Original Research Article

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

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ABSTRACT

Aims: Drainage of peatlands is known to decrease overall CH_4 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 Dubnensky 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 archeal16S rRNA genes. FISH method was 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 acetothrophic 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 CH_4 oxidation in the drained peats.
Conclusion: This study indicates drastic changes in structure of CH_4-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 (CH_4) [1].

Methanogenic Archaea produced CH_4 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 CH_4 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 CH_4 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 CO_2 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 CH_4 emission, and the site may be turned from a methane source into a small sink [8]. Previous have shown the effects of drainage on composition and functioning of the microbial community in peatlands [9] but until recently CH_4-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 older-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 CH_4 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 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. MATERIAL 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; 56.70^0N , 37.83^0E, (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 3.8^0C 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 4^0C in the field. Subsamples for molecular analysis were frozen within 8 h of collection, firstly at -20^0C,

before transfer to -80^{0}C. Subsamples for FISH analysis were fixed by paraformaldehyde at the same time.

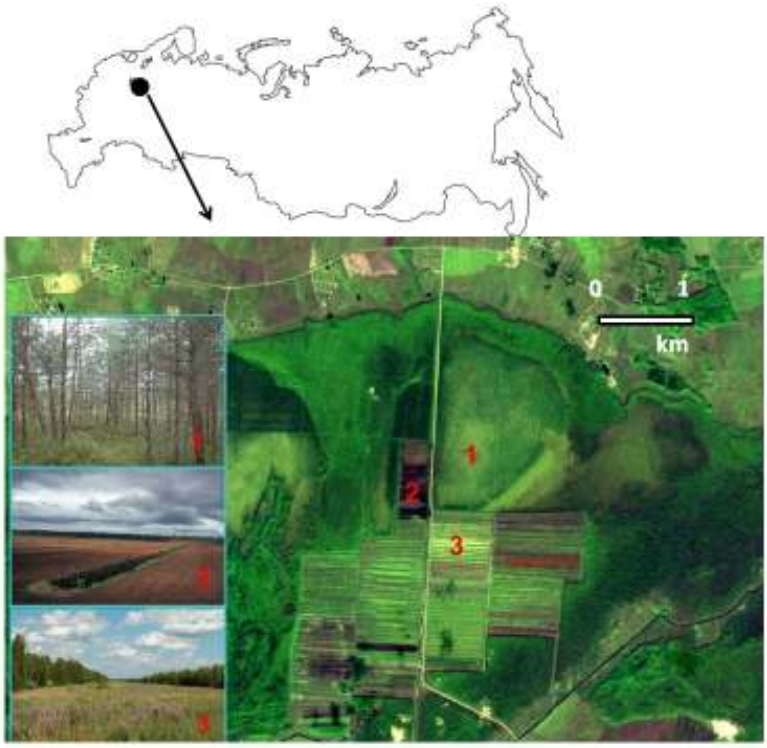

Fig 1. Map of the Taldom study site indicating three measurement plots along the drainage system and landscape topography. The inset shows the location in north-east Russia.

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 *Sphagnum* bog	Open peat surface	Haying grassland
*Water Table, cm	15	10	12
Peat layer, cm	250	140	145
pH	3.8	4.2	4.4
$N\text{-}NH_4^+$, mg/kg	2.3	4.6	6.3
$N\text{-}NO_3^-$, mg/kg	3,0	1,9	4.3
$C\text{-}CH_4$ emission rate, mg/(m^2×h)	0.8	0.2	0.03

* Below the peat surface

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 paraformaldehyde solution4% (wt/vol) [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 (Gammaprotepbacteria) and M-450 for type II (Alphaproteobacteria) MOB was used in this study [12] All oligonucleotide probes were sintesized by Syntol (Moscow, Russia).

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℃) 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., 2012 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℃) and voltage (200 V), as describ ed in Kizilova et al, 2012. 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

About a third of terrestrial soil carbon is stored in Northern peatlands 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].

Drainage impact on structure and function of peatland microbial community has previously been studied in some oligotrophic bogs and minerotrophic fen peatlands [18-21]. However, bogs and fens fundamentally differ in plant composition hydrology, pH, nutrient availability, and soil chemistry.

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 $0.9 – 3.1\times10^8$ cells, that comprised from 1.8 to 3.9 % of the total DAPI cell counts. The number of archaral 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.

