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Evidence of in situ microbial activity and sulphidogenesis in perennially sub-0 °C and hypersaline sediments of a high Arctic permafrost spring

Guillaume Lamarche-Gagnon · Raven Comery · Charles W. Greer · Lyle G. Whyte

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Abstract The lost hammer (LH) spring perennially discharges subzero hypersaline reducing brines through thick layers of permafrost and is the only known terrestrial methane seep in frozen settings on Earth. The present study aimed to identify active microbial communities that populate the sediments of the spring outlet, and verify whether such communities vary seasonally and spatially. Microcosm experiments revealed that the biological reduction of sulfur compounds (SR) with hydrogen (e.g., sulfate reduction) was potentially carried out under combined hypersaline and subzero conditions, down to −20 °C, the coldest temperature ever recorded for SR. Pyrosequencing analyses of both 16S rRNA (i.e., cDNA) and 16S rRNA genes (i.e., DNA) of sediments retrieved in late winter and summer indicated fairly stable bacterial and archaeal communities at the phylum level. Potentially active bacterial and archaeal communities were dominated by clades related to the T78 *Chloroflexi* group and *Halobacteria* species, respectively. The present study indicated that SR, hydrogenotrophy (possibly coupled to autotrophy), and short-chain alkane degradation (other than methane), most likely represent important, previously unaccounted for, metabolic processes carried out by LH microbial communities. Overall, the obtained findings provided additional evidence that the LH system hosts active communities of anaerobic, halophilic, and cryophilic microorganisms despite the extreme conditions in situ.

Keywords Cryophile · Halophile · Sulfur/sulfate reduction · Microbial ecology · Anaerobic activity · 16S rRNA

Abbreviations

cmbs	cm below the sediment surface
SR	Sulfur reduction, (bio)chemical reduction of a sulfur compound (e.g., sulfate reduction)
SRR	Sulfide release rates; the rates of sulfide (H_2S) production resulting from SR

Introduction

The environmental conditions that define the habitability window of life are continuously being reassessed with the discovery of organisms or communities living in natural settings previously considered hostile to biology. The Earth's cryosphere, this portion of the biosphere that encompasses microorganisms inhabiting constantly cold or frozen places (i.e., cryoenvironments), has recently been recognized to comprise biologically active ecosystems (Priscu and Christner 2004). This emergent view of an active cryosphere has global repercussions on environmental fluxes and cycles considering the widespread

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G. Lamarche-Gagnon · R. Comery · L. G. Whyte (✉)
Department of Natural Resource Sciences (NRS), McGill University, Montreal, Canada
e-mail: lyle.whyte@mcgill.ca

C. W. Greer
National Research Council Canada, Montreal, Canada

distribution and extent of cold environments, but too little is known regarding the microbial assemblages that make up the cryosphere to fully assess its importance.

Cryoenvironments are normally found in polar and alpine regions and mainly consist of ice formations such as glaciers, ice shelves, and sea ice, as well as permafrost (Goordial et al. 2013). In line with the extent and diversity of cryoenvironments, the cryosphere exhibits a wide range of microbial diversity normally reflective of the environments themselves. Some environmental selection nonetheless appears to shape cryoenvironment communities with successful colonization and survival mostly limited to microorganisms bearing at least some degrees of cold adaptation (Margesin and Miteva 2011). Considering that the presence of liquid water in cryoenvironments often depends on freezing point depression, cryophilic microorganisms (i.e., those capable to metabolize below 0 °C) also often require to cope with high concentrations of salts or solutes (Goordial et al. 2013). For example, Deep Lake in the Vestfold Hills, Antarctica, is hypersaline (32 % salt), remains ice-free year-round with an average annual temperature of -15 °C, and is dominated by halophilic archaea (DeMaere et al. 2013). Lake Vida is an almost entirely frozen lake, the bottom of which consists of an unfrozen anoxic brine (~20 % salinity, −12 °C) and a recent study of the site revealed that active bacteria most likely populate the brine despite the extreme conditions in situ (Doran et al. 2003; Murray et al. 2012). More extreme is the Don Juan Pond in Antarctica; considered the most saline water body on Earth, this hypersaline playa is composed of a eutectic $CaCl_2$ brine of about 40.2 % salt, in which microbial activity has never been detected (Samarkin et al. 2010).

The Lost Hammer (LH) spring in the Canadian high Arctic discharges perennial sub-0 °C hypersaline brines through >500–600 m of permafrost, and so far accounts for the coldest terrestrial methane seep on the planet (Niederberger et al. 2010). The subzero, hypersaline, anoxic, and sulfate- and methane-rich nature of the LH spring arguably makes it an excellent analog to alien environments described on Mars, Enceladus, or Europa (McKay et al. 2012). The likely presence of liquid briny water, reports of spring-like structures, the large distribution of chloride deposits, and the discovery of sulfate minerals on the Martian surface support the LH spring's relevance as an analog site and the importance of studying and better understanding its microbial populations (Allen and Oehler 2008; Andersen et al. 2002; Davila et al. 2010; Gendrin et al. 2005; Osterloo et al. 2010; Rossi et al. 2008). The perennial nature of the site also makes LH a good natural laboratory to probe microbial adaptations and responses to combined subzero temperatures and salinity, set in a permafrost-dominated environment that normally lacks significant amounts of unfrozen water.

Previous investigations raised the possibility that LH is populated by metabolically active indigenous communities despite the extreme in situ conditions (Lay et al. 2013; Niederberger et al. 2010; Steven et al. 2007). These studies have, however, mostly been restricted to molecular surveys and laboratory experiments that did not necessarily reflect the LH natural environment; whether the LH system truly hosts active communities remains somewhat speculative. The ability to accurately link microbial activity and metabolic function to sequencing surveys continues to be a challenge in microbial ecology studies, particularly in environments prone to long-term nucleic acid preservations such as cryo- and hypersaline systems (Blazewicz et al. 2013; Borin et al. 2008; Charvet et al. 2012; Tehei et al. 2002). The present research built on previous LH investigations and aimed at ascertaining that actively metabolizing populations are present in situ, via a combination of sequencing of both 16S rRNA genes (DNA) and transcripts (cDNA), as well as anaerobic and subzero incubation experiments targeting previously reported LH methanogen, anaerobic methane oxidizing archaea (ANME), and sulfate/sulfur-reducing populations (Lay et al. 2013; Niederberger et al. 2010). It also constitutes the first report on the microbiology of the LH spring outlet sediments collected during the late Arctic winter allowing to infer the spatial and seasonal stability of the LH microbial communities. Lastly, the cryophilic potential (i.e., capacity to metabolize below 0 °C) of LH populations was also assessed in colder-than-in situ- incubations by taking advantage of the low eutectic point of the LH brine.

Materials and methods

Site description

The LH spring is located in a valley off the shores of Strand Fjord on Axel Heiberg Island (AHI) in the Canadian high Arctic in a region of diapiric uplift (79°07′N, 90°21′W) derived from buried carboniferous anhydrite-salt evaporites (Pollard 2005). Because it is part of the Wolfe diapir, the spring is sometimes referred to as the "Wolfe spring" (Battler et al. 2013); for consistency with previous microbiology work on the site, this paper will still refer to it as "Lost Hammer". The LH outlet is characterized by a cone-shaped salt tufa of ~2 m in height and 3 m in diameter (Fig. S1). The spring discharges perennially subzero (~−5 °C), nearly anoxic, reducing brines very rich in sulfate (0.05 and 1.04 M for the brine and underlying sediments, respectively); the brine's geochemical characteristics are summarized in Table S1. The LH spring also constantly releases gases, comprised mainly of methane (CH_4; ~50 %), nitrogen (N_2; ~35 %), and carbon

dioxide (CO_2; ~10 %), as well as smaller amounts of hydrogen, helium, and C_2–C_4 alkanes (Niederberger et al. 2010). The mineral composition of the spring sediments mainly consists of Na-sulfates (mainly thenardite and mirabilite), halite, gypsum, elemental sulfur, as well as quartz and clays (Battler et al. 2013). Previous investigations of the LH site have indicated that the spring geochemistry is fairly stable between years and sampling seasons, with few observed changes in water chemistry and gas discharges (Lay et al. 2012; Niederberger et al. 2010). The water level of the spring outlet does, however, fluctuate seasonally; the LH outflows are blocked during the colder winter months, resulting in a periodic filling and emptying of the salt tufa during winter and summer months, respectively (Fig. S1).

