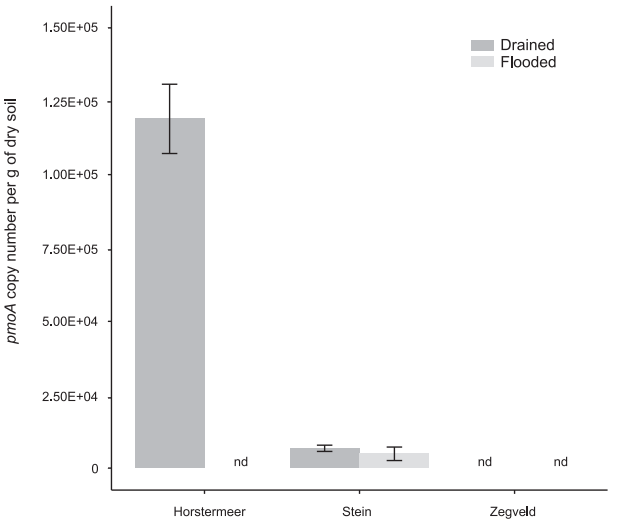

Figure 2. *pmo*A gene copy numbers of the USC-α MOB subgroup at different study sites (error bar shows standard deviation). Dark gray bars represent the drained reference site and light gray bars the restored or short-term flooded site (in case of Zegveld). nd indicates that the target gene was below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

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 *pmo*A 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 MOB detected were the putative atmospheric CH_4 oxidizers USC-α and related, but there was also a small fraction of other putative atmospheric CH_4 oxidizers such as tropical upland soil cluster (TUSC; Fig. 1). The qPCR assay specifically targeting USC-α *pmo*A genes revealed a low abundance of those organisms at the studied environments (Fig. 2). Similarly to the results of *pmo*A gene clone libraries, the 454 pyro-sequencing revealed a large proportion of *pmo*A sequences belonging to the genus *Methylocystis*, except for the drained reference site at Stein (Fig. 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 (Fig. 3). The qPCR data of the three major groups of MOB revealed temporal variability with a peak of all three sub-populations in samples taken in June (Fig. 3). Overall, the abundance of type II was a 200-fold higher compared with type Ia and Ib MOB (Fig. 3). The qPCR assay specifically targeting USC-α *pmo*A genes showed that USC-α were below the detection limit over the whole growing season (Fig. 2).

We performed an additional Sanger sequencing of *pmo*A gene clone libraries using only the reverse primer mb661r (Fig. 1) to get