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

Sample ID	No. of cells (10^7) 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 number of type I MOB cells (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 CH_4 flux and *Archea* abundance (r^2=0.99). In contrast, the CH_4 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 *Clonothrix* [22] as well as for the extremely acidophilic methane-oxidizing *Verrucomicrobium* [23]. In bacterial cells, methane conversion to methanol is performed by methane monooxygenase 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 [24-26] Furthermore, good correlation between phylogenies inferred using *pmoA* and 16S rRNA genes has been demonstrated [27]. 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 monooxygenase gene fragments, as a functional marker for characterization of the methanotrophic communities. No amplification product for soluble methane monooxygenase (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.

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 [28-30]. Phylogenetic analysis of Pmoa demonstrated that sequences formed two separate clusters within *Methylocystis* genera (Fig. 2) distantly 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 [29] and wetland in Finland [31].

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.

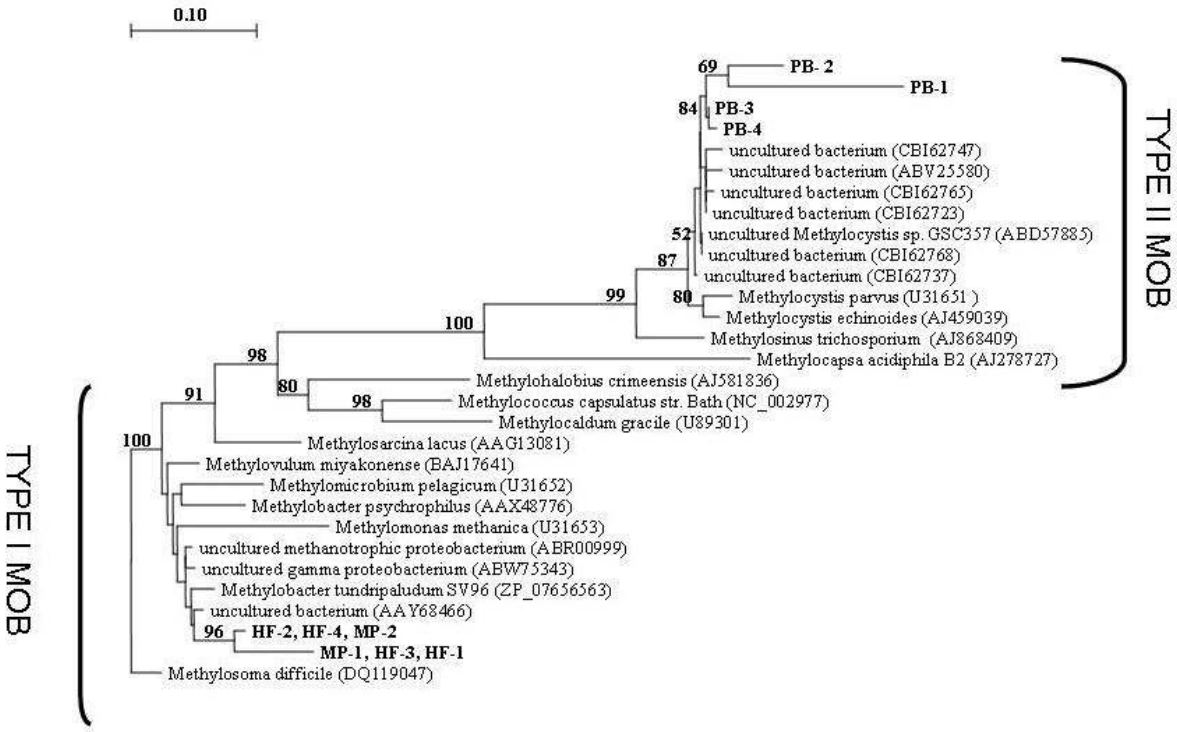

Fig.2 Phylogenetic tree based on the deduced partial Pmoa amino acid sequences from peatland DGGE bands soils (in bold) and reference sequences. The dendrogram was constructed with a neighbor-joining analysis. Scale indicates 10% sequence divergence. The maximum likelihood tree is rooted with *Nitrosomonas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

