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Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s00792-011-0417-9
Extremophiles, 16, 2, pp. 177-191, 2012-01-14

Extremophiles (2012) 16:177–191
DOI 10.1007/s00792-011-0417-9

ORIGINAL PAPER

Microbial diversity and activity in hypersaline high Arctic spring channels

Chih-Ying Lay · Nadia C. S. Mykytczuk · Thomas D. Niederberger · Christine Martineau · Charles W. Greer · Lyle G. Whyte

Received: 3 August 2011 / Accepted: 13 December 2011 / Published online: 14 January 2012

Abstract Lost Hammer (LH) spring is a unique hypersaline, subzero, perennial high Arctic spring arising through thick permafrost. In the present study, the microbial and geochemical characteristics of the LH outflow channels, which remain unfrozen at ≥ -18°C and are more aerobic/less reducing than the spring source were examined and compared to the previously characterized spring source environment. LH channel sediments contained greater microbial biomass ($\sim$100-fold) and greater microbial diversity reflected by the 16S rRNA clone libraries. Phylotypes related to methanogenesis, methanotrophy, sulfur reduction and oxidation were detected in the bacterial clone libraries while the archaeal community was dominated by phylotypes most closely related to THE ammonia-oxidizing *Thaumarchaeota*. The cumulative percent recovery of ^{14}C-acetate mineralization in channel sediment microcosms exceeded $\sim$30% and $\sim$10% at 5 and -5°C, respectively, but sharply decreased at -10°C ($\leq$1%). Most bacterial isolates (*Marinobacter*, *Planococcus*, and *Nesterenkonia* spp.) were psychrotrophic, halotolerant, and capable of growth at -5°C. Overall, the hypersaline, subzero LH spring channel has higher microbial diversity and activity than the source, and supports a variety of niches reflecting the more dynamic and heterogeneous channel environment.

Keywords Cryomicrobiology · Arctic spring · Methane seep · Polar microbial ecology · *Thaumarchaeota* · Hypersaline

Communicated by A. Oren.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-011-0417-9) contains supplementary material, which is available to authorized users.

C.-Y. Lay · N. C. S. Mykytczuk · C. Martineau · L. G. Whyte (✉)
Department of Natural Resource Sciences, McGill University, Montreal, Canada
e-mail: Lyle.Whyte@mcgill.ca

T. D. Niederberger
College of Marine and Earth Studies, University of Delaware, Newark, USA

C. Martineau · C. W. Greer
Biotechnology Research Institute, National Research Council Canada, Montreal, Canada

Introduction

Cryoenvironments are defined as permanently frozen or subzero environments including permafrost, glaciers, ice sheets, multi-year sea ice, high-elevation Antarctic dry valleys, and glaciers as well as their associated microhabitats such as brine veins in sea ice and permafrost. In addition to prolonged exposure to subzero temperatures, microbial communities existing in such cryoenvironments must overcome extremely low rates of nutrient and metabolite transfer, high solute concentrations, low water activity, and potentially high background radiation (Ayala-del-Rio et al. 2010; Bakermans 2008; Steven et al. 2006). Nevertheless, microbial diversity, ecology and activity have been recently described in numerous cryoenvironment habitats and generally indicate that viable microbial communities consisting of bacteria, archaea, viruses, and eukaryotes exist in these extreme habitats (Bakermans 2008, 2012; Priscu and Christner 2004; Steven et al. 2006; Wells and Deming 2006; and reviewed in Margesin and Miteva 2011) and are capable of both growth and metabolic activity at ambient subzero temperatures (Anesio et al. 2007; Bakermans 2012; Bottos et al. 2008; D'Amico et al. 2006; Niederberger et al.

2010; Steven et al. 2008). Cold-adapted microorganisms inhabiting such environments exhibit a variety of modifications to their proteins, nucleic acids, and membranes, which allow them to maintain their fluidity, flexibility and associated activity at low temperatures, as well as other adaptations including cryoprotectant production, and highly efficient regulation of growth (Ayala-del-Rio et al. 2010; Bakermans 2008). However, the means by which microorganisms survive and even sustain active metabolism warrants further investigation. For example, it is still not clear what the cold temperature limits of microbial life are in terms of growth, metabolism/maintenance and survivability, whether the microbial communities inhabiting cryoenvironments are active microbial ecosystems or merely microbial survivors, and what contributions these microorganisms make to global biogeochemical cycles (Bakermans 2012; Bakermans and Skidmore 2011; Price and Sowers 2004; Steven et al. 2009).

The cold saline springs on Axel Heiberg Island (AHI) in the Canadian High Arctic are among the only known cold springs in permafrost cryoenvironments on Earth and represent a unique opportunity for expanding our knowledge of microbial life in extreme cold environments. These springs occur in an area with an average annual air temperature of −15°C that can dip below −40°C during the winter months. The microbial communities of two moderately extreme High Arctic spring systems, Gypsum Hill (GH) and Colour Peak (CP) were found to contain active microbial communities capable of existing in an extreme environment that experiences prolonged periods of continuous light or darkness, low temperatures (−1 to 8°C), and moderate salinity (~8 to 15%), and where life seems to rely on sulfur-based chemolithoautotrophy (Niederberger et al. 2009; Perreault et al. 2007, 2008).

We recently described the microbial communities inhabiting Lost Hammer (LH) spring source, a hypersaline (24% salinity), subzero (−5°C) perennial spring that is the only known terrestrial hypersaline CH_4 seep in a cryoenvironment on Earth arising through thick permafrost (Niederberger et al. 2010). Numerous sources of methane seeps including deep-sea marine margins where methane hydrates occur (Valentine 2002; Valentine and Reeburgh 2000) and terrestrial thermokarst lakes (Anthony et al. 2010; Isaksen et al. 2011; Matveeva et al. 2003) are known to exist and have been described in both Arctic and sub-Arctic regions, however, the microbial communities have rarely been explored. Our initial microbial characterization of LH spring sediments revealed a novel low diversity, low biomass microbial community capable of metabolic activity at in situ subzero, saline conditions. Molecular analyses (bacterial and archaeal 16S rRNA gene clone libraries, CARD-FISH) detected bacterial phylotypes related to microorganisms previously recovered from cold, saline habitats. Archaeal phylotypes were related to those found in hypersaline deep-sea methane-seep sediments and were dominated by the ANaerobic MEthane group 1a (ANME-1a) clade of anaerobic methane oxidizing archaea indicating that the thermogenic methane exsolving from the LH spring source may act as an energy and carbon source for sustaining anaerobic oxidation of methane-based microbial metabolism under ambient hypersaline, subzero conditions (Niederberger et al. 2010).

The springs on AHI are regarded as Martian analog sites due to their unique geology, climate, and geomorphology which mimic conditions that did once, or currently exist, on Mars (Pollard et al. 2009). For example, a gully which formed during the past decade on Mars provides compelling evidence that liquid water (or brine) may exist on Mars (Malin et al. 2006; McEwen et al. 2011), while the trace amounts of methane in the Mars atmosphere (Formisano et al. 2004) may originate from localized 'hot spots' or 'plumes' of methane arising from the frozen terrestrial Martian surface (Mumma et al. 2009). The origin of Martian atmospheric methane is under extensive debate (Lefevre and Forget 2009) and could be attributable to either geological or biological (methanogenic) sources. As such, LH spring is a unique analog, and provides a model of how a terrestrial methane seep can form in a cryoenvironment and supports a microbial community capable of utilizing the methane as an energy source.

