

# Methanogenic communities in permafrost-affected soils of the Laptev Sea coast, Siberian Arctic, characterized by 16S rRNA gene fingerprints

Lars Ganzert<sup>1</sup>, German Jurgens<sup>2</sup>, Uwe Münster<sup>3</sup> & Dirk Wagner<sup>1</sup>

<sup>1</sup>Alfred Wegener Institute for Polar and Marine Research, Potsdam, Germany; <sup>2</sup>Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Helsinki, Finland; and <sup>3</sup>Tampere University of Technology, Institute of Environmental Engineering and Biotechnology, Tampere, Finland

**Correspondence:** Dirk Wagner, Alfred Wegener Institute for Polar and Marine Research, Telegrafenberg A43, D-14469 Potsdam, Germany; Tel.: +49 331 288 2159; fax: +49 331 288 2137; e-mail: dwagner@awi-potsdam.de

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## Keywords

archaea; methanogenic diversity; 16S rRNA gene; DGGE; methane; permafrost soils.

## Abstract

Permafrost environments in the Arctic are characterized by extreme environmental conditions that demand a specific resistance from microorganisms to enable them to survive. In order to understand the carbon dynamics in the climate-sensitive Arctic permafrost environments, the activity and diversity of methanogenic communities were studied in three different permafrost soils of the Siberian Laptev Sea coast. The effect of temperature and the availability of methanogenic substrates on CH<sub>4</sub> production was analysed. In addition, the diversity of methanogens was analysed by PCR with specific methanogenic primers and by denaturing gradient gel electrophoresis (DGGE) followed by sequencing of DGGE bands reamplified from the gel. Our results demonstrated methanogenesis with a distinct vertical profile in each investigated permafrost soil. The soils on Samoylov Island showed at least two optima of CH<sub>4</sub> production activity, which indicated a shift in the methanogenic community from mesophilic to psychrotolerant methanogens with increasing soil depth. Furthermore, it was shown that CH<sub>4</sub> production in permafrost soils is substrate-limited, although these soils are characterized by the accumulation of organic matter. Sequence analyses revealed a distinct diversity of methanogenic archaea affiliated to *Methanomicrobiaceae*, *Methanosarcinaceae* and *Methanosaetaceae*. However, a relationship between the activity and diversity of methanogens in permafrost soils could not be shown.

## Introduction

Arctic tundra wetlands are an important source of the climate-relevant greenhouse gas methane (CH<sub>4</sub>). The estimated methane emissions from these environments varies between 20 and 40 Tg year<sup>-1</sup> CH<sub>4</sub>, which corresponds to up to 8% of the global warming (Cao *et al.*, 1996; Christensen *et al.*, 1999). The degradation of organic matter is slow, and large amounts of organic carbon have accumulated in these environments as a result of the extreme climate conditions with long winters and short summers (Gorham, 1991), and the wet conditions in the soils during the vegetation period. Arctic wetlands could therefore be significant for the development of the Earth's climate, because the Arctic is observed to heat up more rapidly and to a greater extent than the rest of the world (Hansen *et al.*, 2005). In particular, the melting of permafrost and the associated release of climate-relevant trace gases driven by intensified microbial turnover of

organic carbon represent a potential environmental hazard (IPCC, 2001). However, the control mechanisms of methane production, oxidation and emission from tundra environments are still not completely understood.

Permafrost relates to permanently frozen ground with a shallow surface layer of several centimetres (the active layer) that thaws only during the short summer period. The seasonal freezing and thawing of the active layer, with extreme soil temperatures varying from about +18 °C to -35 °C, leads to distinct geochemical gradients in the soils (Fiedler *et al.*, 2004). During the short arctic summer, permafrost soils also show a large temperature gradient along their depth profiles, and this is one of the main environmental factors that influence the microbial communities in these extreme habitats (Kotsyurbenko *et al.*, 1993; Wagner *et al.*, 2003). Water is another important factor for microbial life in these environments. The seasonal thawing of the upper permafrost promotes water saturation of the

soils, leading to anaerobic degradation of complex organic matter to simple compounds, such as acetate, H<sub>2</sub>, CO<sub>2</sub>, formate and methanol, by fermentative bacteria. These compounds serve as substrates for methanogenic archaea, which are responsible for the production of CH<sub>4</sub> (Garcia *et al.*, 2000).

Methanogenic archaea, which belong to the kingdom *Euryarchaeota*, are ubiquitous in anoxic environments. They can be found both in moderate habitats such as rice paddies (Grosskopf *et al.*, 1998a), lakes (Jurgens *et al.*, 2000; Keough *et al.*, 2003) and freshwater sediments (Chan *et al.*, 2005), as well as in the gastrointestinal tract of animals (Lin *et al.*, 1997) and in extreme habitats such as hydrothermal vents (Jeanthon *et al.*, 1999), hypersaline habitats (Mathrani & Boone, 1995) and permafrost soils and sediments (Kobabe *et al.*, 2004).

Several studies have revealed the presence of methanogens in high-latitude peatlands by finding sequences of 16S rRNA gene and methyl coenzyme M reductase (*mcrA*) genes affiliated with *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriaceae* and *Methanomicrobiales* (Galand *et al.*, 2002; Basiliko *et al.*, 2003; Galand *et al.*, 2003; Kotsyurbenko *et al.*, 2004; Høj *et al.*, 2005). It has recently been shown using FISH and phospholipid analyses that the active layer of Siberian permafrost is colonized by high numbers of bacteria and archaea with a total biomass comparable to that of temperate soil ecosystems (Kobabe *et al.*, 2004; Wagner *et al.*, 2005).

The present investigation is part of a long-term study on carbon dynamics and microbial communities in permafrost-affected environments in the Lena Delta, Siberia (Hubberten *et al.*, 2006). The overall purpose of this study was a basic characterization of the methanogenic communities in different extreme habitats of the Laptev Sea coast using both physiological and molecular ecological methods. DNA was extracted from the active layer of three vertical permafrost profiles and analysed by PCR with primers specific for 16S rRNA genes of methanogenic archaea and by denaturing gradient gel electrophoresis (DGGE) followed by sequencing of DNA bands reamplified from the gels. In addition, the potential methane production was analysed under various temperature and substrate conditions.

