Methane Cycling Microbial Communities in Natural and Drained Sites of Taldom Peatland, Moscow Region, Russia

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Authors’ contributions

The work was carried out in collaboration between all authors. Authors IK and AS conceived and designed the study, organized and performed field sampling. Author AK and EM designed and performed laboratory experiments. Author IK wrote the manuscript and author AS reviewed it. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2015/14978
Editor(s):
(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Anonymous, Universiti Teknologi Malaysia, Malaysia. 
(2) Anonymous, Universiti Teknologi Malaysia, Malaysia.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?id=797&id=32&aid=7297

Received 30th October 2014
Accepted 20th November 2014
Published 15th December 2014

Original Research Article

ABSTRACT

Aims: Drainage of peatlands is known to decrease overall CH4 emission, but the effect on methane cycling microbes is poorly described. For this reason we aimed to reveal the differences in methanogenic and methanotrophic communities between pristine peatlands and its counterpart that was drained in 1979.

Study Design: Comparative molecular analysis of microbial communities involved in methane cycling.

Place and Duration of Study: Peat samples were collected in July 2012 in Taldom natural and drained sites of Dubnenisky peatland massif.

Methodology: Total DNA was extracted from fresh peat samples and analyzed by PCR-DGGE technique in order to evaluate diversify of key functional genes for methanotrophy (pmoA) and methanogenesis (mcrA) as well as the phylogenetic archael16S rRNA genes. FISH method was

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applied to count Bacteria and Archea.

**Results:** Our results demonstrated that in natural peatlands hydrogenotrophic methanogens dominated, but in the drained peats both hydrogenotrophic and acetotrophic methanogens were found. It was revealed striking difference between methanotrophs of natural and drained peatlands. Sequence analysis of marker pmoA gene, suggested that Methylocystis –like methanotrophic Alphaproteobacteria were dominants in natural peatlands. Conversely Methylobacter was found to be actively involved in CH₄ oxidation in the drained peats.

**Conclusion:** This study indicates drastic changes in structure of CH₄-cycling microbial communities, affected by drainage and can be applied as environmental indicators in monitoring of anthropogenic influence on peatland ecosystems.

**Keywords:** DGGE; peatlands; methanotrophs; methanogens; microbial communities.

1. INTRODUCTION

Peatlands have regulating functions influencing especially climate and catchment hydrology. They cover only 3% of the terrestrial surface, but are significant players in the global carbon (C) cycle and the climate system, since they store one quarter of the global soil C and are the most important global sources of methane (CH₄) [1].

Methanogenic Archaea produced CH₄ in the terminal step of the anaerobic decomposition of organic matter in reduced environment by a community consisting of hydrolytic, fermentative, acetogenic and methanogenic microorganisms [2]. The opposite process is CH₄ oxidation which takes place in the presence of oxygen, and methane conversion to methanol is performed in cells of methane oxidizing bacteria (MOB) by methane monooxygenase enzymes, particulate (pMMO) and soluble (sMMO) methane monooxygenase [3]. Both CH₄ production and oxidation in peatland soils are variable and dependent on temperature, substrate supply, community structure and activity of microbes [4]. Because of its control on the zonation of methanogenesis and methanotrophy, water table position is usually the dominant regulation factor for methane emissions from wetland soils [5].

Peatlands are actively used for human needs by drainage, agricultural use and peat extraction, and this management shifts pristine peatlands from green house gases sinks into sources. It was shown that emissions in intensively managed peatlands can reach more 40 t CO₂ equiv. ha⁻¹ year⁻¹ [6]. On the other hand, the restoration of degraded peatlands usually reduces these emissions towards climate neutral levels [7]. Drainage of peatlands is known to decrease CH₄ emission and the site may be turned from a methane source into a small sink [8]. Previous investigations have shown the effects of drainage on composition and functioning of the microbial community in peatlands [9], but until recently CH₄-cycling microbial communities have not been documented.

The Dubna wetland is composed of 40,000 ha and located in the northern part of Moscow region, Russia. It is situated at the south part of the Russian plain, and belongs to the zone of temperate continental climate. The main native vegetation types of the area are mixed coniferous forests and wetlands, including oldet-birch swamps, raised pine-moss and transitional bogs. The significant part of Dubna peatland massif was drained and nowadays utilized for peat extraction or for agriculture. Field studies from 2005 at drained Taldom part of the Dubna wetland have revealed high methane emission effluxes from some drained peatlands used for peat extraction or as hayfield, and the most intensive emission was registered from the surface of drainage ditches [10].