In 2005, during our first winter expedition to the LH spring site, we discovered that the outflow spring channels downstream from the LH spring source remained unfrozen, due to high salt concentration in the sediment pore water, and contained gas bubbles exsolving from the channel sediments despite ambient sediment temperatures as low as −18°C (Fig. 1b). The objectives of the present work were to characterize the microbial biodiversity, ecology, and activity of the microbial communities present in runoff channel sediment of the LH spring using culture-dependent, molecular-based (CARD-FISH—catalyzed reporter deposition fluorescence in situ hybridization and 16S rRNA gene clone libraries), and activity analyses. Our primary goals were to determine if the LH channel sediments were microbially active at subzero, hypersaline conditions (down to −20°C) and to compare the more heterogeneous channel features with the source to fully describe the range of geochemical and microbial characteristics that exist within the LH spring system.

Materials and methods

Sample site description and geochemical analyses

A total of three sediment samples (C1–C3) were collected from the outflow channel (79°04.608N; 90°12.739W) of

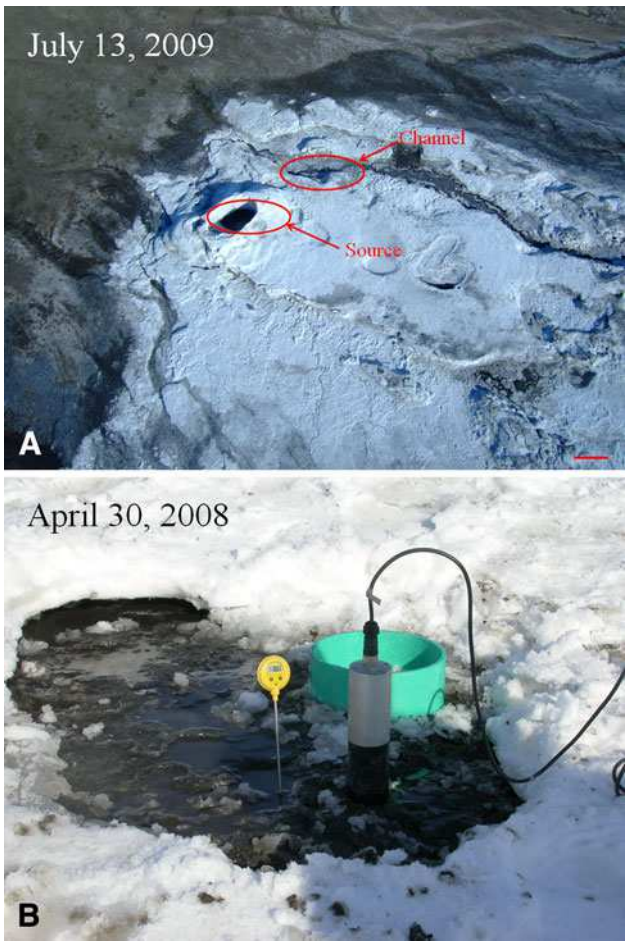

Fig. 1 **a** Photograph showing the position of Lost Hammer spring source and channel (*scale bar* 2 m). **b** Measurements being taken in LH Spring channel; the sediments remained unfrozen at −18°C

LH Spring (Fig. 1a): sediment samples C1 and C2 were collected on 30 April 2008; sediment sample C3 was collected on 4 May 2007. Samples were obtained using sterile scoopula and material down to 5 cm depth was collected. Sediments were placed into 500 ml sterile sample bottles and the remaining volume filled with LH spring channel water. The samples were transported under 4°C and stored at −20°C for future analyses. When possible, parallel geochemical measurements were taken. Due to the remoteness of the LH spring site, logistical challenges and difficult weather often either prevented adequate time at the site or prevented planned field investigations completely. Therefore, it was not possible to acquire complete in situ geochemical data in each sampling campaign on an annual basis for both later winter (early May) and summer (July). Multiple geochemical parameters including temperature, pH, salinity, total dissolved solids and redox potential (ORP) were measured using the YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH, USA). Hydrogen sulfide and dissolved oxygen concentrations were measured by colorimetric assay, as per manufacturer's instructions (CHEMetrics, Calverton, VA, USA). For geochemical analysis sediments were dried at 60°C and finely ground to pass through a 1-mm mesh sieve. Carbonate content was determined using a subsample of each oven-dried sediment that was acidified using 1 M HCl and then dried at 50°C to remove the carbonates (Hedges and Stern 1984). The original sediments were analyzed for total carbon and total nitrogen, and the acidified sediments were analyzed for organic carbon by combustion at 900°C with a Carlo Erba Flash EA NC Soils Analyzer [Carlo Erba, Milan, Italy (Lim and Jackson 1982)]. Ammonia, nitrite and nitrate concentrations were measured in the aqueous phase extracted from sediments following centrifugation at 2,000*g* for 10 min. Sediment-bound ammonia concentrations were determined by washing 30 g of sediment with 30 ml milli-Q water and then extracting with 30 ml of 2 M KCl (Maynard and Kalra 1993). Ammonia and nitrate/nitrite concentrations were analyzed on a multi-channel Lachat AE Quik-Chem auto-analyser (Lachat Instruments; Milwaukee, WI, USA).

CO_2 and CH_4 concentrations and flux measurements

To determine in situ CO_2 flux only from LH channels, an automated static-chamber Li-Cor model Li-8100 (Li-Cor Bioscience, Lincoln, Nebraska, USA) was used as described by the manufacturer. To measure both in situ CO_2 and CH_4 flux rates from the LH spring source sediment, an alternative method employing manual static chambers (Hoover et al. 2008) was also applied. Existing methodologies for manual static flux chambers were adapted as follows: collars with a diameter of 24 cm were installed in the channel sediments or over bubbling hot spot/loci in the spring; 7 L chambers were placed on the collars and the system was allowed to equilibrate ∼20 min to 1 h. For source measurements, the air in the chambers was mixed prior to sampling using a 50 ml syringe and 40 ml samples were collected and stored in 20 ml evacuated vials every 5 min for a 20-min period. CO_2 and CH_4 concentrations in the gas samples were determined by gas chromatography as previously described (Roy and Greer 2000) and fluxes were calculated based on linear regression. For the channel, flux chambers were set up with fixed sampling rates of 60 ml/min for 20 min, and then the total CO_2 and CH_4 were determined on a Picarro CRDS (Picarro, California). To measure both CO_2 and CH_4 concentrations within the sediments from LH spring and channel, the protocol described by Wagner et al. (2003), with some modifications was employed. Briefly, 10 g of sediment was added to a 60 ml vial containing 20 ml of saturated NaCl solution to reduce the solubility of gases in the liquid phase

(Yamamoto et al. 1976). The vial was then crimp-sealed, vortexed for 30 s and incubated for 1 h at 80°C to allow for the transfer of gases from the sample to the headspace of the vial. The resulting CO_2 and CH_4 concentration in the headspace of the vial was determined by gas chromatography as described by Roy and Greer (2000).

