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Environmental transcription of *mmoX* by methane oxidizing *Proteobacteria* in a Sub-Arctic palsa peatland

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Abstract

Methane oxidizing bacteria (MOB) that possess the soluble form of methane monooxygenase (sMMO) are present in various environments, but unlike the prevalent particulate methane monooxygenase (pMMO) the *in situ* activity of sMMO has not been documented. Here we report on the environmental transcription of a gene (*mmoX*) for this enzyme which was attributed mainly to MOB lacking a pMMO. Our study indicates that the sMMO is an active enzyme in acidic peat ecosystems but its importance for the mitigation of methane releases remains unknown.

Methane oxidizing (methanotrophic) prokaryotes hold an important function in ecosystems around the globe because they mitigate the release of the greenhouse gas methane (CH_4). Most methanotrophs belong to the *Proteobacteria* and *Verrucomicrobia* (28). Additionally, *candidatus Methylomirabilis oxyfera* (14) and some methanogenic archaea (3) can oxidize CH_4 in the absence of oxygen using nitrate and sulfate as electron acceptors, respectively. The key enzyme, methane monooxygenase (MMO), which oxidizes methane to methanol at the expense of NAD(P)H, exists in two forms. A particulate, membrane bound enzyme (pMMO) occurs in nearly all methane oxidizing bacteria (MOB) with the exception of *Methyloferula* and *Methylocella.* These latter two genera feature only a soluble, cytoplasmic enzyme (sMMO). While the pMMO has a narrow substrate range, oxidizing only C1-C4 alkanes and alkenes, the sMMO can also utilize C5-C9 alkanes, alkenes, alicyclic and

aromatic compounds, and the chlorinated compounds trichloroethylene (TCE) and chloroform (6, 17, 33) exposing it to interest for bioremediation and biotechnology applications. The well conserved genes *pmoA* and *mmoX* encode subunits of the pMMO and sMMO, respectively, and are used as functional marker genes for both enzymes (27). To date, environmental transcripts of *mmoX* have failed to be detected, even in acidic peatlands (5, 20) where pMMO lacking methanotrophs thrive (8, 12, 13). This has led to the suggestion that the pMMO may be involved in methane oxidation here (21), leaving the ecological relevance of the sMMO unclear.

Various MOB also possess genes encoding a nitrogenase and can carry out N_2-fixation (1). The *nifH* gene is highly conserved, widely used as a functional marker gene for N_2-fixation, and suitable for phylogenetic analysis. Among the four phylogenetic clusters of *nifH* sequences, MOB related *nifH* sequences fall into the 'conventional' Mo-containing *nifH* cluster I (4). Despite the widespread ability among MOB species to fix nitrogen, little attention has been devoted to N_2-fixation by MOB in nitrogen-limited, remote ecosystems.

In this study we investigated the presence of functional transcripts for the genes *pmoA*, *mmoX*, and *nifH* among MOB along the succession of permafrost in a palsa peatland. Palsa peatlands occur at the marginal zone of permafrost distribution and have been widespread throughout Scandinavia until a very few decades ago (31). These usually pristine ecosystems offer heterogeneous substrate conditions for microbial activity and display frozen peat mounds (palsas), thermokarst ponds, hollows and hummock like structures in close proximity. At present, palsa formation and collapse are no longer in their natural balance since the permafrost is thawing faster than new palsas can build up. In some areas of northern Scandinavia, climate changes had already 10 years ago caused a decrease in the palsa area by two thirds (26) and palsa regression still continues to be significant.

Our study site was located in northern Norway (UMT: 69.694 N; 29.383 E) at the transition from the Arctic to the Sub-Arctic using the 10 degrees July isotherm as the boarder. Annual average temperatures and precipitation from 1965 to2011 were -0.6 °C and 435 mm, respectively (Norwegian Meteorological Institute, Stations Veines/Neiden and Kirkenes Airport). In this period, a positive trend of both annual mean temperature and precipitation was observed (Fig S1). Three different successional palsa stages were selected as sites for sampling and analysis. They covered a currently degrading palsa (DP), a thermokarst pond (TP) adjacent to a DP and a hollow, which represents an old successional stage of a previously collapsed palsa (CP). At the palsas (elevated sites), the vegetation was dominated by *Ledum palustre*, *Empetrum* sp., *Pleurozium sp.* and *Rubus chamaemorus* while the mire

