Assessing anaerobic activity in a perennial subzero hypersaline spring of the high Arctic: Focus on methanogenesis, anaerobic oxidation of methane, and sulphur reduction

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Abstract/Résumé

The Lost Hammer (LH) spring in the Canadian high Arctic perennially discharges subzero (-5°C) hypersaline (24% salt) brines through thick layers of permafrost (> 500 m), and so far accounts for the only described terrestrial methane seep in frozen settings on Earth. The present thesis aimed to ascertain that actively metabolising, indigenous, microbial communities do populate the sediments of the LH spring outlet despite the extreme conditions *in situ*. Incubation experiments with LH sediments could not confirm that microbial consortia undergo anaerobic methane metabolism but revealed that the reduction of sulphur compounds (SR) with hydrogen, most likely hydrogenotrophic sulphate reduction, was potentially carried out by some cryophilic populations under combined hypersaline and subzero (down to -20°C) conditions. Unusual H_2S releases from LH sediments were also detected at high temperatures (80°C); the biogenicity of these releases could however not be confirmed and could alternatively reflect abiotic processes. Pyrosequencing analyses of both 16S rRNA (i.e. cDNA) and 16S rRNA genes (i.e. DNA) on 30 cm layers of LH sediments retrieved in April 2012 and July 2012 indicated fairly stable bacterial and archaeal communities at the phylum level, but a greater bacterial diversity at the species level (> 97% sequence similarities). The composition of the LH communities however differed significantly from previous surveys of the site, either reflecting site's heterogeneity and/or differences in sequencing coverage. Potentially active bacterial and archaeal communities were respectively dominated by clades related to the T78 *Chloroflexi* group and *Halobacteria* species, as indicated by 16S rRNA results; no sequence related to ANME-1 archaea were detected unlike in previous investigations of the site. The present study indicated that SR, hydrogenotrophy (possibly coupled to autotrophy), and hydrocarbon degradation (other than methane), most likely account for important metabolic processes carried out by LH microbial communities. Overall, the obtained findings provided additional evidence that the LH system host active communities of anaerobic, halophilic, and cryophilic microorganisms despite the extreme conditions *in situ*.

La source d'eau Lost Hammer (LH), située dans l'extrême arctique canadien, déverse des eaux hypersalines (salinité de 24 %) et froides, ayant une température constante avoisinant les -5°C, à travers d'épaisses couches de pergélisol (> 500 m). LH est considérée comme le seul suintement terrestre de méthane documenté à ce jour se situant en milieu continuellement gelé sur Terre. Cette thèse visait à déterminer si les communautés microbiennes indigènes aux sédiments de la source LH sont métaboliquement actives *in situ*, malgré les conditions extrêmes de la source. Des expériences d'incubations de sédiments de LH n'ont pu confirmer que les consortia microbiens métabolisent du méthane de façon anaérobique, mais ont révélé que des populations cryophiles sont probablement capables de réduire des composés de soufre, probablement la réduction de sulfate, sous des conditions hypersalines et jusqu'à -20°C. Des échappements de H_2S des sédiments ont aussi été détectés à haute température (80°C); l'authenticité biologique de ces échappements nécessite d'être confirmée et pourrait alternativement refléter des processus chimiques abiotiques. Des analyses de pyroséquençage du 16S ARNr (ADNc) et du gène du 16S ARNr (ADN) sur des couches de 30 cm de sédiments collectés en avril 2012 et juillet 2012 ont indiqué que les communautés d'archées et de bactéries de LH sont assez stables au niveau du phylum, mais que la diversité entre les communautés de bactéries est plus variable au niveau de l'espèce (similarité des séquences > 97 %). La composition des communautés de LH différait par contre significativement de celle décrite lors d'études antérieures du site, reflétant possiblement une hétérogénéité du site, ou des différences de couverture de séquençage. Les résultats de pyroséquençage du 16S ARNr ont indiqué que les communautés de LH de bactéries et d'archées potentiellement actives étaient dominées respectivement par des clades reliés au groupe T78 des *Chloroflexi* et à des espèces de *Halobacteria*; aucune séquence reliée aux archées ANME-1 ne fut détectée contrairement à ce qui fut observé lors d'investigations précédentes du site. La présente recherche a indiqué que la réduction de composés de soufre, l'hydrogénotrophie (possiblement couplée à l'autotrophie), et la dégradation d'hydrocarbures (autres que le méthane) sont probablement d'importants processus métaboliques chez les communautés microbiennes de LH. Dans l'ensemble, les résultats obtenus ont fourni des évidences additionnelles que la source LH abrite des microorganismes anaérobiques, halophiles, et cryophiles actifs, malgré les conditions *in situ* extrêmes.

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Contribution of authors

Sections 2.2.2 to 2.2.5, as well as 4.3, correspond to relevant sections written by myself of the studies:

- "Defining the Functional Potential and Active Community Members of a Sediment Microbial Community in a High-Arctic Hypersaline Subzero Spring**"** published in Applied and Environmental Microbiology, April 2013. **79**(12): 3637-3648.
- "Left Out in the Cold: Life in Cryoenvironments" published in Polyextremophiles, 2013, **79**: 335-363.

Part of the borrowed text was slightly modified in order to improve its flow and to better connect the different sections of the present thesis. I also acknowledge that Jacqueline Goordial and Dr. Lyle Whyte contributed to critical editing of the entire thesis.

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Nomenclature and abbreviations

16S rRNA gene: Gene coding for the ribosomal small subunit RNA; the 16S rRNA is used a phylogenetic marker.

AOM: Anaerobic oxidation of methane.

Astrobiology: The study of the origin, evolution, and distribution of life in the universe.

cDNA: complementary DNA; DNA that have been generated by the reverse transcription of a RNA molecule.

Chemolithoautotroph: A microorganism that obtains energy through chemical oxidation (chemo), and uses inorganic compounds as an electron (litho) and carbon (auto) sources.

cmbs: cm below the sediment surface.

Cryoenvironment: Environments which temperature is continuously below 0°C, or which is continuously surrounded by frozen settings.

Cryomicrobiology: The study of cryophiles.

Cryophile: A cold-adapted microorganism capable of growth below 0°C, regardless of its optimum growth temperature.

Cryosphere: Portion of the biosphere that lives in cryoenvironments.

Halophile: A microorganism capable of metabolizing under hypersaline conditions.

OTU: Operational taxonomic unit; a classification of DNA sequences that have been binned as unique taxonomic units based on similarity/dissimilarity to other sequences.

Psychrophile: The term psychrophile is mostly used in the literature to describe cold-adapted microorganisms that have growth optima below 20°C; some, but not all, psychrophiles can grow below 0°C.

SR: Sulphur reduction; (bio)chemical reduction of a sulphur compound (e.g. sulphate reduction).

SRB: Sulphate reducing bacteria.

SRM: Sulphur reducing microorganism; microorganisms undergoing the reduction of a sulphur compound (e.g. sulphate) to sulphide (e.g. SRB).

SRR: Sulphide release rates; the rates of sulphide (H_2S) production resulting from SR.

Sulphidogenesis: The production of sulphides, either chemical or biological.

Chapter 1: Introduction

1.1 Extremophile research and the cryosphere

The environmental conditions that define the habitability window of life on Earth are continuously being reassessed, with the discovery of organisms or communities living in natural settings previously considered hostile to biology. The label "extremophile" has been applied to (those) microorganisms that inhabit environments bearing conditions that fall outside of the range traditionally considered normal by anthropomorphic standards. Extremophile research has not only expanded our views regarding terrestrial habitability, but also of potential habitats, either past or present, that exist elsewhere in our solar system. Of special interest is the study of the Earth's cryosphere, this portion of the biosphere that encompasses living microorganisms inhabiting constantly cold or frozen places (i.e. cryoenvironments), which only recently has been recognised to comprise biologically active ecosystems. This emergent view of an active cryosphere has global repercussions on environmental fluxes and cycles considering the widespread 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. Considering that most current astrobiology targets (e.g. Mars and the moons Europa and Enceladus) experience very cold temperatures, the study of extremophilic microbial communities autochthonous to cryoenvironments also bears high relevance in the search for signs of life outside the Earth.

The present research centered on the study of the microbiology of the Lost Hammer (LH) spring, a perennially subzero hypersaline spring discharging through ~ 500-600 m of permafrost in the Canadian high Arctic. Given the extreme geochemical conditions of the site (cold, hypersaline, anoxic, etc.), the investigation aimed at assessing whether indigenous and active microbial communities populate the anoxic LH sediments, with a focus on anaerobic microbial clades expected to metabolize the abundant methane and sulphate present on site (Niederberger et al. 2010).

1.2 The Lost Hammer spring system

Manipulations were performed on sediment and water samples of the permafrost-associated Lost Hammer (LH) spring of the Canadian high Arctic. It is located in a valley off the shores of Strand Fjord on Axel Heiberg Island (AHI) in a region of diapiric uplift (79°07'N, 90°21'W). No continuous meteorological data is available for the LH site; atmospheric data for the nearby (79°24′N, 90°45′W) McGill Arctic Research Station (MARS) however indicates that regions in the vicinity of MARS can be considered as polar deserts where evaporations exceed precipitations (Andersen et al. 2002) and with annual mean atmospheric temperatures of ~ -15°C, with a maximum of 25°C recorded in July 2009, and minima of -50°C (Wilhelm et al. 2011). The LH spring is sometimes referred to as the "Wolfe spring" considering it being part of the Wolfe diapir (Battler et al. 2013); for consistency with previous microbiology work on the site however, the present document will still refer to it as the "Lost Hammer spring".

The LH outlet is characterised by a cone-shaped salt tufa of ~ 2 m in height and 3 m in diameter (Figure 1.1). The spring discharges perennially subzero (~ -5°C), nearly anoxic, reducing brines very rich in sulphate (0.05 M and 1.04 M for the brine and underlying sediments respectively); some of the brine geochemical characteristics are summarised in Table 1.1. In addition to the perennial brine, the LH spring also constantly discharges gases that are mainly comprised of methane (CH_4; ~50%), nitrogen (N_2; ~35%), and carbon dioxide (CO_2; ~ 10%), as well as smaller amounts of hydrogen, helium, and heavier hydrocarbons (i.e. ethane, propane, butane, etc.) (Niederberger et al. 2010). Previous investigations of the LH site have indicated that the spring geochemistry is fairly stable over the years and sampling seasons, with little observed changes in water chemistry and gas discharges (Lay et al. 2012; Niederberger et al. 2010). The water level of the spring outlet is however known to 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 (Figure 1.1).

The subzero temperature of the LH brine results from the cooling down of the spring water by the surrounding permafrost as it travels upward to the spring surface, previously heated-up at depth by geothermal warming . The origin of the spring water is not known but

is thought to differ from those of other permafrost springs present on the island, which have been hypothesised to originate from subglacial lake-water and glacial melt (Andersen et al. 2002; Heldmann et al. 2005; Pollard et al. 1999). A glacial or lacustrine origin is not thought to apply to the LH site which lacks glaciers and large sub-glacial lakes in its proximity (Battler et al. 2013).

Previous microbiology investigations of LH sediments revealed that they contained viable microbial populations. Biomineralization assays with ^{14}C-labelled glucose showed that LH communities were capable of aerobic respiration down to -10°C, although this assay revealed very little activity compared to samples from other Arctic sites (Steven et al. 2007b). Salt-tolerant bacteria capable of growing at -5°C were also isolated from the spring's sediments (Niederberger et al. 2010). Stable isotopic and compositional analyses of exsolving LH gases pointed at a thermogenic (as opposed to methanogenic) origin for the venting methane, characterised by a relatively heavy methane isotopic signature, and alkane composition and stable isotope signatures typical of thermogenic processes (Niederberger et al. 2010). Microscopy (i.e. CARD-FISH) and 16S rRNA analyses of LH sediments detected DNA and RNA sequences related to a clade of anaerobic methane-oxidizing archaea, the ANME-1 clade, suggesting anaerobic methane oxidation *in situ* (Niederberger et al. 2010). More recent metagenomic and 16S rRNA pyrosequencing analyses of the site revealed, among others, the presence of methanogen sequences and suggested the important role of sulphur cycling in driving the metabolism of the spring communities (Lay et al. 2013).

Research on brine channels outflowing from the LH tufa indicated a change in microbial community composition and structure with respect to the spring outlet sediments (Lay et al. 2012). Notable distinctions included greater microbial biomass and diversity in the channel sediments, as well as higher aerobic activity as assessed by radiolabelled substrate experiments. Similar to studies on the LH outlet communities, LH outflow channels were dominated by bacterial populations over archaeal ones; *Bacteroidetes* and *Thaumarchaeota* accounted for the most abundant bacterial and archaeal clades respectively (Lay et al. 2012).

Figure 1.1 The Lost Hammer (LH) spring outlet in April (left) and July (right) 2012.

Table 1.1 Geochemical parameters of the LH spring water and microbial counts of the spring sediments

Sampling year	**Jul-05**	**Jul-06**	**Apr-07**	**Apr-08**	**Jul-11**	**Apr-12**	**Jul-12**
Temperature (°C)	-4.8	-4.9	-4.7	-5.9	-3.13	-7.7	-4.8
pH	6.3	5.96	6.37	7.38	5.95	6.77	ND
D.O. (p.p.m.)	0.2	0.1	ND	1	0.117	1.12	0.04
S^{2-} (p.p.m.)	25-50	0-20	20-50	20-30	<20	0.017	0.57
ORP (mV)	-154	-187.4	-159	-171.8	-223	-224	-165
TDS (gL^{-1})	ND	241.72	175	176	171	203.7	ND
Salinity (%)	22-23	26	22.4	22.1	22.3	26	24
Total cell counts	$4.3\pm0.23X10^5$	$5.5\pm0.41X10^5$	ND	ND	ND	$3.14 \pm 1.82 x10^9$	ND

Abbreviations: D.O., dissolved oxygen; N.D. not determined; O.R.P. oxido-reduction potential; TDS, total dissolved solids
Data reported before 2011 was taken from Niederberger et al. 2010.
Details regarding 2011-2012 data are described in section 2.1.

1.3 Research objectives

The cold saline springs that flow on the surface of Axel Heiberg Island (AHI) in the Canadian high Arctic represent great natural laboratories to study the combined effects of cold temperatures and elevated salinities on microbial populations. The present research built on previous investigations on the coldest of these saline springs, the Lost Hammer (LH) spring, which hinted at the existence of an autochthonous microbial community populating the spring outlet sediments (Niederberger et al. 2010, Steven et al. 2007, Wilhelm 2009). The general goal of the present study aimed at ascertaining that actively

metabolizing populations are present *in situ* despite the extreme conditions of the LH site (i.e. combined subzero temperatures, hypersalinity, and anoxia). A special focus was put on specific clades of anaerobic microorganisms either previously detected (i.e. the archaeal anaerobic-methane-oxidizers ANME-1), or expected to be capable of chemolithotrophic metabolisms (e.g. hydrogenotrophic sulphate reduction). A secondary objective consisted of assaying the stability of the LH sediment communities, again focusing on potentially active members. The LH outlet sediments had never been studied under late winter conditions when the salt tufa is completely filled with spring water, and potential variations in community structures were hypothesised to occur seasonally, especially regarding the surface-sediment populations, which may be affected by the recurrent filling and emptying of the LH salt tufa.

More specifically, the research objectives consisted of:

i) Ascertaining if the large pools of thermogenic methane and sulphate salts at LH could fuel microbially mediated anaerobic oxidation of methane (AOM) under *in situ* conditions.

ii) Enriching for potential halophilic and cryophilic methanogenic and/or sulphate reducing populations in laboratory incubation experiments with LH sediments.

iii) Determining the range of cold temperatures that the LH populations identified in i) and ii) can withstand.

iv) Identifying potentially active microbial communities present within LH sediments *in situ* via community pyrosequencing of both 16S rRNA genes (DNA) and transcripts (RNA).

v) Determining the stability of the LH sediment microbial communities with respect to seasonality and sediment depth based on 16S rRNA community profiles.