Microscopy and CARD-FISH

To determine the numbers of bacterial and archaeal cells, CARD-FISH was applied to the sediment samples and were prepared according to Pernthaler et al. (2001). In brief, 0.5 g of sediment from each sample was fixed using 4% para-formaldehyde overnight at 4°C, and then the samples were washed in PBS buffer 3 times and then stored in PBS/ethanol (1:1) solution at −20°C. The fixed samples were filtered through polycarbonate filters of 0.22 µm pore size which were then embedded in 0.1% (w/v) low melting point agarose. The dried filters were then treated with lysozyme solution for 1 h at 37°C to increase the permeability of the microbial cell walls. Subsequently, the filters were hybridized with horseradish peroxidase (HRP)-labeled probes (50 ng/µl) EUB338 (Amann et al. 1990), ARCH915 (Medina-Sanchez et al. 2005), ANME-350 (Boetius et al. 2000), and NON338 (Wallner et al. 1993) for bacteria, archaea, ANME-1, and a negative control, respectively. Filters were treated with formamide—55% for EUB338 and ANME-350, 35% for ARCH915, 20% for NON338, and incubated at 35°C overnight. Fluorescently labeled tyramide and H_2O_2 were used for the catalyzed reporter deposition for 15 min at 46°C (Niederberger et al. 2010). Filters were viewed (10 fields of cell counting per slide) using a fluorescent Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) at an excitation wavelength of 568 nm (Furukawa et al. 2006) under a 100× immersion oil objective.

To determine the total cell numbers in the sediments, DAPI (4′,6-diamidino-2-phenylindole) staining was performed. The protocol was modified based on Porter and Feig (1980). In brief, 0.5 g of sediment was fixed using 3.7% formaldehyde at 25°C for 1 h. The fixed samples were then washed by PBS buffer and then diluted 100-fold in PBS. The diluted sediment was filtered through polycarbonate 0.22 µm filters. Dried filters were then washed in water and 100% ethanol. Each slice of the cut filters was incubated with 10 µL of 20 µg/ml DAPI solution at 25°C for 10 min. The filters were again washed in PBS for 30 min at 25°C and then dehydrated with 100% ethanol. The filters were viewed (20 fields of cell counting per slide) using a fluorescent Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) at an excitation wavelength of 350 nm under a 100× immersion oil objective.

Microbial cultivation and characterization

To evaluate culturable heterotrophic microorganisms, a total of 5 g of sample was used to prepare serial dilutions in tetrasodium pyrophosphate solution (0.1 w/v $Na_4P_2O_7 \cdot 10H_2O$, pH 7.0) followed by spreading of 100 µL of each suspension (undiluted, 10^{-1}, 10^{-2} and 10^{-3} dilutions) onto R2A plates with 7% and 12% NaCl (Difco™ R2A Agar, Beckton, Dickenson Co., Sparks, MD, USA). This medium has been successfully used for culturing and enumerating heterotrophic microorganisms from Arctic hypersaline springs as described in previous studies (Niederberger et al. 2010; Perreault et al. 2008). All plate counts were performed in triplicate. The plates were incubated at room temperature for 2 weeks and 5°C for 2 months followed by colony counts. From the colonies that appeared and subsequent re-streaking, 22 isolates exhibiting unique colony morphology from R2A plates with 7% NaCl were selected. Cold temperature tolerance was tested by sub-culturing onto R2A plates supplemented with 7, 12, 20 and 25% NaCl incubated at 37, 25, 5, −5, and −10°C for 2–6 months. For culturing of archaea, undiluted suspensions were also streaked onto plates containing DSMZ 371 media as modified by Walsh (Niederberger et al. 2010).

DNA from isolated cells was extracted using a phenol/chloroform DNA extraction method (Barrett et al. 2006). Partial 16S rRNA fragments for the isolates were amplified by polymerase chain reaction (PCR) using the primer pair 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 758R (5′ CTACCAGGGTATCTAATCC 3′) (Bottos et al. 2008; Lane 1991; Woese 1987). The conditions for PCR reactions were as described by Steven et al. (2008). PCR products were sequenced using a 16-capillary genetic analyzer, ABI Prism 3130XL at the University of Laval Sequencing Facility (Plate-forme d'Analyses Biomoléculaires, Québec, QC, Canada). The sequences were compared against the Genbank database using the BLASTn algorithm (Altschul et al. 1990) and the Classifier tool of the RDP II (Cole et al. 2003).

Bacterial and archaeal 16S rRNA gene clone libraries

Total genomic DNA was isolated from 0.5 g of sediment from LH channel samples using the Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) as per manufacturer's instructions. DNA was eluted in 50 µL of sterile distilled H_2O and stored at −20°C. The 16S rRNA gene was amplified from the total isolated genomic DNA by PCR using primer pairs 27F and 758R for bacteria, and 109F (5′ ACKGCTCAGTAACACGT 3′) and 934R (5′ GTGCTCCCCCGCCAATTCCT 3′) for archaea (Baker et al. 2003; Whitehead and Cotta 1999). Each PCR contained 25 µl volumes with 1× PCR buffer, 0.2 mM of each dNTP, 3.5 mM $MgCl_2$, 0.5 µM of each primer, 6.25 µg bovine serum albumin, 1 U of *Taq* polymerase and 2 µl of

template DNA. Thermo-cycling conditions for archaeal PCR consisted of 94°C for 5 min followed by 20 cycles of 94°C for 30 s, 62°C for 30 s decreasing 1°C per cycle until 52°C, 72°C for 1 min and 30 s followed by 15 cycles of 94°C for 1 min, 52°C for 30 s, 72°C for 1 min and 30 s and a final extension of 5 min at 72°C. For bacterial 16S rRNA genes, PCR conditions were the same as those used in the amplification of partial fragments of the 16S rRNA genes of the 22 isolates above (Steven et al. 2008). PCR products were cloned into the pGEM-T easy vector system (Promega, Madison, WI, USA) and the ligation products transformed into competent DH5α cells (Invitrogen, Carlsbad, CA, USA). Clone screening was carried out using amplified ribosomal DNA restriction analyses (ARDRA) (Niederberger et al. 2010; Steven et al. 2007a). Identical ARDRA patterns were considered as one OTU (operational taxonomic unit) and one or two representative clones were selected for sequencing. 16S rRNA sequences were compared against the Genbank database using the BLASTn algorithm (Altschul et al. 1990) and the Classifier tool of the RDP II (Cole et al. 2003). All sequences from each clone library were aligned using ClustalW software and neighbor-joining phylogenetic trees built within the MacVector 7.2 software package (Oxford Molecular Ltd., Oxford, UK) using Jukes–Cantor modeling with 1,000 bootstrap re-samplings. The clone libraries were examined both in terms of total species richness for the channel communities as a whole (the unique sum of all clone libraries) and as individual profiles depicting changes in the relative species abundance between samples.