## Materials and methods

### Study sites and sample collection

Soil samples were collected at various sites on the Laptev Sea coast, northeast Siberia during two Russian–German expeditions in 2002 and 2003. The investigation sites were located in the Lena River Delta on Samoylov Island (72°22'N, 126°28'E) and in the Lena–Anabar lowland on the Nuchcha Dzhielle river near Cape Mamontovy Klyk

(73°36'N, 117°20'E). Both study sites are located in the zone of continuous permafrost and are characterized by an Arctic continental climate with a mean annual air temperature of  $-14.7^{\circ}\text{C}$  ( $T_{\text{min}} = -48^{\circ}\text{C}$ ,  $T_{\text{max}} = 18^{\circ}\text{C}$ ) and a mean annual precipitation of about 190 mm. Further details of the study sites can be found in Schwamborn *et al.* (2002) and Wagner *et al.* (2003).

Soil and vegetation characteristics show great variation over small distances owing to the geomorphological situation of the study sites (Fiedler *et al.*, 2004; Kutzbach *et al.*, 2004). Two sites were chosen for sampling on Samoylov Island, a floodplain (FP) and a polygon centre (PC). The floodplain was characterized by recent fluvial sedimentation, whereas the polygon centre was characterized by peat accumulation with interspersed sand layers. The vegetation at the floodplain site was dominated by *Arctophila fulva*. In the polygon centre, typical plants were *Sphagnum* mosses, *Carex aquatilis* and lichens. The sampling site at Cape Mamontovy Klyk (MAK) was located in a small low-centre polygon plain. The vegetation here differed from that of the polygon centre on Samoylov Island and was dominated by *Eriophorum* spp., *Carex aquatilis*, some *Poaceae* and mosses.

For soil sampling, vertical profiles were arranged and samples were taken from defined soil horizons for physico-chemical (e.g. CH<sub>4</sub> concentration, dissolved organic carbon and total organic carbon contents) and microbiological (e.g. potential CH<sub>4</sub> production, DNA-based analyses) analyses. The samples for microbiological analyses were placed in 250-mL sterile Nalgene boxes, which were immediately frozen at  $-22^{\circ}\text{C}$ . For detailed investigations, horizons with a thickness of more than 10 cm were divided and subsamples were taken. Continuous cooling at  $-22^{\circ}\text{C}$  was guaranteed for the sample transport from the Lena Delta (Siberia) to Potsdam (Germany). Samples were thawed at  $4^{\circ}\text{C}$  and used directly for the analyses, or subsamples were separated and refrozen for later analyses at  $-22^{\circ}\text{C}$ .

### Soil properties

The investigated soils were classified according to US Soil Taxonomy (Soil Survey Staff, 1998). The depth of the permafrost table was measured by driving a steel rod into the unfrozen soil until frozen ground was encountered. The water table was measured in perforated plastic pipes that were installed in the active layer. Soil temperature measurements (a Greisinger GTH 100/2 equipped with a Ni–Cr–Ni temperature sensor) were carried out in each horizon before soil sampling.

Vertical profiles of soil CH<sub>4</sub> concentrations were obtained by extracting CH<sub>4</sub> from fresh soil samples by adding 10 g of soil to saturated NaCl solution, shaking the solution, and subsequently analysing the CH<sub>4</sub> headspace concentration with gas chromatography.

Dissolved organic carbon (DOC) was extracted from various horizons of the soil profiles. Fresh soil material (9 g) was taken from each horizon, weighed into glass jars (50 mL) and mixed with 45 mL of distilled water. The bottles were closed and shaken for 1 h in the dark. Afterwards, the suspension was filtered (0.45- $\mu$ m mesh, Gelman Science) and the clear solution was inactivated by the addition of sodium azide. The DOC analysis was carried out with an Elementar High-TOC-II. Total organic carbon (TOC) and total nitrogen (TN) were analysed with an element analyser (Elementar Vario EL) using dried and homogenized soil samples. Prior to analysis the samples were treated with HCl (10%) at 80 °C for carbonate removal.

### CH<sub>4</sub> production rates

The influence of temperature, as well as of different substrates (no substrate, methanol or H<sub>2</sub>/CO<sub>2</sub>), on microbial CH<sub>4</sub> production was determined for each horizon. The substrates were chosen according to previous results obtained for the same study site, which showed that hydrogen is more important than acetate for methanogenesis in permafrost soils (Wagner *et al.*, 2005), while no information is available on the importance of methanol as a methanogenic substrate in permafrost environments. Under anoxic conditions, 30-mL glass bottles were filled with 10 g of soil material, and 3 mL of sterile water was added. All bottles were sealed with sterile butyl rubber stoppers. In the case of methanol as additional substrate, 0.1 mL of 1 M methanol stock solution was added to reach a final methanol concentration of about 30 mM. Afterwards, all jars were flushed with N<sub>2</sub>/CO<sub>2</sub> (80:20 v/v). For growth with hydrogen, samples were flushed a second time with H<sub>2</sub>/CO<sub>2</sub> (80:20 v/v, pressurized to 150 kPa). Three replicates were used for the different experiments. The incubation temperatures were 5 °C and 18 °C. CH<sub>4</sub> production was measured daily over a period of one week by sampling the headspace using a Hamilton gastight syringe. Gas analysis were performed with an Agilent 6890 gas chromatograph equipped with a Carbonplot capillary column ( $\phi$  0.52 mm, 30 m) and a flame ionization detector (FID). Helium was used as carrier gas. The injector, oven and detector temperatures were set at 45, 45 and 250 °C, respectively. CH<sub>4</sub> production rates were calculated from the linear increase in CH<sub>4</sub> concentration. Samples were dried after incubation at 55 °C, and the methane production was calculated to the dry weight.

### DNA extraction and PCR amplification

DNA was extracted directly from 0.75 g of soil material using an UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories Inc.), following the manufacturer's instructions. The quality and quantity of DNA were controlled on 0.8% agarose gels with SYBR Gold staining.

16S rRNA gene fragments with a length of approximately 350 bp were amplified using PCR with the primer pair GC\_357F-691R specific for methanogens (Watanabe *et al.*, 2004). The 50- $\mu$ L PCR mixture contained 1  $\times$  PCR reaction buffer, 0.25 mM of each dNTP, 2 mM MgCl<sub>2</sub>, 0.4  $\mu$ M of each primer, 2.5 U HotStarTaq DNA Polymerase (Qiagen) and 1–3  $\mu$ L of DNA template, depending on the quality and quantity of extracted DNA. In some cases, extracted DNA from permafrost soils was diluted 10-fold. PCR was performed using an iCycler Thermal Cycler (Bio-Rad). The amplification conditions consisted of an initial activation step for the HotStarTaq at 94 °C for 10 min, followed by 35 cycles of 94 °C for 60 s, 53 °C for 60 s and 72 °C for 2 min, with a final elongation step of 8 min at 72 °C. PCR products were checked on 2% agarose gels stained with SYBR Gold (Molecular Probes).