2. Literature Review

2.1 Overview of literature review

The first section of the literature reviewed below focuses on the habitability of cryoenvironments and intends to expose how cryophilic microbial life (i.e. life capable of subzero activity) accounts for a significant portion of the Earth's biosphere despite being present in environments that are permanently cold or frozen. A special focus is put on the microbiology of cold saline springs that have similar conditions to the studied site. The relevance of cryomicrobiology research in astrobiology is also reviewed. The second part of the literature review covers microbial methanogenesis, anaerobic oxidation of methane (AOM), and sulphate reduction under cold and/or hypersaline conditions. The intention is to provide the reader with background on these metabolisms under these specific conditions considering that their study is the focus of this MSc thesis.

2.2 Cryoenvironments and microbial life below the freezing point (of water)

Most of Earth is considered cold, with ~ 90% of the world's ocean volume having temperatures never rising above 5°C (below ~ 1000 m) and ~ 25% of all landmasses being permanently frozen and/or covered by ice (Margesin and Miteva 2011; Pikuta et al. 2007). Cryoenvironments, herein defined as environments permanently below 0°C, mainly consist of permafrost (i.e. soil that has remained frozen for at least two consecutive years) and ice formations such as glacial ice, ice shelves, and sea ice, all of which are mainly found in polar and alpine regions. Consistent with their wide distribution, cryoenvironments are known to contain a very large reservoir of microbial cells (e.g. glacier and ice sheets are estimated to withhold an order of 10^{25} cells (Margesin and Miteva 2011)) yet microbial abundance is known to be sporadic and will vary with the type of environments. Microbial biomass in permafrost can range from ~10^{6}-10^{8} cells g^{-1} with the higher end of this range found in high Arctic permafrost and the lower one in the Dry Valleys of Antarctica (Goordial et al. 2013). Ice wedges and massive ground ice, which consist of permafrost-associated ice environments, normally show abundances in the order of 10^{4}-10^{5} cells mL^{-1} (Steven et al. 2008; Wilhelm et al. 2012). Sea ice constitutes a more plastic portion of the

cryosphere and will see its microbial biomass fluctuates with seasons and depths; upper winter sea ice for example can contain as little as 10^3 cells mL^{-1} whereas 10^7 cells g^{-1} are normally found in bottom summer ice (Collins et al. 2008; Deming 2010).

In line with their extensive heterogeneity, cryoenvironments exhibit a wide range of microbial biodiversity, normally reflective of the environments themselves. Terrestrial ice systems are mainly seeded by aerial depositions during snow events (Margesin and Miteva 2011), permafrost normally contains endemic soil or sediment communities that have been trapped upon freezing (Gilichinsky et al. 2007; Steven et al. 2009), and sea ice is essentially comprised of marine genotypes (Deming 2010). Some environmental selection nonetheless appears to shape cryoenvironment communities with successful colonisation and survival mostly limited to microorganisms bearing at least some degrees of cold adaptation (Margesin and Miteva 2011). As such, somewhat specific community signatures tend to characterise different cryoenvironments. For example, *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* are now recognised as typical permafrost phyla; the *Actinobacteria* are well characterised cold-adapted bacteria and the spore-forming nature of several *Firmicutes* and *Bacteroidetes* members are hypothesised to allow such clades to persist in the permanently frozen permafrost environment (Gilichinsky et al. 2007; Steven et al. 2007a; Steven et al. 2008). For extensive reviews on the extent and microbial composition of cryoenvironments, see Goordial et al. (2013) and Margesin and Miteva (2011).

2.2.1 Oases in frozen deserts: liquid water in cryoenvironments

Considering the obligate water-requirement of all life as we know it on Earth, the apparent lack of liquid water in cryoenvironments arguably represents the major challenge to cryophilic microbes. Despite their frozen state, however, cryoenvironments normally still contain a certain amount of water that remains unfrozen even below 0°C. Brine channels in sea ice, for example, are derived from the freezing of sea water. Upon freezing, most salts – and cells – are extruded from the growing ice crystals; the concentrated solutes then decrease the local freezing point of water, resulting in the formation of brine channels, or veins, within the sea ice environment (Junge et al. 2001). The size and salinity of these channels is directly dependent on the surrounding temperature, with smaller and more

concentrated veins formed under colder temperatures. As such, the extent and composition of sea-ice brines fluctuate annually and spatially, ranging from several millimeter thick channels of near-seawater salinity (~3 %) in the summer, to micrometer-sized veins or inclusions that can reach 23.7% salt towards the surface of winter sea ice (Collins et al. 2010; Junge et al. 2004; Mock and Junge 2007).

Glaciers are also thought to contain some degree of unfrozen water, though the extent of their vein systems is considered less extensive than in their sea-ice counterparts considering the lower solute content of glacial ice (Doyle et al. 2012). The smaller vein systems consequently select for smaller cells to populate the vein or film network. Mader et al. (2006) showed that particles larger than about 5 µm becomes entrapped within ice crystals but that those less than 2 µm are mostly partitioned into the liquid portion of artificial polycrystalline ice at -10°C. Still, Rohde and Price (2007) proposed that even within the ice crystals themselves, cold-adapted cells could potentially undergo metabolic redox reactions with small molecules diffusing through the crystal grains.

The extent of glacial veins is essentially dictated by the composition of glacial impurities. Sulphate constitutes the main ion found within Antarctic ice; the eutectic point of sulphuric acid is about -73°C. It is thus expected that even the coldest reported ice in Antarctica (i.e. -56°C) should contain certain amounts of liquid veins. Lastly, thin films of water can also remain liquid at very cold temperatures as a result of the ordering effect of clay minerals, a feature expected to exist in dirty ice but also within permafrost environments (Anderson 1967; Jakosky et al. 2003).

2.2.1.1 Bodies of water in terrestrial cryoenvironments

Larger water bodies are also found in cryoenvironments and essentially include (subglacial) lakes, ice streams, and permafrost- or glacier-associated saline springs or outflows. Despite being surrounded by frozen settings, these relatively large water masses can remain liquid as a result of high pressures, freezing point depression by elevated salts or solutes, or geothermal warming. Subglacial lakes probably account for the most studied of these systems, amounting to nearly 400 in Antarctica (Shtarkman et al. 2013), with Lake Vostok arguably the most notorious, being the 7th largest and 4th deepest lake on Earth and overlaid by approximately 4 km of ice. Studies on Lake Vostok have however been

restricted to its overlaying accretion ice. A recent meta-genomic/-transcriptomic study of Lake Vostok's accretion-ice sections reported sequences related to a large diversity of bacterial clades (ranging from known thermophiles to psychrophiles, halophiles, aerobes and anaerobes, etc.) as well as multicellular eukaryotes, indicating that the lake may host a whole range of different communities (Shtarkman et al. 2013). Deep Lake and Organic Lake of the Vestfold Hills, Antarctica, are two hypersaline lakes (mostly ice-free) with respective salinities and average temperatures of 32% and 20%, and -15°C and -7°C. Microbiology surveys of these sites revealed a dominance of halophilic archaea in Deep Lake but a much more diverse microbial community was found in Organic Lake, made up of heterotrophic and mixotrophic members which metabolisms probably center around nitrogen and sulfur cycling, especially of the highly abundant dimethylsulfoniopropionate (DMSP) compounds (Bowman et al. 2000; DeMaere et al. 2013; Yau et al. 2013). Lake Vida is an almost entirely frozen lake which bottom's 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 of the site (Doran et al. 2003; Murray et al. 2012).

Ice streams have only been recently recognised to constitute a major part of Earth's hydrological system, comprising teraliters of glacial meltwater that connect lakes, freshwater sediments, and drainage pathways below ice sheets (Lanoil et al. 2009; Wadham et al. 2010). These waters remain liquid at an *in situ* temperature of about -1.5°C due to basal melting (high glacial pressure); recent investigations also suggest that they too most likely host indigenous active microbial populations, such as chemolithotrophic methanogens and sulphate reducers (Wadham et al. 2012; Wadham et al. 2004).

2.2.1.2 Permafrost-associated saline springs and subglacial discharges

Perennial saline discharges in cryoenvironments are relatively rare occurrences and mostly documented in Arctic settings. They can be associated with glacier systems, such as sulphur-rich seeps found at Borup Fjord Pass on Ellesmere Island in the Canadian high Arctic, or Blood Falls in the Antarctic, a sulphur and iron-rich subglacial discharge of which its red colour gives the site its name (Gleeson et al. 2011; Mikucki and Priscu 2007). Perennial springs arising through thick permafrost are extremely rare as permafrost is normally considered impermeable to the mixing of groundwater with sub- and supra-

permafrost (Heldmann et al. 2005). Permafrost-associated springs are mainly found on Axel Heiberg Island (AHI) in the Canadian high Arctic and on the Svalbard Norwegian archipelago, though reports also exist of permafrost springs in Greenland and on the Qinghai-Tibetan Plateau located in the Mediterranean-Himalayas tectonic zone (Li et al. 2012; Pollard et al. 1999; Worsley and Gurney 1996). Despite being located in Arctic settings, the Svalbard springs are found in a hydrothermally active region, percolating through ~ 200 to 450 meters of permafrost, and surface at relatively warm temperatures (6-15°C) (Reigstad et al. 2011).

The coldest permafrost-associated springs are found on AHI in regions of thick permafrost (>600 m) and where there is no evidence for recent magmatic geothermal activity (Andersen et al. 2002). They flow in a region of diapiric uplift derived from buried carboniferous anhydrite-salt evaporites (Pollard 2005). The hydrology of these systems is poorly understood but the source of some of the springs' water has been suggested to originate from a combination of subglacial flow and lake water (Andersen et al. 2002). AHI spring brines surface at constant temperatures that range between ~ -7°C to 7°C in a region where air temperatures drop below -40°C during the winter months and where the surrounding permafrost temperature averages -16°C (Anderson et al. 2002; present study). Discharge brines bear salinities that range from ~ 7.5 to 26 %, are nearly to completely anoxic, highly reducing (~ -160 to -325 mV), and rich in sulphate salts (Perreault et al. 2007; present study). The coldest and saltiest of these springs, the LH spring, is described in section 1.2 and its microbial communities are the subject of the present investigation.

2.2.1.3 Cold spring microbial diversity

Despite elevated salinities and low temperatures, several viable, and potentially active, microorganisms have been associated with nearly all perennial brine discharges in cryoenvironments, with the exception of the Don Juan Pond in Antarctica. This shallow hypersaline playa, considered the most saline water body on Earth, 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). In general, cold spring microbiology reflects the sites' biogeochemistry, and chemolithotrophic members normally account for significant portions of the indigenous populations. For example, chemolithotrophic sulphur-oxidizing microbial

filaments have been reported in both the AHI Gypsum Hill (GH) spring system and the Svalbard Trollosen and Fisosen springs (Niederberger et al. 2009; Reigstad et al. 2011); sulphur oxidizing bacteria also populate other sulphidic brines such as the Blood Fall system, the Colour Peak (CP) springs of AHI, as well as at the Borup Fjord Pass (Gleeson et al. 2011; Mikucki and Priscu 2007; Perreault et al. 2008). Sequences related to methanogens and sulphate reducing bacteria (SRB) were also found at the GH and/or CP sites on AHI, consistent with the elevated sulphate concentrations of these springs' discharges and the methane gas detected on site (Perreault et al. 2008). Furthermore, most of the strains isolated from the colder AHI springs exhibited degrees of pshychro- and halo- tolerance (Niederberger et al. 2010; Perreault et al. 2008).

2.2.2 Cryoenvironments, more than biological freezers

The preservative properties of cold environments is well established; for example, "freezing" of bacterial isolates in glycerol at -80°C is a routine method for storing bacteria in the laboratory. A fundamental question that remains, however, is whether the microorganisms identified in cryoenvironments are actually active *in situ*. For example, the potential for cryopreservation of nucleic acids in cold, dry environments makes it especially difficult to differentiate between active, dormant, or dead populations based on molecular surveys (Ah Tow and Cowan 2005; Willerslev et al. 2004). There is, however, a growing body of evidence that indicate that cryoenvironments are more than natural freezers, and sustain an actively metabolizing population of bacteria. Work on both bulk environmental samples and on microbial isolates in the lab show measureable microbial metabolism at subzero temperatures. Additionally, the finding of anomalous CO_2, N_2O and CH_4 gas concentrations and isotopic compositions in ice environments is indicative of active cryophilic life *in situ*.

2.2.3 Measuring activity from bulk environmental samples

Activity assays performed on bulk environmental samples have the advantage of targeting whole sample communities without the bias of working on specific isolated strains, and by preserving, at least in some part, the community integrity of the original sample. Rivkina et al. (2000) assayed activity on permafrost samples using ^{14}C-acetate to

show respiration by native microbial populations down to -20°C. Similar techniques have since been used to asses subzero activity on other permafrost samples, as well as cold-spring sediments, and reports of active microbial members in cryoenvironments have become numerous (Lay et al. 2012; Steven et al. 2006; Steven et al. 2007b). However, the use of specific substrates (e.g. ^{14}C-acetate, ^{14}C-glucose) to assess activity is limited to the capability of the microbial community to metabolize such compounds and also constitutes a relatively selective method. Failed attempts to detect measurable amounts of mineralization in permafrost-associated-ice environments (i.e. ice wedges and massive ground ice), for example, cannot rule out the possibility of active indigenous communities (Wilhelm et al. 2012).

Compared to glacial and permafrost ice environments, greater microbial activity has been observed in sea ice. Subzero activity in summer sea ice has in fact been known since the 1970s (Deming 2010). The habitability of the more extreme winter-time Arctic sea ice, however, was only recently shown. By combining microscopy with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) respiration experiments, Junge et al. (2004) exposed how sea ice bacteria and archaea not only populate highly concentrated brines in winter sea ice, but are likely metabolically active *in situ* in liquid veins with salinities of 20% at -20°C. Despite many investigations of subzero activity in cryoenvironments, no clear demonstration to date of microbial activity on environmental samples has been reported below -20°C, a temperature that has been proposed by some to constitute a practical limit for microbial growth and below which evidence of metabolism is still scarce (Bakermans 2008; Beaty et al. 2006; Clarke et al. 2013).

2.2.4 *In situ* measurements of microbial respiration

Independent of laboratory experiments, measurements of putatively microbially produced gases can serve as direct proxies for biological activity *in situ* without the caveats of additional manipulations. Several anomalous CO_2, N_2O, and CH_4 gas concentrations and isotopic signatures have been reported in cryoenvironment studies, hinting at cryophilic life metabolizing *in situ*. For example, the bottom of the Greenland ice sheet has been found to contain elevated concentrations of CH_4 and CO_2 (Miteva et al. 2009; Tung et al. 2006). The findings of high numbers of cells attached on glacial clay minerals at these measured depths,

in concert with F_{420} autofluorescence imaging (as a proxy for methanogenesis), revealed that at least some of the reported cells may be active *in situ* (Tung et al. 2006). Methane concentrations four orders of magnitude higher in silty glacial ice than in clear ice samples, alongside with CH_4 isotopic compositions, were also indicative of biogenic CH_4 production (Miteva et al. 2009). Similarly, isotopic compositions of the occluded gases O_2 and CO_2 found in ice wedges from the Yukon suggested microbial respiration by heterotrophic bacteria *in situ* (Lacelle et al. 2011).

To date, the report of anomalous N_2O concentrations and isotopic ratios consistent with biogenic production within Vostok glacial ice (Antarctica) perhaps represents the most extreme case of possible activity in ice at an *in situ* temperature of approximately -40°C (Miteva et al. 2007; Sowers 2001). Nitrification in ice has since been described in both pure culture experiments as low as -32°C (Miteva et al. 2007), and implied in other glacial environments to potentially occur at -40°C (Rohde et al. 2008). While trapped gas measurements are suggestive of *in situ* metabolism, they do not indicate when or on what timescale these gases may have accumulated. Measurements of CO_2 flux have been used to detect putative microbial respiration in polygon and trough surfaces in Canadian high Arctic permafrost. A net CO_2 flux was observed at significantly higher levels above atmospheric values, with ambient surface soil temperatures of -9ºC and underlying permafrost temperatures of -16ºC, suggesting *in situ* microbial respiration in these soils (Wilhelm et al., 2012).