In this study we investigated the structure of microbial communities related to the CH₄ cycle in natural and degraded peatlands and indicated their response to anthropogenic disturbance. We hypothesized that the peat milling and grassing would have significant effects on peatland microbial community. We expected under higher water table conditions after milling an increase in the relative abundance in anaerobic-tolerant taxa as well as an increase in the relative abundance and diversity of methane producing archaea due to income of organic substrates from peat oxidation in MP and photosynthates in HF.

To test our hypotheses, we analyzed bacterial and archaeal communities in order to elucidate changes in abundance and composition following drainage and grassing manipulations. As microbial communities were originally identical
(pristine bog) any shift in community composition can be considered as a direct result of the anthropogenic disturbance.

2. MATERIALS AND METHODS

2.1 Site Description and Sample Collection

The three sites, namely pristine bog (PB), milled peat (MP) and hayefield (HF) were about 0.5 km apart, located in the drained Taldom site in the Dubna wetland massif, Moscow region, Russia; 56.70°N, 37.83°E, (Fig. 1). They were part of a larger long-term experiment studying the effects of drainage and rewetting on net GHG flux from a typical area. The annual average air temperature (MAT) was 3.8°C and the annual average precipitation (MAP) was 525 mm. The vegetation period was about 170 days. The water table was near the soil surface and the peat layer was at least 140 cm deep. The selected information on sampling sites is providing in Table 1.

10–20 cm depths peat samples were collected from 3 locations up to 5 m apart within each plot, pooled, mixed, divided into two aliquots and immediately stored at 4°C in the field. Subsamples for molecular analysis were frozen within 8 h of collection, firstly at -20°C, before transfer to -80°C. Subsamples for FISH analysis were fixed by paraformaldehyde at the same time.

2.2 FISH analysis

The fixation procedure of peat samples included (i) The sonication for desorption of microbial cells from peat particles, (ii) The separation of the microbial cells fraction from the Sphagnum debris by stomacher treatments, (iii) Extraction of the microbial cells from the peat water by centrifugation and (iv) Cell fixation with freshly prepared 4% (wt/vol) paraformaldehyde solution [11]. A set of Cy3-labeled oligonucleotide probes specific for the domains Bacteria (EUB-338 mix) and Archaea (ARC 915), as well as M-84+M705 for Type I (Gammaproteobacteria) and M-450 for type II (Alphaproteobacteria) MOB was used in this study [12] All oligonucleotide probes were synthesized by Syntol (Moscow, Russia).

![Fig. 1. Map of the Taldom study site indicating three measurement plots along the drainage system and landscape topography. The inset shows the location in north-east Russia](image-url)
Table 1. Selected properties of experimental plots and peat soil at the time of sampling

| Characteristics          | PB (pristine bog) | MP (milled peat) | HF (hayfield) |
|--------------------------|-------------------|------------------|---------------|
| Vegetation cover         | Virgin pine dwarf-shrub | Open peat surface | Haying grassland |
| *Water Table, cm         | 15                | 10               | 12            |
| Peat layer, cm           | 250               | 140              | 145           |
| pH                       | 3.8               | 4.2              | 4.4           |
| N-NH$_4^+$, mg/kg        | 2.3               | 4.6              | 6.3           |
| N-NO$_3^-$, mg/kg        | 3.0               | 1.9              | 4.3           |
| C-CH$_4$ emission rate, mg/(m$^2$×h) | 0.8               | 0.2              | 0.03          |

*Below the peat surface

Hybridization was carried out on pre-coated by gelatin (0.1%, wt/vol) Teflon slides (MAGV, Germany) with eight wells for the samples. The fixed samples were put into these wells, hybridized to the fluorescent probes, and then stained with the universal DNA stain 4, 6-diamidino-2-phenylindole (DAPI) as described earlier [12]. Cells were counted with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with the Zeiss filters No. 20 for Cy3-labeled probes and 02 for DAPI staining.

