The ISME Journal (2008) 2, 37–48

www.nature.com/ismej

ORIGINAL ARTICLE

Effects of temperature on the diversity and community structure of known methanogenic groups and other archaea in high Arctic peat

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Archaeal populations are abundant in cold and temperate environments, but little is known about their potential response to climate change-induced temperature changes. The effects of temperature on archaeal communities in unamended slurries of weakly acidic peat from Spitsbergen were studied using a combination of fluorescent *in situ* hybridization (FISH), 16S rRNA gene clone libraries and denaturing gradient gel electrophoresis (DGGE). A high relative abundance of active archaeal cells (11–12% of total count) was seen at low temperatures (1 and 5 °C), and this community was dominated by Group 1.3b *Crenarchaeota* and the euryarchaeal clusters rice cluster V (RC-V), and Lake Dagow sediment (LDS). Increasing temperature reduced the diversity and relative abundance of these clusters. The methanogenic community in the slurries was diverse and included representatives of *Methanomicrobiales*, *Methanobacterium*, *Methanosarcina* and *Methanosaeta*. The overall relative abundance and diversity of the methanogenic archaea increased with increasing temperature, in accordance with a strong stimulation of methane production rates. However, DGGE profiling showed that the structure of this community changed with temperature and time. While the relative abundance of some populations was affected directly by temperature, the relative abundance of other populations was controlled by indirect effects or did not respond to temperature.
The ISME Journal (2008) **2,** 37–48; doi:10.1038/ismej.2007.84; published online 22 November 2007
Subject Category: microbial population and community ecology
Keywords: archaea; Arctic microbiology; diversity; methane; peat; temperature

Introduction

Most climate models predict that by 2080, summer temperatures in Arctic regions will increase by 4.0–7.5 °C, and there will also be increased precipitation (Anisimov and Fitzharris, 2001). Motivated by the demonstrated importance of temperature on emissions of the greenhouse gas methane from Arctic and subarctic wetlands (Christensen *et al.*, 2003), several studies have addressed the effect of temperature on the methane production potential in these soils (Svensson, 1984; Dunfield *et al.*, 1993; Kotsyurbenko *et al.*, 1996, 2004; Metje and Frenzel, 2005, 2007; Ganzert *et al.*, 2007).

Recently, archaeal community analysis was included in studies of temperature effects on methanogenesis and methanogenic pathways in subarctic permafrost peat from Northern Finland (Metje and Frenzel, 2005) and Siberia (Metje and Frenzel, 2007). Both the archaeal community structure and the dominating methanogenic pathways differed significantly between the studied sites. In samples of acidic (pH 4.1) peat from Northern Finland, the community was dominated by hydrogenotrophic methanogenesis and all methanogenic sequences belonged to *Methanobacteriales* (Metje and Frenzel, 2005). In contrast, in samples of weakly acidic (pH 5–6) peat from Siberia, acetoclastic methanogenesis accounted for about 70% of the methanogenesis, and the archaeal community included *Methanosarcinales* and *Methanobacteriales* (Metje and

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Received 29 May 2007; revised 10 September 2007; accepted 11 September 2007; published online 22 November 2007

Frenzel, 2007). For both sites, increased temperature resulted in few changes in the archaeal community structure, and the methane production rates at 4 °C corresponded to as much as 10 and 17% of the rates at the optimum temperature (25 °C and 26–28 °C, respectively).

The archaeal communities in peat from the weakly acidic, 6.0 in $MgCl_2$ (pH) wetland at Solvatnet, Spitsbergen, include representatives of a wide range of methanogenic groups, including groups with potential for hydrogenotrophic methanogenesis (*Methanomicrobiales*, *Methanobacteriaceae*), acetoclastic methanogenesis (*Methanosaeta*) and both acetoclastic, hydrogenotrophic and methylotrophic methanogenesis (*Methanosarcina*) (Høj *et al*., 2005). Previous studies of rice paddy soils, which harbor similar methanogenic groups, have demonstrated changes in the archaeal community structure with temperature (Fey and Conrad, 2000). No information is however available on how increased temperature will affect the archaeal community structure in Arctic peat where all these methanogenic groups are represented.

Previous studies of archaeal communities in subarctic and Arctic wetlands have repeatedly detected archaeal groups not expected to be involved in methane production (Kotsyurbenko *et al*., 2004; Metje and Frenzel, 2005; Høj *et al*., 2005, 2006). The studies indicate that these groups have a high relative abundance in the archaeal community, though little attention has been given to these findings as the studies were focused on the methanogenic community. In contrast, increasing attention has been given to the relatively high abundance of archaea in cold nonmethanogenic systems such as the Southern Ocean (DeLong *et al*., 1994; Murray *et al*., 1998; Church *et al*., 2003), the Arctic Ocean (Wells and Deming, 2003; Bano *et al*., 2004; Garneau *et al*., 2006; Kirchman *et al*., 2007) and an Arctic river (Galand *et al*., 2006). No information is however available on how temperature affects the diversity and abundance of these nonmethanogenic archaeal populations.

In this study, unamended slurries of peat from Solvatnet were incubated at six temperatures in the range 1–25 °C. After 4 weeks incubation, active archaeal cells were visualized and counted using fluorescent *in situ* hybridization (FISH) and the archaeal community structure was studied in detail for two selected temperatures (5 and 20 °C) by cloning of archaeal 16S rRNA genes. Archaeal community denaturing gradient gel electrophoresis (DGGE) fingerprints for six incubation temperatures and two time points revealed whether apparent temperature trends changed with time, and hence were due to indirect rather than direct temperature effects. The effect of temperature on microbial activities was addressed by monitoring the accumulation of methane, CO_2 and volatile fatty acids.

Materials and methods

Study site and sample collection

The site Solvatnet is located on a marine terrace outside the township of Ny-Ålesund (78°50′N–11°30′E) on the west coast of Spitsbergen. The site and peat characteristics, as well as the *in situ* archaeal community composition at two depths, have been described in detail elsewhere (Høj *et al*., 2005, 2006). The peat is classified as undecomposed (H1) and almost undecomposed (H2) using von Post's scale (Clymo, 1983). The peat has a high organic content (88% of dry weight), a high water content (>600% of dry weight) and is weakly acidic (pH 6.8 in H_2O). The site is grazed by Barnacle Geese (*Branta leucopsis*). Previously recorded CO_2 emissions were in the range 0.18–0.51 $g\,m^{-2}\,h^{-1}$, and methane emissions were in the range 93–2801 $\mu g\,m^{-2}\,h^{-1}$ (Høj *et al*., 2005). Peat samples for this experiment were collected in mid-August 2000 using polyvinyl chloride corers (inner diameter 5 cm, length 10 cm), as previously described (Høj *et al*., 2005). The cores were stored for 4 months at 2–4 °C until the experiment was commenced.