2.2.5 Pushing the limits for life: artificial freezing of psychrophiles

Work on isolated strains have rendered it possible to look at cryophilic life under controlled laboratory settings, allowing us to probe the biological limits to cold and gain insights on responses and adaptations to cryo-related stresses. The lowest recorded temperature for microbial division has recently been pushed down to -15°C in *Planococcus halocryophilus* OR1, 3°C below the previously held record of *Psychromonas ingrahamii* (Breezee et al. 2004; Mykytczuk et al. 2013). Experiments on cryophilic isolates have also succeeded in expanding our view of subzero microbial activity. Recent work on ice isolates (*Paenisporosarcina* sp. B5 and *Chryseobacterium* sp. V3519-10) incubated in frozen M9 medium, for example, showed that viable cells were respiring down to -33°C (Bakermans

and Skidmore 2011a). The same research group compared how a liquid brine media of comparable salinity to that of the liquid veins in frozen M9 media affected microbial growth at -5°C. Diminished activity in ice veins as opposed to briny media was observed (Bakermans and Skidmore 2011b), a trend that has also been observed by others at lower temperatures (Amato and Christner 2009). These findings suggest that the osmotic stress caused by the concentration of solutes during freezing is not the sole constraint to microbial activity in ice.

Extreme subfreezing incubations, down to -80°C and -196°C (in liquid nitrogen), have also been performed on permafrost and marine isolates (Amato and Christner 2009; Junge et al. 2006). Though true that such extremely low temperatures are generally not found on Earth, these are relevant to astrobiological research on other planetary bodies and our understanding of how life may cope with such low temperatures. Interestingly, both studies provided evidence of activity, yet only for relatively short incubation periods (less than a day), followed by virtually inactive states, reflecting probable cold-shock responses and entries into dormancy.

2.2.6 Challenges to life in cryoenvironments and microbial adaptations to cold

Cold temperatures expose cells to several factors of stress both directly and indirectly. The deleterious effects of low temperatures on microorganisms are indeed often accompanied with constraints associated with the milieu in which the microbes are found and include, among others, freezing, desiccation, and high osmolarity (Goordial et al. 2013). The intrinsic effects of cold mainly relate to a decrease in thermal energy (or enthalpy) of the system, which ultimately prevents non cold-adapted microorganisms to undergo metabolic reactions below a specific permissive temperature (Bakermans 2008). Essentially, low temperatures will slow down metabolic rates, decrease enzyme activity and membrane fluidity, and can result in denatured or misfolded proteins or the formation of intracellular ice crystals, as well as increased exposure to reactive oxygen species due to the higher oxygen solubility at low temperatures (Cavicchioli 2006; D'Amico et al. 2006; Pikuta et al. 2007; Steven et al. 2006). Adaptations to cope with decreased, cold, and even subzero temperatures, have been described elsewhere (e.g. Bakermans et al. 2009; Casanueva et al. 2010; Qiu et al. 2009), and specific studies exist on cold-adapted model organisms such as

Psychrobacter cryohalentis, *Psychrobacter arcticus*, *Planococcus halocryophilus* OR1, and *Methanococcoides burtonii* (Allen et al. 2009; Ayala-del-Río et al. 2010; Bakermans et al. 2009; Bakermans et al. 2012; Mykytczuk et al. 2013). Common cold adaptations include increasing the degree of unsaturation and shortening of fatty acids to maintain membrane fluidity and flexibility, the production of cold-acclimation and cold-adapted proteins including an increased production of chaperone proteins to assist in protein folding and maintain mRNA stability (Panoff et al. 1995; Ting et al. 2010), as well as the synthesis of specialised exopolysaccharides which have been found to counteract the effects of ice-crystal formation and result in local freeze-point depression (Goordial et al. 2013; Marx et al. 2009). It should be pointed out however that even though cold adaptations have been the center of several studies, mechanisms by which microorganisms remain active at low, and especially at subzero, temperatures are not fully understood (Bakermans 2008; Bakermans et al. 2009).

2.2.7 Cryophily and halophily

As previously described (section 2.2.1), life at subzero temperatures is often linked with exposure to increased salinities. Consequently, dual adaptations to cold temperature and increased osmotic stress are frequent, if not essential, in psychrophilic and cryophilic microorganisms (Chin et al. 2010; Steven et al. 2006). This dual tolerance to cold and solutes is reflected in the list of cold-adapted strains isolated to date, especially regarding those capable of subzero growth (Goordial et al. 2013). For example, the *Firmicutes* bacterium *Planococcus halocryophilus* Or1, isolated from an Arctic permafrost active-layer, can divide at -15°C in 19% salt media (Mykytczuk et al. 2013). Microorganisms living under high salt concentrations mainly rely on two different strategies to maintain turgor pressure and achieve osmotic balance between the outside milieu and the cell cytoplasm. The "salt-in" strategy involves the accumulation of salts, normally K^+ ions over Na^+, intracellularly in order to balance the osmotic pressure inside and outside the cell. While being the most energy efficient strategy of the two, this type of halophilic lifestyle requires several adaptations and modifications that differ from typical cell machinery and is normally restrained to a limited number of extreme halophilic organisms such as archaea of the family *Halobacteriaceae*, as well as a few aerobic *Salinibacter* and fermentative

Halanaerobiales bacteria (Oren 1999, 2011). The alternative strategy involves the exclusion of salts from the cytoplasmic milieu and accumulation and biosynthesis of compatible solutes to maintain osmotic balance. Though adopted by a wider variety of microorganisms, this type of adaptation is considerably more energy demanding, and is therefore thought to be restrained to microorganisms capable of high energy-yielding metabolisms (e.g. aerobic respiration) at high salinities (Oren 2011).

2.3 Cryomicrobiology and significance to astrobiology

The NASA astrobiology mission aims at answering fundamental questions regarding the origin, evolution, and distribution of life in the universe (Des Marais et al. 2008; Morrison 2001). Due to technological limitations, most astrobiology research in the short term focuses on the search for life within the solar system, with current astrobiological hotspots being Mars, as well as Jupiter's moon Europa and Saturn's moon Enceladus (Goordial et al. 2013). Extreme cold characterise these targets, with average surface temperatures of ~ -190°C on Enceladus, -160°C on Europa, and -60°C on Mars (with minima of -130°C and maxima of 20°C). Earth cryoenvironments are thus considered excellent analogue sites to perform research targeting the habitability of these alien worlds for either extinct or extant life. Despite these cold temperatures, evidence of both past and present liquid water has been documented for these planetary bodies. Jets of salty water vapor and ice emerging from fractures on the surface of Enceladus have been observed by the NASA Cassini mission (Hansen et al. 2006). Jupiter's icy moon Europa is hypothesised to contain a subglacial ocean beneath its ~20-40 km thick ice shell and recent studies have also revealed the likelihood presence of shallower (~ 3 km) water bodies beneath the ice cap that may be more readily accessible by future missions (Schmidt et al. 2011). There is also the possibility that sulphate brines percolate to the surface of the Europa moon as implied by spectroscopic and geophysical interpretations of the non-ice materials present on the several ice fractures on Europa's surface (McCord et al. 2001). Mounting evidence now strongly indicates the presence of past, and even potentially present, liquid water on Mars (Gendrin et al. 2005; Kraal et al. 2008; Mustard et al. 2008; Soare et al. 2012). Of special interest are the observations of potential gully activity reported by the Global Surveyor Mars Orbiter and depicting what appear to be subsurface brines flowing on the surface on Mars (Malin et

al. 2006; McEwen et al. 2011). Moreover, the Phoenix lander in 2009 discovered eutectic brines overlaying subsurface Martian water ice (Hecht et al. 2009; Rennó et al. 2009).

Reports of methane gas in the atmosphere of Mars have sparked the interest of the scientific community regarding prospects of finding signs of life on Mars (Mumma et al. 2010; Price 2010; Zahnle et al. 2011). About 90-95% of Earth's methane is biological in origin, with roughly 65% directly produced by methanogenic archaea (Conrad 2009); methane can also serve as a carbon and energy source to both aerobic and anaerobic microbes (Oremland 2010). As such, methane gas bears a special importance as a potential biomarker in astrobiology research (Mumma et al. 2010). Despite the failure of the Curiosity rover to confirm the presence of methane on Mars (Webster et al. 2013), methane is still considered a prime biological signature in the search for life outside the Earth, and methane and other simple organics have potentially been detected in Enceladus' water plumes (Postberg et al. 2011).

2.4 The Lost Hammer spring as a relevant analogue site for astrobiology research

Considering the likelihood presence of liquid briny water, alongside the detection of methane gas, on other planetary bodies of the Solar System, the subzero, hypersaline, anoxic, and sulphate and methane rich nature of the LH spring arguably makes it an excellent analogue environment to alien habitats described on Mars, Enceladus, or Europa. Reports of spring-like structures, the large distribution of chloride deposits, as well as the detection of sulphate minerals on the Martian surface furthers LH relevance as an analogue 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). Moreover, considering the scarcity of characterised anaerobic, non-heterotrophic, cryophiles, investigations on the putatively active LH chemoautotrophic ANME, chemolithotrophic sulphate reducing microorganisms (SRM), and potential methanogen populations under subzero temperature and hypersaline conditions are highly relevant to increasing our understanding of cryophilic microbiology (Goordial et al. 2013).

2.5 Methanogenesis, anaerobic oxidation of methane, and sulphate reduction under cold and saline conditions

Methanogenesis and dissimilatory sulphate reduction under hypersaline or cold conditions have been reported in several environments, including deep-sea marine sediments, Antarctic lakes, hypersaline soda lakes, glaciers, and permafrost (Foti et al. 2007; Sagemann et al. 1998; Sattley and Madigan 2010; Smith et al. 2008; Sorokin et al. 2012; Tazaz et al. 2012; Tung et al. 2006). Yet few studies have explored both conditions simultaneously, and never below ~ -4°C for SRB (Sattley and Madigan 2010; Tarpgaard et al. 2006).

A classic view regarding the coexistence of SRB and methanogens is that SRB normally out-compete methanogens for electron donors (mainly hydrogen, acetate, and formate) in environments where sulphate is not limited. Under hypersaline conditions however, methanogens relying on the use of methylated compounds (e.g. methanol, methylamines, and dimethylsulfide) are known to coexist with SRBs; methylated compounds are therefore often referred to as "non-competitive substrates" (Ollivier et al. 1994). These compounds are also thought to act as osmoregulators in hypersaline environments; as such, methanogenesis above ~ 12% salt is thought to be restrained to methylotrophic clades of methanogens (Oren 2011). In contrast, hydrogenotrophy is favoured by SRBs over less exergonic sulphate reduction reactions under high salt concentrations (Oren 2011).

Metabolic specificities of cryophilic methanogens are less documented than their halophilic counterparts considering that most reports of methanogenesis below 0°C were inferred from the measurement of methane gas from glacier and permafrost environments as opposed to incubation experiments (e.g. Tung et al. 2006; Rivkina et al. 2004). Of interest, however, is that the sole methanogen strain capable of subzero growth (-2.5°C) in culture, the *Methanococcoides burtonii* DSM 642 methanogen isolated from the hypolimnion of the Antarctic Ace Lake, is methylotrophic and grows on methylamines (Franzmann et al. 1992). This methylotrophic lifestyle agrees with the view that links cryophily with halophily. Reports of potential hydrogenotrophic and acetoclastic methanogenesis in ice at -9°C have however been proposed (Tung et al. 2006) and such methanogenic metabolisms are known to be common in tundra wetlands and Siberian peat bogs under cold (~ 5°C) temperatures

(Kotsyurbenko et al. 2004; Kotsyurbenko et al. 1996). Interestingly, a recent study on the microbiology of the hypersaline Organic Lake in Antarctica (20 % salt, -13°C) failed to show methanogenic activity, and only small amount of dissimilatory sulphate reduction, despite it being the water body with the highest amount of dimethylsulfide (DMS), a known methylotrophic methanogenic substrate, on Earth (Yau et al. 2013). Unlike most documented cryophilic methanogen populations, nearly all documented SRB strains or populations capable of subzero activity originate from Arctic marine sediments, and utilise a wide range of electron donors (e.g. Tarpgaard et al. 2006; Knoblauch et al. 1999) .

The anaerobic oxidation of methane (AOM), though first documented more than 40 years ago (Barnes and Goldberg 1976; Claypool and Kaplan 1974; Davis and Yarbrough 1966), was only confirmed at the end of the twentieth century based on analyses of ^{13}C isotopic content of lipids in marine sediments (Hinrichs et al. 1999). AOM is now recognised to account for the major methane sink on the planet and is most often coupled to sulphate reduction via the syntrophic association of ANME archaea and SRB, typically of the *Desulfosarcina*, *Desulfococcus*, or *Desulfobulbus* genera (Boetius et al. 2009). Research on AOM has also revealed that ANME cells, more specifically of the ANME-1 clade, can sometimes undergo sulphate-mediated AOM without the help of a syntrophic partner (Maignien et al. 2012; Orphan et al. 2002; Thauer and Shima 2008). The mechanism by which ANME cells undergo AOM is not completely understood, but several lines of evidence indicate that they do so via reverse methanogenesis. Molecular investigations indicate that ANME cells are phylogenetically highly related to methanogens, and that they possess most of the genes involved in methanogenesis, including the *mcr* gene coding for the key methanogenic enzyme methyl-coenzyme-M-reductase (MCR) that catalyses the last step of methanogenesis and first of AOM (Hallam et al. 2004; Meyerdierks et al. 2010; Scheller et al. 2010). Though AOM is generally coupled to sulphate reduction, manganese and iron can also act as alternative oxidants (Beal et al. 2009). Unusual denitrifying bacteria can also oxidize methane under anoxic conditions via the more typical aerobic methanotrophy pathway. These methanotrophic, denitrifying, bacteria do so by producing intracellular oxygen as a result of denitrification (i.e. their nitric oxide dismutase enzyme produces N_2 and O_2 as opposed to the more traditional reaction yielding N_2 and CO_2) (Ettwig et al. 2010; Ettwig et al. 2008; Oremland 2010).

Most studied ANME consortia are cold-adapted, metabolising under *in situ* temperatures often below 10°C in deep-sea sediments or methane hydrates (Boetius et al. 2009). More recent investigations have shown that the temperature range permissive of AOM also extends to hydrothermal temperatures (up to at least 75°C) (Holler et al. 2011; Teske et al. 2002); there is no report however of subzero AOM. Investigations of subzero (-1.5°C) Haaken Mosbon Mud Volcano sediments in the Atlantic did not reveal signs of AOM at *in situ* temperatures but AOM was observed from these samples at 4°C (Krüger et al. 2005). The AOM under hypersaline conditions is considered thermodynamically unfavorable considering that the AOM with sulphate yields only little energy ($\Delta G^{\circ\prime} = -16$ kJ mol^{-1}) (Oren 2011). Regardless of these energetics constraints, ANME-1 cells have been reported in different hypersaline environments (Lloyd et al. 2006; Ziegenbalg et al. 2012) and ANME-1 mediated AOM has recently been confirmed in cold marine sediments of a hypersaline mud volcano at 4°C and at salinities reaching halite saturations (Maignien et al. 2012).

Chapter 3: Materials and methods

3.1 Sample collection and geochemical analyses

Lost Hammer (LH) spring samples were collected during two summer expeditions (July 2011-2012), as well as one in late winter (April 2012). Ethanol-sterilised push cores were used to collect sediments; cores never extended deeper than 30 cmbs (cm below the sediment surface), a depth potentially corresponding to the complete layer of deposited sediments overlaying either permafrost or active layer soil. July 2011 sediment cores were collected using central-vacuum PVC tubing (O.D. 2") capped with PVC plugs and sealed using electrical tape. On site, intact cores were stored vertically inside heat-sealed laminated bags and kept anoxic using gas-generating AnaeroGen sachets (Oxoid, Nepean, On, Canada); anaerobic indicators (Oxoid, Nepean, On, Canada) were also added to visually verify that the environment was kept anoxic after sampling.