Sample collection

Spring samples were collected during three summer expeditions (July 2009, 2011, and 2012) and one in late winter (April 2012). Ethanol-sterilized push cores were used to collect sediments; cores never extended deeper than 30 cmbs (cm below the sediment surface). Immediately after sampling, cores were either stored vertically inside heat-sealed laminated bags (2011 samples) or were transferred into sterile 50 mL tubes, filled completely with spring water, and stored inside portable anaerobic jars (2012 samples); both 2011 and 2012 samples were kept anoxic using with gas-generating AnaeroGen sachets and anaerobic indicators (Oxoid, Nepean, On, Canada). 2009 cores were not stored anoxically but cores were capped full (no headspace) on site. Sediments (~12 g) to be used for molecular analyses (2012 samples) were stored in sterile, nuclease-free, 50-mL tubes pre-filled with 36 mL of LifeGuard Soil Preservation Solution (MoBio Laboratories, Inc., Carlsbad, CA, USA). Sediment and water samples were kept at <5 °C (but not frozen) during transport and within 2 weeks were stored at −5 °C. Samples stored in RNA LifeGuard were frozen at −20 °C within 3 h of collection and subsequently stored at −80 °C until processing. Dissolved sulfide and oxygen concentrations were measured in situ by colorimetric assays using either CHEMetrics visual kits for sulfides (methylene blue method) or a portable V-2000 photometer (CHEMetrics, Calverton, VA, USA) for oxygen (rhodazine D method). Water chemical properties such as conductivity, salinity, total dissolved solids and oxido-reduction potential (ORP) were measured using the YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH, USA).

Anaerobic microcosm incubations

Sediments used in anaerobic incubations were all processed inside an anaerobic chamber (COY Laboratory, Grass Lake, MI, USA) to minimize oxygen exposure. Microcosms consisted of either autoclaved 37.5-mL serum vials or 26-mL tubes crimp-sealed with autoclaved blue butyl-rubber stoppers (2 cm thick) and aluminum caps. Except for experiments using 2011 samples, the water used was natural LH water rendered anoxic and reducing via N_2 gas bubbling and the addition of the reducing agent $Na_2S{\bullet}9H_2O$ (5 mM). The LH water added to microcosms set up with 2009-collected sediments consisted in a mixture of previously collected LH water (years 2006–2009) pooled together. Because of LH water shortage, the incubation experiments using 2011-collected LH sediments were set up with water made of half LH water sampled in July 2011, and half artificial water synthesized in the laboratory. The composition of the synthesized water was a modification of Berges et al. (2001) artificial seawater medium to match that of the natural LH water whenever known (Table S2). In all cases, the atmosphere of each crimped vial was exchanged by flushing the vial's headspace for at least 5 min with the required gas (N_2, N_2:CO_2, CH_4, or H_2:CO_2) before the start of the experiment.

Measurement of anaerobic oxidation of methane (AOM)

Microcosms consisted in 2011 LH sediment (0–30 cmbs) and water slurries in anaerobic culture tubes (Balch tubes). AOM was assessed using both radiotracers ($^{14}CH_4$) similar to Treude et al. (2007) and via methane consumption and sulfide production over time. Details regarding the radiotracer experiment are provided as supplementary material. In the methane consumption experiment, pure CH_4 was added to the sample tubes (~500 ppm final concentration); controls consisted in tubes with a N_2:CO_2 (80:20) atmosphere to which no CH_4 had been added. Both methane and sulfide levels were assessed as proxy for AOM. Methane consumption was determined by gas chromatography using a Shimadzu GC-8A equipped with a flame ionization detector (FID) and connected to a HP 3394 integrator; He was used as carrier gas, supplied to the GC at a flow rate of 30 mL/min, and oven and detector temperatures were set at 50 and 150 °C, respectively. The GC was calibrated using certified methane standards (Matheson, Morrow, GA, USA); 100 µL of headspace was injected into the GC for methane quantification. To avoid a dilution effect over the long incubation periods, a modified version of the methylene blue colorimetric assay designed by Cline (1969) was used to measure sulfide concentrations from the vials' headspace instead of dissolved sulfide concentrations in the slurry samples. In short, 100 µL of headspace was sampled using a high precision gastight syringe pre-filled with 100 µL of 20 % zinc acetate. The sample was then injected into sealed tubes containing cline reagents (dimethyl-p-phenylenediamine sulfate 0.5 g/L, zinc acetate 0.5 g/L, glacial acetic acid

2.5 %). 0.1 mL of 25 % $FeCl_3$ was then added to the tubes. The tubes were inverted to mix, incubated in the dark for 20 min to allow color development, and spectrophotometric measurements were then performed at 670 nm. Technical duplicate measurements were taken for each sample. For both methane and sulfide measurements, tubes were hand-shaken for 2 min before headspace sampling to equilibrate the dissolved gases with the headspace.

Measurement of methanogenesis

Incubations were set up using LH sediments collected in both July 2009 and July 2011 inside serum vials. Samples were amended with 500 µM of either dimethylsulfide (DMS), trimethylamine (TMA), or methanol and incubated at temperatures ranging from −5 to 10 °C under a N_2:CO_2 (80:20) atmosphere (2009 samples), or unamended under a H_2:CO_2 (80:20) atmosphere (2011 samples). Samples were set up in triplicate and negative controls consisted in microcosms either autoclaved twice for 30 min at a 48-h interval (2009 samples), amended with 5 % paraformaldehyde, or, left unamended under a N_2:CO_2 (80:20) atmosphere (2011 samples). After about 100 days of incubation, DMS, TMA, and methanol concentrations were increased to 5 mM. After 170 days of incubation, unamended N_2:CO_2 (80:20) negative control vials (2011 samples) were amended with sodium acetate (10 mM); these later vials were also assayed for H_2S releases in combination to methanogenesis both prior and after acetate addition. Methane concentrations were measured via gas chromatography as described above. Incubations set up to assay for methanogenesis are summarized in Table S3.

Measurement of sulfidogenic activity

Serum vials were amended with ~5 g of wet sediments (~0–15 cmbs, 2012 samples), 10 mL of LH water and a H_2:CO_2 (80:20) atmosphere; formalin-treated slurries (5 % final formaldehyde concentration) were used as negative controls. The pH of the slurries at the start of the experiment was 6.5. Samples were incubated at 5, −5, −10, −15, and −20 °C; sample incubation at 0 °C was not possible due to incubator malfunction and incubation temperatures lower than −20 °C resulted in freezing of the LH water. Sulfide production was measured as for the AOM experiments. Sulfide release rates (SRR) were calculated using regression on the linear section of the curves showing sulfide concentration over time (Fig. 1b).