Preparation and incubation of soil slurries

Soil from the inner part (5–7 cm depth) of three cores was mixed to create a pooled composite sample, which was subsequently used to make slurries. The peat slurries were made under N_2 atmosphere by mixing 50 g wet peat (corresponding to 4.5 g dry peat) with 15 ml sterile, anoxic water in a 125 ml serum bottle, which was then sealed and capped. The slurries were gassed with N_2 and incubated in the dark without shaking in precisely thermostated (±0.01 °C) water baths at temperatures 1, 5, 10, 15, 20 and 25 °C. Two parallel slurries were made for each temperature. One parallel was terminated in week 4 to perform analyses of the archaeal community structure, while the other parallel was terminated in week 13. Due to the labor intensity associated with sample processing, samples from no more than two temperatures were processed per day. All temperatures were sampled within 5–6 days, with the highest temperatures being sampled first.

Analytical techniques

Before sampling the headspace gas, bottles were shaken vigorously to allow equilibration between the liquid and gas phases. The methane concentrations in 1 ml of headspace gas was measured using a Shimadzu GC-14A gas chromatograph equipped with a Porapak Q stainless steel column (1.5 m × 1/8 inch) and a flame ionization detector, with N_2 as carrier gas. Injection, detection and column temperatures were 200, 240 and 45 °C, respectively. The hydrogen concentration in 2 ml headspace gas was measured using a Varian Aerograph model 920 gas

chromatograph equipped with a Stainless Steel Washed Molesieve (Alltech, Deerfield, IL, USA) 60/80 mesh column (2.0 m × 1/8 inch) and a thermal conductivity detector (125 mA), with Argon as carrier gas. Injection, detection and column temperatures were 100, 100 and 50 °C, respectively. Carbon dioxide concentration in 2 ml headspace gas was determined using an infrared CO_2 analyzer (Binos 100, Rosemount GmbH & Co., Geschaftsbereich Analysentechnik, Hanau, Germany).

Samples of slurry water (1 ml) were stored frozen at −20 °C until analysis for volatile fatty acids by a Schimadzu GC 14A gas chromatograph equipped with a flame ionization detector, with N_2 as carrier gas. Thawed samples were acidified by addition of 10 µl HCl so that C2–C5 volatile fatty acids could be separated as free acids on a 6 ft × 2 mm i.d. glass column packed with 10% SP-1200 and 1% H_3PO_4 on 80/100 Chromosorb W AW. Injection, detection and column temperatures were 200, 200 and 125 °C, respectively.

Calculations

Calculations of methane accumulation were based on the concentration in the gas phase, since the amount of dissolved methane can be assumed to be low (Wilhelm *et al.*, 1977). The partial pressure was calculated based on the amount of methane in the headspace using the ideal gas law. The CO_2 partial pressure in the headspace was calculated as described above for methane.

Total counts and FISH analysis

Soil samples (2.5 g) were fixed in 4% paraformaldehyde (10 ml) at 4 °C with shaking overnight. The fixed soil was transferred to a 250 ml Waring-blender steel jar (Waring, CT, USA) with 12.5 ml ice-cold 0.2 µm filtered water and homogenized 10 times for 1 min, with 5 min cooling between each blending. After the last blending, the homogenate was allowed to sediment for 1 min, and two parallels of 1 ml samples were taken with a 1 ml plastic syringe without needle. The maximum amount of sample that could be filtered while still keeping the linear relationship between PicoGreen counts and sample volume was determined for each sample (Fægri *et al.*, 1977). Appropriate amounts of soil homogenate was then filtered onto white polycarbonate filters (pore size 0.2 µm, 25 mm diameter; Osmonics Poretics Products, Minnetonka, MN, USA), and air-dried filters were stored at −20 °C (Glöckner *et al.*, 1999). For each sample, two parallel filters from each parallel were processed, giving a total of four filters analyzed per sample. The dry weight of soil samples was determined gravimetrically.

The DNA-specific stain PicoGreen (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was chosen for determination of total counts of bacteria and archaea. This was based on initial experiments, which showed that PicoGreen was superior to 4′-6-diamidino-2-phenylindole (Porter and Feig, 1980) in discriminating between cells and soil particles, and caused less background on white polycarbonate filters than SYBRGreen I (Molecular Probes). Filter sections were stained with PicoGreen (1:100 dilution in 1 × Tris-acetate-EDTA) in the dark for 15 min (Weinbauer *et al.*, 1998) and mounted with a drop of antifade solution (0.1% *p*-phenylenediamine, 50% glycerol, 0.5 × PBS) (Noble and Fuhrman, 1998).

The oligonucleotide probes used in this study were labeled with a CY3 fluorochrome on the 5′ end (Interactiva Biotechnologie, Ulm, Germany). The probes and used stringency conditions are presented in Table 1. For each probe the stringency conditions were optimized using control cultures of *Escherichia coli*, *Archaeoglobus fulgidus*, *Methanococcus voltae*, *Methanosaeta concilii*, *Methanosarcina siciliae*, *Methanobacterium formicicum*, *Methanospirillum hungatei* and *Sulfolobus solfataricus*. Each filter was cut into six sections prior to hybridization with the labeled oligonucleotide probes. All hybridizations were performed at 42 °C overnight. Otherwise the hybridization and washing steps were performed as outlined by Glöckner *et al.* (1999). The NaCl concentrations of the washing buffer was adjusted according to the stringency condition of each probe as described in Pernthaler *et al.* (2001). Filter sections were mounted with antifade solution as described above.

Table 1 Oligonucleotide probes used for FISH

Probe	*Sequence*	*Specificity*	*Stringency (% formamide)*	*Reference*
EUB338	5′-GCTGCCTCCCGTAGGAGT	*Bacteria*	30	Amann *et al.* (1990)
NON338	5′-ACTCCTACGGGAGGCAGC	Negative control	30	Wallner *et al.* (1993)
ARCH915	5′-GTGCTCCCCCGCCAATTCCT	*Archaea*	25	Amann *et al.* (1990)
EURY498	5′-CTTGCCCRGCCCTT	*Euryarchaeota*[a]	15	Burggraf *et al.* (1994)
MSMX860	5′-GGCTCGCTTCACGGCTTCCCT	*Methanosarcinales*	35	Raskin *et al.* (1994)
MB310	5′-CTTGTCTCAGGTTCCATCTCCG	*Methanobacteriaceae*	35	Raskin *et al.* (1994)

[a]This probe has limited coverage of the phylum *Euryarchaeaota* (Jurgens *et al.*, 2000). Inspection of retrieved clone 16S rRNA sequences revealed a perfect match for methanogenic archaea and most of cluster LDS. In contrast, EURY498 had mismatches with most 16S rRNA sequences from cluster RC-V.

Filter sections were inspected using a Zeiss Axioplan epifluorescence microscope (Zeiss, Jena, Germany) equipped with a 50 W high pressure mercury bulb. The specific filter set Chroma HQ 41007 (Chroma Tech. Corp., Rockingham, VT, USA) was used for CY3-labeled probes and the Zeiss filter set 09 was used for PicoGreen. For each sample and probe combination, at least 10 fields of view were counted per filter, corresponding to a total of at least 40 fields of view. For determination of PicoGreen total counts at least 2500 cells were counted per sample. The statistical significance of differences in total counts and fractions of cells detected by individual FISH probes were analyzed using the nonparametric Mann–Whitney *U*-test (confidence level 0.05). Counts with FISH probes were regarded as statistically significant if they (on average) were significantly higher than the negative control probe counts (NON338, Mann–Whitney *U*-test, $P > 0.05$).