To reach the sediment layer in April 2012 when the spring dome was full, a customized coring device consisting of a series of commercially available galvanized pipe

(3/4" O.D. X 24" long) mounted with a welded rotary handle was used to reach the sediment layer, overlain by approximately 2 meters of spring water (Figure 1.1). The same stainless steel cores were used in July 2012; in both cases hammering of the coring device with a rubber mallet was necessary to reach 30 cmbs. Immediately after sampling, sediments were pushed out of the stainless steel cores, transferred into sterile 50 mL conical tubes, filled completely with spring water, and stored inside portable anaerobic jars with gas-generating AnaeroGen sachets and anaerobic indicators (Oxoid, Nepean, On, Canada) to maintain the jar anoxic. Spring water was collected in pre-autoclaved plastic bottles. Sediments to be used for molecular analyses were stored into sterile, nuclease-free, 50 mL conical tubes pre-filled with LifeGuard soil preservation solution (MoBio Laboratories, Inc., Carlsbad, CA, USA) to a final volume of 50 mL. Sediment and water samples were kept at <5°C (but not frozen) during transport and stored at -5°C at the university laboratory within two weeks of sampling. Samples stored in RNA LifeGuard were frozen at -20°C within three hours after collection and stored at -80°C at the university laboratory until further processing. LH sediments previously collected in July 2009 were also used in some incubation experiment (see section 3.2.2 below). These samples consisted in sediments (0-30 cmbs) that had been stored at <5°C during transport to the laboratory in 2009 and subsequently at -20°C in the laboratory. No special care to keep the sediment anoxic had however been taken.

Dissolved sulphide and oxygen concentrations were measured *in situ* by colorimetric assays as per manual instructions using either visual kits or a portable V-2000 photometer (CHEMetrics, Calverton, VA, USA). 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).

3.2 Anaerobic microcosm experiments

Sediments used in anaerobic incubations were all processed inside an anaerobic chamber (COY Laboratory, Grass Lake, MI, USA) to minimize oxygen exposure. Unless mentioned otherwise, the water used consisted in natural LH water rendered anoxic and reducing via N_2 gas bubbling and the addition of the reducing agent $Na_2S•9H_2O$ (5 mM[1]). Autoclaved blue butyl-rubber stoppers (2 cm thick), crimped sealed with aluminium caps,

[1] Unless stated otherwise, all reported concentrations correspond to final concentrations.

were always used to seal vials and tubes during anaerobic incubations. 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; 2012 microcosms were set-up with LH sediment and water collected during the same 2012 sampling campaign. 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 synthesised in the laboratory. The composition of the synthesised water was a modification of Berges et al. (2001) artificial seawater medium where the concentration of nutrients and major water elements was changed to match that of the natural LH water whenever known. Table 3.1 summarises the concentration of the nutrients and major elements that were modified from those of Berges et al. (2001).

Table 3.1 Salts in the artificial LH water medium which concentrations differ from Berges et al. (2001) artificial seawater medium

	g/L stock solution
Salt solution I – *anhydrous salts*	
NaCl	174
Na_2SO_4	16
KCl	3.89
$NaHCO_3$	0.3549
KBr	0.1761
H_3BO_3	0.0469
Salt Solution II – *hydrated salts*	
$MgCl_2\ 6H_2O$	50.88
$CaCl_2\ 2H_2O$	99.74
$SnCl_2\ 6H_2O$	0.3498

3.2.1 Anaerobic oxidation of methane (AOM)

Two 30 cm LH sediment cores collected in July 2011 were cut in half using an ethanol-sterilised hand saw inside an anaerobic chamber. Sediment samples from the two halves of the cores (i.e. 0-15 cmbs and 16-30 cmbs) were kept separated and transferred into sterile Whirl-pack bags (Nasco, Fort Atkinson, WI, USA) on ice. Samples were

homogenised by hand and transferred into 26 mL Hungate tubes on ice. Pre-chilled 1:1 natural:synthetic LH water was then added to each tube. The water had previously been bubbled with N_2 gas and reduced with $Na_2S•9H_2O$ (5 mM) to ensure reducing conditions and the absence of dissolved oxygen; the water pH was adjusted to 7.0 with 1 M NaOH. Amended tubes were then sealed with 2 cm blue butyl stoppers and crimped sealed. Inoculated tubes were divided into two experiments to assess AOM either with the use of radiotracer methane (i.e. $^{14}CH_4$) or non-labelled methane.

3.2.1.1 Anaerobic oxidation of methane – radiotracer experiment

AOM microcosms were set-up similarly to Treude et al. (2007) inside 26 mL Hungate tubes. In brief, the tubes' headspace was exchanged to 100% methane and supplemented with 50 µL of $^{14}CH_4$ (~220 kBq, gaseous) using a 100 µL gastight syringe. Tubes contained ~ 6 g of wet sediments and 12 mL of water and were set-up in triplicates, with 5%-formaldehyde-killed controls set-up in parallel. The tubes were incubated horizontally at 5°C, 0°C, and -5°C in the dark. Enough tubes were prepared to allow sacrifice sampling (3 time point measurements). Tubes were gently shaken bi-monthly to allow mixing of the headspace with the sediment slurry. Converted $^{14}CH_4$ as $^{14}CO_2$/ $H^{14}CO_3^-$ was measured following the protocol describe in Treude et al. (2003) with slight modifications. In short, about 6 mL of headspace, as well as 10 mL of slurry, were transferred to a pre-evacuated serum vial sealed with a 2 cm butyl blue stopper using a 10 mL gastight syringe. As a trap for $^{14}CO2$, the pre-evacuated serum vials contained a glass tube filled with a folded glass microfibre Whatman filter, and 0.75 mL of phenylethylanine. 0.1 mL of antifoam, 1 mL of bromothymol blue, and 5 mL of 5M HCl were then added to acidify the slurry sample; about 10 mL of air was also added to equilibrate the vials' headspace. Vials were left undisturbed for 48 hours and the pH of the slurry was checked visually to ensure that it had not become alkaline. $^{14}CO_2$-traps were then transferred to scintillation vials filled with 20 mL of scintillation fluid (SX 18-4 ScintiVerse™ BD cocktail, Fisher Scientific, On, Canada), and the amount of converted $^{14}CH_4$ to $^{14}CO_2$ was quantified via scintillation counting on a Beckman Coulter (CA,USA) LS 6500 Multi-purpose Scintillation Counter.

3.2.1.2 Anaerobic oxidation of methane – methane consumption experiment

Hungate tubes were amended with ~ 5 g of wet sediments and 8 mL of water, set-up in triplicate, and incubated horizontally at 5°C, 0°C, and -5°C in the dark. 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 sulphide 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°C 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 and because the incubation experiments were expected to run for long periods of time (i.e. over a year), a modified version of the methylene blue colorimetric assay designed by Cline (1969) was used to measure sulphide concentrations from the vials' headspace instead of dissolved sulphide concentrations in the slurry samples. In short, 100 µL of headspace was sampled using a high precision 500 µL gastight syringe pre-filled with 20% zinc acetate. The sample was then injected into tubes sealed with black butyl stoppers and pre-filled with 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 minutes to allow colour development, and spectrophotometric measurements were then performed. Technical duplicate measurements were taken for each sample. Sulphide concentrations were derived from standard curves generated using diluted amounts of 0.5 mM $Na_2S•9H_2O$ standard solutions. For both methane and sulphide measurements, tubes were hand-shaken for 2 minutes before headspace sampling in order to equilibrate the dissolved gases with the headspace.

3.2.2 Measurement of methanogenesis

Methanogen-enrichment incubations were set-up using LH sediments collected in both July 2009 and July 2011. Microcosms with 2009 samples consisted of 10 g of wet sediments (0-30 cmbs) and 10 mL of anoxic LH water reduced with $Na_2S_9H_2O$ (0.5 mM),

inside 37.5 mL serum vials. Samples were amended with 500 µM of either dimethylsulfide (DMS), trimethylamine (TMA), or methanol. Samples were set-up in triplicate and negative controls consisted in microcosms autoclaved twice for 30 minutes at a 48 hour interval. Microcosms were incubated upside down in the dark at either 5°C or 10°C. Methane levels in the headspace were measured by gas chromatography as described above (section 3.2.1.2). After about 100 days of incubation, DMS, TMA, and methanol concentrations were increased to 5 mM. Microcosms amended with 2011-collected sediments were also assayed for H_2S releases in combination to methanogenesis in microcosms amended with sodium acetate (100 mM) or having a H_2:CO_2 (80:20) atmosphere as an alternative carbon and electron source. These later microcosms were incubated upside down in the dark at both -5°C and 5°C, and were also assayed for sulphide production; their set-ups are described below (section 3.2.3). Table 3.2 summarises the incubation set-ups used to assay methanogenesis from LH sediments.

Table 3.2 Summary of attempted methanogenesis experiments performed on LH sediments.

Electron/carbon source added	Year of sediment/water collection	Incubation temperatures (°C)
Methanol (0.5 and 5 mM)	2009	5, 10
DMS (0.5 and 5 mM)	2009	5, 10
TMA (0.5 and 5 mM)	2009	5, 10
*Acetate (100 mM)	2011	-5, 5
*H_2:CO_2 (80:20)	2011	-5, 5

*Microcosms amended with these substrates were also assayed for H_2S production (see section 3.2.3).

3.2.3 Measurement of sulphate and/or sulphur reduction (SR)

3.2.3.1 SR incubation set-ups

SR microcosms were set-up using LH sediments collected in July 2011 or April 2012. The 2011 microcosms consisted of 7.5 mL of water and ~ 15 g of wet sediments of either 0-15 cmbs or 16-30 cmbs and incubated at either 5°C or -5°C inside 37.5 mL serum vials; samples were set-up in triplicates. The headspace of the vials was either replaced with

N_2:CO_2 or H_2:CO_2 (80:20); two sets of controls were designed for the H_2:CO_2 microcosms: formalin-treated controls (5% final formaldehyde concentration) and sodium molybdate controls (1 M). N_2:CO_2 vials served as negative controls for biological hydrogen-dependent sulphidogenesis, formalin treated vials served as killed controls, and sodium molybdate amended vials served as negative controls of biological SR. Sodium molybdate was added in such high amounts considering it being a competitive inhibitor for sulphate and therefore following recommendations for adding it at equimolar concentrations to sulphate (Fleming et al. 2006; Oremland and Capone 1988). After about 180 days of incubation, sodium acetate (100 mM) was added to the vials with N_2:CO_2 headspaces to test for acetoclastic sulphate reduction; acetoclastic methanogenesis was also assessed from those same vials as described above (section 2.2.2).

The 2012 microcosms were set-up similarly as above, with ~5 g of sediments (~0-15 cmbs), 10 mL of LH water and a H_2:CO_2 (80:20) atmosphere; only formalin-treated sediments were used as negative controls. Samples were incubated at 5°C, -5°C, -10°C, -15°C, and -20°C; sample incubation at 0°C was prevented because of incubator malfunction and incubation temperatures lower than -20°C resulted in the freezing of the used LH water. SR was assayed by the measurement of gaseous H_2S as described above for both the 2011 and 2012 incubations (see section 3.2.1.2).

Rates of sulphide releases (SRR) were calculated using the regression of the linear portion of the curves of sulphide concentration over time (Figure 4.4 B). The apparent activation energy (Ea) for sulphide releases, potentially reflecting microbial sulphate reduction, was estimated as Robador et al. (2009) by plotting the natural logarithm of rate versus the inverse of temperature as follow:

$$\ln(k) = \ln(A) + \left(\frac{-Ea}{R} \cdot \frac{1}{T}\right)$$

where Ea is the activation energy (J mol^{-1}), k the reaction rate (nmol cm^{-3} day^{-1}), A is the Arrhenius constant, R is the gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature in Kelvin.

3.2.3.2 Incubation of LH sediments using cysteine-HCl as an alternative reducing agent

In order to verify whether or not the use of $Na_2S•9H_2O$ as a reducing agent contributed to H_2S emissions, incubation experiments with the alternative reducing agent cysteine-HCl were also performed with LH sediments. The microcosms were set-up as above in duplicate with ~ 5 g of July-2012-collected surface LH sediments and 10 mL of LH water. Another set of incubation vials consisted of only 10 mL of LH water (i.e. without sediments) that had been filtered-sterilised on-site using a 0.22 µm pore size filter and kept at -5°C in the laboratory. The used water was rendered anoxic and reducing as described above; half of the amended vials (both with and without sediments) were reduced with $Na_2S•9H_2O$ (5 mM) and the other half with cysteine HCl (0.05%). The headspace of all incubated vials was exchanged to H_2:CO_2 (80:20) and all vials were incubated upside down at 5°C. H_2S concentrations were assayed as described above.

3.2.3.3 Testing for hyperthermophilic SR

As a result of suspected sulphide releases from LH sediments subjected to autoclaving in an experiment independent from this MSc thesis (sulphide concentrations were not measured), a set of LH microcosms were incubated at high temperatures in order to measure potential hyperthermophilic SR from LH sediments. Serum vials contained 10 g of July 2012 collected sediments and 10 mL of LH water reduced with $Na_2S•9H_2O$ (5 mM) and were incubated in a water bath maintained at 80°C. Four different treatments were used as negative controls and included the addition of the following killing or inhibitory agents to the sediment slurries: formalin (2% final formaldehyde concentration), sodium hydroxide (2.8%), bleach (10%), and sodium molybdate (20 mM). Sediment slurries without killing agents were incubated in triplicates and negative control vials in duplicate; the atmosphere of all incubated vials was H_2:CO_2 (80:20). Considering the concern of $Na_2S•9H_2O$ being a source of the measured sulphides, additional control experiments were also designed using the alternative reducing agent cysteine-HCl. These additional control experiments and results are summarised in Appendix Table 1.

3.2.3.3 Total cell count by DAPI staining of sulphidogenic sediments incubated at 5°C for 8 months

One of the three replicate vials incubated at 5°C under a H_2:CO_2 atmosphere with 2012-collected sediments (see section 3.2.3.1) was assayed for a potential increase in cell number by total cell counts. Some 2012-collected sediments had previously been fixed in 2% formaldehyde during vial inoculation and intended to represent the LH-sediment-community size at the beginning of the H_2:CO_2 incubation (i.e. time = 0). Total cell counts were determined by DAPI (4',6-diamidino-2-phenylindole) counts using a modified protocol based on Kepner and Pratt (1994) and Epstein and Rossel (1995).

Formalin solution (37% formaldehyde) was first injected using a syringe and a needle into the serum vial to a final formaldehyde concentration of 2% and the vial was incubated at room temperature for 1 hour in order to fix the sample's cell community. To dislodge cells from sediment particles, 5 mL of sediment slurry from both the vial and the pre-fixed time 0 control were transferred into a 50 mL conical tubes containing 2.5 g of glass beads (3 mm diameter) and 15 mL of sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$ pH 7.0, 0.001 M); pyrophosphate was added to act as a dispersant. Samples were then vortexed at maximum speed for 2 minutes and 5 mL of the vortexed samples were transferred into a new 50 mL tube on ice. On ice, the 5 mL samples were sonicated twice for 1 minute with 2 minute breaks between bursts using a microtip sonicator probe vibrating at an amplitude of 109 µm (Epstein 1995). An extra 5 mL of 1XPBS and 0.001M pyrophosphate (final concentration) was added to the sonicated samples and tubes were then shaken thoroughly and vortexed for 10 seconds in order to homogenise the samples. Samples were then diluted by serial dilution in PBS pyrophosphate buffer. 10 µL of the desired dilutions were pipetted onto wells of an epoxy-coated multiwell slide pre-coated with gelatin; the desired dilutions were sampled in triplicates. Slides were air dried and dehydrated in 50%, 80%, and 100% ethanol baths for 3 minutes. 10 µL of 2µg/ml DAPI solution was pipetted onto each sample well and slides were incubated in the dark for 15 minutes. Slides were then washed 3 times for 10 minutes in PBS buffer, air dried, mounted with Citrifluor:Vectashield (4:1) and covered with coverslips. Negative controls were also prepared in parallel using the same solutions but without sediment addition. Mounted samples were observed under a fluorescent Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) at an excitation

wavelength of 350 nm under a 100X immersion oil filter. Cell counts always corresponded to averages obtained from at least 15 random observation fields of all 3 replicate wells; only dilutions that yielded between 30-300 cells per field were counted.