Biodiversity indices and statistical analysis of 16S rRNA gene clone libraries

Sampling coverage of clone libraries was calculated as defined by Good (1953) using the formula $C = (1 - \mathrm{nl}/N) \times 100$, where nl is the number of phylotypes which only appeared once in the sample, and N is the size of the library. Estimates of the biodiversity, richness, and evenness, the Shannon index, Simpson's index, and Chao1 (Chao 1984) were determined using DOTUR software (Schloss and Handelsman 2005). The reciprocal value of Simpson's index ($1/D$) was used in this study for showing the numbers of the most abundant phylotypes. The evenness was calculated by the formula: $E = e^{H'}/N$, where H' is the value of Shannon index, and N is the total numbers of the phylotypes (Krebs 1989).

Microbial activity at cold temperatures

To examine microbial activity at cold temperatures, microcosms containing 5 g of sediment from the LH Spring channel were prepared as described by Steven et al. (2008). Each microcosm was performed in triplicate. Sterile controls were autoclaved twice for 30 min at 120°C, with a 24-h period between sterilizations. Each microcosm was supplemented with 0.045 mCi/ml of [1-^{14}C] acetate (specific activity 57.0 mCi/mmol; Amersham Biosciences, NJ, USA) and incubated at 5, −5, −10, −15 and −20°C in temperature-monitored incubators with ±1°C temperature control. CO_2 traps in microcosms consisted of 1 M KOH (for 5 and −5°C) or 1 M KOH + 15% v/v ethylene glycol (for −10, −15 and −20°C) to prevent freezing during incubation. The CO_2 traps were sampled at timed intervals (1 month) and radioactive counts determined by liquid scintillation spectrometry on a Beckman Coulter (CA, USA) LS 6500 Multi-purpose Scintillation Counter (Steven et al. 2007b).

Nucleotide accession numbers

Partial 16S rRNA sequences were obtained from all clones and strains for building phylogenetic trees as described above and have been deposited in the NCBI database under accession numbers HQ444225-HQ444250 and HQ625077 (bacterial clones), HQ444251-HQ444262 (archaeal clones), and HQ625055-HQ625076 (bacterial strains).

Results

Geochemical analyses

Geochemical characteristics of the LH channel water and sediments varied seasonally and showed some distinct features and more dynamic range of conditions compared to the LH source characteristics (Table 1). The water pH was near-neutral and had ~25% salinity which are similar to the water characteristics previously reported for the LH source in 2005–2008. Due to seasonal periods of both flowing water and dry conditions in the channel, the temperature of these sediments experienced much more pronounced variation than LH source sediments, ranging from −18°C to above 0°C and as depicted in Fig. 1b, the channel sediments remained unfrozen at −18°C. The total carbon content of the channel samples was also considerably higher than the total carbon content in the LH source (Table 1). The upper range of ammonia content of LH channel water (6.57 mg/kg) sampled during low flow conditions in July 2009 was as concentrated as the source water (6.87 mg/kg) (Table 1). However, samples analyzed from a period of high precipitation as observed in July 2010, exhibited much lower ammonia content in the channel waters at (0.62 mg/kg). The range of carbon and nitrogen values for the LH channel indicated a seasonally

Table 1 Physical and geochemical characteristics for Lost Hammer (LH) spring source and channel determined during both Arctic winter and summer

	LH source[a]	Channel[b]
Temperature (°C)	−5.9 to −4.7	−18 to 9.2
pH	5.96 to 7.38	6.52 to 7.28
DO (ppm)	0.1 to 1.0	>1.0
H_2S (ppm)	0 to 50	0 to 20
ORP (mV)	−187.4 to −154.0	−29.9 to 125.5
TDS (g/L)	175.0 to 241.7	61.5 to 95.7
Salinity (%)	22 to 26	22 to 26
Total viable cell count on 7% NaCl R2A media (CFU/g sediment)	ND	$1.25 \pm 0.59 \times 10^5$
Total viable cell count on 12% NaCl R2A media (CFU/g sediment)	ND	$2.4 \pm 0.60 \times 10^3$
Bacterial cells (CARD-FISH) (cells/g sediment)[c]	$3.61 \pm 0.11 \times 10^5$	$4.51 \pm 0.65 \times 10^7$
Archaeal cells (CARD-FISH) (cells/g sediment)	$1.63 \pm 0.11 \times 10^4$	$3.99 \pm 0.44 \times 10^6$

ND not determined

[a] Data represent the range of measurements from the source determined between 2005 and 2008 described in Niederberger et al. (2010)

[b] Data represent the range of measurements from the channel region determined between 2008 and 2010

[c] Data for CARD-FISH from the LH source were converted from the percentage of total DAPI-stained cells

variable nutrient supply. The reduction potential in the channel sediments ranged between moderately reducing to moderately oxidizing conditions (−29.9 to 125.5 mV) while the source remained highly reducing. These values correspond to higher dissolved oxygen levels in the channel water (>1.0 ppm) compared to the source.

CO_2 and CH_4 concentrations and flux measurements

Results for the sediment CO_2 and CH_4 concentrations and fluxes from the LH channel and spring are presented in Table 2. CH_4 concentrations were an order of magnitude greater in the sediments from the source than in the sediments from the channel, while CO_2 concentrations were found to be similar at both sites (Table 2). Estimated CH_4 and CO_2 fluxes for the LH source as a whole (based on 4 actively bubbling seep spots), using the static-chamber technique were 11.1 and 11.9 g/day, respectively. Using the constant flux rate method, CH_4 flux was measured as 33.4 mg/m^2/day and 15.0 g/m^2/day for CO_2 from the LH channel. Using a LiCor-8100, in situ CO_2 flux in the channel was determined at different locations both during spring and summer and were found to range between

Table 2 Carbon and nitrogen analyses for LH Spring source and channel

	LH source	Channel
Total carbon (%)	0.48[a]	0.92–1.08
Organic carbon (%)	0.45[a]	0.77–0.93
Total nitrogen (%)	ND	0.02–0.08
Water dissolved nitrite/nitrate (mg/kg)[b]	0.13	0.04–0.17
Water dissolved ammonia (mg/kg)	6.87 (381 μM)	0.62–6.57 (91.6–365 μM)
Sediment nitrite/nitrate (mg/kg)	2.87	0.09–0.12
Sediment ammonia (mg/kg)	2.55	0.76–0.88

ND not determined

[a] These values are taken from Niederberger et al. (2010)

[b] The nitrite/nitrate/ammonia concentrations in the water and sediments were analyzed separately

152 mg/m^2/day and 38.2 g/m^2/day. The variability in flux within the channels appeared spatially heterogeneous and possibly driven by the degree of saturation of the sediments and not temperature, as both low and high CO_2 flux rates were measured in spring and summer conditions while sediment temperatures varied from −16 to 9.2°C. Most CO_2 flux rates for the channel were lower than those estimated for the source with the exception of occasional bursts of gas exceeding source values.

Cell enumeration

Three methods were used to enumerate sediment microbial populations: DAPI, CARD-FISH, and viable plate counts. The total abundance of microbial cells in the channel sediments had a mean value of $4.14 \pm 1.58 \times 10^7$ cells/g sediment according to DAPI counts. The abundance of bacteria in channel sediments as determined by CARD-FISH enumeration had a mean value of $4.51 \pm 0.65 \times 10^7$ cells/g sediment while the mean abundance of archaea was $3.99 \pm 0.44 \times 10^6$ cells/g sediment. The ratio of bacteria to archaea was approximately 9:1. The bacterial and archaeal numbers showed no significant differences between the different samples used in the analyses ($p > 0.05$). ANME-1 viable cells were below detection by CARD-FISH.