sites were dominated by *Eriophorum vaginatum*, *Andromeda polifolia*, *Carex rotundata*, *Carex cannescens*, *Carex lapponica*, *Sphagnum riparium* (TP) and *Sphagnum lindbergii* (CP). We sampled duplicates of pore water for the analysis of vertical concentration profiles of methane, ammonium, and nitrate and triplicates of soil cores/blocks per site for the determination of carbon and nitrogen content and for molecular analysis. In addition, plot scale methane emissions were conducted in triplicates per site. The procedures for sampling and analyzing pore water and for methane emission measurements are described in detail elsewhere (24). Ammonium concentrations were determined by an analytical laboratory (TosLab, Tromsø, Norway). For molecular analysis, the top layer of fresh plant material was removed and the blocks were sectioned into an upper 10 cm layer and a lower ~15-25 cm layer. Subsamples of those sections were pooled, distributed to sterile 50 ml tubes and stored in a liquid nitrogen saturated dry shipper on site. The environmental data of the sampling sites are presented in Table 1. Briefly, the pH varied between 4.2 and 4.6 and methane emissions and soil methane concentrations were significant in the thermokarst pond, lower in the collapsed palsa site and negligible from the palsa itself. Nitrogen was grossly limited in particular in the top 10 cm layer of the wet sites dominated by *Sphagnum*. Here, the C/N ratios varied between 65 and 97 (compared to 42 in the palsa), and pore water ammonium and nitrate concentrations were below the detection limit of 0.56 µM and 2.4 µM, respectively. Below a depth of 10 cm, the C/N ratios varied between 30 and 62. Unlike nitrate, ammonium could be detected here but did not exceed 2.6 µM.

The samples preserved for RNA analysis were ground in liquid N_2 to a fine powder. Subsequent extraction of total nucleic acids was carried out in duplicates. Approximately 0.3 g of sample were mixed with 0.5 ml of extraction buffer (5% CTAB/120 mM K_3PO_4, pH8) and subjected to bead beating for 45 s. After phenol-chloroform extraction, nucleic acids were precipitated by incubation with linear acrylamide and 2 volumes of 30% PEG-8000 for 120 min at room temperature, collected subsequently by centrifugation for 60 min at 4 °C and resuspended in DEPC treated water. To retrieve only the RNA for downstream cDNA generation and analysis, the solutions were treated with RNase inhibitor while the DNA was digested with the TURBO DNA-free kit (Ambion). Synthesis of cDNA was carried out using 100-500ng RNA as template, random hexamers (Invitrogen) at a final concentration of 500 nM, and SuperScript™ III Reverse Transcriptase (Invitrogen). The obtained cDNA was first used as template for universal bacterial 16S rRNA gene amplification using RNA as NTC to check for successful cDNA synthesis and complete DNA digestion. Then, the different functional genes were targeted. Amplification of *pmoA*, *mmoX*, *nifH*, *pxmA* and 16S rRNA

gene fragments, cloning, sequencing and bioinformatics was performed as described in the supplementary material.

We were able to show that *pmoA* gene products are present in all sites, while environmental transcripts of *mmoX* and *nifH* were found only in the two wet sites (TP and CP). This is, to our knowledge, the first study that reports the detection of environmental transcripts of *mmoX*. Considering the presence of transcripts as an indication for activity, this strongly points at an environmental relevance of the soluble methane monooxygenase in acidic peat ecosystems. The majority of *mmoX* transcripts were assigned to the group of *Beijerinckiaceae*, presumably to relatives of *Methylocella* (Fig. 2B) and thus to species that lack a pMMO. *Methylocystis* was the dominant group based on total number of both *pmoA* and *mmoX* sequences retrieved from DNA, but did not transcribe *mmoX*. In contrast, the identification of *Methylocystis* related *pmoA* transcripts that belong to a cluster of *pmoA* sequences originating solely from peat ecosystems (2) (Fig. 1B) indicates that *Methylocystis* utilize the pMMO rather than the sMMO. This was expected for MOB hosting both forms of MMOs. In addition to *mmoX* transcripts from the *Beijerinckiaceae*, transcripts of this gene detected in the collapsed palsa were assigned to an OTU distantly related to *Methylomonas* and thus belong to the group of type I MOB. There are two possible scenarios that could explain this exciting finding. The first is that in contrast to current beliefs, type I MOB that lacks a pMMO does exist. The alternative explanation would be that methanotrophs preferentially transcribe *mmoX* under certain conditions, although they host both the soluble and the particulate enzyme. Thus, our findings pose interesting questions regarding the competition between 'sMMO dependent' methanotrophs and MOB possessing a pMMO with regard to enzyme kinetics, *in-situ* substrate preferences and the general importance of species lacking a pMMO for mitigating methane emissions. Recalling its broad substrate range and the diverse pool of potential compounds in northern peatlands (39), pMMO lacking MOB could even utilize alternative substrates rather than methane. Similarly to *Methylocystis*, *Methylobacter* and *Methylobacter* related sequences (type Ia) were detected in all sites and one OTU was represented also by *pmoA* transcripts. Furthermore, active species were identified among *Methylomonas* and *Methylocapsa* in the thermokarst pond and in the collapsed palsa, respectively. In the palsa site (DP), cDNA synthesis and detection of *mmoX* was not successful. Also, the amplification of *pmoA* and 16S rRNA was problematic with only a nested approach yielding *pmoA* products. This is indicative for a low abundance of MOB in the palsa site where methane concentrations and emissions were negligible and it is consistent with qPCR data (unpublished data).