Because the high concentration (5 mM) of the reducing agent sodium sulfide ($Na_2S•9H_2O$) used during the experiment could have potentially affected the measured SRR, additional control experiments using an alternative reducing agent (i.e., cysteine-HCl 0.05 % final

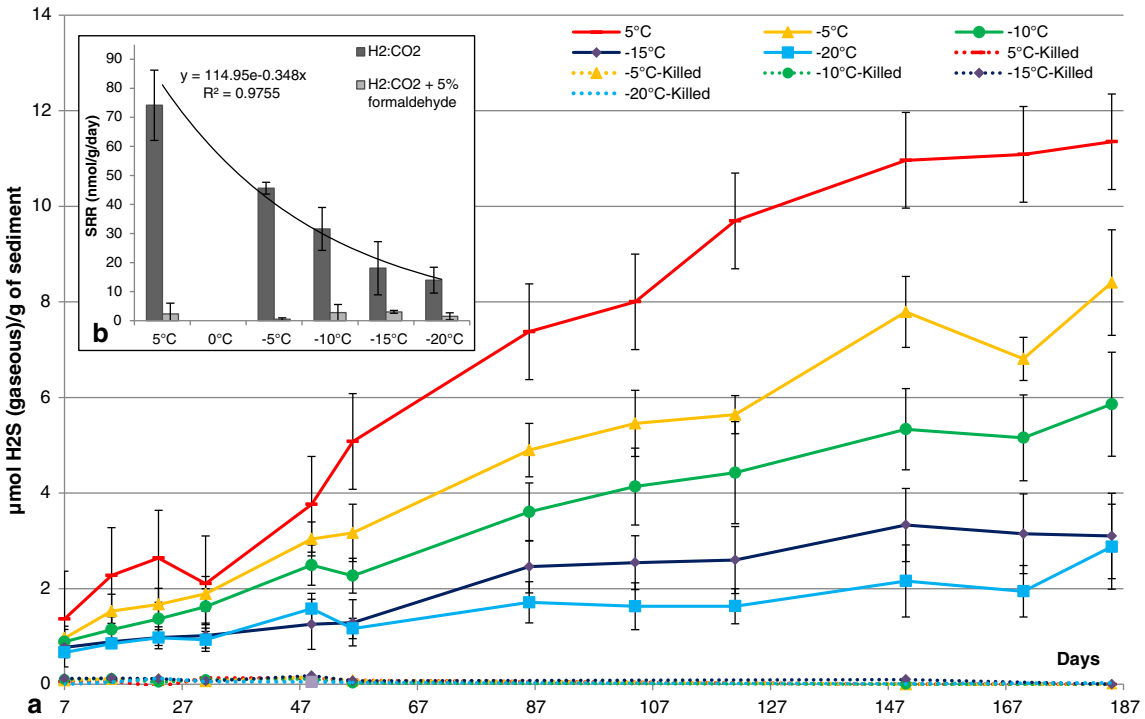

Fig. 1 Concentration of gaseous sulfide emitted from LH sediments incubated under a H_2:CO_2 atmosphere at different cold temperatures (**a**) and corresponding sulfide release rates (**b**). *Dashed lines* represent formaldehyde (5 %) killed controls (**a**). *Error bars* show standard deviations of biological triplicates

concentration) were performed at 5 °C under a H_2:CO_2 atmosphere and compared to parallel Na_2S•$9H_2O$-reduced samples to verify for potential abiotic SR. These results are provided as supplementary material.

Nucleic acid extraction, 454-pyrosequencing, and 16S rRNA operational taxonomic unit (OTU) analysis

For both April and July 2012 cores, DNA and RNA were extracted from ~2 g of sediments coming from 3 different depths: surface sediments (0–10 cmbs), shallow sediments (11–20 cmbs), and bottom sediments (21–30 cmbs). Extractions were performed using a RNA PowerSoil total-RNA isolation kit in combination with an RNA PowerSoil DNA elution accessory kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) with modifications described in the supplementary material. cDNA was synthesized using an iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and the provided random primers. The cDNA reactions were then purified using 0.5 mL Amicon tubes (Amicon 30 K, Millipore, Ireland). DNA and cDNA samples were sequenced at the Research and Testing Laboratory (Lubbock, TX, USA) using a Roche 454 GS FLX Titanium sequencer system (454 Life Sciences, Branford, CT, USA) with bacterial (28F, 5′GAGTTTGATCNTGGC TCAG3′; 519R, 5′GTNTTACNGCGGCKGCTG3′) (Handl et al. 2011) and archaeal (ARCH571F, 5′GCYTAAAGS RNCCGTAGC3′ (Baker et al. 2003); ARCH909R (also known as 890aR), 5′TTTCAGYCTTGCGRCCGTAC3′ (Burggraf et al. 1997)) primers. 16S rRNA sequences were processed using the Mothur platform v. 1.30.2 (Schloss et al. 2009) as described in Bell et al. (2013), which mostly followed the standard operating procedure outlined in Schloss et al. (2011); details on the Mothur commands and parameters used are described in the supplementary material. Sequences sharing >97 % identity were binned into OTUs. An OTU was considered to represent an active population if the relative percentage of reads present in its cDNA library was greater than those of its DNA library (i.e., if its cDNA:DNA ratio was greater than 1). The small amount of reads in most archaeal DNA libraries and some cDNA ones prevented and/or limited their analyses (see Table S4).

The original standard flowgram format (sff) files have been deposited in the NCBI Sequence Read Archive (SRA) database; the SRA submission was designated the Bioproject number PRJNA240206 and separated into two BioSamples (accession numbers SRS582754 and SRS585013 for the April and July samples, respectively), each assigned four experiments corresponding to the bacterial and archaeal 16S rRNA transcript (cDNA) and gene (DNA) libraries (of respective accession numbers SRX505245, SRX505712, SRX505717, and SRX505722 for the April samples, and SRX505728, SRX505740, SRX505747, and SRX505750 for the July ones).

Results

Assessment of cryophilic sulfate/sulfur reduction (SR)

Incubations under a H_2:CO_2 atmosphere resulted in H_2S releases down to −20 °C (Fig. 1). The incubation period of 187 days was too short to observe any leveling off of sulfide releases; although the sulfide levels appear to plateau in the 5 °C-incubated samples at ~150 days of incubation, later measurements (384 days) showed that sulfide concentrations in the headspace continued to increase and had almost tripled (31 μmol g^{-1} sediments). Towards the end of the incubation period, it was noted that H_2S levels in the formaldehyde-killed controls may have been underestimated. Formaldehyde, though commonly used as a killing agent for negative controls of microbiology studies, including those assaying SR (e.g., Zhang et al. (2009)), has been reported to react with H_2S to form a complex mixture of mercapto derivatives, and, therefore, should be avoided in future studies (Aleev et al. 2002; Rafikov et al. 1982). Even if there is a possibility that formaldehyde interaction with sulfide affected H_2S concentrations in the killed-control vials (Fig. 1), incubations of LH sediments at 5 °C under a N_2:CO_2 atmosphere (Table S3) without any killing agent failed to trigger any H_2S release supportive of the biogenicity of the observed hydrogen-dependent reactions.

Parallel incubations set up at 5 °C with an alternative reducing agent to sodium sulfide, cysteine-HCl, also indicated that the measured sulfide emissions did not originate from sodium sulfide degassing out of solution. Incubations of LH sediments using either reducing agent indeed resulted in almost identical SRR (Fig. S2). It should be noted, however, that the recorded rates in this separate experiment were significantly lower than those of the parallel 5 °C incubations (Fig. 1). As the control experiment with different reducing agents was set up several months following sample collection in the field, the marked decrease in rates between the 5 °C incubations depicted on Figs. S2 and 1 may be reflective of negative effects caused by prolonged sample storage before inoculation, such as oxygen contamination or nutrient depletion.