Plate count enumeration was used to determine the numbers of viable heterotrophic colonies with an average of $1.25 \pm 0.59 \times 10^5$ CFU/g sediment on R2A media with 7% NaCl and $2.4 \pm 0.60 \times 10^3$ CFU/g sediment on R2A media with 12% NaCl at room temperature. Viable counts on R2A media with 20% NaCl incubated at 5°C were more variable with a mean of $1.05 \pm 1.4 \times 10^3$ CFU/g of channel sediment.

Table 3 CO_2 and CH_4 sediment concentrations and fluxes from LH Spring source and channel determined during both Arctic winter and summer

Site	Sediment concentration[a]		Flux	
	CH_4 (nmol/g)	CO_2 (μmol/g)	CH_4	CO_2
LH source	102 ± 17.7	41.1 ± 0.53	11.1 g/day[b]	11.9 g/day[b]
Channel	9.36 ± 1.70	35.5 ± 4.82	ND	18.3–84.0 g/m^2/day[c]

ND not determined

[a] Values are means of triplicates; standard error of the mean is presented

[b] Values are an estimate of the CH_4 and CO_2 fluxes for the entire spring, based on the assumption that four hotspots are continuously bubbling

[c] The values were determined using the LiCor 8100. These values were converted from 0.48 to 2.21 μmol/m^2/s

Identification and characterization of isolates

A total of 22 unique bacterial strains were isolated from the R2A plates with 7% NaCl, identified by 16S rRNA sequencing, and characterized in terms of growth temperature ranges and salinity tolerance (Table 3). Isolated strains were grouped within four different phyla: the *Firmicutes*, *Actinomycetes*, *Alphaproteobacteria* and *Gammaproteobacteria*. The isolates were all related to known halophilic or psychrophilic representatives (Table 3). The majority of the strains (15/22) were growing on R2A media with 7% NaCl at −5°C (Table 3, supplemental Table S1). None of these cold-adapted isolates grew at 37°C on R2A media with 0 and 20% NaCl. All of the isolates grew on R2A media with 7% NaCl at 25 and 5°C. A total of 6 strains were considered obligate halophiles and grew with 20% NaCl but were unable to grow on media without a minimum of 7% NaCl. However, no strain grew on the media with 25% NaCl, at any temperatures and no strain grew at −10°C (data not shown). In general, increasing NaCl concentration and decreasing temperature inhibited growth of the isolates (supplemental Table S1). Most of the strains (19/22) were pigmented (supplemental Table S1). No archaea were successfully isolated.

Bacterial and archaeal 16S rRNA gene clone libraries

A total of 486 bacterial clones and 184 archaeal clones were obtained from LH channel samples and then analyzed as combined clone libraries for bacteria and archaea, respectively; the microbial composition of the individual clone libraries are shown in Table S2 and S3. The clone libraries were examined both in terms of total species richness for the channel community as a whole (the unique sum of all clone libraries) and as individual profiles depicting natural variation in the relative species abundance between samples. According to the statistical analyses conducted in DOTUR (OTUs > 97%), these clone libraries indicated large variation in the bacterial diversity ranging between 16 and 76 unique bacterial phylotypes and less variation for the archaea having between 3 and 6 unique phylotypes, respectively (Table 4). The most abundant groups in the bacterial clone library were the *Bacteroidetes* (46.1% of all clones), followed by similar amounts of *Actinomycetes* (18.3%), *Alphaproteobacteria* (16.5%), and *Gammaproteobacteria* (11.1%) (Fig. 2a). The most common genus among the *Bacteroidetes* clones was related to *Gillisia* spp (32.3%). Among the other phylotypes, species involved in methanotrophy/methylotrophy and sulfur cycling were present in the 16S rRNA gene clone library and their phylogenetic comparisons are shown in Fig. 3. Among the methanotrophs, one clone (LHCbac-24) had a top BLASTn match (91% similarity) with *Crenothrix polyspora* (DQ295898), a filamentous aerobic methane oxidizer (Stoecker et al. 2006). Several clones affiliated with methylotrophs were also detected including close matches to *Methylophaga sulfidovorans* (91% similarity to NR_026313) and *Methylophaga thiooxidans* (90% identity to DQ660915), which are both able to oxidize dimethylsulfide (DMS) (Boden et al. 2010; de Zwart et al. 1996). Two other clones, LHCbac-15 and LHCbac-19, were affiliated with methylotrophic bacteria *Methylobacterium* sp. and *Methylibium* sp., respectively. Sulfur-cycling phylotypes, such as sulfur reducing species including *Desulfuromonas* (LHCbac-25) and sulfur oxidizing bacteria including *Thiobacillus* (LHCbac-16) were also detected and are shown in Fig. 3.

Amongst the archaeal 16S rRNA clone libraries, representatives were classified within four phyla with the most abundant being among the newly defined archaeal phylum *Thaumarchaeota* (70.2%) (Fig. 2b) (Brochier-Armanet et al. 2008). The remainder were all classified within *euryarchaeal* phylotypes including *Halobacteria* (15.9%), *Methanobacteria* (12.4%), and a small percentage of unclassified Euryarchaeota (1.5%). According to the phylogenetic analyses, several clones (LHCarc-9, LHCarc-10, LHCarc-7, LHCarc-8) grouped with the ammonia-oxidizing *Thaumarchaeota* species 'Candidatus Nitrososphaera gargensis' (Spang et al. 2010) (Fig. 4). Although the classification of two clones, LHCarc-11 and LHCarc-12, was not entirely conclusive, they appeared to match *Thaumarchaea* more than any other archaeal phyla with

Table 4 Characteristics of 22 bacterial strains isolated from LH channel sediments

Numbers of unique strains	Salinity range (% NaCl)	Temperature range (°C)[g]	Closest cultured BLAST hit (accession #)	Origin of BLAST relative	Similarity to BLAST sequence (%)	RDP classifier (>80% confidence)
7[a]	0–20	−5 to 37	*Planococcus* sp., NP 19 (EU196338)	Axel Heiberg Island, Perennial spring	98–99	*Planococcaceae* (family, 100%)
4[b]	7–20	−5 to 25	*Marinobacter* sp. ZS1-16 (FJ889664)	Antarctic sandy intertidal sediments	97–100	*Marinobacter* (genus, 100%)
2[c]	0–12	−5 to 25	*Psychrobacter* sp., E59 (DQ667083)	Antarctic sea water	98–99	*Psychrobacter* (genus, 100%)
5[d]	0–20	5 to 37	*Nesterenkonia* sp. 35/46, (AY571802)	Antarctica soil	98–100	*Nesterenkonia* (genus, 100%)
1[e]	0–12	−5 to 37	*Planomicrobium psychrophilum* strain 4-5-26 (GQ505362)	Mud volcanoes in Xinjian	99	*Planococcaceae* (family, 100%)
1[f]	0–7	5 to 25	*Fulvimarina* sp. NP 28 (EU196328)	Axel Heiberg Island, Perennial spring	100	*Aurantimonadaceae* (family, 100%)