Only type I methanotrophs were detected in MP and NF sites (Fig. 2) and demonstrated distant relation to *Methylobacter tundripaludum* isolated from Norwegian arctic bog [32]. 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. It was shown that type II methanotrophs (*Methylocystaceae)* were tolerant for long periods of oxygen starvation and also were able to synthesize poly-β-hydroxybutyrate, their characteristic storage compound. Conversely, the ability to survive during long periods of oxygen depletion has never been demonstrated for type I methanotrophs (*Methylococcaceae)* [33]. It has to be also noted that type II methanotrophs are sensitive against inorganic nitrogen load. The responses of methanotrophs against nitrogen have be related to community composition since type I methanotrophs have been stimulated whereas type II methanotrophs have been inhibited by nitrogen [34]. Changes in vegetation could also modify the composition of the population of methanogens and methanotrophs, and recent molecular evidences support this idea [35]. Recently, more specific detection methods such as mRNA-based microarrays [36] and SIP-PLFA [29] 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.

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) CO_2 or CO fixation using H_2, formate, or certain alcohols (e.g. methanol, ethanol, 2-propanol, 2-butanol) as electron donors, (2) methylotrophic; methyl groups of C_1 compounds (e.g. methanol, mono-, di- andtrimethylamine, dimethylsulfide, methylmercaptan) are cleaved and reduced to methane, and (3) aceticlastic; acetate is fermented to methane and CO_2 [37].

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) [38] and phylogenetically conserved [39]. 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.

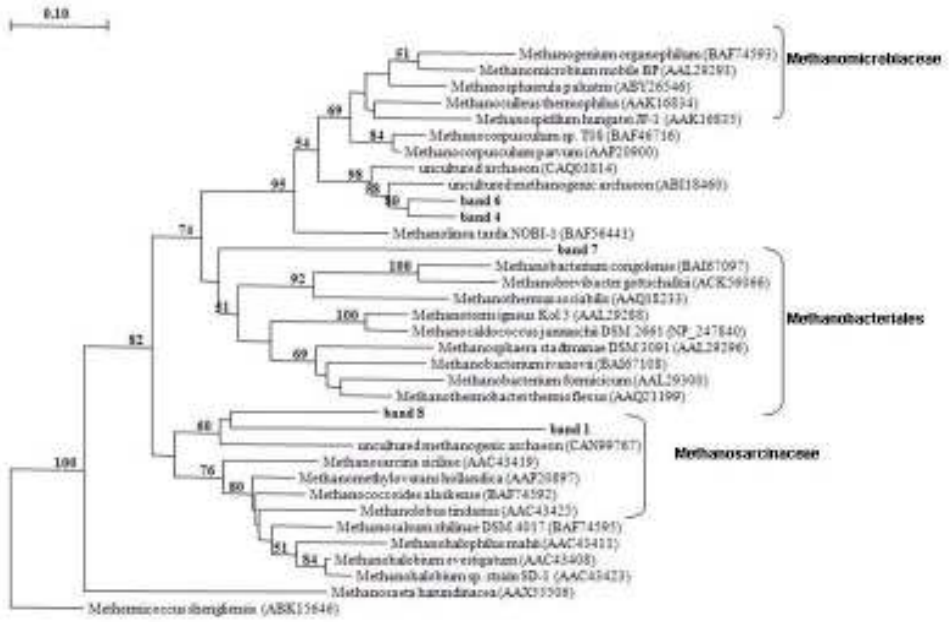

Fig. 3 Phylogenetic tree based on the deduced partial McrA amino acid sequences from peatland DGGE bands soils (in bold) and reference sequences. The dendrogram was constructed with a neighbor-joining analysis. Scale indicates 10% sequence divergence.

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 [40] and acid oligotrophic fen [41].

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 rhibosomal gene to analyze Archea diversity in peat samples.

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

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. ll 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).

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 *Methanomicrobi*a archaeon clone LPBBA93 (FJ902710)	91	Limestone sinkholes
	13	Uncultured archaeon clone ZA_P5_C01 (GQ328162)	89	Thermal pools

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 [42, 43] and hydrogenotrophic *Methanomicrobiales* and *Methanobacteriales* dominate in acid bogs. Metagenomes studies of ombrotrophic peatland revealed thet dominant methanogenic genera were associated both with hydrogenotrophic and

acetoclastic pathways [44]. *Methanosarcinales* are metabolically more diverse than *Methanomicrobiales* and *Methanobacteriales* and can carry out acetoclastic, hydrogenotrophic, and methylotrophic methanogenesis [45]. 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.

A number of unclassified Euarchaeote, Crenarhaeote and Thaumarchaeote were found in peat samples. These Archea 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 CH_4 oxidation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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