Data are expressed as the mean of technical replicates (n = 3) and SEs. Simple linear regression analysis was performed to describe the relationships of number of probe target cells with CH$_4$ flux data.

2.3 Extraction of Nucleic Acids

Before the extraction of nucleic acids, frozen peat samples were cooled in liquid N$_2$ and homogenized by hand milling. Extraction and purification of nucleic acids from 0.25 g of wet peat (n=5) were carried out using Power Soil DNA isolation kit (MoBio, USA) according to the manufacturer’s protocol with minor modifications. To enhance the yield of microbial DNA the lysis step was carried out at high temperature (80°C) and agitation.

2.4 End-point PCR and Analysis of Methanogen and Methanotroph Communities by DGGE of Marker Genes

Fingerprinting of the methanogen and methanotroph diversity was performed by DGGE (denaturing gradient gel electrophoresis) of key functional genes mcrA and pmoA, respectively. Fragments of pmoA, encoding β-subunit a of particulate methane monooxygenase, were PCR targeted with the A189f/A682r primers [13] with a GC-clamp attached to the reverse primer, and fragments of mcrA, encoded methyl coenzyme M reductase, were amplified with the ML primers of Luton et al. [14] (see Kizilova et al. [12] for details).

DGGE was performed using the DCode Universal Mutation Detection System (BioRad, United States) for 6 h in a denaturing gradient (formamide and urea, 35–60%) at a constant temperature (60°C) and voltage (200 V), as described in Kizilova et al. [12]. Stained with ethidium bromide gel slabs were digitalized with a GelDoc gel imaging and documentation system (BioRad, United States). Single DGGE bands of interest were excised from the gel, purified and sequenced.

2.5 Sequence Analysis and Phylogeny

mcrA and pmoA sequences were compared with database sequences with BLAST analysis of NCBI (National Center for Biotechnology Information). Deduced amino acid sequences of McrA and PmoA together with selected reference sequences were aligned with ClustalW.

Maximum likelihood trees were constructed with TREECONW software package. Bootstrap values were generated from 100 replicates.

3. RESULTS AND DISCUSSION

Northern peatlands store about a third of terrestrial soil carbon in the form of partially decayed peat while also emitting 9-18% of all global atmospheric methane [15]. Historically, peatlands have served as net carbon sinks but could change for the carbon sources as a result of climate change or anthropogenic drainage [16,17]. Shifts in the environmental and physicochemical features could alter carbon
cycling and methane production/consumption processes [9].

3.1 FISH Studies of Bacterial Community Structure

The number of DAPI-stained cells in peat sampled decreased in milled peat and grassland as compare with pristine bog and was of 13.1, 2.57 and 8.62×10^8 cells per g of wet peat, correspondently (Table 2). In FISH with EUB338-mix, the number of bacterial cells varied in 0.9 – 3.1×10^5 cells, that comprised from 1.8 to 3.9 % of the total DAPI cell counts. The number of archaean cells (probe ARCH915) comprised up to 10.5% of the total cell number. Thus, the proportion of cells detectable with domain-specific probes did not exceed 15% of the DAPI stained cells.

The number of type I MOB (probe M-84+M705) in pristine bog was very low – 4×10^4 cells per g of wet peat, but was hundredfold in MP and HF sites up to 7 × 10^6 cells per g of wet peat. Vise versa, the number of type II methanotrophs (M-405 probe) was high in PB site and decreased in drained sites (Table 2). Almost all currently known strains of Methylosinus and Methylocystis are targeted by M-405 probe, thus, our study suggests that type II MOB were the predominant in pristine bog, but drainage gave rise to shift to increase of type I methanotrophs population level up to two orders of magnitude.

A significant positive correlation was found for the relationship between CH4 flux and Archea abundance (r^2=0.99). In contrast, the CH4 flux rate was inversely related to type II MOB abundance (r^2=0.72) suggesting the direct influence of this group of methanotrophs on the modulation of surface methane flux.