Methanogenesis and anaerobic oxidation of methane (AOM)

$^{14}CH_4$ experiments failed to unambiguously demonstrate signs of AOM after 2 years of incubation, regardless of the incubation temperature (i.e., −5, 0, or 5 °C); only very low

levels of $^{14}CO_2$ could be recovered and no significant difference was observed between formalin-treated (i.e., killed controls) and non-killed samples (data not shown). Similar results were also observed for the LH sediment samples incubated in microcosms under a N_2:CO_2:CH_4 atmosphere, where the levels of methane present in the vials' headspace remained unchanged throughout the course of the experiment. As it was hypothesized that AOM would be coupled to sulfate reduction, sulfide levels in the headspace were also monitored in conjunction with methane measurements; no sulfide could, however, be detected above background (data not shown). None of the different incubation set-ups summarized in Table S3 resulted in significant methane production from LH sediments throughout the course of the experiments and no significant differences in methane levels could be seen between non-killed samples and killed controls regardless of the methanogenic substrate utilized.

Sampling coverage of the 16S rRNA libraries

Sequencing results indicated that most likely only a small portion of the total LH communities was sampled and sequenced. This partial sampling coverage was reflected by the limited number of sequences in archaeal DNA libraries (i.e., too small sample size), the absence of plateaus for most bacterial rarefaction curves, as well as the relatively large differences between bacterial Chao1 estimators (reflecting the theoretical minimum of phylotypes in a sample) and observed bacterial richness (Fig. S3; Table S4). The incompleteness of the LH community profiles was further highlighted by the small portion of shared OTUs (>97 % sequence identity) between cDNA and DNA libraries, and the fact that more OTUs were present in the cDNA library in comparison to the DNA one (Fig. S4).

Overall, the discrepancies between DNA and cDNA libraries, as well as the incomplete community sequencing coverage for most samples, restrain the interpretations of the LH 2012 16S rRNA results to the most abundant taxa. Regarding the archaeal libraries, the lower abundance of archaeal cells over bacteria (Niederberger et al. 2010) and the poor quality of the nucleic acid extractions may have accounted for some of these discrepancies. Figure S3 and the coverage indices listed in Table S4 do not suggest that an increased sequencing depth on the archaeal samples would greatly increase community coverage (i.e., curves appear to be plateau despite incomplete sampling coverage).

Stability and phylogenetic composition of the site: bacterial communities

The bacterial community profile of the LH outlet sediments remained relatively stable at the phylum level, both seasonally and with depth, with *Chloroflexi*, *Proteobacteria* (mainly gamma and beta), *Synergistetes*, and some unclassified clades accounting for the main phyla identified. Some notable differences could nonetheless be seen between cDNA and DNA libraries, such as the higher relative abundance in cDNA than in DNA libraries of *Chloroflexi* taxa across all samples, and of *Synergistetes* in the July bottom as well as shallow (11–20 cmbs) samples. In contrast, *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria* were present in higher relative abundances in the DNA libraries than in their cDNA counterparts, as well as *Alphaproteobacteria* in the surface samples (Fig. 2).

The observation that less abundant clades may account for the most active members of the LH communities is further highlighted at the OTU level. A clear separation between DNA and cDNA libraries can be seen on Fig. 4, as well as by the fact that several (abundant) cDNA OTUs were completely absent from the DNA libraries (Fig. S4; Table 1). These differences in community structure between DNA and cDNA libraries suggest a clear distinction between background (dormant/dead cells) and potentially active (cDNA) communities, where LH most active populations may only account for a fraction of the total microbial biomass present on site, hinting at an active LH community that is distinct from the background DNA pool.

Taxonomic classification of putatively active (cDNA) bacterial communities

Despite being relatively stable at the phylum level, LH bacterial communities (cDNA) exhibited a higher degree of diversity at the OTU level. Though still compositionally similar, a decrease in diversity with sediment depth characterized the April libraries; in July, the LH sediment profile also exhibited the lowest biodiversity for the deepest sample, though species richness and evenness were highest in the shallow (11–20 cmbs) rather than the surface library (Fig. 3; Table S4). A striking feature, however, was the dominance of a single *Chloroflexi* OTU (light blue OTU bar in Fig. 3) among all cDNA samples, which accounted for at least 45 %, and up to more than 80 %, of total bacterial communities. This *Chloroflexi* OTU was related to the unclassified clade T78 of the *Anaerolineales* family based on Greengenes taxonomy. BLAST searches against the NCBI nt database revealed close resemblances to sequences isolated from anaerobic digesters, but the T78 LH OTU was only distantly related to cultured strains, having for closest representative the non-photosynthetic *Chloroflexi Longillinea arvoryzae* (Table 1) (Yamada et al. 2007).

Other major OTUs, of the phyla/classes *Synergistetes*, *Beta-* and *Deltaproteobacteria*, as well as some

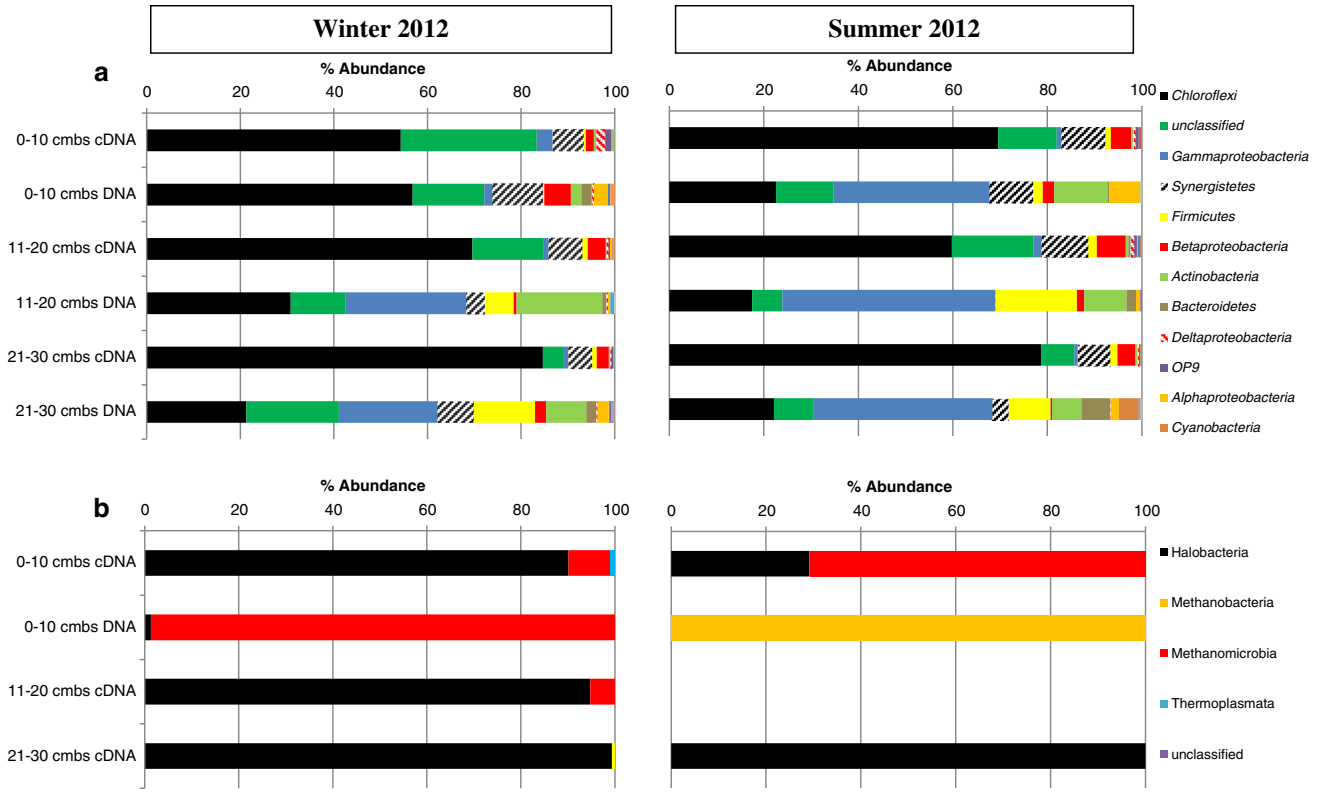

Fig. 2 Relative abundance of LH bacterial phyla and *Proteobacteria* classes (**a**) and archaeal classes (**b**) of the 16S rRNA gene (DNA) and transcript (cDNA) pyrosequencing libraries. Graphs on the *left* correspond to April 2012 samples and graphs on the *right* to July 2012 ones. In **a**, only the most numerous bacterial clades are referenced in the figure legend; *dashed bars* correspond to taxa containing close representatives to sulfur- and/or sulfate-reducing bacteria. In **b**, samples with too small numbers of reads were not shown (see Table S4)