### Denaturing gradient gel electrophoresis and sequencing

All samples were separated on 8% polyacrylamide gels in 1  $\times$  TAE buffer using a D-Code System (Bio-Rad). The denaturing gradient ranged from 30 to 60% (100% denaturant consisted of 7 M urea and 40% (v/v) deionized formamide). The gels were run at 60 °C, at a constant voltage of 100 V for 14 h. After electrophoresis, the gels were stained for 30 min with SYBR Gold (1:10 000 dilution) and visualized under UV light using a GeneFlash system (SynGene).

DNA bands that appeared sharp and clear in the gel were cut out with a sterile scalpel and were transferred to sterile 0.5-mL Eppendorf tubes. DNA was eluted overnight in 30  $\mu$ L of sterile milliQ water at 4 °C. Reamplified products with the expected migration in a new DGGE gel were reamplified again without GC clamp. After purification, using a QIAquick PCR Purification Kit (Qiagen), the DNA bands were sequenced. Sequencing was done by AGOWA GmbH (Berlin, Germany) with forward and reverse primers.

### Phylogenetic analysis

Sequences were compared with those in the GenBank database using the BLAST ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)) and FASTA3 ([www.ebi.ac.uk](http://www.ebi.ac.uk)) tools in order to find and include in the analysis all closest relatives. The phylogenetic analysis of partial 16S rRNA gene sequences was performed using the ARB software package ([www.arb-home.de](http://www.arb-home.de); Ludwig *et al.*, 2004) and RAXML-IV (Stamatakis *et al.*, 2005). The ARB\_EDIT tool of the ARB was used for automatic sequence alignment, and the sequences were then corrected manually. A 50% invariance criterion for the inclusion of individual nucleotide sequence positions in the analysis was used to avoid possible treeing artifacts during construction of the 'backbone' trees. 'Backbone' trees were inferred using an

algorithm of the RAXML-IV program with 685, 625 and 602 informative nucleotide positions for *Methanosarcinaceae*, *Methanosaetaceae* and *Methanomicrobiales*/Rice cluster II trees, respectively. Next, 'backbone' trees were exported back to ARB and the partial sequences of the DGGE bands were added to the trees using the parsimony addition tool of the ARB program package. The partial 16S rRNA gene sequences of the DGGE bands obtained in this study are available in the EMBL/GenBank/DBJ database under accession numbers AM259179–AM259207.

## Results

### Soil environmental conditions

The physicochemical soil properties of the investigated sites showed a large vertical gradient and high small-scale variability in dependence of microrelief of the different permafrost soils (Table 1).

The soil of the polygon centre on Mamontovy Klyk was classified as a *Typic Aquiturbel*. The water level reached about 1 cm below the soil surface and the perennially frozen ground started at 44 cm. The comparable centre soil on Samoylov Island was a *Typic Historthel*, with a water table

near the soil surface and the permafrost beginning at 33 cm depth. Both soils are characterized by peat accumulation, with extremely high contents of organic carbon in the upper soil layers (> 18%) which decrease with increasing depth of the active layer.

The floodplain soil on Samoylov Island was classified as a *Typic Aquorthel*. The water table was near the soil surface and the permafrost started at 54 cm depth. In contrast to the other two permafrost soils, the floodplain soil on Samoylov Island was characterized by a silty soil texture with organic carbon contents between 0.8 and 3.1%.

The DOC concentration varied between 4.7 and 12.8 mg L<sup>-1</sup> on Samoylov Island polygon centre and between 2.7 and 7.8 mg L<sup>-1</sup> on Samoylov Island floodplain. The CH<sub>4</sub> concentration increased with increasing soil depth and showed values from 0.15 to 541.71 µmol g<sup>-1</sup> and from 0.002 to 0.411 µmol g<sup>-1</sup> in the polygon centre and floodplain, respectively.

In general, the active layer of permafrost was characterized by a strong temperature gradient from top to bottom, which ranged from 6 to 1 °C in the polygon centre on Mamontovy Klyk, and from 17.8 to 0.8 °C and from 7.5 to 0.4 °C, respectively, for the two investigated soils on Samoylov Island.

**Table 1.** Selected soil properties of the investigated permafrost soils

Sample ID	Horizon	Depth (cm)	T (°C)	CH <sub>4</sub> conc. (µmol g <sup>-1</sup> )	DOC (mg L <sup>-1</sup> )	TOC (%)	TN (%)
Polygon centre ( <i>Typic Aquiturbel</i> ), Mamontovy Klyk							
221	Oi1	0–6	6			39.4	1.42
222	Oi2	6–12	5			28.1	1.23
223	Bjg1	12–17	4	ND	ND	11.2	0.73
224	Bjg2	17–22	3			14.1	0.90
225	Bjg3	22–29	2			7.6	0.52
226	Bjg4	29–36	2			5.5	0.40
227	Bjg5	36–44	1			4.5	0.27
Floodplain ( <i>Typic Aquorthel</i> ), Samoylov Island							
6941	Ai	0–5	17.8	0.004	4.5	3.1	0.4
6942	Ajj	5–9	14.2	0.004	3.8	1.1	0.2
6943	Bg1	9–18	8.8	0.002	2.7	2.2	0.3
6944	Bg2	18–20	ND	ND	4.4	3.0	ND
6945	Bg3	20–35	4.0	0.035	7.8	2.5	0.4
6946	Bg4	35–40	1.9	0.114	4.9	2.0	0.3
6947	Bg5	40–52	0.8	0.411	4.8	0.8	0.2
Polygon centre ( <i>Typic Historthel</i> ), Samoylov Island							
6968	Oi1	0–5	7.5	0.15	11.7	18.3	0.51
6969	Oi2	5–10	5.8	13.19	8.8	13.8	0.43
6970	Ajj1	10–15	4.0	24.37	4.7	13.7	0.36
6971	Ajj2	15–20	2.7	70.50	9.5	9.3	0.23
6972	Bg1	20–23	1.2	ND	ND	7.0	0.19
6973	Bg2	23–30	0.4	163.24	11.9	4.7	0.16
6974	Bg2	30–35	< 0.4	328.87	12.8	3.6	0.15
6975	Bg3	35–40	< 0.4	541.71	ND	4.3	0.18
6976	Bg3	40–45	< 0.4	ND	ND	4.9	0.22

Horizon nomenclature and soil classification according to Soil Survey Staff (1998).

T, *in situ* temperature; DOC, dissolved organic carbon; TOC, total organic carbon; TN, total nitrogen; ND, not determined.