### 3.2 Diversity of Aerobic Methanotrophs Based on DGGE Analysis of pmoA genes

Aerobic bacteria, utilizing methane as the source of carbon and energy (methanotrophs), are described in phyla Alpha- and Gammaproteobacteria [3]. Also, the ability to oxidize methane was demonstrated for filamentous bacteria Crenothrix and Cloacibacterium [18] as well as for the extremely acidophilic methane-oxidizing Verrucomicrobiium [19]. In bacterial cells, methane conversion to methanol is performed by methane monoxygenase enzymes, sMMO and pMMO. The gene pmoA is encoding the 27 kDa subunit of pMMO, and is present in all currently known methanotrophs, except Methylocella and Methyloferula. This gene has been successfully used to detect and identify methanotrophs in various environmental samples [20-22] Furthermore; good correlation between phylogenies inferred using pmoA and 16S rRNA genes has been demonstrated [23]. PCR-DGGE analysis of pmoA was applied to investigate the diversity of aerobic methanotrophic bacteria in peatland soils.

We have used the primer system A189/A682, which is known to target methane monoxygenase gene fragments, as a functional marker for characterization of the methanotrophic communities. No amplification product for soluble methane monoxygenase (sMMO) was obtained for any of investigated soils. Thus sMMO is likely to provide the minor contribution to methane oxidation in the large range of studied samples.

Methanotrophs communities showed a quite low diversity with only 3-4 clearly visible bands in DGGE fingerprint. A total of 10 bands in the DGGE gel was excised for further analysis, the pmoA re-amplified, sequenced and the encoded amino acid sequence comparatively analyzed by phylogenetic treeing.

### Table 2. Microbial cell numbers in peat samples from experimental plots determined by direct microscopy methods of detection

| Sample ID | No. of cells (10^3) per g of wet peat determined with: |
|-----------|-----------------------------------------------|
|           | DAPI-staining | EUB-338 mix | ARCH 915 | M-84+M705 | M-405 |
| VB        | 131.0±4.2     | 3.13±0.49   | 1.5±0.9 | 0.004±0.1 | 0.36±0.8 |
| MP        | 25.7±5.1      | 0.99±0.53   | 2.7±1.2 | 0.2±0.1   | 0.03±0.007 |
| HF        | 86.2±9.4      | 1.58±0.26   | 2.2±0.7 | 0.7±0.2   | 0.18±0.06 |
The analyses allowed the detection in pristine bog of pmoA sequences related only to the type II methanotroph *Methylocystis* and this result was consistent with previous studies of pristine bogs. Dominance of type II *Methylocystis*-like MOB is in line with previous studies of boreal *Sphagnum* peatlands [24-26]. Phylogenetic analysis of Pmoa demonstrated that sequences formed two separate clusters within *Methylocystis* genera (Fig. 2) distinctly related with cultivated strains. These sequences may represent a novel lineage since they were grouped with a large set of pmoA of uncultured methanotrophs from acidic peatland in Northern England [25] and wetland in Finland [27].

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The abundance of type II methanotrophs in peatlands could be explained by the characteristic of *Methylocystaceae* (*Methylocystis*) to be acidophilic or acidotolerant. In addition, copper limitation in nutrient-pure ombrotrophic bogs may favor non-copper-dependent type II methanotrophs.

Only type I methanotrophs were detected in MP and NF sites (Fig. 2) and demonstrated distant relation to *Methyllobacter tundripaludum* isolated from Norwegian arctic bog [28]. The drainage impact brought about a peculiar shift in methanotroph populations and alters MOB community structure. It was shown that drainage of peatlands can alter methanotrophs community structure and cause succession in methanotroph community. Type II methanotrophs of the family *Methylocystaceae* are known to have the ability to tolerate long periods of oxygen starvation and also to ferment their characteristic storage compound, poly-β-hydroxybutyrate. By contrast, for type I methanotrophs of the family *Methylococcaceae* the ability to survive during long periods of oxygen depletion has never been demonstrated [29]. It has to be also noted that type II methanotrophs are sensitive against inorganic nitrogen load. The responses of methanotrophs against nitrogen have been related to community composition since type I methanotrophs have been stimulated whereas type II methanotrophs have been inhibited by nitrogen [30]. Changes in vegetation could also modify the composition of the population of methanogens and methanotrophs, and recent molecular evidences support this idea [31].

Recently, more specific detection methods such as mRNA-based microarrays [32] and SIP-PLFA [25] have been applied to study MOB communities in environmental samples. These advanced techniques detect active MOB populations and are precise and comprehensive tools to future investigations of peatland MOB.