Targeting PmoA, altogether 12 OTUs were assigned, with the highest species richness in the latest successional stage of palsa degradation (CP) and the lowest in the palsa itself (Fig 1A). The largest number of OTUs based on MmoX was also detected in the late successional stage of CP (Fig. 2A). A summary on the number of DNA and cDNA sequences of each site used for phylogenetic and diversity analysis and the respective amount of OTUs is given in Table S2. Targeting the 16S rRNA gene using MOB specific primers, only 8 OTUs were revealed, indicating primer based failure of detecting some MOB. Nevertheless, all dominant groups that were identified based on functional genes were also found by targeting the 16S rRNA gene (Figs. S2 and S3) and rarefaction analysis in general revealed a good coverage of species richness (Fig. 3). 6 OTUs were assigned based on the MmoX of which at least 3 most likely lacks a pMMO (*pmoA* gene), increasing the total number of detected MOB to 15. In order to define the MmoX OTU cutoff on the species level, we used a distance of 4% which was based on the correlation between MmoX and 16S rRNA gene sequence distances of selected species (Fig. 3B). Plotting of pairwise distances also proved that *mmoX* is an appropriate phylogenetic marker within MOB. In comparison with *mmoX* homologues (Fig. 3C), *mmoX* seems to have evolved within MOB species and is presumably an essential enzyme. Overall, the diversity of palsa MOB is moderate and ranges between MOB species numbers of rice paddies (25) and Arctic soils (16, 23, 38). However, only a very few species were observed to be active which most likely is a result of the low pH (15, 22, 30).

In general, the composition of the MOB community of this palsa peatland and the dominance of *Methylocystis* related sequences is representative for what has been reported for acidic *Sphagnum* dominated peat (5, 7-10, 12, 13, 19, 20, 32). Most cultivars from acidic *Sphagnum* peat are known to be capable of N_2-fixation which was reported for *Methylocapsa acidiphila* (12), *Methylocella tundrae (8)* and *Methylocella palustris* (13). Also species of the genotypes *Methylomonas*, *Methylocystis* (1) and *Methylobacter* (e.g.(37)) are known to carry out N_2-fixation. This set of MOB reflects the palsa community. Thus it supports the assumption that nitrogen availability influences soil bacterial communities (29), in particular since in our study MOB related *nifH* sequences made up $\geq$ 10% of all sequences both on the DNA and on the cDNA level (Fig. 4). The presence of MOB related *nifH* transcripts suggests a direct compensation for nitrogen deficiency through N_2-fixating MOB. So far, environmental transcripts of *nifH* related to MOB were neither reported in *Sphagnum* peat nor in any other pristine and oligotrophic habitat. Our findings point at an important ecosystem function carried out by MOB both in the carbon and the nitrogen cycle of acidic peatlands. The contribution of MOB to N_2-fixation in acidic peatlands could be substantial considering

the pronounced number of MOB related *nifH* transcripts detected and should attract more attention.

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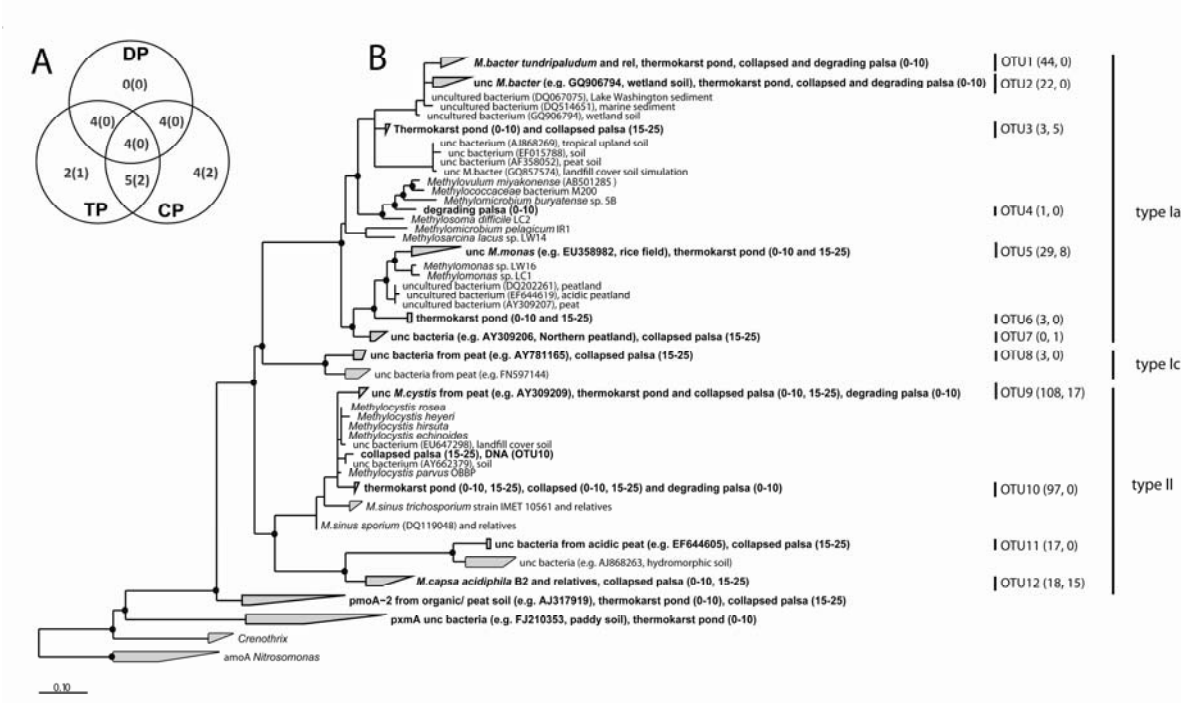