unclassified ones present in the LH cDNA libraries, are summarized in Table 1. All closest BLAST hit sequences originated from environments bearing similar geophysical conditions to LH; most of the hits originated from anoxic environments almost all related to either hydrogen, methane, or other hydrocarbon-rich milieus (Table 1). Of special interest were some unclassified OTUs which, even though only distantly related, were closest to the halophilic, hydrogenotrophic, sulfate-reducing bacterial strain *Desulfohalobium retbaense* DSM 5692, isolated from a hypersaline lake (data not shown). Dominant cDNA OTUs also had 16S rRNA cDNA:DNA OTU ratios of more than 1 for most of the LH samples. Highest ratios were observed among the *Chloroflexi*, *Synergistetes*, and, especially, for a *Betaproteobacteria* OTU (Table 1). These elevated ratios indicate that the dominant LH phylotypes characterized in the cDNA libraries appeared relatively active under in situ conditions, both during winter and summer, and, throughout the 30 cm sediment layer.

LH archaeal communities

LH archaeal communities exhibited less diversity than bacterial ones, being comprised of a few different taxonomic classes, all of which belonging to the *Euryarchaeota* phylum. Aside from the July-surface sample, of which ~70 % was composed of *Methanomicrobia*, *Halobacteria* dominated all of the LH cDNA libraries, with an apparent increase in abundance with sediment depth (Fig. 2). In contrast, *Halobacteria* were either absent or only accounted for a small portion of the upper sediment DNA libraries, which were mainly comprised of either *Methanomicrobia* (April) or *Methanobacteria* (July) (Fig. 2b). It should be reiterated, however, that the 0–11 cmbs July libraries only contained a few reads and, therefore, that the taxonomic profiles depicted here are most likely incomplete. The differences between cDNA and DNA surface samples point toward active archaeal communities at the LH site comprised mainly of *Halobacteria*.

Table 1 Taxonomic information of the representative sequences of the two most abundant bacterial OTUs among all 16S rRNA cDNA libraries for the dominant clades of bacteria as well as the *Deltaproteobacteria*

Mothur classification (Greengenes database)								Information of closest BLASTn representative (sequences from uncultured strains)			
	Closest taxonomic ID[a]	cDNA:DNA ratios[b]						Isolation location	E-value	Identities (%)	Acc. Number
Chloroflexi	g: T78 (98)	0.8	**2.3**	**4.1**	**3.5**	**4.1**	**7.5**	Full-Scale Anaerobic Digesters	6 E-101	100	JQ117024.1
	g: WCHB1-05 (90)	nD	**1.1**	0.4	nD	**1.3**	0.1	Anaerobic sludge	4 E-98	99	CU924139.1
Unclassified	k: Bacteria (100)	**2.6**	**2.7**	0.7	0.9	**3.5**	0.9	Hydrogen production in a microbial electrolysis cell	2 E-100	100	GQ152935.1
	k: Bacteria (100)	0	0.7	0.1	nD	nD	nD	Anaerobic enrichment involved in microsystin-LR degradation	1 E-103	100	AB896655.1
Synergistetes	g: HA73 (100)	**6.8**	**3.3**	0.4	0.5	nD	nD	Full-scale Anaerobic Digesters	0.E + 00	99	AB780941.1
	g: *Aminobacterium* (100)	nD	0.3	nD	nD	nD	nD	*Aminobacterium colombiense* DSM 12261	0.E + 00	99	NR074624.1
Betaproteobacteria	g: *Ralstonia* (100)	0.5	**16.8**	0.6	**5.2**	nD	**7.8**	South China Sea	0.E + 00	98	GU940717.1
	g: *Pelomonas* (95)	0	nD	**1.8**	1	nD	nD	Soil around a coal gangue dump	0.E + 00	99	KF506873.1
Deltaproteobacteria	s: *Syntrophus* sp. (100)	nD	nD	0.1	nD	nD	nD	Anaerobic digester	4.E-154	100	JQ167107.1
	s: *Desulfovibrio aminophilu* (100)	0.2	nD	nD	**1.6**	nD	nD	Low temperature oil reservoir fluids	4.E-164	99	JQ256500.1

	Information of closest BLASTn representative (sequences from cultured strains)				
	Closest culture representative	Isolation location	E-value	Identities (%)	Acc. Number
Chloroflexi	*Longilinea arvoryzae*	Methanogenic propionate-degrading consortia	4 E-53	86	NR_041355.1
	Leptolinea tardivitalis	Methanogenic sludge granules	1 E-62	89	NR_040971.1
Unclassified	*Aminivibrio pyruvatiphilus*	Rice field soil	5.E-170	91	AB623229.1
	Bacteroidales Strain CF	Chloroform-Dechlorinating Enrichment Culture	2.E-70	90	CP006772.1
Synergistetes	*Syntrophomonadaceae* bacterium 11bR	Gangxi Oil Bed	4 E-82	95	GU129077.1
	Aminobacterium colombiense DSM 12261	Anaerobic dairy wastewater lagoon	7 E-105	100	NR_074624.1
Betaproteobacteria	*Ralstonia* sp. W7	Alpine-hydrocarbon-contaminated soil	0.E+00	98	KF560393.1
	Beta proteobacterium ASRB1	Phyllosphere of an arsenic-hyperaccumulating fern	0.E+00	99	AY612302.1
Deltaproteobacteria	*Syntrophus* sp.	Anaerobic consortia producing methane from long-chain alkanes	4.E-119	93	AJ133795.1
	Desulfovibrio sp. VKM B-2200	Anaerobic microbial community capable of degrading p-toluene sulphonate	2.E-156	99	FJ606758.1

nD: Refers to the lack of the corresponding OTU among all 16S rRNA DNA libraries (no DNA)

[a] The letters in front of the "Closest taxonomic ID" define the closest taxonomic rank assigned to each sequences according to Greengenes classification (s for species, g for genus, c for class, etc.). The number in parentheses defines the percentage bootstrap confidence level that the classified sequence matches the given taxonomic ID

[b] cDNA:DNA OTU ratios are reported, from left to right, for the April and July 2012 surface, shallow, and bottom 16S rRNA libraries, respectively; ratios higher than 1 are in bold