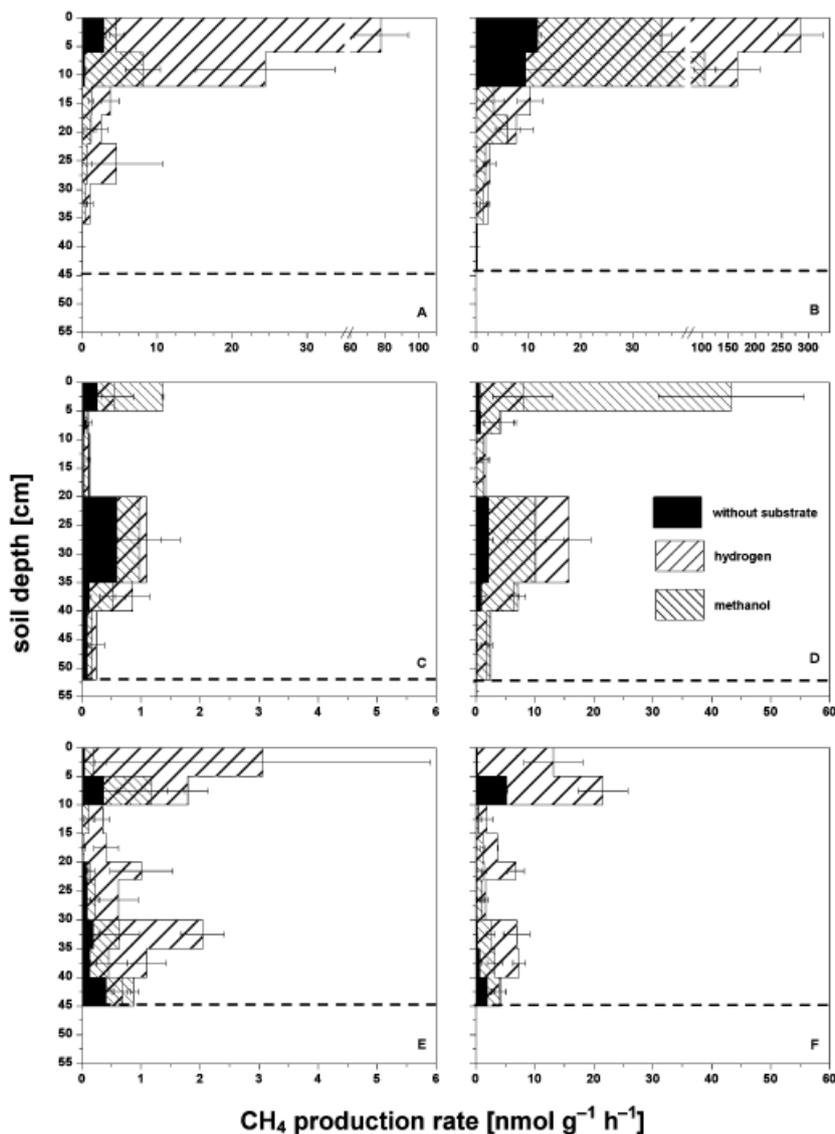
### Temperature and substrate effect on methanogenesis

The  $\text{CH}_4$  production of the three different soils showed significant differences in the rate of activity and vertical distribution (Fig. 1). In general, the activity in each profile was higher with hydrogen or methanol as additional substrate than it was without any substrate. Furthermore, the  $\text{CH}_4$  production was much higher at 18 °C than at 5 °C.

The highest  $\text{CH}_4$  production rate within the centre soil (*Typic Aquiturbel*) on Mamontovy Klyk was found with hydrogen as substrate, followed by methanol as substrate. Without any substrate addition, only a limited activity was detectable (Fig. 1a and b). The activity was highest in the two upper horizons, and decreased with increasing soil depth.

The activity pattern of the other two studied sites on Samoylov Island was different from that for Mamontovy Klyk. The floodplain soil (*Typic Aquorthel*) showed two maxima of  $\text{CH}_4$  production, one in the upper soil horizon and a second in the zone with the highest root density at a depth between 20 and 35 cm (Fig. 1c and d). Here, the highest activity was measured in the upper soil horizon with methanol, while the  $\text{CH}_4$  production rates in all other horizons were higher with hydrogen as substrate.

The soil (*Typic Historthel*) of the polygon centre on Samoylov Island was characterized by the highest  $\text{CH}_4$  production taking place in the upper soil horizons. This was also observed for the comparable soil on Mamontovy Klyk (Fig. 1e and f). However, in contrast to the latter soil, high activity also occurred in the polygon centre on Samoylov Island in the bottom zone of the active layer close to the



**Fig. 1.** Vertical profiles of  $\text{CH}_4$  production for the three study sites at 5 °C (left column) and 18 °C (right column) without any substrate as well as with hydrogen and methanol as additional methanogenic substrates. (a and b) *Typic Aquiturbel* (polygon centre on Mamontovy Klyk); (c and d) *Typic Aquorthel* (floodplain on Samoylov Island); and (e and f) *Typic Historthel* (polygon centre on Samoylov Island). Dashed lines indicate the permafrost tables.

permafrost table with a temperature near to the freezing point of water. With the exception of the bottom horizon, where the highest CH<sub>4</sub> production occurred with methanol, the preferred substrate in all other horizons was hydrogen.

The effect of increasing temperature was different for the three sites, as well as in the vertical profile of each soil. Compared with the CH<sub>4</sub> production at 5 °C on Mamontovy Klyk, CH<sub>4</sub> production at 18 °C was about three times higher, that in the floodplain soil on Samoylov Island was at least 10 times higher, and that in the polygon-centre soil on Samoylov Island was at least two times higher. In general, the methane production activity in the upper part of the active layer of all soils rose after the increase of temperature more strongly than it did within the bottom part of the profiles near the permafrost table.

### DGGE analysis of permafrost soil samples

Three permafrost sites on the Laptev Sea coast were compared with regard to variation in the community structure of methanogenic archaea from the top to the bottom of the investigated soil profiles. DGGE profiles showed up to nine well-defined bands per depth, and a shift within the vertical profiles of Samoylov Island polygon centre (*Typic Historthel*) and Mamontovy Klyk polygon centre (*Typic Aquiturbel*). In the polygon centre on Samoylov Island (*Typic Historthel*), the number of DNA bands increased to a depth of 23 cm (zone of highest root density) and then decreased again (Fig. 2a). The number of bands in the polygon-centre soil on Mamontovy Klyk was constant to a depth of 22 cm, with about four DNA bands in each lane (Fig. 2b). Most DNA bands were observed in the middle of the profile (22–29 cm soil depth) and this number decreased with increasing soil