**Fig. 2.** Phylogenetic tree based on the deduced partial Pmoa amino acid sequences from peatland DGGE bands soils (in bold) and reference sequences. The dendrogram was constructed with a neighbor-joining analysis. Scale indicates 10% sequence divergence. The maximum likelihood tree is rooted with *Nitrosomonas europaea* amoA as the out group.
3.3 Diversity of Methanogens Based on DGGE Analysis of mcrA genes

Methanogens are a large and diverse group of Archea. Currently recognized five Orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales) have distinctive characteristics, and recently a novel Order of methanogens, Methanocellales, was proposed. A limited number of substrates may be used by methanogenic archaea to obtain energy for growth with the concomitant production of methane gas. Based on known pathways methanogenesis fall into three groups: (1) CO₂ or CO fixation using H₂, formate, or certain alcohols (e.g. methanol, ethanol, 2-propanol, 2-butanol) as electron donors, (2) Methylotrophic; methyl groups of C₁ compounds (e.g. methanol, mono-, di-, and trimethylamine, dimethylsulfide, methylmercaptan) are cleaved and reduced to methane, and (3) Aceticlastic; acetate is fermented to methane and CO₂ [33].

Methanogens are difficulty isolate or culture under laboratory conditions, so communities are often examined through culture-independent techniques such as the amplification and sequencing of target DNA from environmental samples. The mcrA gene is considered unique to methane-producing Archaea (methanogens) and anaerobic methane oxidizing Archaea (methanotrophs) [34] and phylogenetically conserved [35]. It is a suitable marker gene for the study of natural populations of methanogens, as it is found in all known methanogens and anaerobic methanotrophs, has never been found in any other metabolic groups. Methanogenesis was found to be a widespread process in wetlands, but until now studies focused on methanogen distribution in managed wetlands are very scarce and their diversity remains uncharacterized.

The DGGE analysis of methanogenic populations didn’t revealed methanogens in pristine bog. The reason may be the aerated conditions of upper peat layer or presence of novel methanogens. Low degree of richness of phylotypes (i.e., bands) was found in samples of managed wetlands (MP and HF) and the average number was 4-5. Samples from MP and HF had similar DGGE profiles, and the variation was mostly observed in the intensity of the bands. It is practically impossible to quantify data from DGGE gel. Differences in PCR amplification kinetics, quantities of DNA loaded and other factors can lead to bands of different intensity.

Results of the phylogenetic analysis of the mcrA gene sequences from MP and HF samples (Fig 3) indicated a high relative abundance of uncultured methanogens closely grouped with Methanosarcinales, Methanobacteriales and Methanomicrobiales. All sequences were identified as unclassified environmental taxa only distantly related to validated methanogens (86-96% of similarity) and demonstrated the most analogy with uncultured clones from bulk paddy soil [36] and acid oligotrophic fen [37].

3.4 Diversity of Archea Based on DGGE Analysis of 16S rRNA gene

Low diversity of methanogens evaluated by mcrA analysis may be explained by the low number of this functional gene sequences deposited in the database, especially originating from peatland areas. We have additionally applied PCR-DGGE analysis of ribosomal gene to analyze Archea diversity in peat samples.

Based on 16S rRNA analysis only 1-2 archeal phylotypes were found in pristine bog samples (line 1 and 4, Fig. 4). Conversely, higher degree of richness of phenotypes was shown in disturbed sites. The average number of phylotypes observed in samples from peat extraction site (MP) and grassland (HF) was 7 and 9, correspondently. Altogether, 10 out of 18 DGGE bands were extracted and sequenced successfully (Table 3 and Fig. 4). Results of the BLASTn of the 16S rRNA gene sequences confirmed a high relative abundance of versatile Methanosarcinales in peat of samples of managed wetlands and revealed presence of hydrogenotrophic Methanomicrobiales pristine bog. Il sequences were identified as unclassified environmental taxa only distantly related to validated methanogens and demonstrated the most analogy with uncultured methanogens from lake and marine sediments (Table 3).