Figure 1: A) Venn diagram comparing OTUs at the PmoA level. Numbers in brackets refer to cDNA. B) Neighbor joining tree of partial *pmoA* sequences based on deduced amino acid residues retrieved from the three sampling sites, degrading palsa, thermokarst pond, and collapsed palsa (in bold), compared with public database sequences. Numbers in brackets next to the OTU assignment refer to the number of DNA and cDNA sequences retrieved, respectively. Closed circles mark nodes that were verified by a maximum likelihood tree.

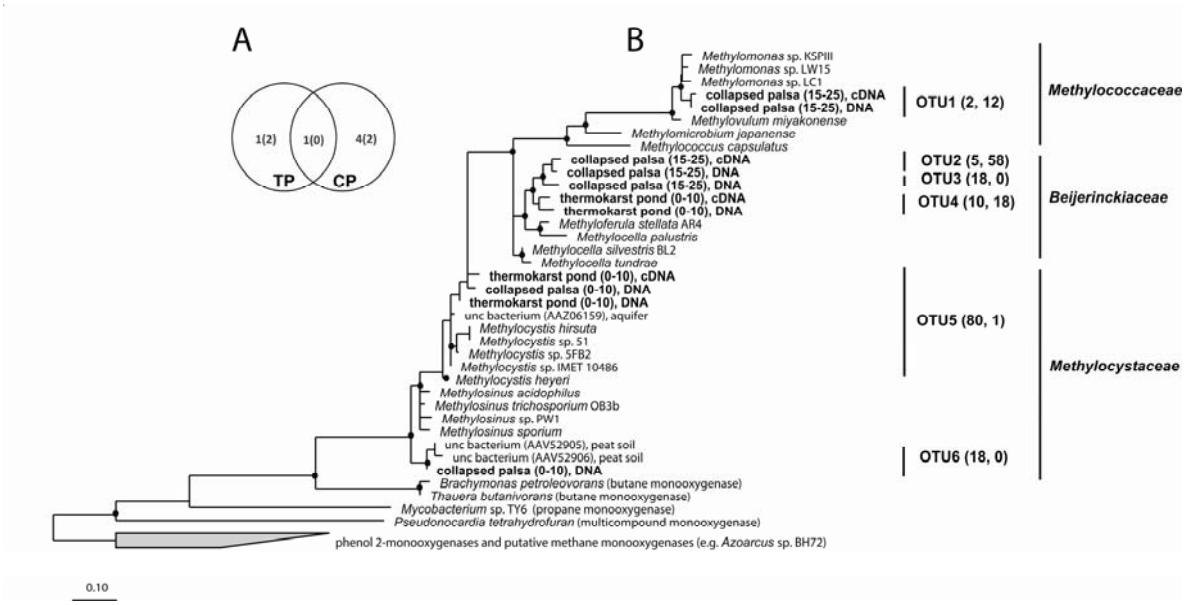

Figure 2: A) Venn diagram comparing OTUs at the MmoX level. Numbers in brackets refer to cDNA. B) Neighbor joining tree of partial *mmoX* sequences based on deduced amino acid residues retrieved from the three sampling sites, degrading palsa, thermokarst pond, and collapsed palsa (in bold), compared with public database sequences. Numbers in brackets next to the OTU assignment refer to the number of DNA and cDNA sequences retrieved, respectively. Closed circles mark nodes that were verified by a maximum likelihood tree.