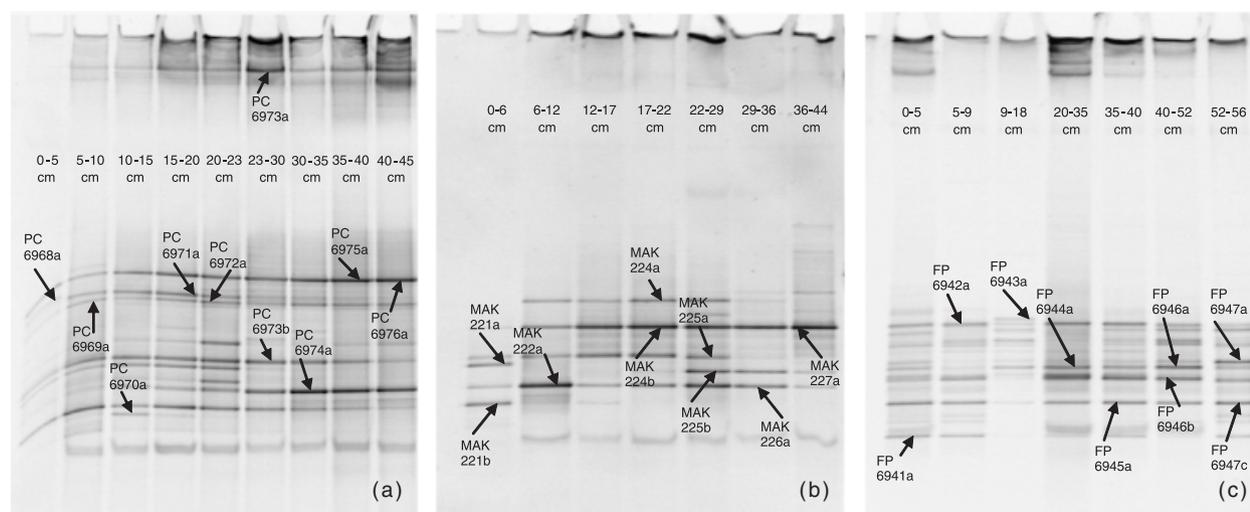
depth, as was also observed for the soil of the polygon centre on Samoylov Island (Fig. 2a). Interestingly, the floodplain on Samoylov Island showed a completely different pattern. Here, the number of bands did not decrease with increasing depth (Fig. 2c). Even the soil horizon close to the permafrost table showed a diversity of methanogens comparable with the highest diversity in the middle of the two other profiles.

Besides the number of bands, the distribution pattern showed distinct differences, particularly within the vertical profiles of the polygon-centre soils on Samoylov Island (soil depth 20–23 cm compared with the bottom of the active layer) and Mamontovy Klyk (the first horizon in comparison with the bottom of the active layer).

Some DGGE bands were found only in certain horizons, such as PC 6970a (*Methanosarcinaceae*), PC 6943a, MAK 221a and MAK 221b (all *Methanomicrobiaceae*). Beside these unique bands, some other bands that did not occur throughout the whole soil profile could also be seen. For example, DGGE bands corresponding to MAK 224a (*Methanomicrobiaceae*) were found only in the middle of the soil profile at a depth of 6–29 cm, and bands corresponding to MAK 225b (*Methanosarcinaceae*) were found only in the deeper regions of the soil profile.

### Phylogenetic analysis of permafrost sequences

A total of 36 DGGE bands from three soil profiles were sequenced. Eight sequences were excluded from further analysis because of their short (< 200 nucleotides) length. All sequences can be differentiated at the genus level. Twenty-eight sequences of 16S rRNA gene fragments obtained from the investigated permafrost environments fell within known euryarchaeotal lineages belonging to the