3.3 Catalyzed Activated Reporter Deposition-Fluorescent In Situ Hybridization (CARD-FISH)

CARD-FISH analyses were performed on the 2009-collected sediments accordingly to Wendeberg (2010), with slight modifications. Sediment samples of 0.5 g were suspended into a 4 % paraformaldehyde solution for one hour at room temperature, followed by pelleting via centrifugation (5 min at 13,000 g). Sediment pellets were re-suspended and washed 3 times in PBS buffer and stored in PBS:ethanol (1:1) at -20°C until further processing. To dislodge cells from sediment particles, samples were sonicated once for 20 seconds on ice at minimum power using a microtip sonicator probe. Sonicated sediment suspensions were mixed with low gelling point agarose (0.1% w/v) in PBS containing 0.0001% SDS, incubated at 55°C for 3 minutes. 10 µL of suspension was pipetted into the slides' wells and dried at room temperature; slides were then dehydrated in ethanol baths of increasing concentrations (50%, 80%, and 96% ethanol) for 1 minute. Cell wall permeabilization was performed using either a 10 mg/mL lysozyme solution or 1.5, or 15, µg/mL proteinase K solution at 37°C for 60 min when targeting bacterial, archaeal, or ANME-1 cells respectively (Knittel and Boetius 2009). Samples were subsequently washed in water and incubated for 20 minutes in 0.01 M HCl at room temperature to inactivate endogenous peroxidases (Teira et al. 2004). Slides were then dehydrated in 100% ethanol and dried at room temperature. Slides were hybridized with horseradish peroxidase (HRP)-labeled probes (50 ng/µL) targeting conserved regions of the 16S rRNA of: Bacteria, EU338 probe (Amann et al. 1990), Archaea, ARCH915 probe (Stahl and Amann 1991) or ANME-1, ANME-1 350 probe (Boetius et al. 2000). Hybridization was performed overnight (~ 10 hours) in the dark at 46°C in hybridization buffer (5 M NaCl, 1M Tris HCl, 20% SDS, 10% blocking reagent, 10% dextran sulphate, and either 35%, 40%, or 55% formamide for ARCH915, ANME-1 350, or EUB338 respectively) containing appropriately labelled nucleotide probes. The catalyzed reporter deposition (CARD) was performed at 46°C for 15 minutes in amplification buffer (20 X PBS, blocking reagent, 5 M NaCl, 10 % w/v dextran

sulphate) containing tyramide (fluorescently-labelled with carboxyfluorescein succinimidyl ester) and H_2O_2 and washed with PBS buffer, water, and 50% ethanol. Slides were then counterstained with DAPI (4',6-diamidino-2-phenylindole) (2 µg/mL), washed in PBS, dried, and mounted with a 4:1 mix Citifluor (Citifluor Ltd., London, U.K.) and Vectashield (Vector Laboratories, Inc., CA) solutions; Vectashield antibleaching properties served to reduce bleaching effects caused by certain electromagnetic wave exposures when observing samples under an epifluorescent microscope (Pernthaler et al. 2001). Mounted samples were observed under a fluorescent Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) at a wavelength of 568 nm and using a 100X oil immersion objective.

3.4 Nucleic acid extraction and 454-pyrosequencing

Several methods were trialed for the extraction of nucleic acid (RNA and DNA) from LH sediments but all failed to yield good quality extracts. The trialed methods included modifications from Griffiths et al. (2000), the use of commercial kits with and without modifications from the manufacturer instructions, as well as a combination of the above with added steps based on Chomczynski and Sacchi (2006). Several attempts were also made to wash some of the salts and other potential dissolved contaminants present in the LH samples prior to nucleic extractions. All manipulations were performed inside a safety cabinet using certified nuclease-free solutions and tubes, and all equipment used and surfaces had been sterilised with 70% ethanol solution and cleaned with RNase AWAY (Molecular BioProducts, Mexico) prior to manipulations to diminish the risk of nuclease contamination and consequent nucleic acid degradation.

The latest trial of nucleic acid extractions were performed on July 2012 and April 2012 LH sediments coming from 3 different depths: surface sediments (0-10 cmbs), shallow sediments (11-20 cmbs), and bottom sediments (21-30 cmbs). About 2 g of sediments were processed from each sample 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 the following modifications. Frozen samples in LifeGuard solution (see section 3.1) were thawed into 50 mL conical tubes on ice and the thawed sediment slurry was aliquoted into different 50 mL tubes (~ 2 mL per tube). 5 mL of DEPC-treated water was then added to each tube and these were sonicated at low power as described above (see

section 3.2.3.2) in order to dislodge cells from sediment particles. Tubes were then centrifuged at low speed at 4°C for 5 minutes in order to pellet larger sediment particles but to keep cells in suspension. Following manufacturer's instructions, the supernatants were pooled and concentrated using 15 mL Amicon tube-filters (Amicon Ultracel 30K, Millipore, Ireland) and centrifuged at 4°C in order to capture the suspended cells and nucleic acids while discarding the supernatant. The sonication and concentration steps were repeated twice on the same sediment samples. The concentrated samples were then re-suspended from the Amicon filters with 1 mL of DEPC-treated water and transferred into 7 mL polypropylene bead-beating vials (Cat. No. 3205, Biospec Products, Bartlesville, OK, USA) that had been pre-treated according to Chomczynski and Sacchi (2006) to remove any nuclease contaminants. Samples were freeze-dried and processed using MoBio kit reagents and recommended volumes. The first 4 steps of the protocol were performed inside the 7 mL bead tubes containing 0.1-mm glass beads (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and bead-beating (step 4 of the MoBio protocol) was performed using a Mini-beadbeater 24 (Biospec Products, Bartlesville, OK, USA) with two 30 second bursts and 1 minute rest on ice between bursts. The second nucleotide precipitation step was performed overnight at -20°C. Large white pellets (potentially salts) often co-precipitated with nucleotides during the first precipitation step (step 12 of the MoBio protocol) but were absent in the second precipitation. To protect extracted RNA samples from RNAse degradation, 2.5 µL of RNAseOUT (Invitrogen, Carlsbad, CA, USA) was added to 50 µL of RNA extracts. RNA samples were then treated with TurboDNAse (Ambion, Grand Island, NY, USA) as per manual instruction to remove any left-over DNA present in the RNA samples. The DNAse was inactivated using the provided DNAse inactivation reagent (Ambion, Grand Island, NY, USA) as per manual instruction. DNA and RNA samples were quantified using a NanoDrop-1000 spectrophotometer and the absence of DNA in the RNA sample was tested by PCR (see section 3.5). cDNA was synthesised using an iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using random primers provided in the kit. The cDNA reactions were then purified using 0.5 mL Amicon tubes (Amicon 30K, Millipore, Ireland).

It should be noted that none of the abovementioned techniques yielded DNA or cDNA extracts readily amplifiable by regular PCR in the laboratory. Spiking PCR positive

controls with nucleic acid extracts did not inhibit the PCR reactions indicating that the presence of PCR inhibitors were likely not the cause of the failed amplifications, but that the quality of the extracts may have been at fault (the used DNA and cDNA samples had concentrations of less than 10 ng/µL with 260/280 absorbance ratios of less than 1.00). 16S rRNA amplification by 454-pyrosequencing however yielded positive amplifications. 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'GAGTTTGATCNTGGCTCAG3'; 519R, 5'GTNTTACNGCGGCKGCTG3') (Handl et al. 2011) and archaeal (ARCH571F, 5'GCYTAAAGSRNCCGTAGC3' (Baker et al. 2003); ARCH909R (also known as 890aR), 5'TTTCAGYCTTGCGRCCGTAC3' (Burggraf et al. 1997)) primers.

3.5 Polymerase Chain Reaction (PCR) of 16S rRNA genes

PCR targeting both conserved bacterial and archaeal 16S rRNA coding sequences were performed using the following primer pairs: the Steven et al. (2007a) 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 758R (5'-CTACCAGGGTATCTAATCC-3') primers for bacterial targets as well as the Grosskopf et al. (1998) A109F (5'-ACKGCTCAGTAACACGT-3') and A934R (5'-GTGCTCCCCCGCCAATTCCT-3') primers for archaeal genes. PCR reactions (25 µL) were performed with the Qiagen HotStarTaq Plus Master Mix kit (Qiagen, Venlo, Netherlands), and comprised 12.5 µL of Master mix buffer, 2 µL of each forward and reverse primer (0.2 µM), 0.5 µL of bovine serum albumin extract (BSA), and 2 µl of template DNA. In all PCR manipulations, water was used as negative control and archaeal and bacterial DNA were used as positive controls. Thermocycling programs were described by Steven et al. (2007a) for bacterial samples and Niederberger et al. (2010) for archaeal ones.

3.6 Classification and operational taxonomic unit (OTU) analysis of 16S rRNA sequences generated by 454-pyrosequencing

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 sequence-processing pipeline outlined in Schloss et al. (2011). Original .sff files were separated into

.fasta and .qual files and filtered with "sff.multiple" with the following parameters: minflows=300, maxflows=720, minlength=200, maxhomop=8, bdiffs=1, pdiffs=2. Unique sequences were aligned against the Silva bacterial and archaeal databases with "align.seqs" (ksize=9, align=needleman, gapopen=1). Alignment of the overlapping region between sequences was ascertained using the "screen.seqs" command (criteria=95) and "filter.seqs". Chimeras were removed with "chimera.uchime" prior to clustering the aligned sequences into operational taxonomic units (OTUs) using the average-neighbour clustering with "dist.seqs" and "cluster.split" commands. Both archaeal and bacterial sequences were classified against the Greengenes database (DeSantis et al. 2006) with "classify.seqs". Taxonomic information was assigned to representative sequences of each OTU using the "classify.otu" command. Manual classification was also performed on representative sequences of major OTUs via BLASTn searches (Mount 2007) against the GenBank nt database (http://www.ncbi.nlm.nih.gov/GenBank/) with default settings, and by excluding non-cultured/environmental sequences from the target database. Alpha diversity indices were calculated using the "summary.single" command and diversity between samples was calculated using the Bray-Curtis metric and non-metric multidimensional scaling (NMDS) in Mothur. Only samples with more than 1000 sequences were considered for statistical analyses; bacterial and archaeal samples were subsampled using the "sub.sample" command to 1326 and 1051 sequences which corresponded to the lowest amount of sequences above 1000 in bacterial and archaeal libraries respectively. Shared OTUs between DNA and cDNA libraries were generated with the Mothur "venn" command. 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 original .sff files have been deposited in the NCBI Sequence Read Archive (SRA) database under the BioProject accession number PRJNA240206 in two different BioSamples, SRS582754 (April 2012 samples) and SRS585013 (July 2012 samples).

3.7 Metagenomic mining of key methanogenesis and methane-oxidation genes

Below is the relevant section written by myself of the metagenomic study "Defining the Functional Potential and Active Community Members of a Sediment Microbial

Community in a High-Arctic Hypersaline Subzero Spring" published in Applied and Environmental Microbiology, April 2013. 79(12): 3637-3648. DNA extractions from LH sediments as well as metagenome assembly are described in Lay et al. (2013).

The presence of key genes involved in methanogenesis and methane-oxidation in the metagenomics dataset of LH sediment was assessed in order to gain more genetic information on putative methane metabolisers present within the LH microbial community. In addition to automated annotations by MG-RAST (Lay et al. 2013), the complete LH metagenome was subjected to additional screenings targeting marker genes of (reverse) methanogenesis (i.e. the alpha subunit of the methyl coenzyme M reductase, *mcrA*), and methane oxidation (i.e. the alpha subunit of the particulate and soluble methane monooxigenase, *pmoA* and *mmoX*). Amino acid sequences of MCRA, PMOA, and MMOX, were recovered from the NCBI protein database (on February 16 2013) and used as target databases for alignments with the LH metagenome. BLASTX alignments were performed using the BLAST command line application (version 2.2.27+) with default algorithm parameters and an E-value cut-off of 10^{-5}. Results were then visualised and proofread in MEGAN (version 4.70.4) and hits with Bit Scores higher than 50 were considered significant (Huson et al. 2011). Reads of significant hits were then extracted and subjected to a second set of BLASTX alignments against the complete GenBank non-redundant (nr) database to ascertain their function and were finally re-annotated in MEGAN.

Chapter 4: Results

4.1 Anaerobic metabolic activity: Incubation-dependent assessments of anaerobic oxidation of methane (AOM), methanogenesis, and sulphate/sulphur reduction (SR) from LH-collected sediments

4.1.1 Anaerobic oxidation of methane

Experiments using radiolabelled methane (i.e. $^{14}CH_4$) failed to unambiguously demonstrate signs of anaerobic oxidation of methane (AOM) after two years of incubation, regardless of the incubation temperature (i.e. -5°C, 0°C, or 5°C), or the original depth of the samples (i.e. 0-15 cmbs or 16-30 cmbs). The very low levels of recovered $^{14}CO_2/^{14}CO_3^{2-}$, inferred from the CPM values on Figure 4.1, as well as the overlapping CPM values between formalin-treated (i.e. killed controls) and non-killed samples, indicate that $^{14}CH_4$ was not oxidized to $^{14}CO_2/^{14}CO_3^{2-}$ as a result of AOM metabolism.

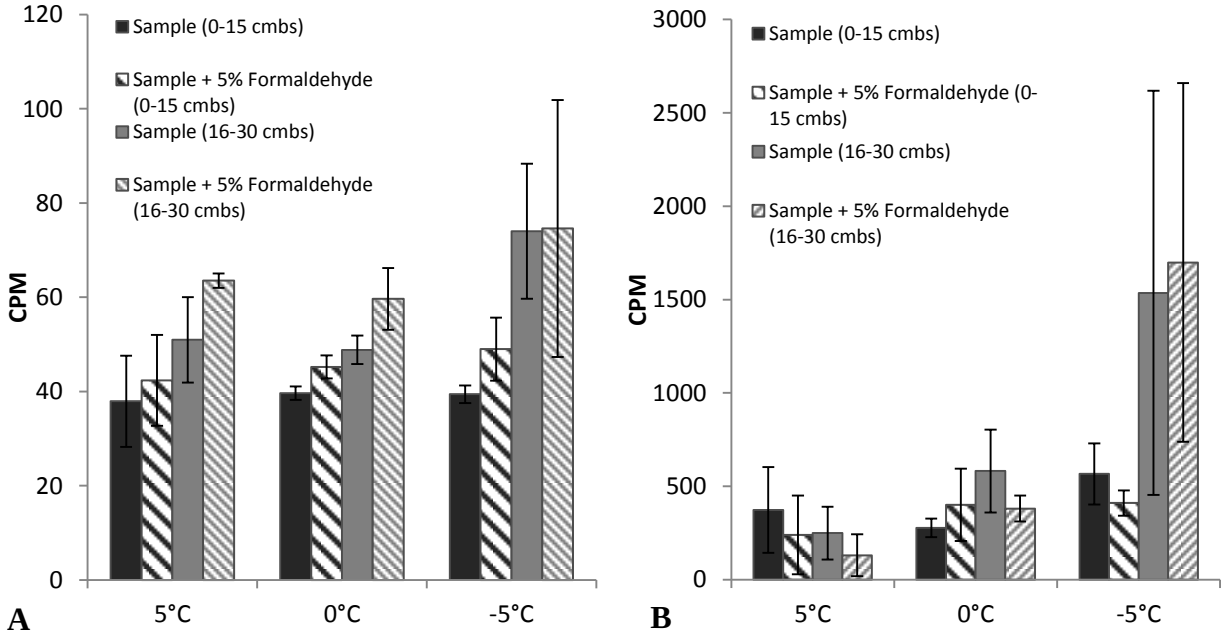


Figure 4.1 Mineralization of $^{14}CH_4$ from LH sediments collected in July 2011 and incubated for 6 months (A) and 2 years (B) at different temperatures. Error bars show standard deviations of biological triplicates.