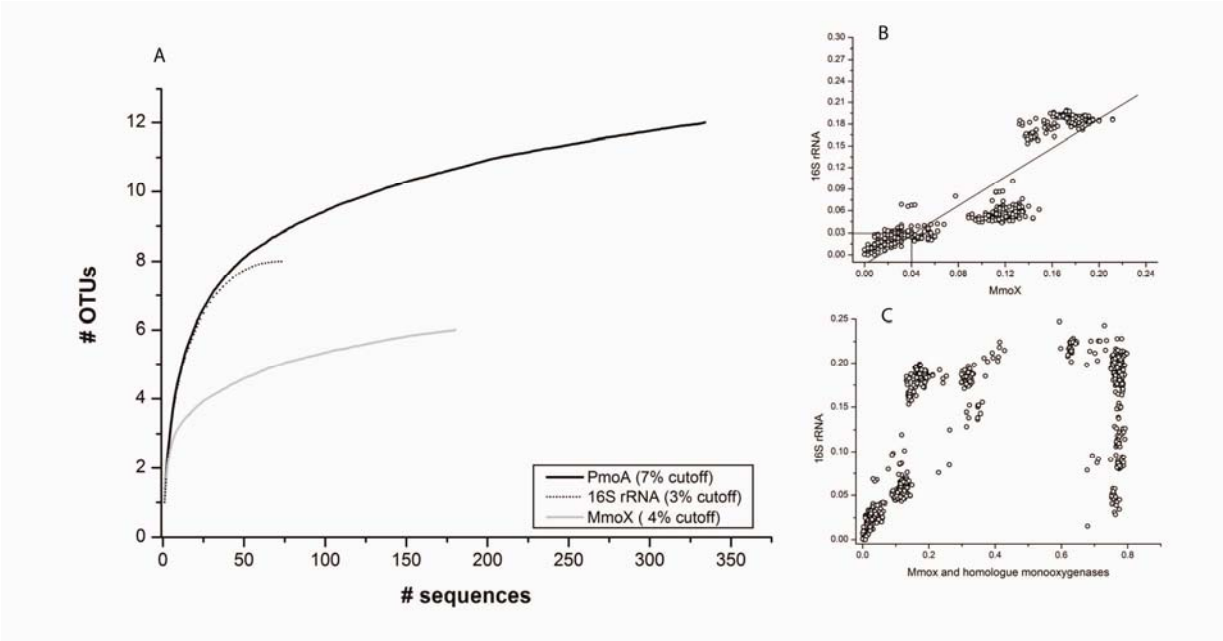

Figure 3: A) Rarefaction analysis of deduced PmoA, MmoX, and *16S rRNA* gene sequences. B) Correlation of MmoX versus *16S rRNA* gene sequence distances of 32 methanotrophic species and C) of 43 species including MmoX homologues.