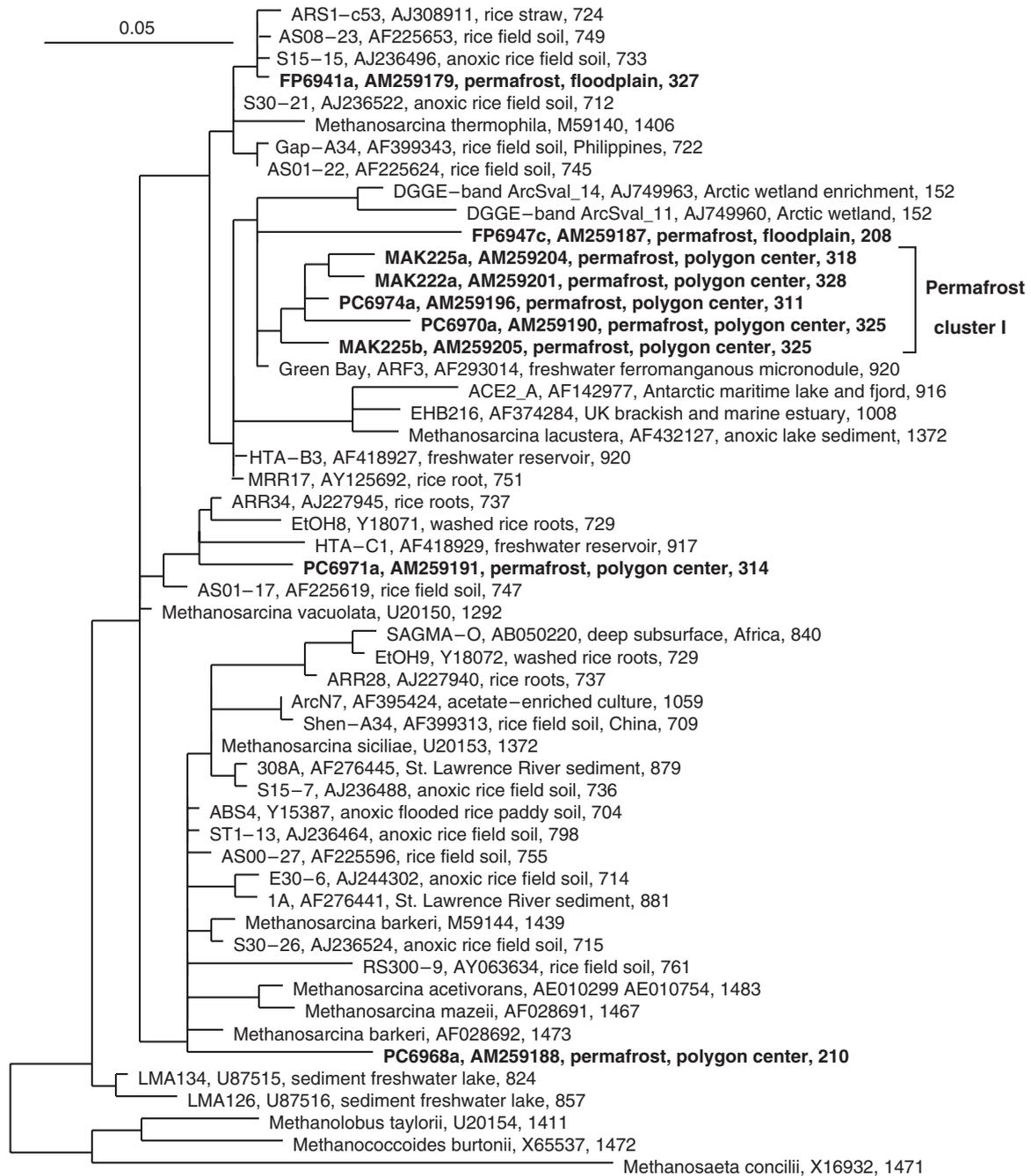


**Fig. 2.** DGGE profiles of 16S rRNA genes amplified from permafrost community DNA obtained from various horizons (thickness in centimetres from top to bottom) of the active layer. (a) *Typic Historthel* (polygon centre on Samoylov Island); (b) *Typic Aquiturbel* (polygon centre on Mamontovy Klyk); and (c) *Typic Aquorthel* (floodplain on Samoylov Island). Selected bands marked with arrows and sample IDs were used for sequence analyses.

major methanogenic groups *Methanosarcinaceae* (11 sequences, 98–99% homology over 312–316 nucleotides; including three, which were 100% identical), *Methanosaeta* (two sequences with 97% homology over 308 nucleotides), and *Methanomicrobiales* (12 sequences, 91–99% homology

over 300–321 nucleotides), and three sequences fell within (97% similarity) an as yet uncultivated archaea lineage named Rice cluster II (Fig. 3a and b).

Sequences affiliated to *Methanosarcinaceae* and *Methanomicrobiaceae* were found in all studied soil profiles, whereas



**Fig. 3.** Phylogenetic trees illustrating the affiliation of methanogenic 16S rRNA gene sequences reamplified from DGGE bands. The sequences recovered from permafrost belong to *Methanosarcinaceae* (a), and *Methanomicrobiales* together with Rice cluster II (b). The 'backbone' trees are based on maximum likelihood analysis of the dataset made with RAxML-IV, and partial sequences of the permafrost DGGE bands (shown in bold) were added to these trees using the parsimony addition tool of the ARB program package. The scale bar represents 0.05 changes per nucleotide. Identification of the bands is shown in Fig. 2. Clone name, accession number, environment and length of each sequence are indicated.

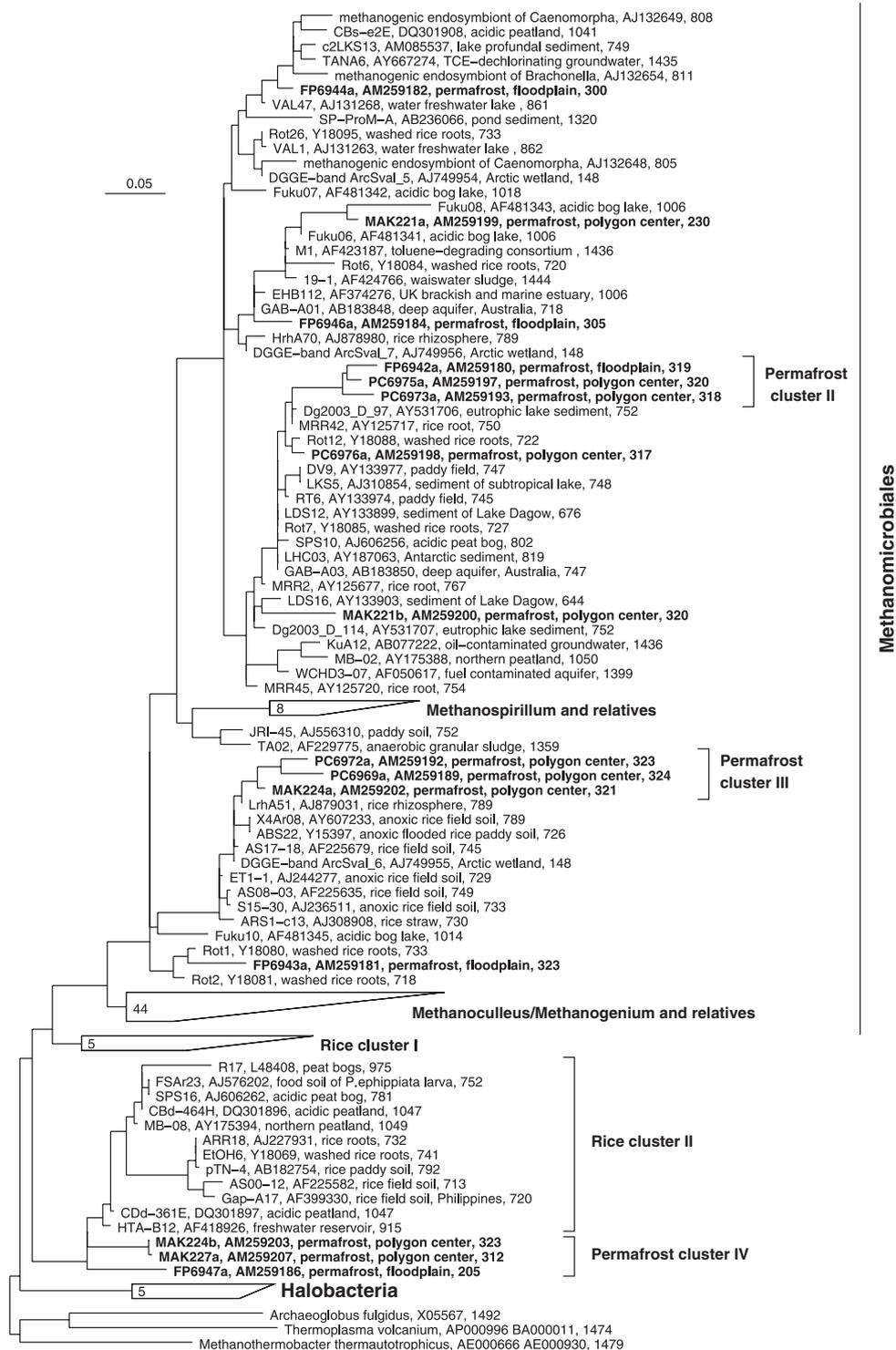


Fig. 3. Continued.

sequences associated with *Methanosaeta* were found only in the floodplain and in the polygon centre on Samoylov Island, but not in the polygon centre of Cape Mamontov Klyk.