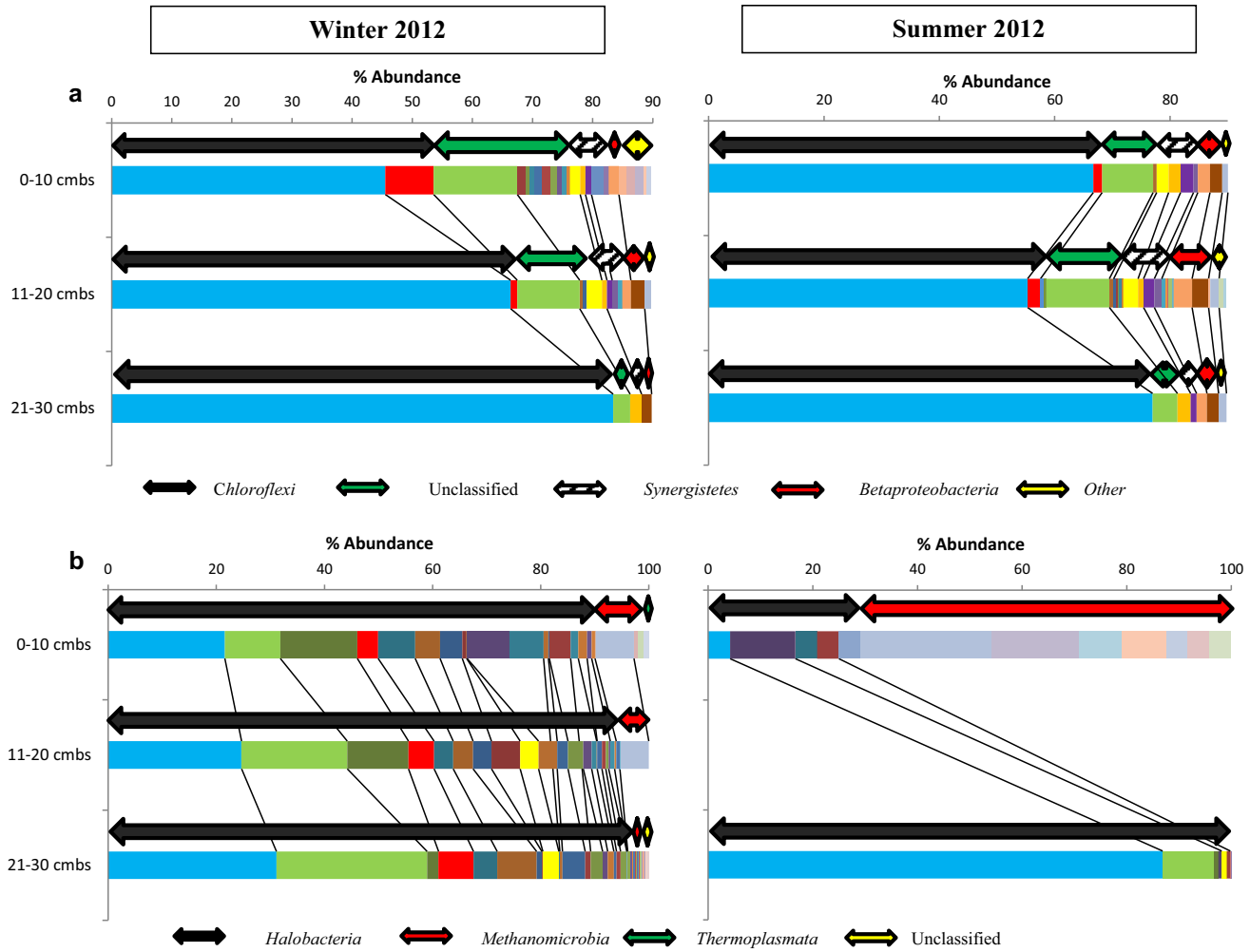

Fig. 3 Relative abundance of the archaeal (**b**) and top 90 % most abundant bacterial (**a**) OTUs (> 97 % sequence identity) in the LH 2012 pyrosequencing cDNA libraries. *Black lines* connect same OTUs present in different libraries of the same sampling season. *Colored arrows* represent the range of OTUs belonging to the corresponding phyla or class. *Graphs* on the *left* correspond to April 2012 samples and *graphs* on the *right* to July 2012 ones

Archaeal April communities were also more stable than bacterial ones at the species level (OTU level of 97 % similarities) and throughout the whole 30 cm LH sediment layers (Fig. 3b). A decrease in species richness occurred between the late winter and summer 21–30 cmbs cDNA samples, with a marked increase in relative abundance of the major *Halobacteria* OTU present in both samples (Fig. 3b). Differences in community structure are further highlighted in Fig. 4b where April cDNA samples clearly form an outgroup distinct from the July cDNA samples and April DNA ones.

The major *Halobacteria* OTUs were associated to cultured representatives of *Haloquadratum*, *Halorubrum*, *Halobellus*, and *Halobacterium* from various hypersaline environments (Table 2). The main *Methanobacteria* OTU present in LH cDNA archaeal libraries was related to a *Methanosaeta* methanogen isolated from a hydrocarbon-contaminated aquifer, consistent with the high levels of hydrocarbons at the LH site. However, the cDNA:DNA ratio of less than 1 for the *Methanosaeta* OTUs of the surface April samples indicates that, even though *Methanosaeta* populations accounted for a major component of the total archaeal LH community, they might not be active at the LH site, unlike their *Halobacteria* counterparts (Table 2).

Discussion

Biogenic sulfidogenesis from LH sediments

The recorded hydrogen-dependent H_2S releases from LH sediments in microcosm incubations (Fig. 1) so far account for the best indication that at least some LH populations are

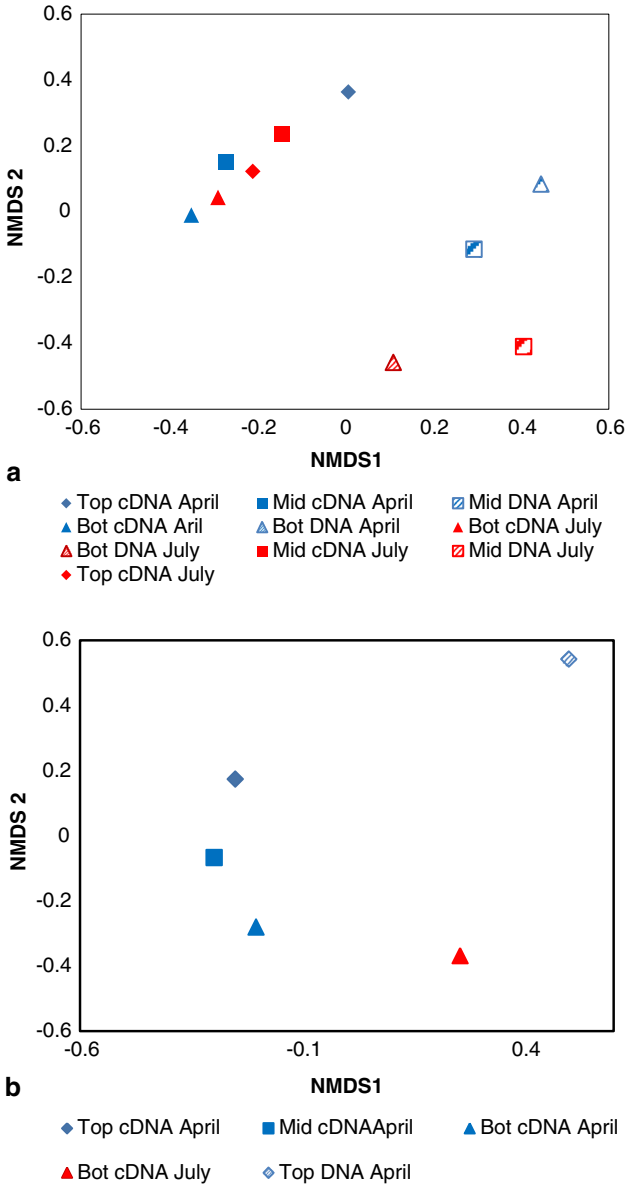

Fig. 4 NMDS ordinations of Bray–Curtis community dissimilarity of bacterial (**a**) and archaeal (**b**) LH 16S rRNA libraries

metabolizing under natural subzero temperatures and hypersaline and hydrogen-rich (~1 % of total venting gases) conditions at the LH site, potentially accounting for at least part of the sulfide detected in situ (Table S1). The exact nature of this reaction could not be confirmed but most probably involves the dissimilatory reduction of some sulfur compound, such as (thio) sulfate or sulfur reduction.