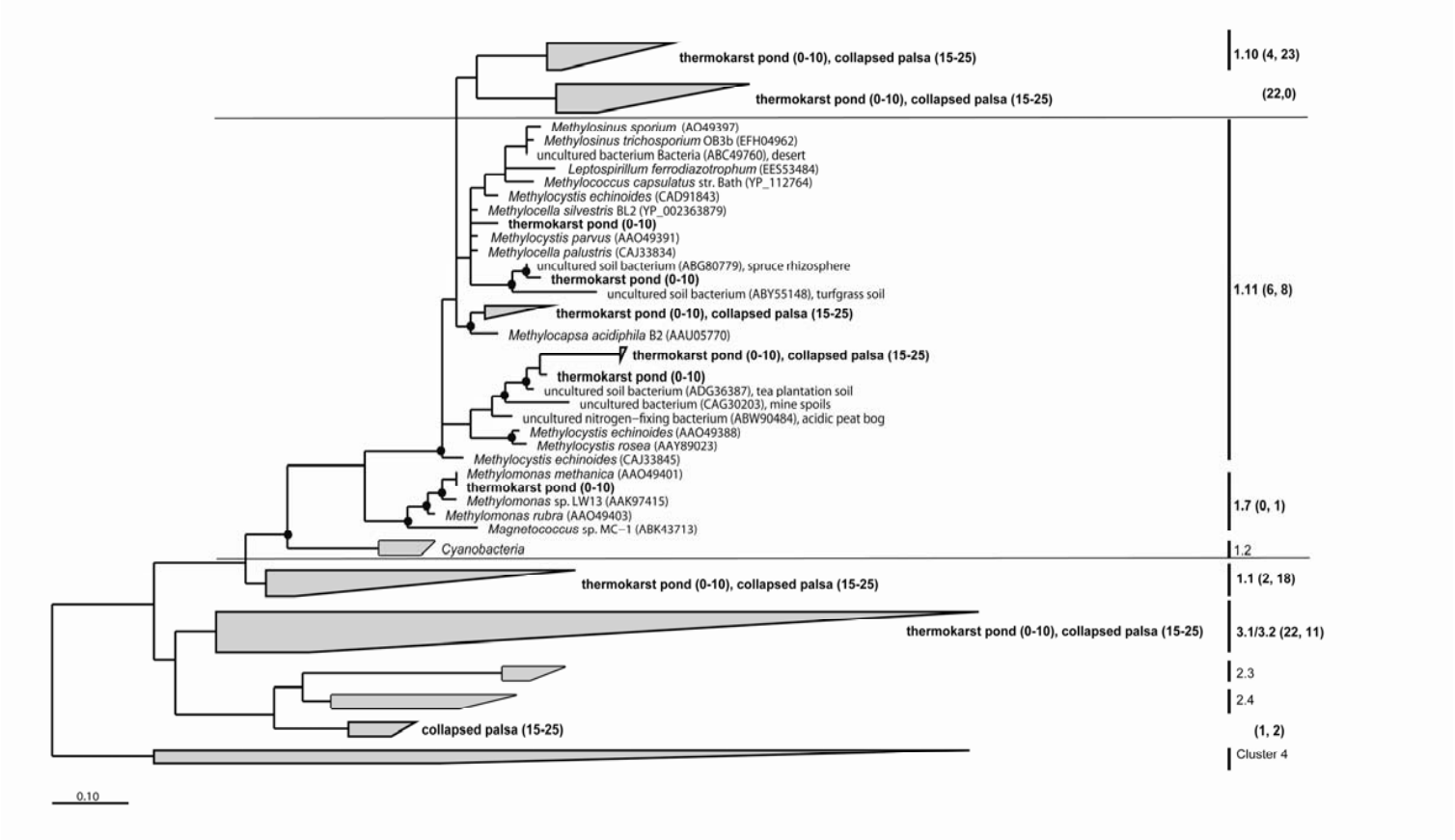

Figure 4: Neighbor joining tree of partial *nifH* sequences based on deduced amino acid residues retrieved from the studied palsa peatland (in bold) compared with public database sequences. Numbers in brackets next to the cluster labeling refer to the numbers of DNA and cDNA sequences retrieved, respectively, and indicate affiliation with existing and new clusters. The detailed view shows clusters 1.11 and 1.7 which primarily consists of methanotroph retrieved *nifH* sequences. Closed circles mark nodes that were verified by a maximum likelihood tree.

Table 1: Environmental data of the three sampling sites, degrading palsa (DP), thermokarst pond (TP), and collapsed palsa (CP), obtained in July 2010.

	pH	CH_4 (µM)	CH_4 emission (mg m^{-2} d^{-1})
DP	**4.2** (±0.11, n=5)[1]	**0.06** (±0.08, n=5) (0-10cm)[2]	**b.d.**
		b.d. (20-25cm)[2]	
TP	**4.2** (±0.3, n=4)	**9.15** (±7.76, n=4) (0-10cm)[3]	**623** (±419, n=3)
		403.51 (±58.01, n=4) (20-35cm)[3]	
CP	**4.6** (±0.6, n=4)	**0.04** (±0.04, n=4) (0-10cm)[3]	**31** (±2, n=3)
		96.15 (±106.83, n=4) (20-35cm)[3]	

[1] measured in July 2011
[2] values refer to pore gas concentrations
[3] values refer to pore water concentrations
b.d.=below detection

Supplementary material

Table S1: Targeted genes, primer details and PCR conditions of this study

Gene	Primer combination	Primer sequence (5'-3')	Annealing Temp (°C)	Reference
pmoA	A189f	GGNGACTGGGACTTCTGG	62-52 (TD)	(4)
	A682	GAASGCNGAGAAGAASGC		
	Mb661r	CCGGMGCAACGTCYTTACC		(2)
pxmA	pxmA 230f	GGCARTGGTGGCCNTTGGT	59	(10)
	pxmA 732r-1	TGGCGAACCATTTACCGATGTAC		
	pxmA 732r-2	TSGCAAACCACTTGCCGATRTRC		
mmoX	mmoX-206f	ATCGCBAARGAATAYGCSCG	60	(5)
	mmoX-886r	ACCCANGGCTCGACYTTGAA		
nifH	Pol-f	TGCGAYCCSAARGCBGAC TC	57	(8)
	Pol-r	ATSGCCATCATYTCRCCGGA		
***16S rRNA gene*, type I MOB specific**	MethT1dF	CCTTCGGGMGCYGACGAGT	56.5	(11)
	MethT1bR	GATTCYMTGSATGTCAAGG		
***16S rRNA gene*, type II MOB specific**	typeII-f	GGGAMGATAATGACGGTACCWG	60	(1)
	typeII-r	GATCAARAGCTGGTAAGGTTC		

Table S2: Total and site specific numbers of DNA and cDNA sequences used for phylogenetic tree construction and diversity analysis and number of assigned OTUs of the different genes targeted.