DNA extraction from soil slurries

Samples for DNA extraction were frozen at −80 °C until processed. DNA extraction and purification was performed with the FastDNA SPIN Kit for soil (BIO 101/Qbiogene, Irvine, CA, USA) in combination with a freeze-thaw lysis protocol (Nakatsu *et al.*, 2000; Høj *et al.*, 2005). Wet peat (0.4 g) and 1 ml lysis buffer (0.12 M NaP (pH 8), 5% sodium dodecyl sulfate) were combined in a MULTIMIX 2 Tissue Matrix Tube provided in the kit. The tube was vortex mixed for 1 min, incubated at 65 °C for 45 min, followed by three quick freeze-thaw cycles (−80 and +65 °C). After this combined mechanical and freeze-thaw lysis cycle, the DNA was purified using the kit solutions as described by the manufacturer. Two parallel extractions were performed per sample, and the resulting eluates were mixed.

PCR amplification, cloning and sequencing

Two clone libraries were constructed using the DNA extracts from slurries incubated for 4 weeks at 5 and 20 °C, respectively. A portion of the 16S rRNA gene was amplified using the *archaea*-specific primers A109f and A934b (Grosskopf *et al.*, 1998a). The optimal template concentration was found using 10-fold serial dilutions of the DNA extracts. The reaction mixture of 25 μl contained 1 μl, 0.2 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate, 0.1% (w/v) bovine serum albumin, PCR buffer 1× (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7) (Qiagen, Hilden, Germany) and 1.25 U μl^{-1} DNA polymerase (Qiagen). Amplification was performed with the following protocol: initial melting at 94 °C for 3 min, followed by 32 cycles of 94 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were purified by gel extraction using the Qiaquick Gel Extraction Kit (Qiagen), following the protocols recommended by the manufacturer. Purified PCR products were cloned using a TOPO TA cloning kit, version K2 (Invitrogen), as recommended by the manufacturer. For each library, 50 clones were randomly selected, and the cloned insert was PCR amplified using the M13 primer supplied with the TOPO vector. Purified PCR products were sequenced using the ABI 3730XL Genetic Analyzer with A109f as sequencing primer.

Analysis of phylogeny and diversity of clone sequences

To identify potentially chimeric molecules the sequences were analyzed using the computer program Mallard version 1.02. (Ashelford *et al.*, 2006). Sequences were initially aligned with 16S rRNA gene sequences of their closest relatives available in the GenBank database using the BLAST search facility of the National Center for Biotechnology Information (NCBI). Sequences derived from this study and their relatives with the highest BLAST scores were added to a prealigned database of 936 complete and partial (>1000 bp) archaeal 16S rRNA gene sequences using the aligning tools from the ARB program package (Technical University of Munich, Munich, Germany; http://www.arb-home.de). The aligned sequences were checked and corrected manually where required. Sequences were added to a preexisting maximum likelihood tree supplied with the ARB database using a special parsimony tool, and nodes not to be included in the tree were removed. Sequences with 97% or higher sequence identity were assigned to the same phylotype, and one representative sequence for each phylotype was included in the presented tree. Library coverage was calculated as $(1-(a/b)) \times 100$, where a was the number of phylotype represented in the library, and b was the number of sequences in the library. Diversity indices were calculated based on the defined phylotypes using the computer program Estimates 8.0.0.

PCR amplification and DGGE analysis

A nested protocol was used for amplification of the 16S rRNA gene of *archaea* using the primers PRARCH112F and PREA1100R in the first reaction, and the primers PARCH340-GC and PARCH519R in a second touchdown reaction as previously described (Høj *et al.*, 2005). DGGE and sequencing of bands was performed as described previously (Høj *et al.*, 2005). Sequences were analyzed using the BLAST tool at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Peak histograms for all profiles were determined using the computer program Gel2k (Svein Norland, Department of Biology, University of Bergen, Norway). Relative band intensities (P*i*) were calculated for each profile as the relative peak area in the profile (Pi = ni/Ni, where ni is the area under peak *i* and Ni is the sum of all peak areas in the profile). For double bands, the relative band intensity was determined by relating their combined

peak area to the sum of all peak areas in the profile. Potential correlations between relative band intensities and temperature were analyzed statistically using Pearson's product moment correlation (confidence level 0.05). The statistical significance of other trends in relative band intensities was analyzed using Student's *t*-test (confidence level 0.05).

Nucleotide sequence accession numbers
All unique sequences generated in this study have been deposited in the EMBL database under accession numbers AM712493–AM712556.

Results

Process-related data
Unamended slurries of Solvatnet peat showed increasing methane partial pressures with temperature (Figure 1). The length of the apparent lag phase before onset of methane accumulation and the period with almost exponential methane accumulation decreased with increasing temperature (Figure 1), as previously reported for slurries of rice soil (Fey *et al.*, 2004) and temperate peat (van Hulzen *et al.*, 1999). The ratio between CO_2 and CH_4 accumulating in the headspace decreased with increasing temperature and with time (Table 2), indicating that the contribution of methanogenesis to the overall organic matter decomposition increased with temperature and time.

At the lowest temperature (1 °C) no fatty acids were present at concentrations above the detection limits throughout the experiment (Table 2). At 5 °C, isobutyrate was the only fatty acid detected, accumulating to 1430 μM at the end of the experiment.

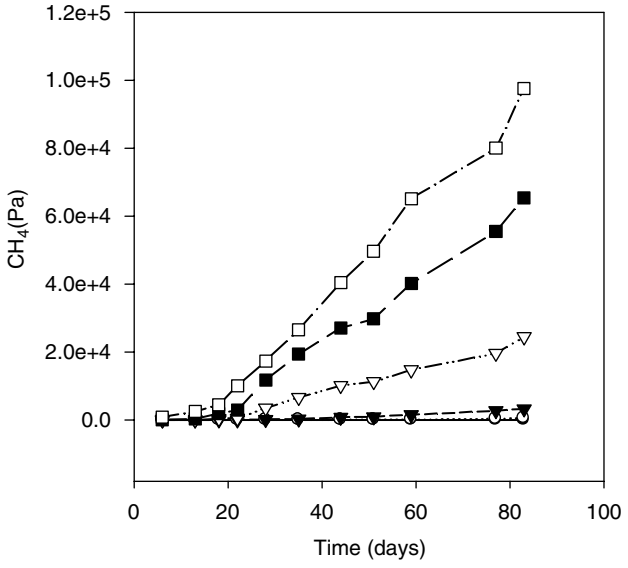

Figure 1 Methane accumulation in slurries of Solvatnet peat incubated at 1 °C (●), 5 °C (○), 10 °C (▼), 15 °C (▽), 20° (■) and 25 °C (□). The accumulation curves for 1 and 5 °C could not be distinguished on this plot.