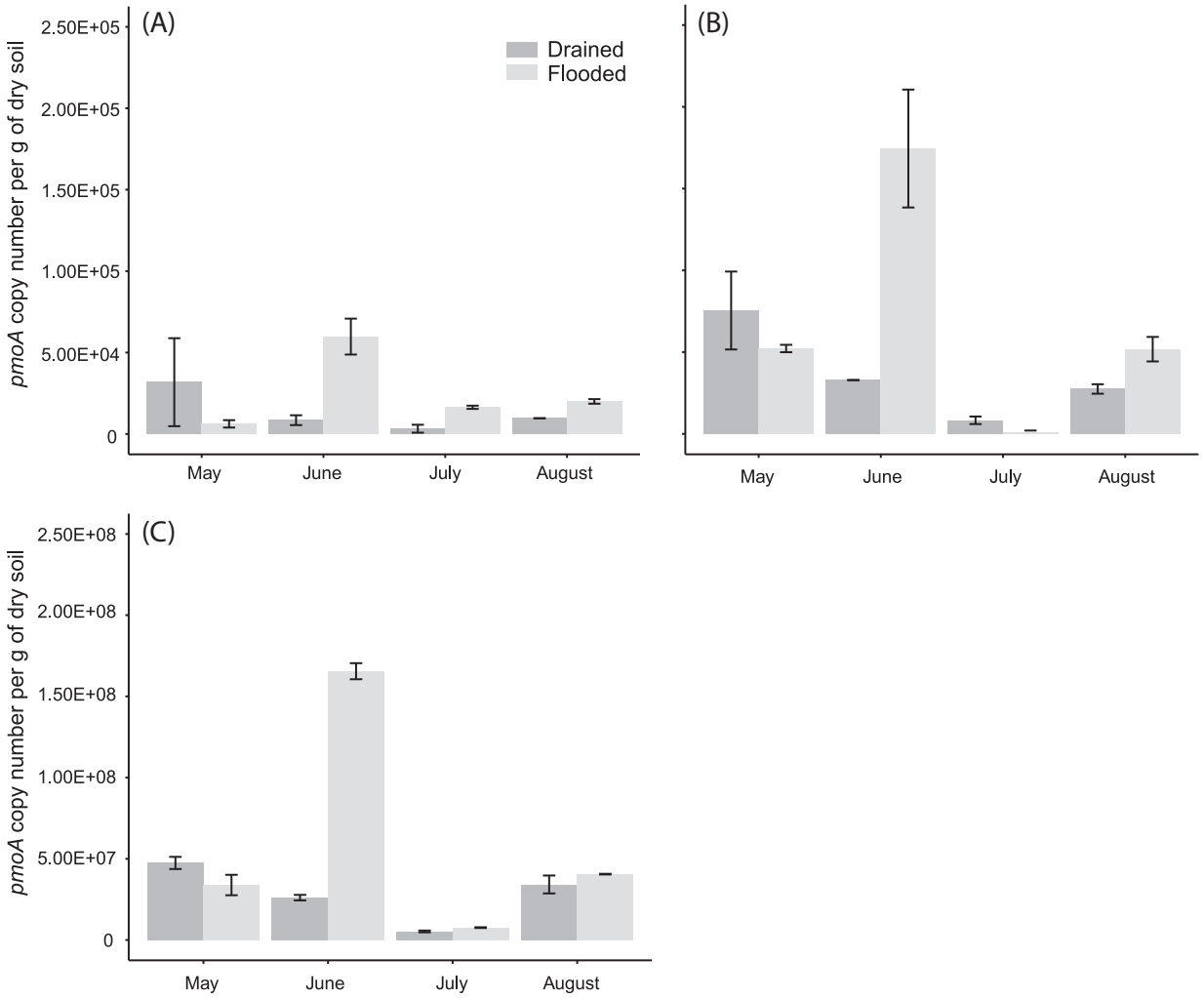

Figure 3. *pmoA* gene copy numbers of type Ia (**A**), type Ib (**B**) and type II (**C**) MOB subgroups over the growing season at drained sites with and without short-term flooding ($n = 2$; error bar shows standard deviation). Dark gray bars represent the drained reference site and light grey bars the short-term flooded site.

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* (Fig. 1). Next to the dominance of type II MOB, sequences were found belonging to type Ib MOB affiliated to the genus *Methylocaldum* at the drained site (Fig. 1). In addition, we detected type Ia MOB affiliated to the genus *Methylosarcina* at the short-term flooded site.

CH_4 oxidation kinetics

CH_4 oxidation kinetics (i.e. $K_{m(app)}$ and V_{max}) were measured once at the long-term hydrological restored and drained sites at Horstermeer and Stein and were followed over the growing season at the drained sites with and without short-term flooding in Zegveld. Overall, V_{max} was comparable between all sites, but $K_{m(app)}$ was lower at Horstermeer and Stein (Tables 1 and 2). Kinetics measured over the growing season in the short-term flooded soil in Zegveld showed very high variability and were not significantly different based on Student's *t*-test. Similarly, calculated specific affinities showed no significant differences but displayed a large variability (Tables 1 and 2).

Short-term flooding increased CH_4 emissions significantly compared to a drained reference site (Table 3). Once the short-term flooded site was re-drained CH_4 emissions decreased continuously and approximated values measured at the reference site approximately 4 weeks after drainage (Table 3).