Gene	#sequences DNA				#sequences cDNA				#OTUs DNA				#OTUs cDNA			
	DP	TP	CP	total	DP	TP	CP	total	DP	TP	CP	total	DP	TP	CP	total
***pmoA* (PmoA)**	27	139	179	**345**	n.d.	19	26	**45**	4	7	9	**12**	-	2	4	**5**
***mmoX* (MmoX)**	n.d.	83	50	**133**	n.d.	19	70	**89**	-	2	5	**6**	-	2	2	**4**
***nifH* (NifH)**	-	34	33	**67**	n.d.	29	34	**63**	-	-	-	**-**	-	-	-	**-**
16S rRNA	-	47	27	**74**	-	-	-	**-**	-	6	4	**8**	-	-	-	**-**

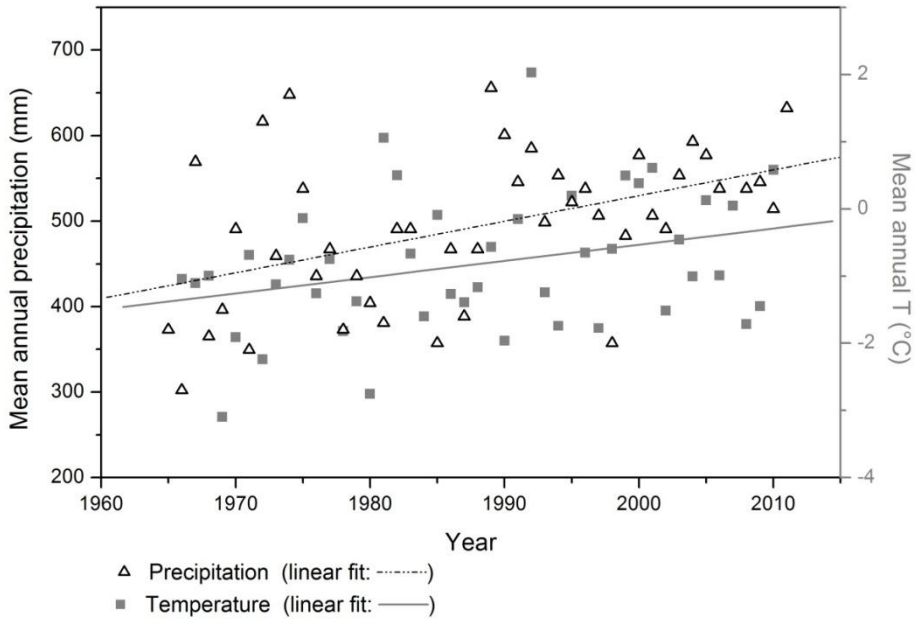

Figure S1: Climate data of Kirkenes in the period 1965-2011.

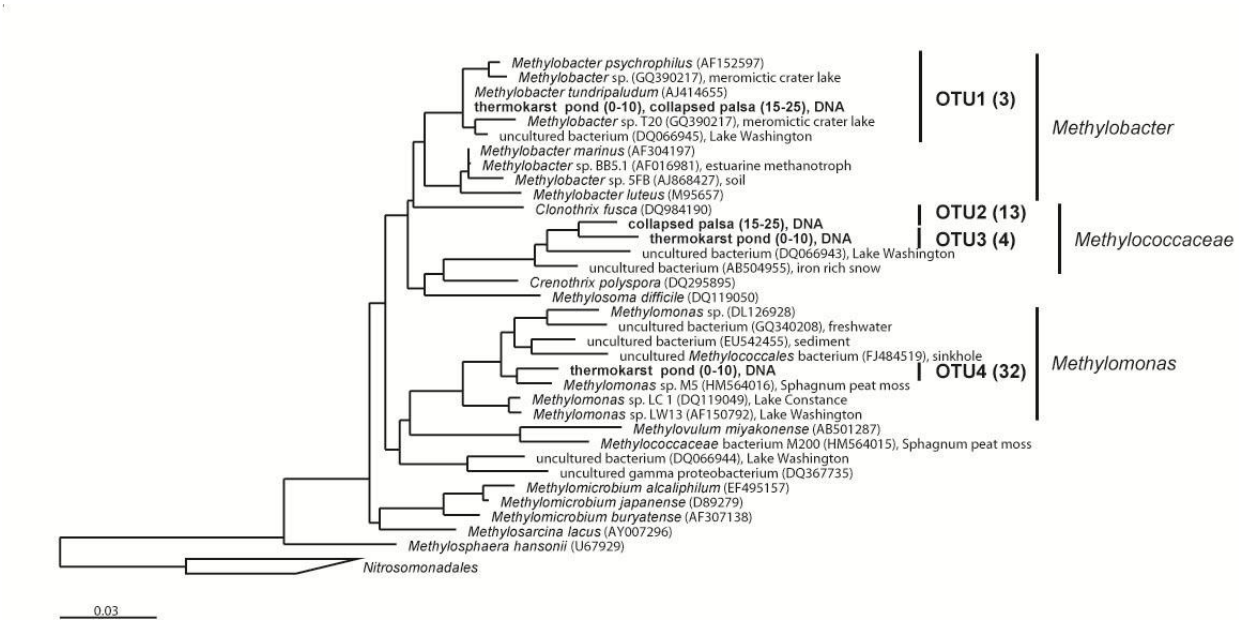

Figure S2: Neighbor joining tree of type I MOB specific 16S rRNA gene sequences retrieved from the three sampling sites degrading palsa, thermokarst pond, and collapsed palsa (in bold) compared with public database sequences.