Accumulation of this fatty acid decreased with increasing temperature. At 10 °C, isobutyrate was still the predominant fatty acid (up to 799 μM), but toward the end of the experiment there was also accumulation of acetate (680 μM) and propionate (95 μM) (Table 2). At higher temperatures (15 °C and higher), acetate and propionate accumulated transiently and maximum values were measured at day 51. Butyrate was detected transiently at low levels (<70 μM) in slurries incubated at 10–20 °C. No hydrogen was detected throughout the experiment, probably due to the low sensitivity of the method (detection limit 18 Pa).

Total counts and FISH-specific counts
The relative abundance of bacteria and archaea in soil slurries incubated for 4 weeks was examined by performing FISH reactions with a bacteria-specific probe (EUB338), an archaea-specific probe (ARCH915) and three probes specific for subsets of the domain *Archaea* (Table 1). FISH revealed little variation in the percentage of cells binding the EUB338 probe between slurries incubated at temperatures of 5 °C and higher (40–50%), but a lower percentage of cells was detected at 1 °C (33%) (Mann–Whitney *U*-test, $P<0.05$) (Table 3). A relatively high proportion of archaea (11–12% of total count) was detected for the two lowest temperatures (1 °C and 5 °C). In the 10 °C slurry, this percentage was significantly lower (1%) (Mann–Whitney *U*-test, $P<0.05$), while at temperatures of 15 °C and higher 3–6% of the microbial cells were identified as archaea (Table 3). The probe EURY498 hybridized

Table 2 Ratio of accumulated CO_2 to CH_4 and accumulation of fatty acids in all slurries after 28, 51 and 83 days incubation

	Day	*Temperature (°C)*					
		1	*5*	*10*	*15*	*20*	*25*
CO_2/CH_4	28	348	270	206	26	4	2
	51	367	254	153	4	1	1
	83	297	73	10	2	1	1
Acetate (μM)[a]	28	<67	<67	<67	299	182	126
	51	<67	<67	<67	612	284	176
	83	<67	<67	680	282	236	<67
Propionate (μM)[a]	28	<26	<26	<26	61	196	243
	51	<26	<26	<26	393	227	327
	83	<26	<26	95	<26	<26	<26
Butyrate (μM)[a]	28	<18	<18	<18	<18	<18	<18
	51	<18	<18	56	53	66	<18
	83	<18	<18	<18	<18	<18	<18
Isobutyrate (μM)[a]	28	<22	395	312	176	79	61
	51	<22	405	426	207	104	53
	83	<22	1430	799	236	136	77

[a]Detection levels were 67 μM for acetate, 26 μM for propionate, 22 μM for isobutyrate and 18 μM for butyrate.

Table 3 Total counts of bacteria and archaea (PicoGreen-stained cells) and percentages of cells detected with FISH probes EUB338, ARCH915 and EURY498

Temperature (°C)	*Total count (cells (g dw)$^{-1}$ × 10^{10})*	*EUB338 (% of total count)*	*ARCH915 (% of total count)*	*EURY498 (% of total count)*	*EURY498 (% of ARCH915)*
1	5.8 (0.6)	32.5 (2.0)	11.1 (3.6)	1.6 (1.2)	14.6 (9.3)
5	7.0 (0.9)	44.7 (2.0)	11.9 (6.3)	2.2 (0.4)	18.6 (3.3)
10	6.7 (0.6)	41.7 (6.9)	1.4 (0.6)	0.9 (0.7)	61.3 (47.1)
15	8.6 (1.4)	44.1 (3.8)	5.6 (2.3)	0.8 (0.3)	13.4 (4.8)
20	6.9 (1.5)	49.8 (3.6)	5.1 (2.9)	2.7 (0.7)	52.4 (14.1)
25	7.2 (1.2)	40.3 (2.6)	2.5 (1.0)	1.6 (1.7)	64.2 (67.5)

Mean values and standard deviations are presented.

Table 4 Diversity statistics for clone libraries (50 clones each) of slurries incubated at 5 °C and 20 °C

Library subset	*Temperature (°C)*	*Library coverage (%)*	*Shannon–Wiener diversity index (H′)*	*Shannon evenness (E)*	*Chao1 richness estimator*
Known methanogenic groups	5	78.6	0.88	0.80	3.0
	20	69.2	1.83	0.88	11.0
Other archaea	5	58.3	2.29	0.85	20.6
	20	65.2	1.68	0.81	18.0

Each library was divided in two subsets (known methanogenic groups and other archaea) before further analysis. A chimeric sequence identified by the computer program Mallard was removed from the 20 °C library. Statistical analyses were performed on basis of phylotypes defined as sequences with 97% or higher sequence identity.

with 1–3% of the microbial cells in all slurries (Table 3). For some slurries the counts with this probe were at or below (10 °C) the detection limit of the method and some counts had large standard deviations (Table 3). Hence, differences in the percentage of EURY498-positive cells were in most cases not statistically significant. The fraction of archaea binding the EURY498 probe was however shown to be higher at 20 °C (52%) than at 5 °C (19%) (Mann–Whitney *U*-test, $P<0.05$).

The morphology of cells detected by the probes ARCH915 and EURY498 differed with temperature. Rods dominated the community at 1 °C and 5 °C, while cocci, sarcina-like clusters and highly organized spheres dominated at higher temperatures. The number of cells binding the group-specific methanogen probes MSMX860 (*Methanosarcinales*) and MB310 (*Methanobacteriaceae*) (Raskin *et al.*, 1994) was below the detection limit of the method. Nevertheless, by examining a large number of fields of view, a few positive cells were identified in slurries incubated at temperatures of 10 °C or higher. The probe MB310 detected rod-shaped cells, and the probe MSMX860 detected sarcina-like and sphere-formed cell aggregates. The size and the probe signal intensity of the aggregates were lower at 10 °C than for the higher temperatures.

Clone libraries

A total of 100 archaea-specific 16S rRNA clones were analyzed from slurries incubated at 5 °C and 20 °C for 4 weeks (50 for each). The computer program Mallard identified one chimeric sequence in the 20 °C library, which was removed from further analyses. In the subsequent statistical analyses clones representing known methanogenic groups and other archaea were separated since they displayed contrasting trends with temperature (Table 4). For simplicity the two library subsets are referred to as the methanogenic subset and the nonmethanogenic subset, although the second subset can theoretically include organisms with a hitherto unrecognized capability of methane production.

The fraction of clones representing known methanogenic groups differed between the libraries (28% at 5 °C and 54% at 20 °C) (Figure 2). The methanogenic subset had a relatively low diversity (H′ 0.88) and species richness (Chao1 3.0) in the 5 °C library as compared to the 20 °C library (H′ 1.83, Chao1 11.0) (Table 4). Hence, the coverage of the methanogenic subset was higher in the 5 °C library (78.6%) than in the 20 °C library (69.2%) despite a lower number of clones in this subset (Table 4). In contrast, the nonmethanogenic subset had highest diversity in the 5 °C library (H′ 2.29 at 5 °C, H′ 1.68 20 °C) (Table 4). The rarefaction curve for the nonmethanogenic subset of the 5 °C library did not approach its asymptote showing that further sequencing of clones would have revealed additional diversity (data not shown). For both temperatures the estimated species richness was higher for the nonmethanogenic than the methanogenic library subset (Table 4).