The drainage has a drastic effect in the methanogenic community and shift to acetoclastic methanogenesis. The majority of previous work indicates that hydrogenotrophic methanogenesis to be the dominant pathway in oligotrophic bogs and other northern peatlands [38,39] and hydrogenotrophic Methanomicrobiales and Methanobacteriales dominate in acid bogs. Metagenomes studies of ombrotrophic peatland revealed that dominant
methanogenic genera were associated both with hydrogenotrophic and acetoclastic pathways [40]. *Methanosarcinales* are metabolically more diverse than *Methanomicrobiales* and *Methanobacteriales* and can carry out acetoclastic, hydrogenotrophic, and methylotrophic methanogenesis [41]. They also possess enzymes for detoxification of oxygen, which could favor their occurrence in the dry conditions. Our results indicated the presence of *Methanosarcinales* in managed peatlands, suggesting that acetoclastic methanogenesis was a dominant methanogenic pathway within these peat soils.

![Phylogenetic tree](image1)

**Fig. 3** Phylogenetic tree based on the deduced partial McrA amino acid sequences from peatland DGGE bands soils (in bold) and reference sequences. The dendrogram was constructed with a neighbor-joining analysis. Scale indicates 10% sequence divergence.

![DGGE fingerprints](image2)

**Fig. 4.** DGGE fingerprints of 16S rRNA-defined archaeal communities. Bands with figures were excised, re-amplified and sequenced. Data of BLAST analysis are done in Table 3.
Table 3. Closest matches (name (accession number)) to database sequences of 16s rRNA gene fragment sequences excised from DGGE bands (Fig. 4). Sequences were compared to those held in the GenBank database using the BLASTn search tool

| Sample ID | Band # | Closest relative organism (accession number) | % Similarity | Environmental location of the closest relative organisms |
|-----------|--------|---------------------------------------------|--------------|--------------------------------------------------------|
| PB        | 1      | Crenarchaeote clone Pav-sed-511 (GU135491)  | 96           | Lake sediments                                         |
|           | 7      | Methanomicrobiales archaeon (AB448783)      | 96           | Sediments of a Gulf of Mexico                         |
|           | 8      | Uncultured Euryarchaeote (AJ867613)         | 98           | Alpine lake                                            |
| MP        | 2      | Uncultured Euryarchaeote (AB119617)         | 86           | Estuarine sediments                                    |
|           | 3      | Uncultured archaeon (AM712497)              | 96           | Arctic peat                                            |
|           | 4      | Uncultured Thermoproteales archaeon (AM501889) | 94       | Lagoon sediments                                      |
|           | 5      | Marine group I thaumarchaeote clone HF770_041I11 (DQ300544) | 84       | Ocean water                                            |
|           | 6      | Uncultured crenarchaeote (AM291985)         | 93           | Acidic forest soil                                     |
| HF        | 9      | Uncultured methanogenic archaeon clone PASLSS0.5m_1 (FJ982666) | 96       | Permafrost sediments                                   |
|           | 10     | Uncultured Methanosarcinales archaeon (AB448783) | 88       | Deposition in the Gulf of Mexico                       |
|           | 11     | Uncultured Methanosarcinales archaeon (AB448783) | 95       | Sediments of a Gulf of Mexico                         |
|           | 12     | Uncultured Methanomicrobia archaeon clone LPBBA93 (FJ902710) | 91       | Limestone sinkholes                                    |
|           | 13     | Uncultured archaeon clone ZA_P5_C01 (GQ328162) | 89       | Thermal pools                                          |
A number of unclassified Euarchaeote, Crenarchaeote and Thaumarchaeote were found in peat samples. These Archa may represent novel lineages not yet described in peatland soils and should be further investigated.

4. CONCLUSION

Peatlands are important participants in the carbon cycle of the Earth’s, and managed peatlands are extremely important in GHG emissions. This study revealed differences in bacterial and archaeal community composition between sampling sites, suggesting that bacteria and archaea involved in methane oxidation or production, respectively, are directly influenced by peatland management practice. Our results demonstrated that in natural peatlands hydrogenotrophic methanogens were predominating, whereas in the drained peats both hydrogenotrophs and acetothrophs were almost equivalent. The striking difference was found between methanotrophic communities of natural and managed peatlands. Analysis of key functional pmoA gene revealed that Methylocystis–like Alphaproteobacteria dominate in natural peatlands. In the drained peats Methylobacter substitutes type II methanotrophs and may be responsible for CH₄ oxidation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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