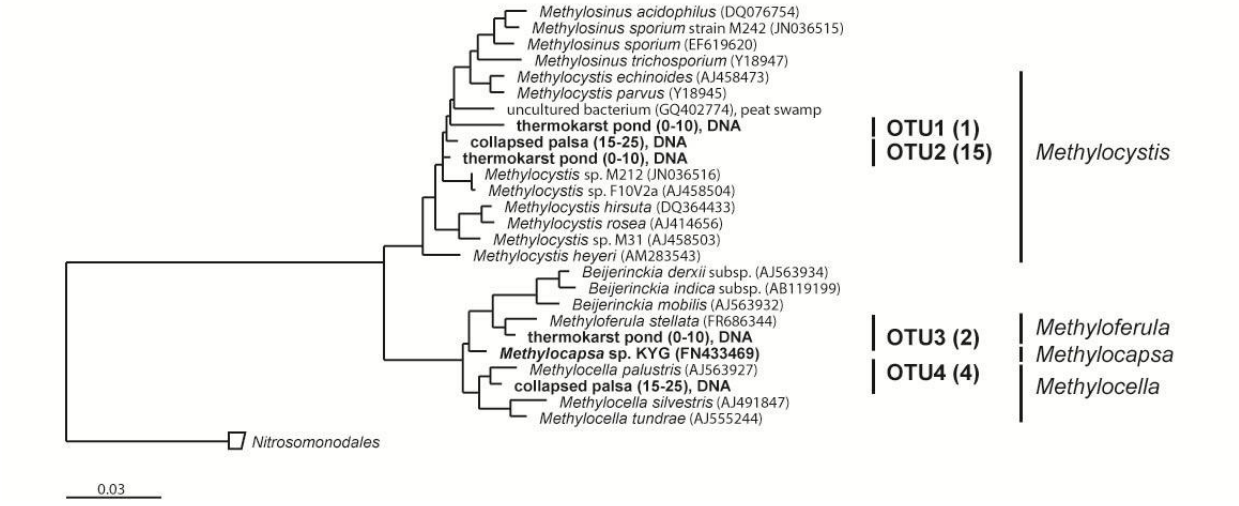

Figure S3: Neighbor joining tree of type II MOB specific *16S rRNA* gene sequences retrieved from the three sampling sites degrading palsa, thermokarst pond, and collapsed palsa (in bold) compared with public database sequences.

PCR on pmoA, mmoX, nifH, pxmA and 16S, cloning, and sequencing

PCR reactions (50 µl) contained 25 ml PremixF (Epicentre Biotechnologies), gene specific primers (Sigma-Aldrich) at a final concentration of 500 nM, 2.5 U Taq polymerase (Invitrogen), and 5-50 ng of template. The final elongation step was extended to 30 min if subsequent cloning was intended. Primer details, targeted genes and PCR programs are summarized in Table S1. The PCR products were excised, purified (QIAquick gel extraction kit, QIAGEN), poly-A ligated into a pCR®4-TOPO® vector (Invitrogen) and transformed via heat-shock into chemically competent TOP10 as described by the manufacturer. Overnight at 37 °C grown colonies were screened for correct inserts using the gene specific primers and vector primers, respectively. Sanger sequencing was performed by MACROGEN.

Bioinformatics

The sequences were processed with the Sequencing Analysis 5.2 software package applying a quality and trimming filter. The post-processed sequences were compared with the GenBank database using the blastn, blastx, and vecscreen algorithms. Using the CLC Sequence Viewer 6.5.1 package, PmoA, MmoX, and NifH sequences were deduced and imported into databases maintained with the phylogenetic software package ARB (6). Thereby, the *nifH* database was obtained from the Zehr lab (http://www.es.ucsc.edu/~wwwzehr/research/database/). Phylogenetic trees of the functional genes were constructed selecting the neighbor joining method implemented in the ARB software using a cutoff and a 30% maximum frequency filter for the amino acid sequences (PmoA: 140 aa, MmoX: 360 aa, NifH: 107 aa). Phylogenetic trees of the *16S rRNA* were constructed selecting the maximum likelihood method also implemented in the ARB software using a preset termini and a bacterial positional variability filter. The *16S rRNA* gene sequences were prior to that aligned with the SINA alignment service (http://www.arb-silva.de/aligner/). Rarefaction analysis and assignment of operational taxonomic units (OTUs) was performed with DOTUR-1.53 fed with distance matrices calculated with ARB using the above mentioned cutoff filters without correction. OTUs were defined using a 7% cutoff for PmoA (3, 7), a 4% cutoff for MmoX (this study, refer to Fig. 4) and a 3% cutoff for the 16S rRNA gene (9). Sequences of *pmoA*, *pxmA*, *16S rRNA*, *nifH*, and *mmoX* have been submitted in the GenBank database under accession numbers xxxx-xxxx.

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