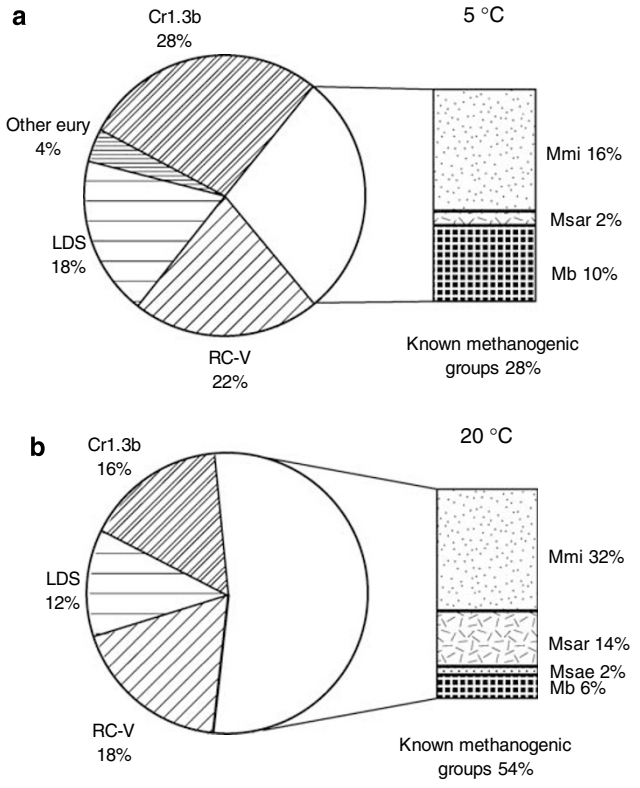

Figure 2 Archaeal community structure in 16S rRNA clone libraries derived from peat slurries incubated at (**a**) 5 °C and (**b**) 20 °C for 4 weeks. The community structure is represented by the relative abundance of clone sequences belonging to different phylogenetic clusters. Abbreviations: Mmi, *Methanomicrobiales*; Msar, *Methanosarcina*; Msae, *Methanosaeta*; Mb, *Methanobacterium*; RC-V, rice cluster V; LDS, Lake Dagow sediment; Cr1.3b, Group 1.3b *Crenarchaeota*.

Sequences not affiliated with known methanogenic groups generally clustered within three clusters: Group 1.3b *Crenarchaeota* (Ochsenreiter *et al.*, 2003) and the euryarchaeal clusters rice cluster V (RC-V, Grosskopf *et al.*, 1998b) and Lake Dagow sediment (LDS, Glissmann *et al.*, 2004) (Figure 3). An exception was seen for phylotype Sv-37, which was not closely related to any sequences in GenBank but was distantly related to clusters RC-V and LDS (Figure 3). In general, the sequences were closely related to sequences previously retrieved from a particle-rich Arctic river (Galand *et al.*, 2006) (Figure 3). The same nonmethanogenic archaeal groups have however been detected also in other wet and cold soils (Kemnitz *et al.*, 2004; Kotsyurbenko *et al.*, 2004; Metje and Frenzel, 2005; Høj *et al.*, 2006). While Group 1.3b *Crenarchaeota* was represented by two closely related phylotypes, clusters LDS and RC-V were represented by 10 and 7 phylotypes, respectively, of which most were represented by only 1–3 sequences (Figure 3). For all of these clusters their relative abundance was higher in the 5 °C than in the 20 °C library (Figure 2). Sequences in the methanogenic subsets were affiliated with the order *Methanomicrobiales* and the genera *Methanobacterium*, *Methanosarcina* and *Methanosaeta* (Figures 2 and 3). In general, the sequences were closely related to sequences previously retrieved from Siberian tundra (Metje and Frenzel, 2005, 2007; Ganzert *et al.*, 2007) and Spitsbergen wetlands (Høj *et al.*, 2005, 2006). *Methanomicrobiales* was the numerically dominant order in both libraries, constituting 57–58% of the methanogenic clones in each library (Figure 2). One of five *Methanomicrobiales* phylotypes (Sv-78) was affiliated with the family *Methanospirillaceae*, while the others did not cluster with any recognized family (Figure 3). Temperature affected the *Methanomicrobiales* phylotypes that were present; in the 5 °C library all *Methanomicrobiales*-affiliated sequences belonged to phylotype Sv-19, while in the 20 °C library only 1 of 15 *Methanomicrobiales* sequences belonged to this phylotype (Figure 3). The genera *Methanobacterium, Methanosarcina* and *Methanosaeta* were each represented by a single phylotype (Figure 3). While the *Methanobacterium* phylotype constituted a larger fraction of clones in the 5 °C library than in the 20 °C library, the opposite trend was seen for the *Methanosarcina* and the *Methanosaeta* phylotypes (Figure 2).

PCR-DGGE analysis of archaea

PCR-DGGE analysis was used to analyze and compare the archaeal communities in slurries incubated at 1, 5, 10, 15, 20 and 25 °C for 4 weeks and again after 13 weeks (Figure 4). The primers used for this analysis had mismatches with most known sequences in clusters RC-V and LDS, so the method mainly provided information on temporal changes in methanogenic populations. The DGGE profiles were similar or identical to profiles retrieved from field samples from the same site (Solvatnet) and sequences recovered from excised DGGE bands (148–152 bp) were identical to previously recovered sequences (Høj *et al.*, 2005). As discussed previously, double bands were present in the profiles, which could not be separated by repeated excisions and reamplification and yielded pure sequences (Høj *et al.*, 2005). All sequences recovered from DGGE bands were represented also in the clone libraries. Banding patterns were relatively stable with temperature, but some bands showed systematic changes in relative intensity with temperature and time (Figure 4 and Table 5). While band 4 (Msae) showed increasing relative intensity with increasing temperature at both time points, the opposite trend was seen for band 5 (Mmi4), which had decreasing relative intensity with temperature at both time points (Figure 4 and Table 5). Band 2 (Mmi3) and band 6 (Msar1) showed more complicated responses to temperature. After 4 weeks both bands showed positive correlations with increasing temperature (up to 25 °C for Msar1 and up to 15 °C for Mmi3) (Figure 4 and Table 5).