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 (data not shown). As it was hypothesised that AOM would be coupled to sulphate reduction, sulphide levels in the headspace were also monitored in conjunction with methane measurements. No sulphide could however be detected above background levels in both 0-15 cmbs and 16-30 cmbs sediment samples incubated at either -5°C or 5°C (data not shown).

4.1.2 Methanogenesis

None of the different incubation set-ups summarised in Table 3.2 resulted in significant methane production from LH sediments throughout the course of the experiments. Even though some slow methane build-up was detected in the headspace of all incubated vials, no significant differences in methane levels could be seen between non-killed samples and killed controls regardless of the methanogenic substrate utilised. The slow increase of methane concentration in all vials is suspected to be the result of equilibration effects with the vial headspaces and dissolved or adsorbed methane present in the LH sediments/water. Background levels of ethane and propane were also observed consistently throughout the course of the incubation experiments (data not shown); considering the high levels of alkanes emanating from the LH outlet *in situ* (Niederberger et al. 2010), it is not surprising that some background alkane levels (methane, ethane, propane, etc.) were still present in the LH water and sediments during incubations.

4.1.3 Hydrogen-dependent sulphidogenesis

4.1.3.1 Assessment of psychrophilic sulphate/sulphur reduction (SR) on 2011-collected LH sediments at -5°C and 5°C

In parallel to methane measurements, microcosm vials containing 2011-collected sediments/water, and amended with either sodium acetate (N_2:CO_2 headspace) or H_2:CO_2 as energy and carbon sources, were also assayed for hydrogen sulphide (H_2S) production by putative sulphate/sulphur reducing microorganisms (SRMs). Whereas no sulphide could be detected in acetate-amended vials, microcosms with a H_2:CO_2 atmosphere evolved sulphide at 5°C and -5°C for both the 0-15 cmbs and 16-30 cmbs sediment samples; no sulphide was

produced in formaldehyde-killed controls. Sulphide release rates (SRR) were comparable for both sample depths at 5°C, yet were markedly diminished at -5°C in the 16-30 cmbs sample (Figure 4.2).

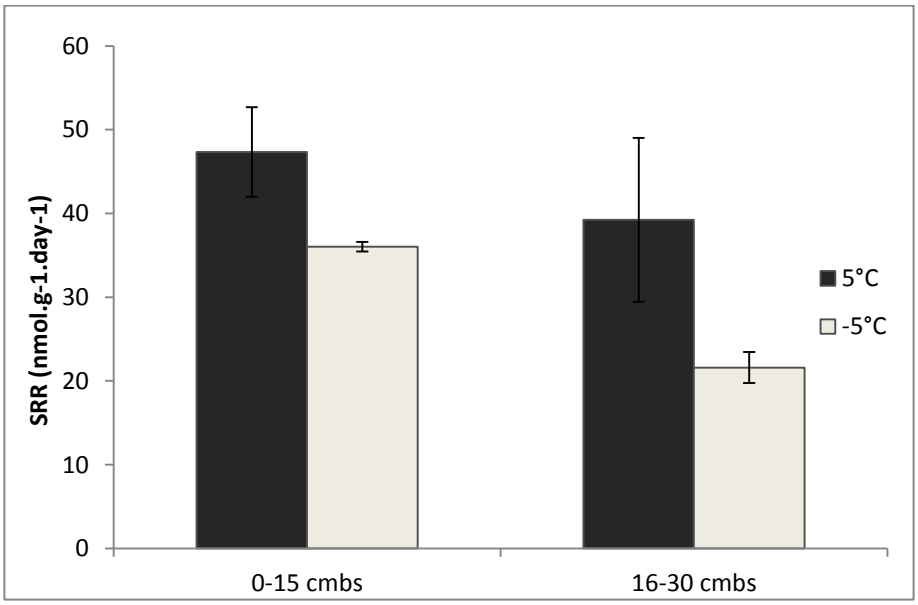


Figure 4.2 SRR from surface (0-15 cmbs) and deeper (16-30 cmbs) LH sediments incubated with H_2:CO_2. No H_2S was detected in the formaldehyde-killed controls over the duration of the incubation experiment. Error bars show standard deviation of biological triplicates.

4.1.3.2 Assessment of SR on 2012-collected LH sediments under a range of subzero temperatures

To further explore the cryophilic potential of the putative LH hydrogenotrophic SRM populations, microcosm experiments were repeated under a broader temperature range with samples collected in April 2012. Figure 4.3 shows that incubations under a H_2:CO_2 atmosphere resulted in H_2S releases down to -20°C, but that relatively little to no sulphide could be detected in formaldehyde-killed controls. The relatively large error bars seen on Figure 4.3 most likely reflect sample heterogeneity, and a better homogenisation process before vial inoculation would have likely reduced the variation in sulphide production recorded in biological replicates. The incubation period of about 190 days was too short to observe any leveling off of sulphide releases; even though the sulphide levels appear to be plateauing in the 5°C-incubated samples at ~150 days of incubation, later time point

measurements (384 days) in one of the triplicate vial showed that sulphide concentrations had roughly tripled in the vial headspace (~ 31 µmol g^{-1} sediments).

Despite the notable increases in sulphide concentrations over the span of the incubation period, no overall increase in cell numbers was observed by DAPI counts between killed (i.e. cell numbers at the beginning of the incubation period; 3.14 ± 1.82 $x10^9$ cells g^{-1} of sediments) and samples incubated for 8 months at 5°C (2.62 ± 0.84 $x10^9$ cells g^{-1} of sediments). Considering the high cell density in the observed samples, the similar counts between the formaldehyde-fixed (time 0) and 8 month samples cannot necessarily rule out an increase in putative SRM populations. It should be noted however that these counts are about four orders of magnitude higher than those obtained previously by Niederberger et al (2010), raising the question of methods replicability in DAPI cell counts.

Figure 4.4A shows that the SRR increased exponentially with temperature and that SRR were always lower in killed than non-killed samples. When graphed on an Arrhenius plot (figure 4.4B), the range of linearity extended across all incubation temperatures (i.e. 5°C to -20°C); the calculated activation energy (Ea) for sulphide production from the LH sediments was about 40.9 $kJmol^{-1}$, similar to the smallest recorded Ea (i.e. 41 $kJmol^{-1}$) for sulphate reducing consortia of Svalbard arctic sediments (Robador et al. 2009).

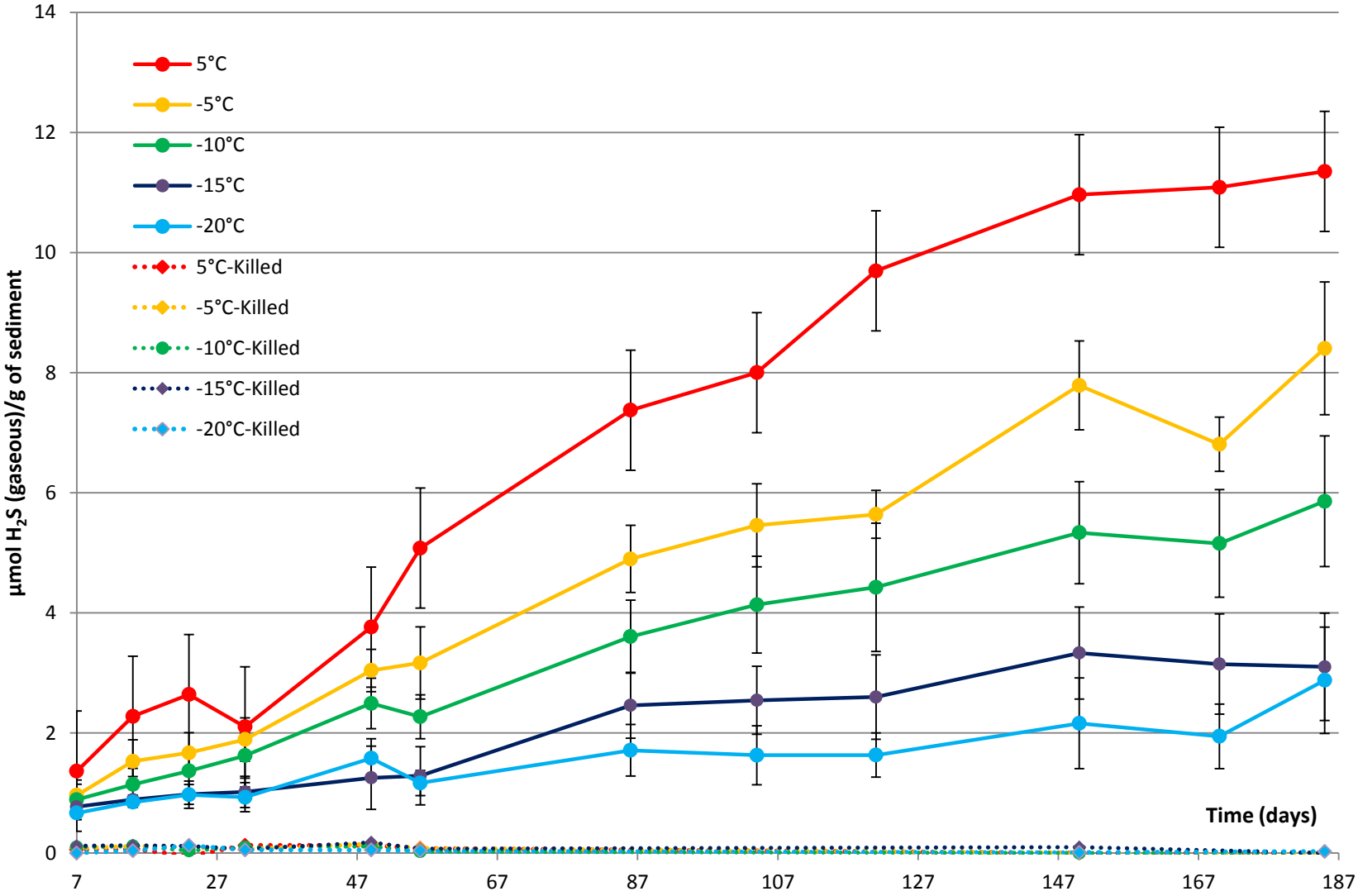


Figure 4.3 Concentration of gaseous sulphide emitted from LH sediments incubated under a H_2:CO_2 atmosphere at different cold temperatures. Dashed lines represent formaldehyde (5%) killed controls. Error bars show standard deviations of biological triplicates.

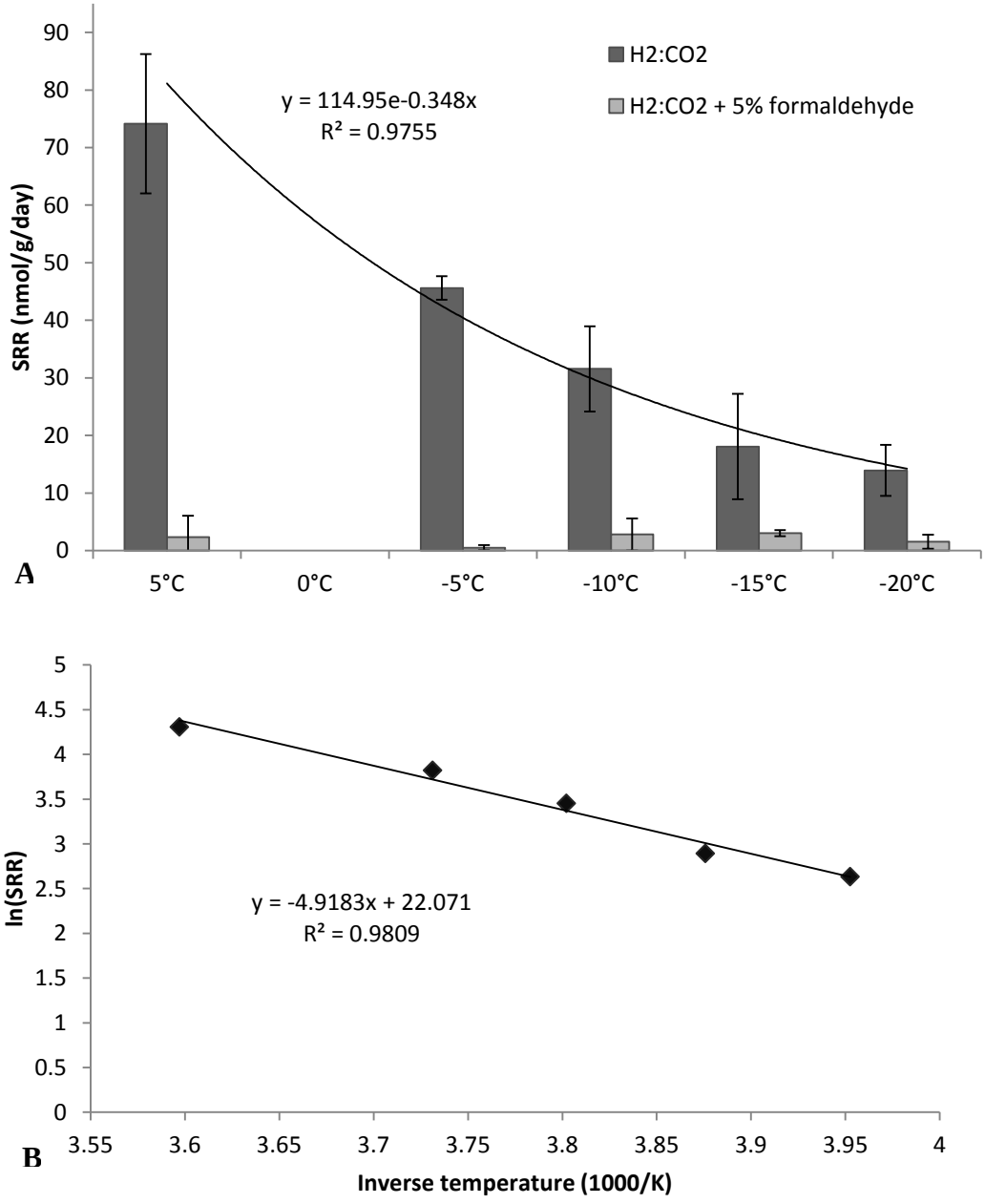


Figure 4.4 Sulphide release rates (SRR) of LH sediments incubated at different temperatures (A) and Arrhenius plot of the data presented in A (B).

4.1.3.3 Negative controls of SR for LH sediments incubated at 5°C

To verify the biogenicity of the sulphide releases, parallel incubations were set-up at 5°C using an alternative reducing agent to $Na_2S•9H_2O$, cysteine-HCl. Doing so was also to ensure that the recorded H_2S concentrations were not resulting from the dissolved reducing agent $Na_2S•9H_2O$ degassing out of solution. When H_2 gas was present in the headspace of the

incubated vials, H_2S was produced in both Na_2S and cysteine-HCl vials containing LH sediments, and, to a lesser extent, in vials without sediments (i.e. only containing filtered LH water). No sulphide was detected in the vials containing filtered water reduced with cysteine-HCl (Figure 4.5). Because of the high fluctuations of sulphide levels recorded for the vials without sediments (only filtered LH water) amended with $Na_2S•9H_2O$ (pale red line on Figure 4.5A), it was impossible to accurately calculate their H_2S emission rate. The similar sulphide production rates (Figure 4.5B) recorded for both reducing agents in sediment-containing vials, alongside the diminished or lack of sulphide production in vials without sediments, support the idea that the produced gaseous sulphide in vials containing LH sediments was biogenic in origin, or at least, that the hydrogen-dependent sulphide producing reaction was not only the result of sodium sulphide ($Na_2S•9H_2O$) degassing (Figure 4.5).