Spatial distribution of CH_4 assimilation in drained peat meadows with and without short-term flooding

Autoradiographic imaging of ^{14}C-labeled soil cores at the same site incubated under elevated (10 000 ppmv) and under near-atmospheric (10 ppmv) CH_4 concentrations showed that CH_4 assimilation was not homogenously distributed but displayed distinct spatial patterns depending on CH_4 supply (Fig. 4). A visual exploration of the autoradiographs displayed a very distinct and actively incorporating community restricted to the top soil under elevated CH_4 concentrations while under near-atmospheric conditions CH_4 assimilation spread over virtually the entire soil profile. These patterns were consistent both for the short-term flooded site and the drained reference site (Fig. 4). However, vertical profiles at the short-term flooded site under near-atmospheric conditions displayed pronounced CH_4

Table 1. Apparent enzyme kinetics and specific affinities from CH_4 oxidation of the methanotrophic community at long-term hydrological restored peat meadows in October 2010 ($n = 5$; means ± standard deviation). $K_{m(app)}$ 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
	$K_{m(app)}$	3.79 ± 1.94	4.94 ± 4.43
Restored site	V_{max}	0.32 ± 0.20	0.45 ± 0.29[a]
	$K_{m(app)}$	7.72 ± 3.90	9.05 ± 5.30[a]
Specific affinity			
Drained site		0.03 ± 0.02	0.10 ± 0.09
Restored site		0.04 ± 0.02	0.05 ± 0.01[a]

[a] $n = 3$; mean ± standard deviation.

uptake more evenly distributed over the whole soil core, reflecting more the soil structure like pore or root channels (Fig. 4).

DISCUSSION

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

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

A possible explanation for the strong resilience of the genus *Methylocystis* can be found in their metabolic flexibility. Dunfield *et al.* (1999) demonstrated consumption of atmospheric CH_4 by *Methylocystis* LR1 after long-term cultivation under low CH_4 availability, later *Methylocystis* SC2 was found, which harbors two isoenzymes of the particulate methane monooxygenase, one oxidizing CH_4 at mixing ratios >600 ppmV and the other oxidizing CH_4 at mixing ratios of <100 ppmV (Baani and Liesack 2008). In addition, some *Methylocystis* species have been shown to be facultative MOB, able to grow on acetate and ethanol (Belova *et al.* 2011; Im *et al.* 2011). It has been suggested that this trait enables them to survive in environments with fluctuating or limited CH_4 supply by using the reducing power obtained from acetate oxidation to keep pMMO functioning at atmospheric CH_4 concentrations.

A second finding from our study is the detection of putative atmospheric MOB (USC-α) in drained peat meadows and in long-term hydrological restored sites. However, a qPCR assay targeting specifically USC-α revealed low signals, suggesting a minor role of this group of organisms. In addition the clade MHP (DNA sequences obtained from Moor House peat samples, UK) has been suggested to be involved in the oxidation of atmospheric CH_4 in acidic upland meadows (discussed in Chen *et al.* 2008a).

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

Next to the long-term hydrological restored and drained sites we also looked at short-term effects of rewetting/flooding

Table 2. Apparent enzyme kinetics and specific affinities from CH_4 oxidation of the methanotrophic community at drained after short-term flooding and drained peat meadows over the growing season in 2011 ($n = 4$; means ± standard deviation). $K_{m(app)}$ 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		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
	$K_{m(app)}$	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
	$K_{m(app)}$	n.d.	35.51 ± 35.95	35.40 ± 6.69	51.61 ± 43.13	12.99 ± 2.74
Specific affinity						
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

Table 3. CH_4 emission (in mg m^{-2} day^{-1}) over the growing season in 2011 at Zegveld sites ($n = 8$; means ± standard deviation). Values in bold are significantly different at $P < 0.05$.