[a] These 7 strains include CY-C3-1, CY-C3-3, CY-C3-4, CY-C3-5, CY-C3-6, CY-C1-11, CY-C2-19-2

[b] These 4 strains include CY-C3-2, CY-C3-9, CY-C2-15, CY-C2-17-1

[c] These 2 strains include CY-C3-7, CY-C3-8

[d] These 5 strains include CY-C1-10, CY-C2-13, CY-C2-14, CY-C2-17-2, CY-C2-19-1

[e] This 1 strain includes CY-C1-12

[f] This 1 strain includes CY-C2-18

[g] No strain grew at 37°C on R2A media with 0 and 20% NaCl. All the strains grew at 25 and 5°C on R2A media with 7% NaCl. Specific characteristics for each strain are presented in the supplementary files (Table S1)

greater than 94% identity (Fig. 4). Phylotypes representing methanogenic archaea, which were all related to the genus *Methanobrevibacter* (12.4%), were also found in the clone library. No clones related to the anaerobic methane oxidizing ANME-1 clade were detected in the clone libraries.

The biodiversity indices (Table 4) for the bacterial and archaeal 16S rRNA clone libraries from the LH channel indicated relatively low diversity of the archaeal clone libraries (Shannon index = 0.78–1.37) compared to the higher diversity within the bacterial clone libraries (Shannon index: 1.69–3.80). Library characteristics were described using multiple indices indicating that a large proportion of the expected bacteria (Chao1 = 44–104, evenness = 34–59%) and archaea (Chao1 = 3–6, evenness = 36–98%) were successfully sampled. The most abundant phylotypes defined by the inverse of the Simpson's index were 3–25 bacterial phylotypes, and 1–4 archaeal phylotypes. The Good's coverage ranged between 67.8 and 80.0 for the bacterial and between 87.5 and 95.0 for the archaeal libraries. Compared to LH source clone libraries, the channel depicts higher bacterial diversity but comparable archaeal diversity in the sediment microbial communities.

Microbial activity at cold temperatures

To detect microbial respiration and activity at cold temperatures, mineralization of ^{14}C-acetate was evaluated within channel sediment microcosms. Following 6 months of incubation, mineralization was observed in triplicate microcosms compared to sterile controls (Fig. 5). The highest cumulative percent recovery of mineralization occurred at 5°C (~30%) but microbial respiration was also detected at −5°C (~10%), −10°C (0.19%), −15°C (1.21%) and −20°C (0.17%) (Fig. 5). Although the percent recovery dropped significantly at temperatures ≤−10°C, levels of microbial respiration were still above background levels measured in the −10, −15 and −20°C sterile controls.

Discussion

Lost Hammer spring represents the first described example of a subzero terrestrial methane-seep ecosystem (Niederberger et al. 2010). The LH outflow channel described here, represents a distinct, more heterogeneous and stochastic environment occurring downstream of this subzero, hypersaline methane seep. The LH channel exhibits many of the extreme conditions found in the spring source but experiences greater seasonal oscillations in physical and geochemical characteristics including water level, much broader temperature ranges, changes in O_2 content, and different carbon and nitrogen concentrations than observed in the source. Most notable are the channels' large variations in temperatures (−18 to 9.2°C), and their less

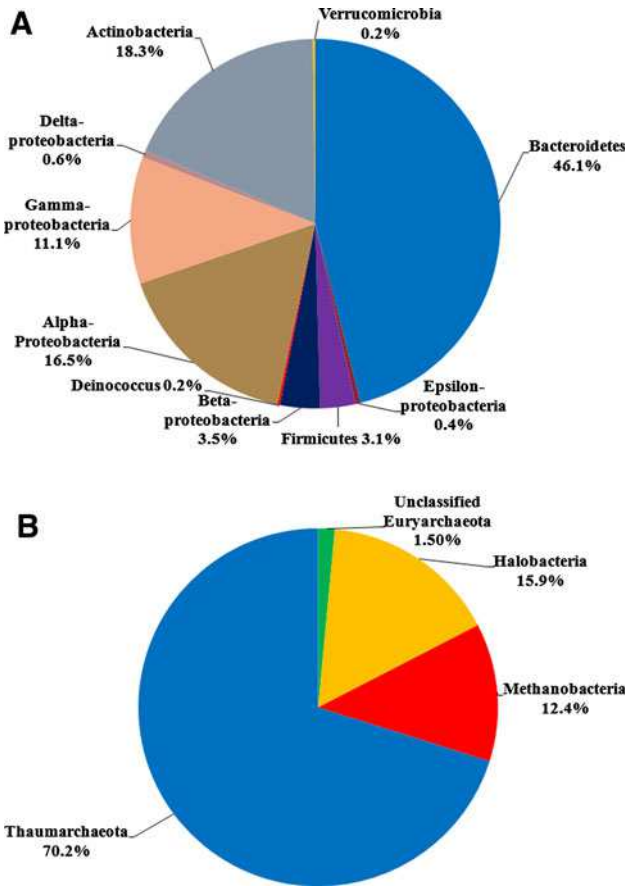

Fig. 2 Phylogenetic composition of sequences from **a** bacterial and **b** archaeal 16S rRNA gene clone libraries constructed from samples from Lost Hammer spring channel sediments. Sequences were grouped using the RDP Classifier function of the Ribosomal Database Project-II release 9 with a confidence threshold of 80%

reducing, more aerobic conditions compared to the seasonally stable source (consistently $\sim -5°C$). There is a sharp decrease in methane concentration in the channel sediments with only $\sim 1/10$ of the methane present than in the source sediments and only moderate CH_4 flux detection over a short time period but remains higher than surrounding permafrost and atmospheric levels.

These variations in the geomorphology of the LH spring system, along with dynamic environmental factors, (i.e. fluid flux, gas concentrations and diffusion, seasonal effects etc.) create a more heterogeneous environment for the channel microbial communities and was reflected by the relative abundance and diversity of different bacterial and archaeal phyla, as described below (Supplementary Table S2). The changing composition of the LH channel microbial communities between samples and over time suggests that species abundance is in fact dynamic, maintaining a simple archaeal composition, common in different marine methane-seep communities (Knittel et al. 2005), but a more diverse and abundant bacterial assemblage occurring in the channel compared to the source. Both these observations suggest that the channel community provides a more favorable environment for microbial growth and has a greater diversity of niches than are present in the source.