The very high concentrations of sulfate present in the LH spring water and sediments (~0.05–1 M), however, make sulfate reduction the most likely metabolism detected in the present study (Fig. 1) (Niederberger et al. 2010). The presence of sequences distantly related to hydrogenotrophic and halophilic sulfate–reducing bacteria (SRB) in the 2012 cDNA libraries is also indicative of SRB populations being active at the LH site, and supports the sulfidogenic incubation experiments performed on LH sediments (Fig. 1). A metagenomic investigation of the spring sediments performed by Lay et al. (2013) also found sequences related to the 16S rRNA gene of *Desulfovibrionales*, as well as the sulfite reductase gene involved in dissimilatory sulfate reduction. Furthermore, sulfate reduction occurring above salinities of about 15 % is normally restricted to autotrophic and hydrogenotrophic processes as other types of sulfate reduction, such as those based on acetate oxidation, are thought to yield too little energy to support halophilic metabolism (Oren 2011); consistently, incubations of LH sediments with acetate and under a N_2:CO_2 atmosphere failed to evolve any detectable sulfide.

Sulfide releases from LH sediments could also be attributed to sulfur- and/or thiosulfate-reducing microorganisms. Though present in lower concentrations than sulfate, elemental sulfur has been detected at LH and could potentially serve as a terminal electron acceptor for sulfur-reducing microorganisms (Battler et al. 2013). The phylum *Synergistetes* was more abundant than the *Deltaproteobacteria* and represented the third most abundant phylum among the 2012 cDNA 16S rRNA libraries, essentially composed of phylotypes of the family *Dethiosulfovibrionaceae* (Figs. 2, 3; Table 1). Several members of the *Dethiosulfovibrionaceae* are known sulfur reducers and have also been found in hydrocarbon-rich, hypersaline, or cold sea sediments (Godon et al. 2005; Magot et al. 2000, 1997; Sorokin and Muyzer 2010; Surkov et al. 2001). Lastly, sulfide production from LH sediments could, at least partly, be derived from *Halobacteria* activity. Although *Halobacteria* typically grows aerobically, some strains are capable of anaerobic growth via nitrate, DMSO, or TMAO reduction, as well as arginine fermentation, and have been reported to produce sulfide from cysteine or thiosulfate metabolism (Cui et al. 2007; Hezayen et al. 2002; Oren et al. 1997; Ozcan et al. 2006; Tomlinson et al. 1986). It should be noted, however, that incubation of LH sediments with cysteine-HCl—added as an alternative reducing agent to sodium sulfide—did not result in increased SRR from LH sediments (Fig. S2) and, therefore, that cysteine metabolism most likely did not significantly account for the observed H_2S production in LH sediments.

To the best of our knowledge, microbial sulfate reduction—or other sulfidogenic metabolism—has never been reported below ~−4 °C (Tarpgaard et al. 2006). As such, the temperatures permissive of SR in LH sediments (i.e., at least down to −20 °C) represent the coldest temperatures ever recorded for SR. Regarding sulfide release rates (SRR), the SRR recorded for the LH sediment consortia incubated between −20 and 5 °C were comparable to those measured in other cold environments such as permanently

Table 2 Taxonomic information of the representative sequences of the ten most abundant archaeal OTUs among all 16S rRNA cDNA libraries

Mothur classification (Greengenes database) Closest taxonomic ID[a]	Information of closest BLASTn representative (sequences from uncultured strains)			
	Isolation location	E-value	Identities (%)	Acc. Number
g: *Haloquadratum* (100)	Solar saltern 'Bras del Port'	0	99	HQ455543.1
g: *Halorubrum* (54)	Solar saltern 'Bras del Port'	0	99	HQ455545.1
g: *Halorubrum* (100)	Aran-Bidgol Salt Lake (Hypersaline Playa in Iran)	0	99	HQ425168.1
g: *Halorubrum* (100)	Aquatic environments of the high altitude Andean Altiplano (northern Chile)	2E-133	99	EF632687.1
Unclassified	Great salt plains of Oklahoma	7E-138	96	FJ696261.1
g: *Halorubrum* (89)	Hypersaline lake	2E-157	96	JQ033974.1
g: *Methanosaeta* (100)	Hydrocarbon-contaminated aquifer	0	99	JQ087754.1
g: *Halorubrum* (100)	Yuncheng salt lake, Shanxi, China	0	97	JN216861.1
g: *Halobacterium* (100)	Salterns of Sfax Tunisia	0	98	JX982770.1
Unclassified	Hypersaline Environments	1E-111	98	JN839744.1
Information of closest BLASTn representative (sequences from cultured strains)				
Closest culture representative	Isolation location	E-value	Identities (%)	Acc. Number
Haloquadratum walsbyi	Solar saltern	0	99	NR_074200.1
Halorubrum sp. DV427	Ancient halite, Death Valley California	5E-149	91	FJ492047.1
Haloarchaeon CSW1.15.5	Magnesium-rich bittern brine from a Tunisian solar saltern	0	99	FN994962.1
Halorubrum arcis strain AJ201	Saline lake on the Qinghai-Tibet Plateau, China	2E-123	97	NR_028226.1
Halobellus salinus	Marine solar salterns	3E-131	95	HQ451075.1
Halorubrum aquaticum	Hypersaline lakes	5E-139	93	AM268115.1
Methanosaeta concilii GP6	Anaerobic sludge–municipal sewage treatment plant	0	99	NR_102903.1
Halorubrum sp. YC-X2	Yuncheng salt lake, Shanxi, China	0	97	JN216861.1
Halobacterium salinarum strain ETD5	Salterns of Sfax Tunisia	0	98	JX982770.1
Halobellus salinus	Marine solar salterns	2E-103	96	HQ451075.1

N.B. All OTUs were absent from all DNA libraries except for the *Methanosaeta* OTU which was present in both cDNA and DNA April surface samples, and had a cDNA:DNA ratio of 0.1

[a] The letters in front of the "Closest taxonomic ID" define the closest taxonomic ranks assigned to each sequences according to Greengenes classification (s for species, g for genus, c for class, etc.). The number in parentheses defines the percentage bootstrap confidence level that the classified sequence matches the given taxonomic ID

cold Arctic sea sediments incubated at 0 °C, or Atlantic coastal sediments incubated at 3 °C (e.g., Robador et al. (2009); Westrich and Berner (1988)). The relatively high SRR at subzero temperatures from LH samples are suggestive of cold-adapted SR populations, and reflect the metabolic plasticity of LH cryophilic SR members to cold (and elevated salinity), with temperature unlikely to be a limiting factor for activity in the perennially subzero LH system.

Spatial and temporal stability of LH microbial communities

Variations in community structure between the different LH samples, if any, were anticipated to be relatively small considering the rather stable geochemical conditions of the perennially subzero and anoxic sample site (Lay et al. 2013; Niederberger et al. 2010). As such, the overall similarities between the 2012 April and July cDNA libraries were to be expected, and similar clades of microorganisms did populate all of the different samples based on 16S rRNA (cDNA) sequencing (Fig. 2), often down to the species level (>97 % sequence similarity), especially in the archaeal April samples (Fig. 3).

Differences were nonetheless observed at the species level among bacterial libraries. Such differences were most notable for the April 0–10 cmbs and July 11–20 cmbs cDNA samples, which exhibited higher richness than the other libraries, with a higher degree of OTUs unique to these samples (Fig. 3). This phylogenetic profile of the LH sediment samples also suggests that LH communities may share functional similarities across the top 30 cm of

sediments, but that niche specialization with depth may account for more subtle genetic differences between related populations (e.g., regarding O_2 sensitivity/requirement). Though site patchiness could also account for these differences (i.e., only one core was collected during each sampling seasons), it could be hypothesized, for example, that the higher biodiversity observed in the April surface, and July shallow, sediment-layer bacterial communities (Table S4; Fig. 3) may relate to the differences in water levels and air exposure between winter and summer months at LH. Emptying of the spring dome during the summer months (where surface sediments become in closer proximity with the atmosphere) may, for example, shift down some LH populations to deeper layers in response to higher concentrations of air gases (e.g., O_2) at the spring surface.