## Discussion

Our results showed differences in the CH<sub>4</sub> production activities and the biodiversity patterns of methanogenic

archaea in the investigated permafrost soils. Activities of methanogenic archaea differed significantly in their rates and distributions among the different soils. While the CH<sub>4</sub> production rates in the active layer on Mamontovy Klyk decreased with increasing soil depth, the two other sites on Samoylov Island showed at least two activity optima. The highest activity occurred in the upper soil horizons, which are characterized by *in situ* temperatures of up to 17.8 °C. The second optimum of methane production was found in the middle or bottom part of the active layer in both soils on Samoylov Island. In the floodplain soil, the highest activity was detected in a horizon constantly exposed to temperatures below 4 °C, which indicates the dominance of methanogens that must be well adapted to the cold conditions observed close to the permafrost table. Here, the second activity optimum correlates with the zone of the highest root density and amount of DOC. It is well known that plants can supply root exudates consisting of low-molecular-weight organic compounds, which can serve as a substrate for methanogens (Chanton *et al.*, 1995; Ström *et al.*, 2003). However, the extraordinarily high CH<sub>4</sub> production rates in the upper layer of Mamontovy Klyk correlates with the high amount of organic carbon in these horizons.

The addition of different substrates led to an increase in the potential CH<sub>4</sub> production in all horizons of all sites. This effect was not confined to horizons with a low content of organic carbon, but could also be observed in horizons with a high amount of organic carbon. Wagner *et al.* (2005) reported that the humification of soil organic matter increased with increasing soil depth. This was shown to be reciprocally correlated with the amount of bioavailable organic carbon. A reduced quantity and quality of organic matter in permafrost soils could lead to a substrate-limited methanogenesis.

The potential CH<sub>4</sub> production at 5 °C was distinctly different from that at 18 °C. A higher incubation temperature resulted in a marked increase of the methanogenic activity in almost all investigated soil horizons. It is noteworthy that the effect of higher temperature on the activity was larger in the upper soil horizons with higher *in situ* temperatures than in the bottom of the active layer with lower *in situ* temperatures. Hence, taking into consideration the physiological studies, we can conclude that the activity of methanogenic archaea in permafrost soils depends on the quality of soil organic carbon, and our results show that methanogens in deep active-layer zones might be better adapted to low temperatures.

Only a few psychrophilic strains of methanogenic archaea have been described so far (Simankova *et al.*, 2003; Cavicchioli, 2006). However, our results indicate a shift in the methanogenic community from mesophilic to psychrotolerant or psychrophilic methanogens with increasing soil depth. Similar results have been obtained from permafrost

soils on Samoylov Island in the context of the methane-oxidizing community (Liebner & Wagner, 2006). An important requirement for microorganisms to adapt to cold environmental conditions is constantly low *in situ* temperatures over a long period of time (Morita, 2000). This is the case in the bottom zone of the active layer close to the perennially frozen ground. A prerequisite for prokaryotes to adapt to low temperatures is that their cell membranes should maintain fluidity. This effect was shown in a related study, carried out for the centre profile on Samoylov Island, which revealed an increase of branched-chain fatty acids in relation to the amount of straight-chain fatty acids with increasing active-layer depth (Wagner *et al.*, 2005).

The DGGE pattern of the investigated permafrost soils showed differences within the depth profile and between the different sites. The number of DNA bands at the floodplain site on Samoylov Island remained fairly constant through the whole profile. While the temperature drastically decreased with soil depth, the carbon (DOC and TOC) and nitrogen concentrations in the profile remained relatively constant. These geochemical profiles can be explained by the fact that the floodplain is periodically flooded by the Lena River. Thus the vegetation is regularly buried by the accumulation of new sediments, which causes the even distribution of organic matter in the profile. Galand *et al.* (2003) reported that vegetation characterizing microsites in a studied boreal fen influences the microbial communities in layers with significant methane production. The similarity of the community pattern for the whole soil profile of the floodplain can probably be attributed to the regular sedimentation at this site, but a significant relationship between this pattern and the methane production as reported by Galand *et al.* (2003) was not determined. This is in accordance with studies in Arctic wetlands in Spitzbergen, which found that methane fluxes depend more on the temperature and thaw depth than on the archaeal community structure (Høj *et al.*, 2005).

In contrast to the profile for the floodplain site, the polygon-centre profiles for Mamontovy Klyk and on Samoylov Island showed a variety of diversity patterns. These soils were characterized by humus accumulation in the upper part of the active layer, with decreasing organic matter content in the underlying mineral soils. However, the number of bands increased until the zone with the highest root density, but started to decrease in the deeper zones of the active layer. The presence of root exudates (Chanton *et al.*, 1995; Ström *et al.*, 2003), as discussed earlier with regard to the methane production activity, seems also to affect the diversity of the methanogenic archaea in permafrost soils. Among the differences in the number of detected DNA bands within the various horizons of the vertical profiles, different band patterns indicated differences in the community structure of methanogens, particularly in the

polygon-centre profiles on Mamontovy Klyk and on Samoylov Island. These differences refer to the bottom zone of the active layer compared with horizons, which lie further above in the respective profiles. A depth-related change of the methanogenic community was also observed in northern peatlands (Galand *et al.*, 2005).

The results of the DGGE analysis indicate changes of the methanogenic community within the vertical soil profiles. Some DGGE bands appeared throughout the whole profile, while others were specific for distinct active-layer depths. Moreover, the band pattern showed distinct differences between specific horizons. On one hand this indicates the presence of methanogenic archaea that can exist under different environmental conditions (temperature, substrate, geochemistry), which are changing within the depth of the active layer. On the other hand, it indicates the presence of methanogens that can exist only under defined environmental conditions. Some sequences, for example those affiliated to *Methanosarcinaceae* (PC 6974a, MAK 225a), were detected only in the cold zones ( $< 3^{\circ}\text{C}$ ) of the active layer.