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

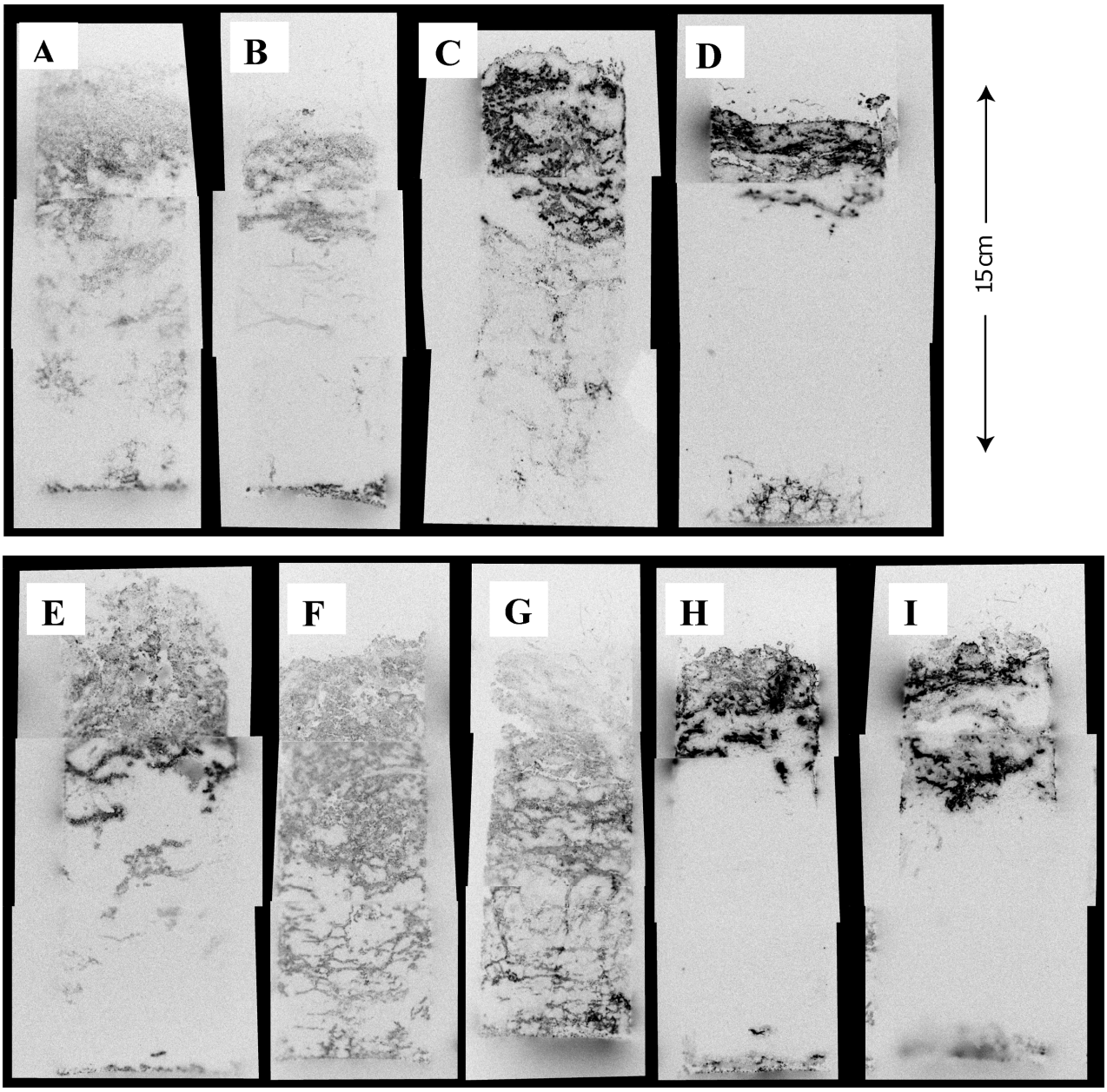

Figure 4. Autoradiographs of $^{14}CH_4$-labeled soil core sections from the drained reference site (**A–D**) and short-term flooded (**E–I**). Soil cores were labeled with 10 ppmv (A–B, E–G) or with 10000 ppmv of $^{14}CH_4$ (C and D, H and I). Cores of 15 cm were used. For each core the three resulting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

of drained peat soils. Monitoring the abundance of different sub-groups of MOB by qPCR showed that the abundance of type II was on average 200-fold higher compared with type Ia and Ib MOB. In line with Sanger sequencing results, this suggests that also in these peat soils type II and *Methylocystis* still persisted after land-use change. The genus *Methylosarcina* can be very responsive within flooding gradients and therefore a short-term flooding pulse may have favored the occurrence of the organisms (Bodelier *et al.* 2012). The occurrence of the genus *Methylocaldum* at the drained reference site is most likely the result 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, Frenzel and Conrad 2008).