LH "active" populations: Inference of metabolism from taxonomical composition of sediment communities

While the use of 16S rRNA as a microbial activity proxy has limitations [see Blazewicz et al. (2013)], it can still be informative in identifying potentially active members of a community. The apparent differences observed between cDNA (i.e., transcript) and DNA (i.e., gene) libraries support the idea that OTUs identified in the cDNA samples truly represent active community members. These pyrosequencing results suggest that the active communities populating the LH sediments are composed partly of rare populations that are absent from the DNA libraries (but present in the cDNA ones). Such trends have also been reported in other marine and hypersaline environments where no clear correlation was observed between cDNA and DNA libraries, with an over-representation of rare DNA taxa in the cDNA samples (e.g., Campbell et al. 2011; Campbell and Kirchman 2013). The small OTU overlap between DNA and cDNA libraries further highlights the probable high proportion of allochtonous cells present in the recovered sample, where DNA signatures of active populations were potentially "diluted out" by high amounts of dead or dormant cells buried within the LH sediments. The presence of putatively dormant populations among DNA samples was also implied at the phylotype level. The relatively high abundance of *Firmicutes*, *Bacteroidetes* and *Actinobacteria* sequences in DNA libraries, and their virtual absence from the cDNA ones, agrees with them not belonging to active LH members (Fig. 2) as such phyla are known to contain a high proportion of spore–former representatives, and are also common to several permafrost environments on Earth (Steven et al. 2009).

The present 16S rRNA profiles of LH sediments somewhat contrasted with those of previous investigations. No archaeal sequences related to *Thaumarchaeota*, or the anaerobic methane oxidizing clade ANME-1, were detected in the 2012 datasets (Lay et al. 2013; Niederberger et al. 2010). Furthermore, where bacterial and archaeal clades normally associated with saline (e.g., *Marinobacter* sp.) or methane-rich (i.e., ANME-1 archaea) environments, respectively, comprised the 2006 LH 16S rRNA clone library survey (Niederberger et al. 2010), cDNA sequencing here revealed archaeal communities dominated by halophiles (Fig. 2b; Table 2) and bacterial ones mostly related to clades either directly or indirectly involved in hydrocarbon and/or hydrogen metabolism (Table 1). The most abundant cDNA OTU, for example, most closely associated with the *Chloroflexi* group T78. T78 populations have been reported to dominate methane-rich Santa Barbara basin sediments underlying sulfate–methane transition zones where ANME-1-mediated AOM was also detected (Harrison et al. 2009); these clades were closely related to other T78 members which dominated Mediterranean deep-sea organic-rich (sapropel) sediments (Coolen et al. 2002). In addition to putative SR LH hydrogenotrophs, the importance of H_2 as an electron source at LH was further illustrated by the high proportion in cDNA libraries of LH phylotypes related to known hydrogen oxidizers (Table 1).

Most cultivated *Synergistes* strains are known to degrade amino acids and *Synergistes* functions in natural settings may center around amino acids turnover (Godon et al. 2005). The exact ecological role of *Synergistes* in anaerobic consortia, however, remains speculative yet has been proposed to relate to hydrogenotrophic thiosulfate or elemental sulfur reduction or to syntrophic associations with hydrogenotrophic methanogens (Godon et al. 2005). Similar methanogen syntrophic associations are also known to occur with *Syntrophus* species; *Syntrophus*–related sequences accounted for the main *Deltaproteobacteria* OTU in the present survey (Table 1) and members of the family *Syntrophaceae* were recently shown to be involved in the degradation of hexadecane via probable syntrophic interactions with hydrogenotrophic methanogens (Cheng et al. 2013; Embree et al. 2013).

The relatively high abundance of bacterial clades related to known hydrocarbon degraders, or syntrophic partners of known hydrocarbon degraders, suggests that hydrocarbon degradation may play an important role in fueling the LH microbial consortia. In addition to methane, short-chain alkanes (ethane, propane, butane) are present on site (Niederberger et al. 2010). Although less documented than anaerobic methane oxidation, anaerobic oxidation of short-chain alkanes has recently been confirmed to play an important role in both carbon and sulfur cycling, often occurring under sulfate-reducing conditions (Adams et al. 2013; Sassen et al. 2004). Even though short-chain alkane oxidation has so far mainly been documented under

mesophilic and thermophilic conditions, it is possible that LH alkanes (other than methane) may represent an unaccounted source of organic carbon in the previously characterized oligotrophic LH spring (Niederberger et al. 2010).

Sulfur cycling, not restricted to sulfidogenic processes, also most likely plays a central role among LH microbial communities, as highlighted by previous research on the site and considering the high concentrations of sulfur compounds present in situ (Lay et al. 2013). The relatively high proportion of *Chloroflexi* and *Betaproteobacteria* sequences among the different LH samples is consistent with 16S rRNA surveys of other sulfur-rich cryoenvironments. For example, *Chloroflexi* accounted for a major portion of the soil microbial community in an alpine-tundra-wet-meadow soil and appeared active at near 0 °C temperatures under likely anoxic conditions in a sulfate-rich environment (Costello and Schmidt 2006). Even though the ecological function of these uncultured *Chloroflexi* could not be ascertain, the authors suggested a likely involvement in sulfide oxidation. The most abundant and apparently active LH *Betaproteobacteria* OTU closely matched members of the *Ralstonia* genus (Table 2). Despite the fact that *Ralstonia* species are typically associated with oxic environments, some *Ralstonia* strains, such as *Ralstonia eutropha* H16, are capable of hydrogenotrophic lithoautotrophic denitrifying metabolism under anoxic conditions (Pohlmann et al. 2006); *R. eutropha* H16 has also recently been shown to possess sulfur oxidation (*sox*) genes (Cramm 2009). For example, *Ralstonia* phylotypes dominated a 16S rRNA clone libraries of a supraglacial sulfur spring in the Canadian high Arctic and indicated a possible role in sulfur cycling at this site (Gleeson et al. 2011).

Conclusion

16S rRNA-based analyses, in concert with sulfide production assays, portrayed the LH spring as a biologically active and relatively stable environment, hinting at which metabolisms can support anaerobic life under combined hypersaline and subzero-temperature conditions. Incubation experiments indicated that the reduction of sulfur compounds (SR) with hydrogen, most likely sulfate reduction, can be carried out by cryophilic and halophilic clades of LH microorganisms both in situ, as well as at much lower temperatures. Of particular interest would be to confirm the autotrophy of the putative LH sulfur/sulfate reducers considering the potential for hydrocarbon metabolism at LH. Finally, the recorded SR at −20 °C brings down the permissive conditions for this microbial metabolism to new temperature records, and raises the possibility that it plays, or had played, important roles in other terrestrial cryoenvironments, including during previous Snowball Earth events; evidence suggests that sulfate reduction played an important role in overall biogeochemical cycling during these time periods (Hurtgen et al. 2002). The combined anaerobic, chemolithotrophic, cryophilic, and halophilic nature of the detected putative SR also is relevant for future space-exploratory missions that intend to look for signs of life on Mars, Europa, and Enceladus, all showing evidence for both past and present liquid brines on their surface (Des Marais et al. 2008). Recent discoveries of sulfate deposits on Mars, and possibly in ice brines on the moon Europa, provide evidence that this oxidant is available on these planetary bodies, and further the importance of sulfate reduction as a possible anaerobic metabolism for potential extraterrestrial microorganisms (Gendrin et al. 2005; McCord et al. 2001).

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