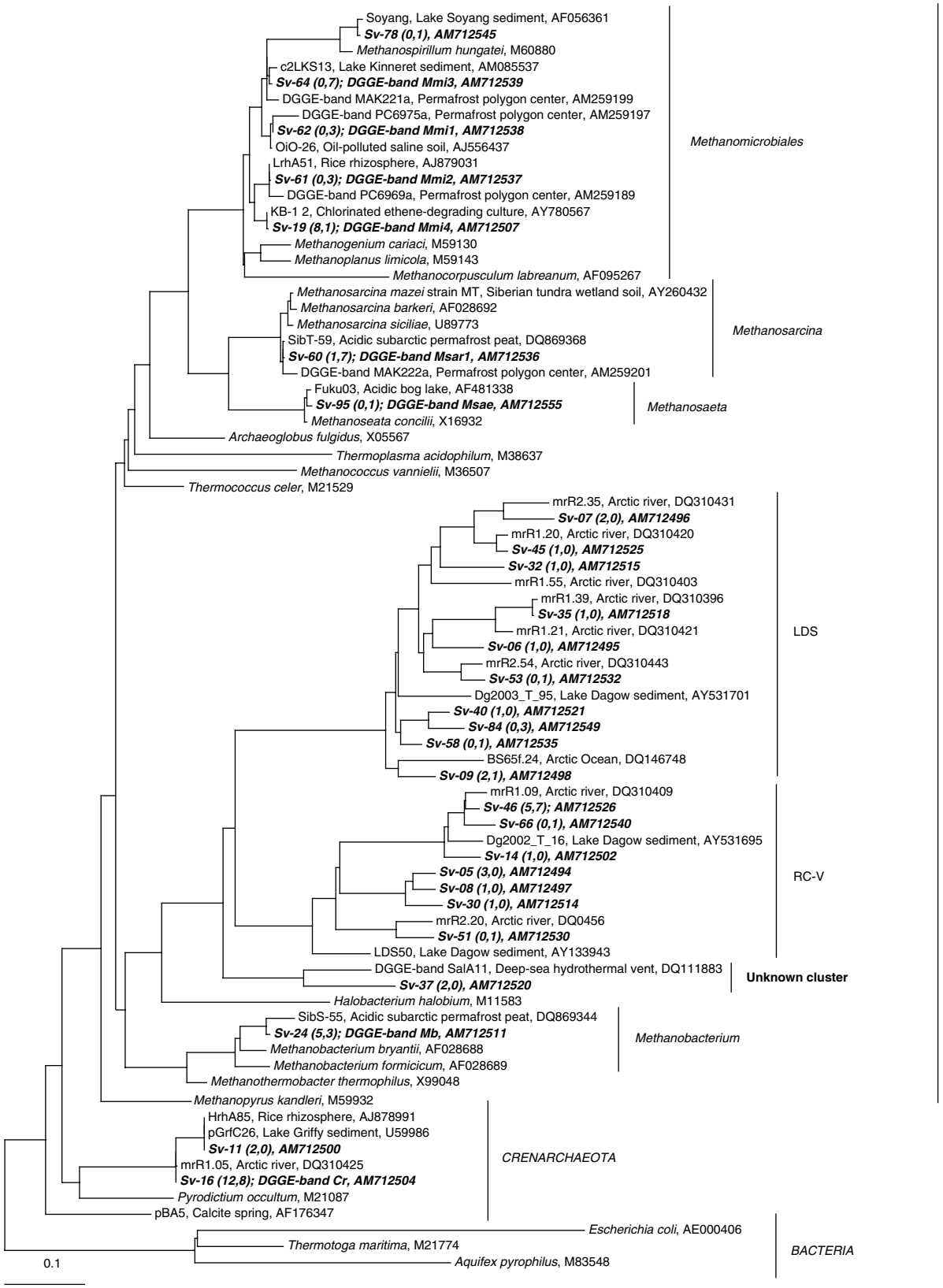

Figure 3 Maximum likelihood tree showing the phylogenetic affiliation of sequences recovered from clone libraries of slurries incubated at 5 and 20 °C and from denaturing gradient gel electrophoresis (DGGE) profiles of slurries and field samples from the same site (Solvatnet) (Høj *et al*, 2005). Each phylotype is represented by one sequence, and the number of unique sequences in the 5 and the 20 °C libraries, respectively, is indicated in parenthesis for each phylotype.

In contrast, there was no longer a direct correlation with temperature for these bands ($P>0.05$) after 13 weeks but their relative intensity was higher at low (1–10 °C) as compared to high (15–25 °C) temperatures (Student's *t*-test, $P<0.05$, Figure 4).

Discussion

This study showed that temperature affected both the diversity and structure of archaeal communities in unamended slurries of high Arctic peat from Solvatnet (Spitsbergen), with contrasting effects seen for known methanogenic groups and other archaea. A polyphasic approach demonstrated that after 4 weeks of incubation, 10 °C was an important temperature for the selection of different archaeal populations. At lower temperatures the archaeal community was dominated by nonmethanogenic groups, while methanogenic groups showed increased diversity and relative abundance at higher temperatures. This was in accordance with increasing methane production rates with increasing temperature.

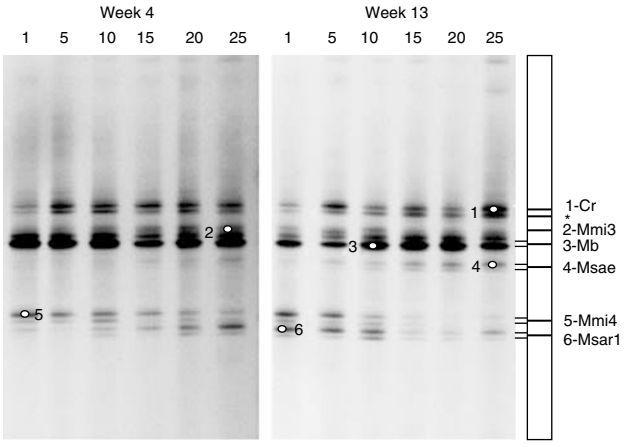

Figure 4 DGGE profiles of archaeal 16S rRNA genes amplified from Solvatnet peat slurries in weeks 4 and 13. Bands marked in the gel were successfully sequenced. The bar on the right shows a schematic diagram of the identified DGGE bands, with double bands indicated. Double bands could not be separated by repeated excisions and reamplification, and they yielded pure sequences. A sequence was successfully obtained for the band labeled * in our previous study (Mmi1) (Høj *et al*., 2005) though a sequence was not recovered in the present study. The band labels are consistent with this previous study of field samples from Solvatnet (Cr, *Crenarchaeota*, Mmi1, Mmi3, Mmi4, *Methanomicrobiales*; Mb, *Methanobacterium*; Msae, *Methanosaeta*; Msar1, *Methanosarcina*).

After incubation for 4 weeks at 1 °C and 5 °C the archaeal community in Solvatnet peat was relatively abundant, representing 11–12% of the total count, or 20–25% of all cells detected by FISH. This high abundance of archaea at low temperatures is in accordance with previous field studies of cold environments such as Siberian tundra soil (Kobabe *et al*. 2004), and marine plankton in Antarctic waters (Murray *et al*, 1998) and the Beaufort Sea (Wells and Deming, 2003; Garneau *et al*., 2006). FISH demonstrated that at these temperatures most of the archaeal cells had rod morphology, while clone libraries showed that at 5 °C the community was dominated by Group 1.3b *Crenarchaeaota* and the euryarchaeal clusters RC-V and LDS. While previous studies have not specifically targeted the ecophysiology of these groups, it has been shown that Group 1.3b *Crenarchaeota* is relatively abundant in permanently flooded or wet soils from Spitsbergen (Høj *et al*., 2006), and that in temperate soils this groups is relatively more abundant during winter (Kemnitz *et al*., 2004). Since the absolute abundance of archaea dropped significantly at 10 °C, this study indicated that some archaeal populations in Solvatnet peat had a competitive advantage in slurries incubated at 1 and 5 °C for 4 weeks. It is not known which physiological attributes that renders this advantage, as populations that are well adapted to low temperatures do not necessarily have a low optimum temperature for growth (Cavicchioli, 2006). It is intriguing however that a similar drop in the number of archaea at 10 °C was reported also for acidic peat from Northern Finland (Metje and Frenzel, 2005), suggesting that 10 °C may be an important temperature for selection of different archaeal populations in Arctic soils.