It should be noted however that the recorded rates in this experiment were significantly lower than those of the parallel 5°C incubations (Figure 4.4). 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 Figure 4.4 and Figure 4.5 may be reflective of negative effects caused by prolonged sample storage before inoculation, such as oxygen contamination or nutrient depletion.

The detectable amount of gaseous sulphide in controls containing only filtered LH water and $Na_2S•9H_2O$ should also be pointed out (Figure 5). Though relatively low, the amount of detected sulphide in the headspace of these vials after 156 days of incubation (i.e. 11.38 ± 9.75 µmol) is significantly higher than the amount of sulphide detected in formaldehyde-killed controls after the same incubation period (ranging from below detection limit to about 1 µmol) (Figure 4.3 and Figure 4.5). This difference in H_2S levels between abiotic controls (i.e. only water + $Na_2S•9H_2O$) and formaldehyde-killed controls suggests that the background sulphide levels recorded in the killed controls on Figure 4.3 were perhaps underestimated and that the addition of formalin to LH slurry may have affected abiotic H_2S releases, perhaps because of dissolved O_2 present in the formalin solution, or of a chemical reaction between sulphide and formaldehyde.

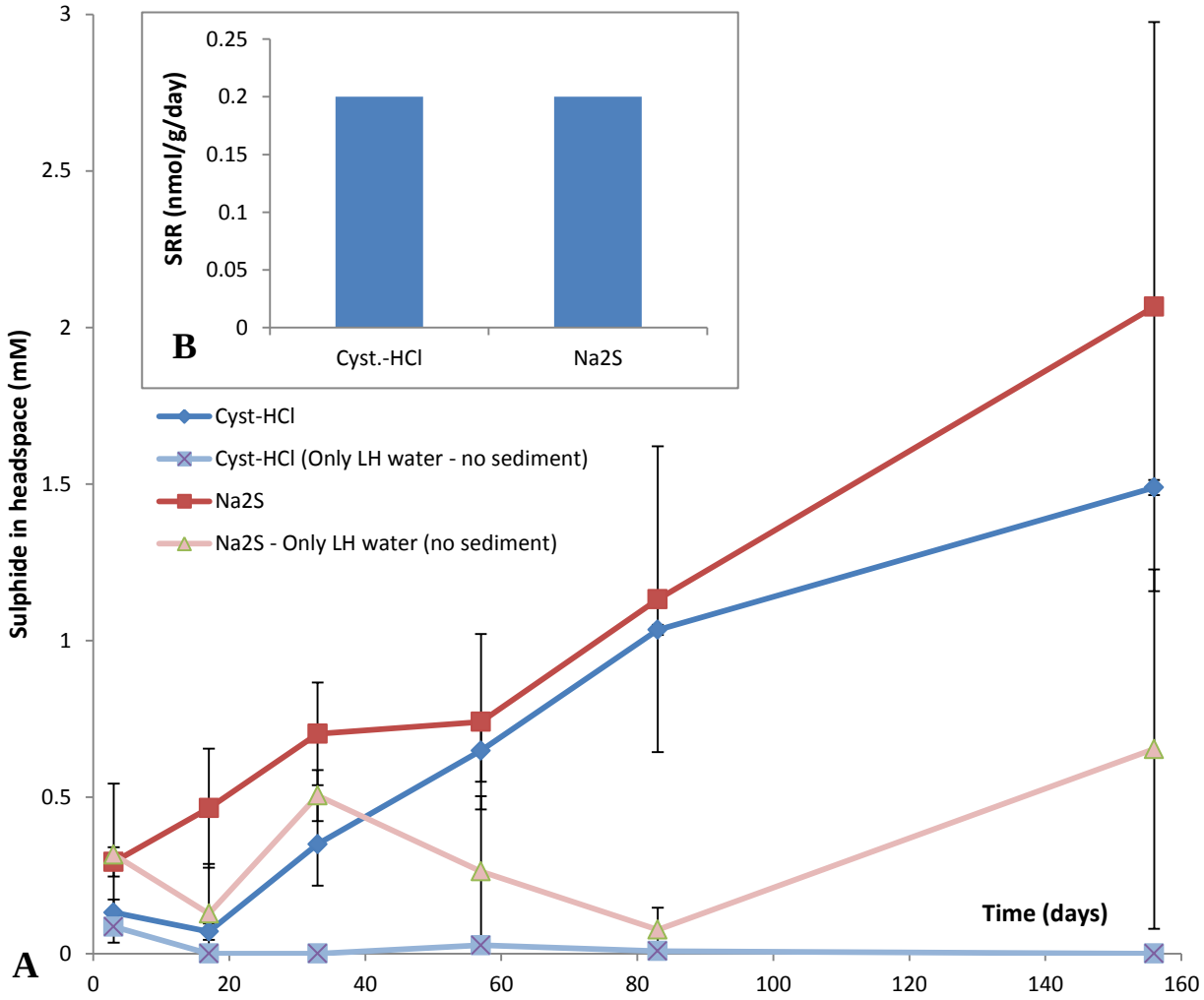


Figure 4.5 Concentrations of gaseous sulphide emitted from vials incubated with different reducing agents with or without sediments at 5°C (A) and the corresponding SRR of the vials in A incubated with sediments (B). Error bars show standard deviation of biological replicates.

4.1.3.4 Hyperthermophilic releases of H_2S from LH sediments

It should be noted that H_2S production from LH sediments was also recorded at high temperatures. As for cold incubations, vials incubated at 80°C only evolved sulphide under a H_2:CO_2 atmosphere, and not when incubated with N_2:CO_2, nor when treated with formalin, bleach, or NaOH (Figure 4.6). 80°C incubations of LH sediments with 0.05% cysteine-HCl also resulted in sulphide releases; incubations without any reducing agents but under a hydrogen atmosphere did not produce any detectable H_2S (Appendix Table 1).While these high temperature releases of H_2S from LH sediments may argue against the biogenicity of the recorded sulphide releases in the cold and/or hot temperature microcosms, solely reflecting an

increased rate of abiotic H_2S production with temperature, they could instead imply that a hyperthermophilic population of SRMs is present in the LH sediments.

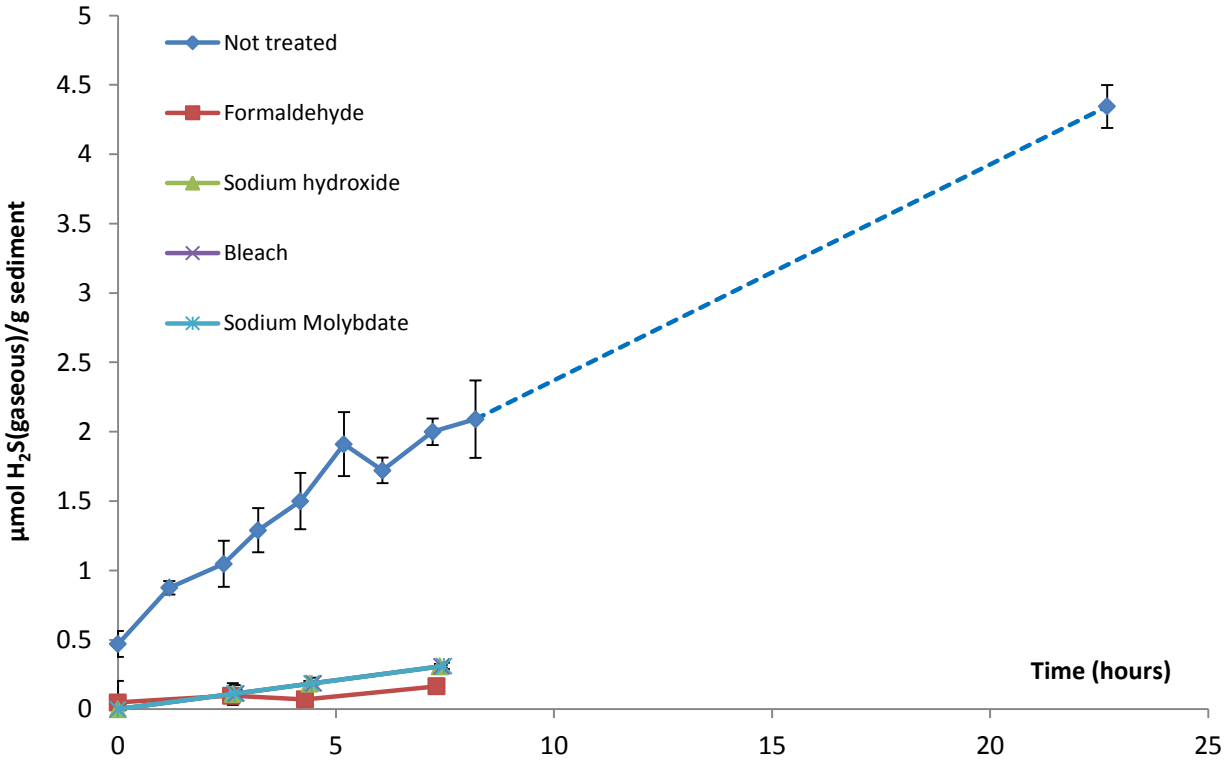


Figure 4.6 H_2S releases from LH sediments incubated under a H_2:CO_2 atmosphere at 80°C and treated with different killing agents.

4.2 16S rRNA 454-pyrotyag sequencing: Snapshots of *in situ* total (DNA) and potentially active (cDNA) LH populations

4.2.1 Sequencing coverage and biodiversity indices

Bacterial rarefaction curves show that the depth of sequencing was insufficient to fully characterise the LH communities at an OTU level of 97% identity (often referred to as the species level (Stackebrandt and Goebel 1994)) (Figure 4.7). The relatively steep shape of the curves on Figure 4.7A, and the lack of apparent plateaus at the "right end" of these curves, indeed reflects incomplete sequencing coverage, where only some of the most abundant phylotypes were most likely captured in the present sequencing survey of the LH bacterial communities. Incomplete coverage is also depicted by the calculated Chao indices (reflecting the

theoretical minimum of phylotypes in a sample) which are significantly higher than the number of OTUs obtained for all bacterial samples (Table 4.1).

Unlike bacterial curves, most archaeal rarefaction curves (except for the April cDNA 21-30 cmbs sample) were plateauing, suggesting that most of the samples' biodiversity had been sampled and sequenced (Figure 4.7 B); Good's coverages also neared 100% for all archaeal libraries further implying sufficient sequencing of archaeal samples (Table 4.1). Still, some archaeal rarefaction curves continued to increase linearly after their initial "leveling-off" indicating that probably a significant portion of rarer phylotypes have not been sampled (Figure 4.7 B). Furthermore, the very small number of archaeal OTUs characterised per sample (sometimes less than 10) raises questions regarding the legitimacy of archaeal rarefaction curves and inferred coverage (Table 4.1).

The incomplete sequencing coverage of the DNA libraries of both archaeal and bacterial communities is further illustrated by the Venn diagrams comparing cDNA and DNA samples (Figure 4.8). The higher amounts of OTUs in the cDNA libraries than DNA ones, as well as the very little amount of OTUs shared between cDNA and DNA libraries, both demonstrate that only a fraction of the LH DNA could be captured and sequenced (i.e. a DNA library should contain as many or more OTUs than its cDNA counterpart). On the other hand, the lack of significant overlap between cDNA and DNA libraries tend to suggest that most (if not all) of the LH populations depicted in the cDNA libraries were members of truly active LH communities, where their relative abundance to the total background of cells present at the LH site was too low to be detected in the DNA libraries, but could clearly be detected at the rRNA transcript level (i.e. are absent from the DNA libraries but present in the cDNA ones).

Overall, the discrepancies between DNA and cDNA libraries, as well as the incomplete sequencing depth for most samples, restrain the interpretations of the LH 2012 16S rRNA results to the most abundant and/or active taxa present in the analysed LH samples. At least regarding the archaeal samples, the quality of the nucleic acid extractions may have accounted for some of those discrepancies considering that Figure 4.7B, as well as the coverage indices listed in Table 4.1, do not suggest that an increased sequencing depth on those archaeal samples would greatly increase community coverage (i.e. curves appear to be plateauing even though not all of the samples' community has been accounted for).

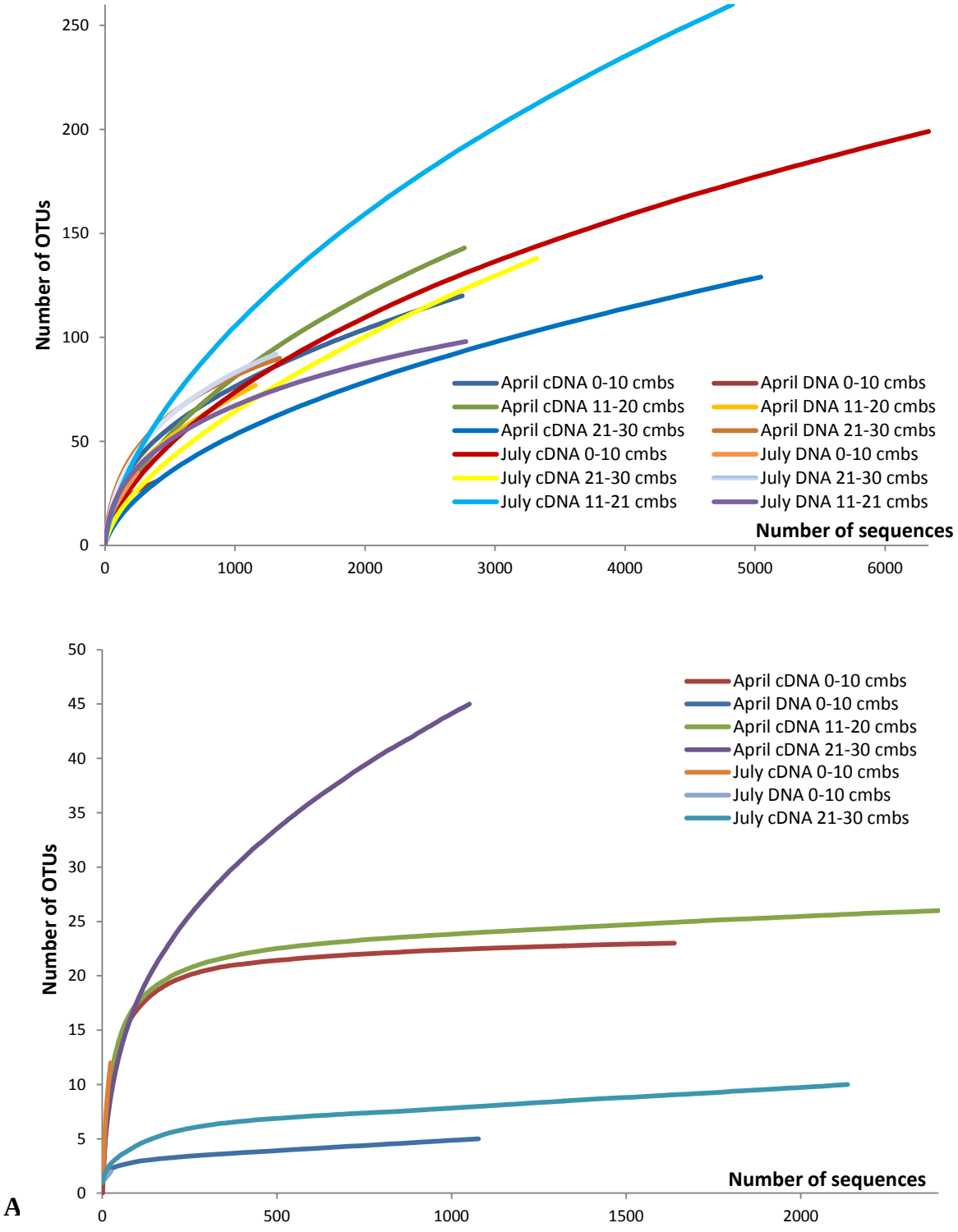


Figure 4.7 Bacterial (A) and Archaeal (B) rarefaction curves of the 16S rRNA libraries at an OTU cut-off of 97% sequence identity.