Our results indicate the presence of hydrogenotrophic, acetotrophic and methylotrophic methanogens in the investigated permafrost soils. Sequences were affiliated with the families of *Methanomicrobiaceae*, *Methanosarcinaceae* and *Methanosaetaceae*, while members of the family *Methanobacteriaceae*, as shown in other studies on archaeal diversity in northern peatlands (Høj *et al.*, 2005; Juottonen *et al.*, 2005), could not be detected. One reason could be the inhomogeneous distribution of microorganisms in soil depending on the distribution of usable organic carbon (Wachinger *et al.*, 2000). Species of *Methanomicrobiaceae* can grow only with hydrogen, formate and alcohols (except methanol), *Methanosarcinaceae* can grow with all methanogenic substrates except formate, and members of *Methanosaetaceae* grow exclusively with acetate as energy source (Hedderich & Whitman, 2005). An important finding is the detection of hydrogenotrophic methanogens in permafrost environments, because several studies have shown that acetate is more important as a substrate in cold than in temperate environments (Chin & Conrad, 1995; Wagner & Pfeiffer, 1997). However, a related study at the polygon-centre site on Samoylov Island showed that the potential methane production in all horizons was lower with acetate as substrate compared with the activity after hydrogen amendment (Wagner *et al.*, 2005). Acetate is likely to be available only to habitats with a significant portion of polysaccharides, which is not the case in Arctic peatlands (Kotsyurbenko *et al.*, 2004). Only representatives of the genera *Methanosarcina* and *Methanosaeta* are able to use acetate as a substrate. In particular, *Methanosarcina* species prefer methanol as carbon and energy source, although methanogenesis via acetate and hydrogen represents the

main pathway of methane production in most environments (Conrad, 2005). The significance of methanol, which is derived from pectin or lignin (Schink & Zeikus, 1982), for methanogenesis in permafrost environments was verified by this study for the floodplain site on Samoylov Island. However, unknown methanogenic archaea could make a contribution to hydrogenotrophic methanogenesis at low temperatures. An indication for this assumption is the presence of sequences affiliated with the order *Methanomicrobiales* that could be detected in deeper layers of all studied sites. One of the few known psychrophilic  $\text{H}_2$ -using methanogens that belongs to this group of methanogens is *Methanogenium frigidum* (Franzmann *et al.*, 1997), which was isolated from an Antarctic sediment.

Detailed phylogenetic analysis showed that two DGGE bands belonging to *Methanosaetaceae* branched very close to each other (data not shown). Both were extracted from relatively deep and cold ( $0.4\text{--}0.8^{\circ}\text{C}$ ) soil horizons on Samoylov Island, but had different  $\text{CH}_4$  production rates. The closest relatives of these sequences have been detected in environments with different physicochemical characteristics, such as rice-field soils (Lueders & Friedrich, 2000; Ramakrishnan *et al.*, 2001), lake sediments (Banning *et al.*, 2005), and an acidic bog lake (Chan *et al.*, 2002).

Four of the *Methanosarcina*-like permafrost sequences (FP6941a, FP6947c, PC6971a and PC6968a) were clustered with cultivated methanogens (e.g. *Methanosarcina barkeri*) and among numerous environmental sequences with the closest relatives from rice-field soils (Lueders & Friedrich, 2000), freshwater environments (Stein *et al.*, 2002) and Arctic wetland (Høj *et al.*, 2005; Fig. 3a). The remaining seven sequences (two of them, FP6945a and MAK226a, were 100% identical to MAK225b) form a cluster with the closest relative sequences ARF3 from Green Bay, recovered from a ferromanganous micronodule (Stein *et al.*, 2001), FP6947c, and sequences ArcSval\_11 and ArcSval\_14 from Arctic wetland (Høj *et al.*, 2005). Sequences in this *Permafrost cluster I* were recovered mainly from cold layers ( $< 4^{\circ}\text{C}$ ) of the medium-depth horizons (6–36 cm) of all the studied sites.

Similar results were obtained for the *Methanomicrobiales*-like permafrost sequences. Six of them were distributed among numerous environmental sequences with closest relatives recovered from rice roots (Lehmann-Richter *et al.*, 1999), PC6976a and FP6943a; acidic bog lake (Chan *et al.*, 2002), MAK221a; freshwater lake (Jurgens *et al.*, 2000), FP6944a; geothermal aquifer GAB-A01 (Kimura *et al.*, 2005), FP6946a; and LDS16 from Lake Dagow sediment (Glissman *et al.*, 2004), MAK221b (Fig. 3b). *Permafrost cluster II*, which consists of three sequences from the floodplain and the polygon centre of Samoylov Island, is closely (97–98%) related to sequences Dg2003\_D\_97 from Lake Stechlin sediment (Chan *et al.*, 2005) and MRR42 from rice roots. Sequences were obtained from soil horizons of

various depths (from 5 to 40 cm) and various temperatures (from  $< 0.4$  °C to  $14.2$  °C). Three sequences (PC6972a, PC6969a and MAK224a) from polygon-centre soils (depth 5–23 cm, temperatures from  $1.2$  to  $5.8$  °C) form *Permafrost cluster III*, related (with about 98% similarity) to sequences recovered recently from rice rhizosphere (Lu & Conrad, 2005). Three other permafrost sequences (MAK224b, MAK227a and FP6947a) recovered from polygon-centre and floodplain soils (depth 17–56 cm) form *Permafrost cluster IV*, related (with about 97% similarity) to Rice cluster II (Grosskopf *et al.*, 1998b). The first representative of Rice cluster II – clone R17 – was found in a peat bog (Hales *et al.*, 1996), but now this cluster consists of about 20 environmental sequences recovered from a broad range of environments (Ramakrishnan *et al.*, 2001; Stein *et al.*, 2002).

The sequences that can be assigned to specific permafrost clusters, might possibly include methanogenic archaea, which are adapted to their extreme habitat by special physiological characteristics. This assumption is supported by the fact that pure cultures of *Methanosarcina*-like species isolated from permafrost soils of the same study site are more persistent to unfavourable environmental conditions (e.g. subzero temperatures, high salinity, dryness) than those from non-permafrost environments (D. Morozova and D. Wagner, pers. comm.). However, further studies that address the activity, diversity and physiological characteristics of methanogenic archaea in permafrost environments should be undertaken.

In conclusion, this study provides the first results concerning the methanogenic communities in three different permafrost soils of the Laptev Sea coast. It has demonstrated methanogenesis with a distinct vertical profile in each studied soil. The results show that  $\text{CH}_4$  production is regulated more by the quality of soil organic carbon than by the *in situ* temperature. We can also say that methanol is an important substrate in these habitats, as indicated by activity tests and by the presence of methylotrophic methanogens. The phylogenetic analysis revealed a distinct diversity of methanogens in the active layer of all study sites, with species belonging to the families *Methanomicrobiaceae*, *Methanosarcinaceae* and *Methanosaetaceae*. There were no restrictions of the detected families to specific depths or sites. Only sequences of *Methanosaetaceae* could not be detected in the polygon-centre soil of Mamontovy Klyk. Out of the 28 sequences, 16 sequences form four specific permafrost clusters. We hypothesize, albeit somewhat speculatively, that these clusters are formed by methanogenic archaea characterized by specific adaptation processes to the harsh permafrost conditions. However, a relationship between the activity and the diversity of methanogens in permafrost soils could not be shown. Molecular ecological analysis of the microbial permafrost communities in combination with process studies on  $\text{CH}_4$  production, oxidation

and emission will be able to improve our understanding of the future carbon dynamics in climate-sensitive permafrost environments.

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