The methanogenic community in slurries of Solvatnet peat was diverse and included members of the order *Methanomicrobiales*, and the genera *Methanobacterium*, *Methanosarcina* and *Methanosaeta*, in accordance with field studies from the same site (Høj *et al*., 2005). Group-specific FISH probes identified a few methanogenic archaea in slurries incubated at 10 °C and above. The signal

Table 5 Correlations with temperature for relative DGGE band intensities

Band	*Affiliation*	*Week*	*Temperature range (°C)*	*Correlation*[a]
2 (Mmi3)	*Methanomicrobiales*	4	1–15	0.9563 ($P<0.05$)
4 (Msae)	*Methanosaeta*	4	1–25	0.9351 ($P<0.01$)
		13	1–20	0.9655 ($P<0.01$)
5 (Mmi4)	*Methanomicrobiales*	4	1–25	−0.9614 ($P<0.01$)
		13	1–25	−0.9534 ($P<0.01$)
6 (Msar1)	*Methanosarcina*	4	1–25	0.9176 ($P=0.01$)

[a]Pearson's product moment correlation.

intensity from positive cells and the size of cell aggregates were generally lower at 10 °C than at higher temperatures, in accordance with relatively low methane production rates in this slurry ($<0.2\,\mu mol\ CH_4\ (g\,dw)^{-1}$ per day). Although the overall diversity and relative abundance of methanogenic archaea increased with temperature, individual methanogenic populations showed differences in their temperature response. While some populations showed little or no response to temperature, other populations showed systematic changes in their relative abundance with temperature and time. This was reflected in clone libraries constructed for slurries incubated at 5 °C and 20 °C for 4 weeks, but was more clearly showed by trends in the DGGE profiles obtained for all slurries (1–25 °C) at two time points (4 and 13 weeks). Since the same inoculum was used for all slurries and all samples were processed in parallel, it could be assumed that changes in relative intensities of DGGE bands were robust. In addition, for samples where both DGGE profiles and clone library data were available, the two methods, which depended on different primer sets, generally showed consistent trends.

The *Methanosaeta* population Sv-95 (Msae) increased in relative abundance with increasing temperature at both time points, in accordance with a previous study of unamended rice soil slurries (Fey and Conrad, 2000). In contrast, one of the *Methanomicrobiales* populations (Sv-19, Mmi4) had an apparent competitive advantage at low temperatures, suggesting that it was well adapted to the conditions prevailing in slurries incubated at low temperatures. This trend was supported both by differences in clone frequencies in the two clone libraries and by DGGE profiles at both time points. Again, it is unknown which traits that give this population a competitive advantage at low temperature as proteomics studies of the cold-adapted methanogen *Methanococcoides burtonii* have identified a range of cellular processes that are important for cold adaptation (Cavicchioli, 2006). DGGE profiles after 13 weeks incubation showed that the *Methanomicrobiales* population Sv-64 (Mmi3) and the *Methanosarcina* population Sv-60 (Msar1) could also grow at temperatures as low as 1 °C despite having relatively low abundance after 4 weeks, and after 13 weeks their relative abundance was highest at the temperatures (1–10 °C) (Student's *t*-test, $P<0.05$). The temporal change in their temperature response suggested that their relative abundance was controlled indirectly rather than directly by temperature. Since the transient accumulation of at least two fatty acids (acetate and propionate) was also delayed at low temperatures, it can be hypothesized that the observed indirect effect of temperature could be related to the availability of substrates or growth factors. This study was however not designed to elucidate the exact mechanism behind indirect temperature effects, and no direct link to a specific fatty acid could be demonstrated.

The results from this study are in accordance with previous studies of rice soil slurries (Chin *et al.*, 1999a, b; Fey and Conrad, 2000), which also showed changes in the archaeal community structure with temperature. In contrast, no significant temperature-induced changes in the archaeal community structure were observed in acidic peat from Northern Finland (Metje and Frenzel, 2005) or from Siberia (Metje and Frenzel, 2007), where *Methanobacteriales* only, or *Methanobacteriales* and *Methanosarcinales*, respectively, were predominant at all incubation temperatures. We note that the stimulating effect of temperature on methane production rates was higher for Solvatnet peat than reported for the acidic peat from Northern Finland (Metje and Frenzel, 2005) and from Siberia (Metje and Frenzel, 2007), but it is unknown whether this can be linked to differences in the temperature characteristics of the methanogenic communities. So far, no studies have been able to link methane emissions or methane production rates to the archaeal diversity and community composition (Høj *et al.*, 2005; Ganzert *et al.*, 2007).

In conclusion, this study demonstrated contrasting effects of temperature on known methanogenic archaea and other archaea present in peat from the high Arctic wetland Solvatnet, Spitsbergen. The diversity and relative abundance of nonmethanogenic archaeal groups were highest at low temperature, and at least some members of Group 1.3b *Crenarchaeota*, and the euryarchaeal clusters RC-V and LDS appeared to have a competitive advantage at low temperatures. The overall diversity and relative abundance of methanogenic archaea increased with increasing temperature, in consistency with a strong stimulation of methane production rates. Nevertheless, individual methanogenic populations showed differences in their temperature response. While the relative abundance of some populations showed the same temperature trend (increase or decrease) after both 4 and 13 weeks, the relative abundance of other populations was controlled by indirect effects or did not respond to temperature.

Acknowledgements

We thank Ingvild Wartiainen and Lars Egil Haugen for collaboration in the field. This work was funded by The Norwegian Research Council (Grant 121458/720) and by grants from Meltzers høyskolefond and Bergen Myrdyrkningsforenings fond to LH. The Norwegian Polar Institute is acknowledged for financial support of the fieldwork.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S ribosomal RNA targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Anisimov O, Fitzharris B. (2001). Polar regions (Arctic and Antarctic). In: McCarthy JJ, Canziani OF, Leary NA, Dokken DJ, White KS (eds). Intergovernmental Panel on Climate Change *Climate Change 2001: Impacts, Adaptation, and Vulnerability*. Cambridge University Press: Cambridge, pp 801–841.

Ashelford K, Chuzhanova N, Fry J, Jones A, Weightman A. (2006). New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72**: 5734–5741.

Bano N, Ruffin S, Ransom B, Hollibaugh J. (2004). Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with antarctic assemblages. *Appl Environ Microbiol* **70**: 781–789.

Burggraf S, Mayer T, Amann R, Schadhauser S, Woese CR, Stetter KO. (1994). Identifying members of the domain *Archaea* with ribosomal-RNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **60**: 3112–3119.

Cavicchioli R. (2006). Cold-adapted archaea. *Nat Rev Microbiol* **4**: 331–343.

Chin K-J, Lukow T, Conrad R. (1999a). Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. *Appl Environ Microbiol* **65**: 2341–2349.