CH_4 oxidation kinetics

The apparent half-saturation constant ($K_{m(app)}$) 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 ($K_{m(app)} > 1\ \mu M$), while in dry, well aerated environments CH_4 is oxidized with a high apparent affinity ($K_{m(app)}$ ranges from 0.03 to 0.05 μM) (Bender and Conrad 1992). In addition, intermediate kinetic values have been observed for several hydromorphic soils with a $K_{m(app)}$ 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 $K_{m(app)}$ values 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 and Dunfield 2005). Observed kinetics values showed no indication of 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 temporarily increased CH_4 emissions. During and immediately after the flooding period, field fluxes were between 45 and 347 mg m^{-2} day^{-1}, which is in a similar range to those 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.* 2010). In addition, observed values were comparable to natural wetlands with measured fluxes between 72 and 1184 mg m^{-2} day^{-1} (Juottonen *et al.* 2012).

Spatial distribution of CH_4 assimilation in drained peat meadows with and without short-term flooding

Restoring agriculturally used peat land back to natural wetlands turned a potential sink into a source of CH_4. To further investigate the response of indigenous MOB to different CH_4 concentrations, we varied CH_4 availability and incubated soil cores under elevated (10 000 ppmv) or near-atmospheric (10 ppmv) CH_4 conditions.

Under elevated CH_4 concentrations, most $^{14}CH_4$ was assimilated in the top soil layers. This suggests the presence of a community with the capacity to consume the offered CH_4 completely at these concentrations occurring usually in soil with an internal CH_4 sources, e.g. when flooded. The MOB present act as an efficient 'filter' that reduces CH_4 emissions. Under near-atmospheric CH_4 concentrations, $^{14}CH_4$ assimilation was found over the entire soil profile, reflecting a community which can assimilate atmospheric CH_4 but with a capacity below the diffusion of CH_4 into the soil. This community presumably is active when the soil water table is lower. Putative atmospheric MOB (e.g. USC-α) are likely not to be the responsible organisms here because a USC-α specific qPCR assay did not give any signals. We think in line with previous studies (Kolb and Horn 2012) that *Methylocystis* species found at these sites may be capable of oxidizing CH_4 over a large range clearly distinguishing this genus from other cultivated MOB.

Critical remarks and conclusions

We applied a comprehensive set of tools to compare diversity and activity of aerobic MOB in drained and rewetted peat meadows with different water table management and different agricultural practice, but we may have missed some groups of MOB. Using our approach we cannot detect methanotrophic Verrucomicrobia and MOB containing only a soluble CH_4 monooxygenases (sMMO). To the best of our knowledge, Verrucomicrobia have only been detected in wetlands with a pH between 1.8 and 5.0 (Sharp *et al.* 2014) but our sampling sites were less acidic (Supplementary Tables S1 and S2), which suggests that they do not play a major role here. From the sMMO-containing MOB we additionally tested for the widely occurring genus *Methylocella* (Rahman *et al.* 2011), but they were not detected by PCR using *Methylocella* specific primers (data not shown). Next to aerobic MOB, recent studies have shown that anaerobic CH_4 oxidation coupled to denitrification is more widely distributed and may play a significant role as an additional CH_4 sink in environments such as wetlands (Hu *et al.* 2014) and lake sediments (Deutzmann *et al.* 2014).

Nevertheless, our findings conclusively show that rewetted and short-term flooded agriculturally used peat meadows are comprised of a fundamentally similar methanotrophic community. The present MOB community members appear to process CH_4 at a wide range of concentrations that naturally occur due to water-table changes and associated changes in O_2 availability and methanogenesis. The strong dominance of type II aerobic MOB (*Methylocystis* sp.) suggests a very pronounced flexibility and persistence of this group under land use change.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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