In similar investigations of gradients along marine methane seeps, differences in geochemical conditions have been shown to correlate with niches occupied by distinct microbial communities including different spatial abundance of ANME archaea (Arakawa et al. 2006; Knittel et al. 2005) and simple assemblages of key functional groups including methanotrophs, hydrocarbon degraders, sulfate-reducers and sulfide-oxidizers (Jørgensen and Boetius 2007; Valentine 2002; Valentine and Reeburgh 2000). The presence of numerous species in the LH channel environment not found in the LH source reflects a greater potential metabolic diversity utilizing a variety of nitrogen and sulfur substrates in addition to methane. The phylotypes previously found within the LH source sediments include *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteria*, and *Firmicutes* (Niederberger et al. 2010). With the exception of *Cyanobacteria*, all LH source phyla were represented in the channel as well as several additional phyla, including an abundance of *Actinobacteria*, that were not detected in the LH source sediments. Phylotypes for *Halomonas*, *Gillisia*, and *Marinobacter*, which are common bacterial genera in cold Arctic and Antarctic environments (Bowman et al. 1997; Bowman and Nichols 2005; Brinkmeyer et al. 2003; Franzmann et al. 1987; Guan et al. 2009; Zhang et al. 2008), were detected in both the LH source (Niederberger et al. 2010) and channel sediments. Representatives of sulfur-cycling bacteria were also detected in channel sediments that were not found in the source sediments indicating that various sulfur intermediates were important metabolic substrates within the LH channel including sulfite (*Sulfitobacter* sp. and *Desulfitibacter* sp.), elemental sulfur (*Desulfuromonas* sp.) and various reduced sulfur compounds for sulfur oxidizers (*Thiobacillus* sp., *Sulfuricurvum* sp., *Sulfurovum* sp.). Most of the closest relatives for these identified phylotypes were found in cold and/saline environments including *Desulfuromonas* sp. identified in subpermafrost saline fracture water at the Lupin mine in the Canadian Arctic (Onstott et al. 2009).

In contrast to finding several similar species in the bacterial profiles of the LH source and channel sediments, the archaeal 16S rRNA clone libraries presented different phylotypes. The most abundant and notable phylotype in the source was related to the anaerobic methane oxidizing archaea group 1 (ANME-1), however, this group was not detected in the channel clone libraries. A few putative cells of ANME-1 homologs (statistically below the detection limit) were detected in the channel sediments by CARD-FISH using an ANME-1 specific probe (ANME-1 350)

Fig. 3 Phylogenetic relationships of representative bacterial 16S rRNA gene sequences obtained from the LH Spring channel clone libraries and strains. The tree was inferred by neighbor-joining analysis of 460 homologous positions of sequence from each organism or clone. *Numbers on the nodes* are the bootstrap values based on 1,000 replicates. *Scale bar* indicates the estimated number of base changes per nucleotide sequence position. *Percentages* indicate the prevalence of the clone types within the clone library with the number of clones indicated in *parentheses*. The titles starting with LHCbac indicate LH channel clone representatives, and the titles starting with CY indicate the strains

(Losekann et al. 2007) suggesting that the much lower concentration of methane and increased oxygen content may not favor ANME-1 in the channel sediments. However, unlike in the source sediments, methanogenic archaea, predominantly *Methanobrevibacter* were detected in the channel suggesting that at least part of the methane produced in the channel, albeit at significantly lower concentrations than the source, may be of biogenic origin. The potential for methane cycling in the channel sediments is also supported by an abundance of aerobic methylotrophic/methanotrophic species including *Methylobacterium* suggesting that methane may be used as both carbon and energy source under the moderately aerobic conditions in the LH channel system.

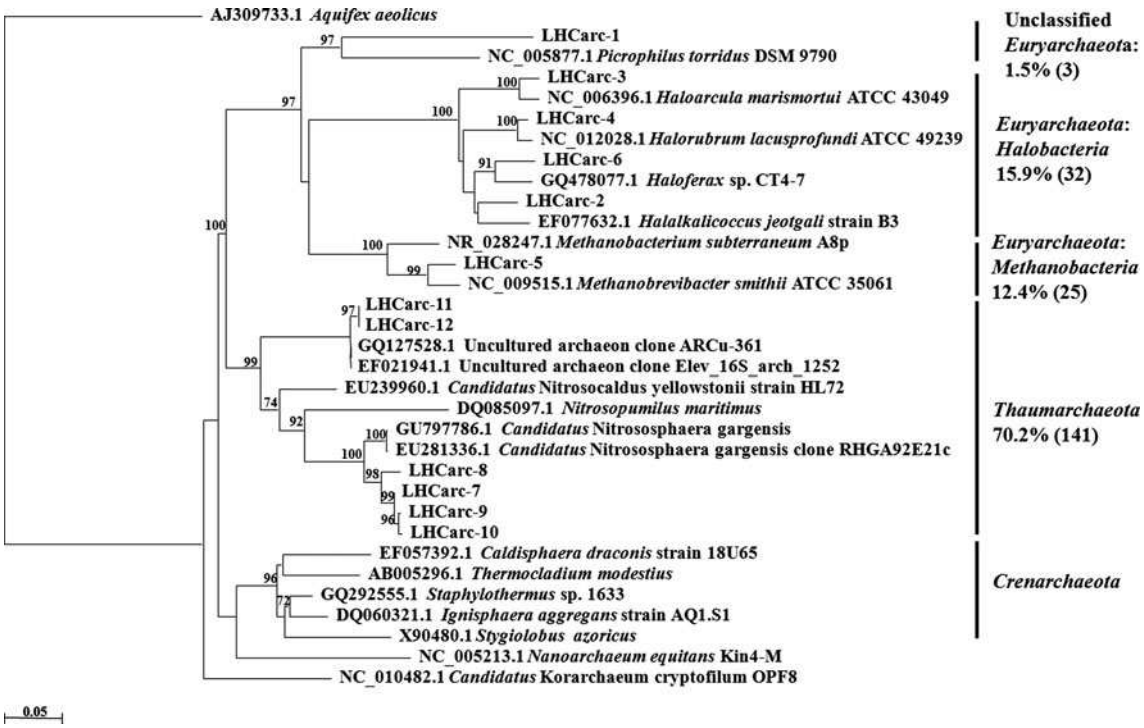

Fig. 4 Phylogenetic relationships of the archaeal 16S rRNA gene sequences obtained from the LH channel clone libraries. The tree was inferred by neighbor-joining analysis of 576 homologous positions of sequence from each clone. *Numbers on the nodes* are the bootstrap values based on 1,000 replicates. The *scale bar* indicates the estimated number of base changes per nucleotide position. *Percentages* indicate the prevalence of clone types within the clone library with the number of clones indicated in *parentheses*. The titles starting with LHCbac indicate LH channel clone representatives

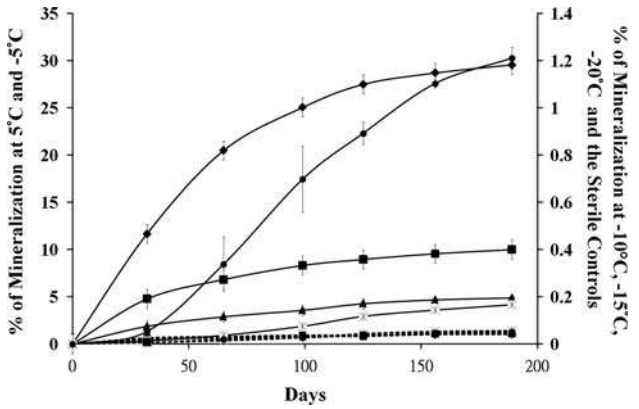

Fig. 5 Mineralization assays of [1-^{14}C] acetate in LH channel sediment microcosms at different temperatures. Each point represents the mean cumulative mineralization (% $^{14}CO_2$ recovered) from triplicate assays. Curves are shown for 5°C (*filled diamonds*) and −5°C (*filled squares*) on the primary axis, and for −10°C (*filled triangles*), −15°C (*filled circles*), −20°C (*asterisks*) and the sterile controls on the secondary axis. The curves of the samples and the sterile controls are shown in the *solid lines* and the *dotted lines*, respectively