Chin K-J, Lukow T, Stubner S, Conrad R. (1999b). Structure and function of the methanogenic archaeal community in stable cellulose-degrading enrichment cultures at two different temperatures (15 and 30°C). *FEMS Microbiol Ecol* **30**: 313–326.

Christensen TR, Ekberg A, Ström L, Mastepanov M, Panikov N, Öquist M *et al.* (2003). Factors controlling large scale variations in methane emissions from wetlands. *Geophys Res Lett* **30**: Art. No. 1414.

Church MJ, DeLong EF, Ducklow HW, Karner MB, Preston CM, Karl DM. (2003). Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula. *Limnol Oceanogr* **48**: 1893–1902.

Clymo RS. (1983). Peat. In: Gore AJP (ed). *Mires: Swamp, Bog, Fen and Moor*. Elsevier Scientific Publishing Company: Amsterdam, The Netherlands, pp 159–224.

DeLong EF, Wu KY, Prezelin BB, Jovine RVM. (1994). High abundance of Archaea in Antarctic marine picoplankton. *Nature* **371**: 695–697.

Dunfield P, Knowles R, Dumont R, Moore TR. (1993). Methane production and consumption in temperate and subarctic peat soils: response to temperature and pH. *Soil Biol Biochem* **25**: 321–326.

Fægri A, Torsvik V, Goksøyr J. (1977). Bacterial and fungal activities in soil: separation of bacteria and fungi by a rapid fractionated centrifugation technique. *Soil Biol Biochem* **9**: 105–112.

Fey A, Claus P, Conrad R. (2004). Temporal change of ^{13}C-isotope signatures and methanogenic pathways in rice field soil incubated anoxically at different temperatures. *Geochim Cosmochim Acta* **68**: 293–306.

Fey A, Conrad R. (2000). Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil. *Appl Environ Microbiol* **66**: 4790–4797.

Galand PE, Lovejoy C, Vincent WF. (2006). Remarkably diverse and contrasting archaeal communities in a large arctic river and the coastal Arctic Ocean. *Aquat Microb Ecol* **44**: 115–126.

Ganzert L, Jurgens G, Münster U, Wagner D. (2007). Methanogenic communities in permafrost-affected soils of the Laptev Sea Coast, Siberian Arctic, characterized by 16S rRNA gene fingerprints. *FEMS Microbiol Ecol* **59**: 476–488.

Garneau ME, Vincent WF, Alonso-Saez L, Gratton Y, Lovejoy C. (2006). Prokaryotic community structure and heterotrophic production in a river-influenced coastal arctic ecosystem. *Aquat Microb Ecol* **42**: 27–40.

Glissmann K, Chin KJ, Casper P, Conrad R. (2004). Methanogenic pathway and archaeal community structure in the sediment of eutrophic Lake Dagow: effect of temperature. *Microb Ecol* **48**: 389–399.

Glöckner F, Fuchs B, Amann R. (1999). Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Appl Environ Microbiol* **65**: 3721–3726.

Grosskopf R, Janssen PH, Liesack W. (1998b). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**: 960–969.

Grosskopf R, Stubner S, Liesack W. (1998a). Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. *Appl Environ Microbiol* **64**: 4983–4989.

Høj L, Olsen RA, Torsvik VL. (2005). Archaeal communities in High Arctic wetlands at Spitsbergen, Norway (78°N) as characterized by 16S rRNA gene fingerprinting. *FEMS Microbiol Ecol* **53**: 89–101.

Høj L, Rusten M, Haugen LE, Olsen RA, Torsvik VL. (2006). Effects of water regime on archaeal community composition in Arctic soils. *Environ Microbiol* **8**: 984–996.

Jurgens G, Glockner FO, Amann R, Saano A, Montonen L, Likolammi M *et al.* (2000). Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent *in situ* hybridization. *FEMS Micobiol Ecol* **34**: 45–56.

Kemnitz D, Chin KJ, Bodelier P, Conrad R. (2004). Community analysis of methanogenic archaea within a riparian flooding gradient. *Environ Microbiol* **6**: 449–461.

Kirchman D, Elifantz H, Dittel A, Malmstrom R, Cottrell M. (2007). Standing stocks and activity of *Archaea* and *Bacteria* in the western Arctic Ocean. *Limnol Oceanogr* **52**: 495–507.

Kobabe S, Wagner D, Pfeiffer EM. (2004). Characterisation of microbial community composition of a Siberian tundra soil by fluorescence *in situ* hybridisation. *FEMS Microbiol Ecol* **50**: 13–23.

Kotsyurbenko OR, Chin KJ, Glagolev MV, Stubner S, Simankova MV, Nozhevnikova AN *et al.* (2004). Acetoclastic and hydrogenotrophic methane production and methanogenic populations in an acidic West-Siberian peat bog. *Environ Microbiol* **6**: 1159–1173.

Kotsyurbenko OR, Nozhevnikova AN, Soloviova TI, Zavarzin GA. (1996). Methanogenesis at low temperatures by microflora of tundra wetland soil. *Anton Leeuw Int J G* **69**: 75–86.

Metje M, Frenzel P. (2005). Effect of temperature on anaerobic ethanol oxidation and methanogenesis in acidic peat from a northern wetland. *Appl Environ Microbiol* **71**: 8191–8200.

Metje M, Frenzel P. (2007). Methanogenesis and methanogenic pathways in a peat from subarctic permafrost. *Environ Microbiol* **9**: 954–964.

Murray AE, Preston CM, Massana R, Taylor LT, Blakis A, Wu K *et al.* (1998). Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal

waters near Anvers Island, Antarctica. *Appl Environ Microbiol* **64**: 2585–2595.

Nakatsu CH, Torsvik V, Øvreås L. (2000). Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Sci Soc Am J* **64**: 1382–1388.

Noble RT, Fuhrman JA. (1998). Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat Microb Ecol* **14**: 113–118.

Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C. (2003). Diversity and abundance of *Crenarchaeota* in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* **5**: 787–797.

Pernthaler J, Glockner F-O, Schonhuber W, Amann R. (2001). Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Meth Microbiol* **30**: 207–226.

Porter KC, Feig YS. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* **25**: 943–948.

Raskin L, Stromley JM, Rittmann BE, Stahl DA. (1994). Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl Environ Microbiol* **60**: 1232–1240.

Svensson BH. (1984). Different temperature optima for methane formation when enrichments from acid peat are supplemented with acetate or hydrogen. *Appl Environ Microbiol* **48**: 389–394.

van Hulzen JB, Segers R, van Bodegom PM, Leffelaar PA. (1999). Temperature effects on soil methane production: an explanation for observed variability. *Soil Biol Biochem* **31**: 1919–1929.

Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Weinbauer M, Beckmann C, Höfle M. (1998). Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. *Appl Environ Microbiol* **64**: 5000–5003.

Wells LE, Deming JW. (2003). Abundance of bacteria, the cytophaga-flavobacterium cluster and archaea in cold oligotrophic waters and nepheloid layers of the northwest passage, canadian archipelago. *Aquat Microb Ecol* **31**: 19–31.

Wilhelm E, Battino R, Wilcock R. (1977). Low-pressure solubility of gases in liquid water. *Chem Rev* **77**: 219–262.