A notable feature of the channel communities is the abundance of the phylum, *Thaumarchaeota* (Brochier-Armanet et al. 2008), which comprised the largest proportion of the archaeal clone libraries but was not detected in the LH source sediments. To date the *Thaumarchaeota*, which are found in a wide range of environments, are thought to be associated with pathways involved in nitrification, and are often referred to as ammonia-oxidizing archaea (AOA) (Spang et al. 2010). Marine AOA species are able to convert ammonia to nitrite in environments of low ammonia concentrations, such as the open sea (<0.03–1 μM) and coastal waters (<0.03–100 μM) (Könneke et al. 2005) both of which are lower than the ammonia concentrations measured in the LH channel sediments. The majority of the currently known species of *Thaumarchaeota* have been identified within mesophilic and thermophilic environments (de la Torre et al. 2008; Hatzenpichler et al. 2008; Könneke et al. 2005; Schouten et al. 2008), however, some phylotypes have also been detected in cold Antarctic bathypelagic sediments (Gillan and Danis 2007). Four channel clones were grouped closely with 'Candidatus Nitrososphaera gargensis' and 'Candidatus Nitrosopumilus maritimus', the latter being a moderately psychrophilic and halophilic species (Könneke et al. 2005). The presence of *Thaumarchaeota* in the LH channel seems plausible as suitable conditions (moderately aerobic, high salinity and low sediment ammonia concentrations) for their autotrophic ammonia oxidizing and nitrification activities exist within the LH channel. This is the first description of

Table 5 Summary of the range of statistics and indices for the 16S rRNA gene clone libraries of LH channel and source sediments

	Channel		Source[a]	
	Bacteria	Archaea	Bacteria	Archaea
No. of clones	80–236	24–80	61	66
No. of phylotypes	16–76	3–6	9	7
Shannon index (H')	1.69–3.80	0.78–1.37	1.65	1.39
Simpson index ($1/D$)	3–25	2–4	ND	ND
Chao1	44–104	3–6	ND	ND
Evenness (E)	0.34–0.59	0.36–0.98	ND	ND
Coverage (%)	67.8–80.0	87.5–95.0	98.5	95.1

ND not determined

[a] Data represent the statistics and indices obtained from Niederberger et al. (2010)

Thaumarchaeota identified within a subzero hypersaline environment (Table 5).

All the strains isolated in this study were related to known cold and salt tolerant species. Interestingly, we were able to culture a portion of the bacterial phylotypes detected within the 16S rRNA clone libraries including *Marinobacter*, *Planococcus* and *Nesterenkonia* with all isolates capable of growth at LH in situ temperatures of (−5°C) and 13 of 22 isolates capable of growth at the high in situ salinity concentrations. Only *Marinobacter* spp. had previously been isolated from the LH source (Niederberger et al. 2010) but most of the isolated species have also been found in nearby GH and CP springs (Perreault et al. 2008), as well as permafrost from Eureka (Steven et al. 2008), and in Antarctic sea ice brine (Junge et al. 1998). The majority of the isolated channel strains were also pigmented; a common adaptive strategy in many cold environment species that may serve several functions including cryo- and solar radiation protection, light-harvesting, and anti-oxidative activity (Dieser et al. 2010; Mueller et al. 2005).

Due to the long-term stability of DNA at high ionic concentration, low temperatures and anoxic environmental conditions (Inagaki et al. 2005) it is difficult to conclude which of the microbial phylotypes detected in the LH channel are active or dormant under in situ conditions. However, the high percent recovery of ^{14}C-acetate mineralization and high viable cell counts suggest that a significant portion of the microbial biomass could be active in situ, including many of the heterotrophic phylotypes detected in the clone libraries and viable isolates. The cumulative percent recovery reported at 5°C are similar to the ones reported for other Arctic samples, such as Eureka permafrost active layer (Steven et al. 2008), the Markham ice shelf, and the Ward Hunt Ice shelf (Steven et al. 2007b). In contrast, microbial activities at −5, −10, −15 and −20°C were lower than permafrost soils but remained similar to measurements in ice shelf material (Steven et al. 2007b). The very low percent recovery of ^{14}C-acetate mineralization at −10°C and below in the channel sediments indicate that microbial activity and growth may be restricted at temperatures ≤−10°C in this hypersaline environment possibly due to the inactivation of protein folding, cell membrane fluidity and DNA/protein synthesis (Bakermans 2008; Price and Sowers 2004). Moreover, no isolates in this study were found to grow at −10°C and could partly explain the low respiration activity at temperatures ≤−10°C resulting from low level metabolism of cells not undergoing active division (Bakermans and Skidmore 2011). However, the percent recovery of ^{14}C-acetate utilization by the LH channel sediments was higher at all temperatures than in ^{14}C-glucose mineralization measurements from the LH source after 6 months (Steven et al. 2007b) and could be related to the 10–100 times greater biomass present in the channel compared to the LH source. Furthermore, the detection of CO_2 and CH_4 flux from the channel sediments does suggest that microbial respiration occurs both in winter and summer within the channel sediments under ambient conditions. It is also likely that some of the measured gas flux represents pockets of gas periodically released from saturated and/or partially frozen sediments. Further analyses are underway to determine the biogenic signature of LH channel CO_2 and CH_4 efflux.

In conclusion, our results illustrate that the LH channel sediments present a dynamic yet extreme habitat supporting microbial community with greater biomass, diversity, and activity than that previously found in the LH spring source. Overall, the findings further our understanding of extreme microbial ecosystems within the context of unique niche formation along chemical gradients within the same spring environment. Future investigations will probe which species are active under in situ conditions through deep sequencing of cDNA libraries of 16S rRNA coupled with metatranscriptomic analyses to identify the active metabolic pathways occurring within the channel relative to the LH source environment. Lastly, examination of the unique and diverse microbial communities present in such a cold, hypersaline cryoenvironment both will increase our knowledge of microbial life in extremely cold habitats on Earth as well as lead to a more realistic understanding for the potential of microbial life to exist in other very cold solar system bodies such as Mars, Europa, or Enceladus (Kerr 2011; Postberg et al. 2011).

Acknowledgments This project is supported by NSERC CREATE Astrobiology Training Program, CSA Canadian Analogue Research Network Grant Program, Polar Continent Shelf Program, NSERC Discovery Grant Program, and Department of Natural Resource Sciences, McGill. We thank Dr. M. Wagner for the information regarding *Thaumarchaeota*, Dr. A. Chao for the questions regarding the indices, Dr. J. Whalen and H. Lalande for the help of C/N/nitrite/nitrate/ammonia analyses, and thanks to the members of the Whyte and Greer Labs for helpful discussions and to Dr. H. Vrionis for critical review of the manuscript.

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