Table 4.1 Summary of biodiversity indices for the LH 16S rRNA pyrosequencing libraries

			Depth (cmbs)	# Sequences	# OTUs*	Chao	Shannon (H')	Simpson (1/D)	E ($e^{H'}/N$)	C (%)
Bacteria	**April**	**cDNA**	0-10	2748	88	188	2.46	4.3	0.13	96.28
			11-20	2764	85	157	1.64	2.1	0.06	95.94
			21-30	5044	54	108	1.00	1.4	0.05	97.58
		DNA	0-10	405	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			11-20	1157	77	151	2.65	7.1	0.18	96.80
			21-30	1343	87	118	3.15	11.6	0.27	97.41
	July	**cDNA**	0-10	6335	82	235	1.75	2.3	0.07	95.68
			11-20	4826	111	258	2.28	3.2	0.09	94.21
			21-30	3320	84	229	1.45	1.8	0.05	95.07
		DNA	0-10	248	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			11-20	2776	66	89	2.62	6.3	0.21	98.01
			21-30	1315	88	128	2.75	6.3	0.18	97.06
Archaea	**April**	**cDNA**	0-10	1636	22	22	2.53	9.4	0.57	99.90
			11-20	2394	24	25	2.48	8.0	0.50	99.81
			21-30	1051	45	67	2.23	5.3	0.21	98.29
		DNA	0-10	1077	5	6	0	1	0	100
			11-20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			21-30	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	July	**cDNA**	0-10	24	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			11-20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			21-30	2134	9	12	0.58	1.3	0.20	99.71
		DNA	0-10	25	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			11-20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
			21-30	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

E, C, D, and N respectively stand for a sample's evenness, Good's sampling coverage, Simpson's index, and total number of OTUs.

N.D. Not determined

*OTU cut-off of >97% sequence identity

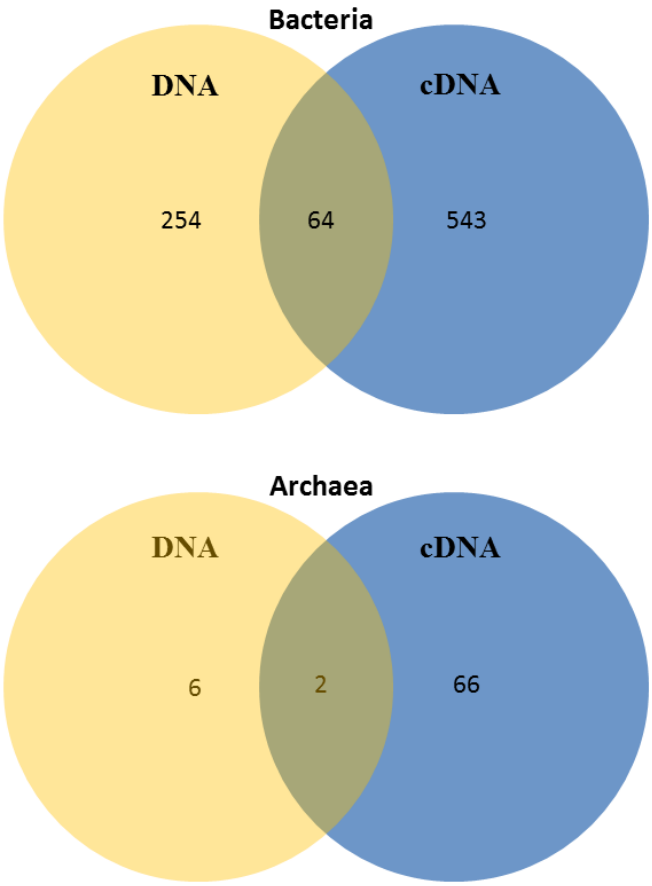


Figure 4.8 Venn diagrams showing the amount of OTUs (>97% identity) shared between all cDNA and all DNA LH 16S rRNA libraries for both bacteria (A) and archaea (B) samples.

4.2.2 Profile and structure stability of LH 2012 bacterial communities

Figure 4.9 portrays the bacterial community profile of the LH outlet sediments as fairly 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 can 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 deep sample as well as mid-layer samples (11-20 cmbs). 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 (Figure 4.9). These differences in community structure between DNA and cDNA libraries suggest a clear distinction between background

(dormant/dead cells) and potentially active (cDNA) communities. It also suggests that LH most active populations may only account for a fraction of the total microbial biomass present on site (i.e. a disproportion of important taxa between DNA and cDNA samples) hinting at an active LH community that is distinct from the background DNA pool.

The observation that less abundant clades may account for the most active members of the LH communities is further highlighted at the OTU level (97% similarity). The absence of most cDNA OTUs in the DNA libraries (and vice-versa) stresses that relatively more active OTUs (i.e. the ones dominating the cDNA libraries) were relatively rare in the LH samples (i.e. absent, or present in very low abundance, in the DNA libraries). This is partially illustrated in the Venn diagrams where only a fraction of sample-overlap exists between both DNA and cDNA samples (Figure 4.8), on Figure 4.11 where cDNA samples are distant from the DNA ones, and also by the fact that several abundant cDNA OTUs were completely absent from the DNA libraries (Table 4.2).

Figure 4.9 Relative abundance of LH bacterial phyla and *Proteobacteria* classes (A) and archaeal classes (B) of the16S 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 sulphur and/or sulphate reducing bacteria.

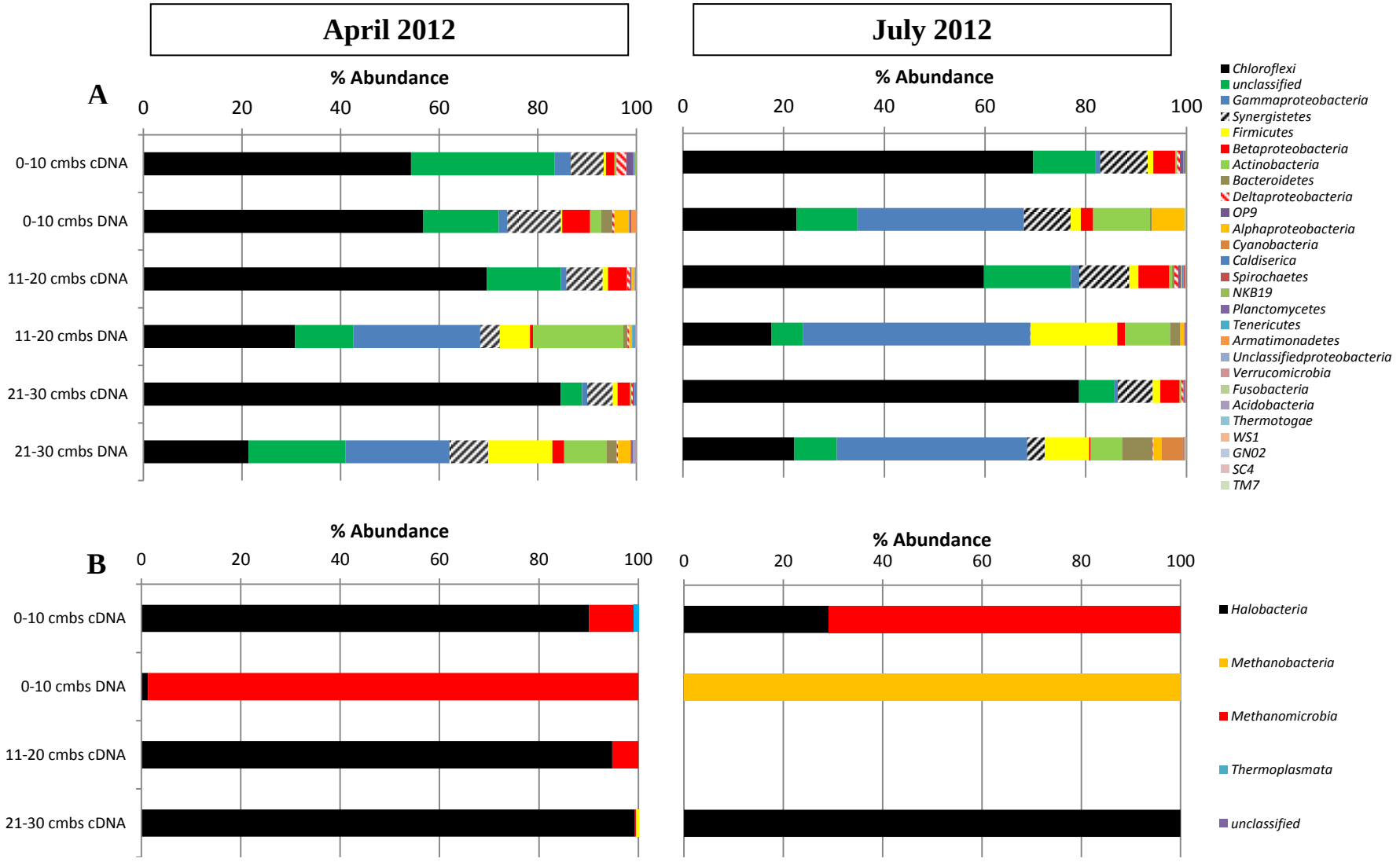
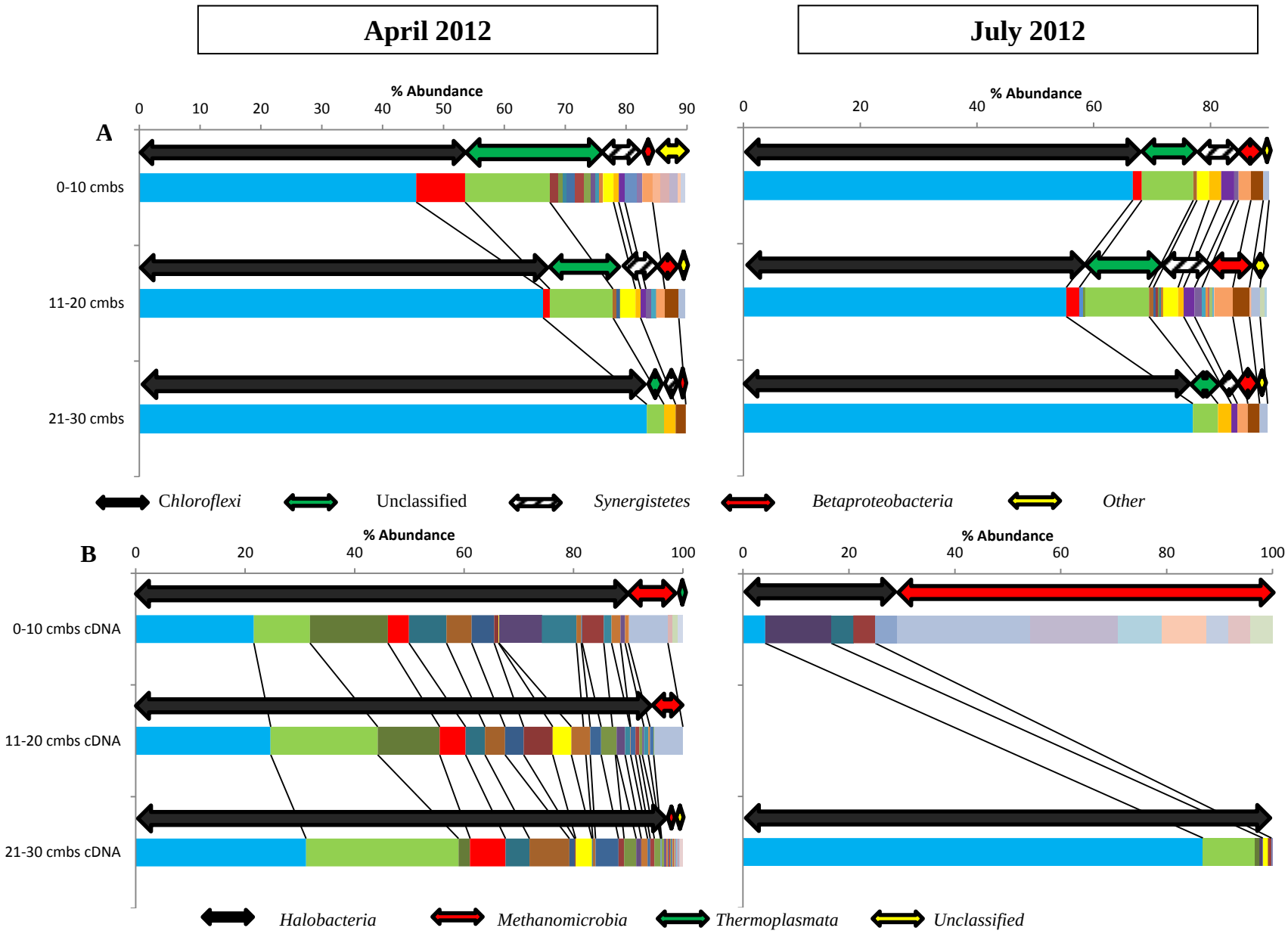

April 2012
July 2012
A
% Abundance
0
20
40
60
80
100
0-10 cmbs cDNA
0-10 cmbs DNA
11-20 cmbs cDNA
11-20 cmbs DNA
21-30 cmbs cDNA
21-30 cmbs DNA
Chloroflexi
unclassified
Gammaproteobacteria
Synergistetes
Firmicutes
Betaproteobacteria
Actinobacteria
Bacteroidetes
Deltaproteobacteria
OP9
Alphaproteobacteria
Cyanobacteria
Caldiserica
Spirochaetes
NKB19
Planctomycetes
Tenericutes
Armatimonadetes
Unclassifiedproteobacteria
Verrucomicrobia
Fusobacteria
Acidobacteria
Thermotogae
WS1
GN02
SC4
TM7
B
Halobacteria
Methanobacteria
Methanomicrobia
Thermoplasmata
unclassified

Figure 4.10 Relative abundance of the archaeal (B) and top 90% most abundant bacterial (A) OTUs (>97% sequence identity) in the LH 2012 pyrosequencing libraries. Black lines connect same OTUs present in different libraries of the same sampling season. Coloured 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


April 2012
July 2012
% Abundance
0 10 20 30 40 50 60 70 80 90
0 20 40 60 80
A
0-10 cmbs
11-20 cmbs
21-30 cmbs
Chloroflexi
Unclassified
Synergistetes
Betaproteobacteria
Other
% Abundance
0 20 40 60 80 100
B
0-10 cmbs cDNA
11-20 cmbs cDNA
21-30 cmbs cDNA
Halobacteria
Methanomicrobia
Thermoplasmata
Unclassified

4.2.2.1 16S rRNA libraries (cDNA): LH potentially active bacterial communities

Despite cladistic similarities between cDNA and DNA libraries (and between samples) at the phylum and class levels (Figure 4.9), more variability could be seen between DNA and cDNA samples at the species level (i.e. >97% sequence identity). The clear separation between potentially active (cDNA) and total (DNA) LH communities is depicted on figure 4.11, with more closely clustered cDNA samples and loosely associated DNA ones. July cDNA communities appeared to be more closely associated than their April counterparts (figure 4.11), a trend also illustrated by their increased numbers of shared dominant OTUs when compared to April communities (figure 4.10). Some depth-related patterns also seemed to emerge for the different cDNA libraries, with an apparent diminution in species richness with depth for the April samples (table 4.1 and figure 4.10). Whereas LH deepest communities appeared relatively stable seasonally (figure 4.10 and 4.11), an apparent seasonal switch in biodiversity was observed for the shallower samples, with more diverse communities switching from the surface sediment layer in April (when the LH tufa is full) to the middle layer in July (when the LH tufa is nearly-empty) (figure 4.10). More replicate samples would however be needed to strengthen these observations. Again, these differences in community composition may represent artifacts of sampling or sequencing depth/coverage, but may also reflect genuine changes in community structure, both seasonally and/or with depth, at the species level (i.e. >97% identity).

These observed seasonal and depth changes in community richness however mostly affected rarer LH phylotypes, and, overall, LH cDNA libraries were compositionally similar (figures 4.10 and 4.11). The most abundant OTUs were indeed generally present across all cDNA libraries for the dominant *Chloroflexi*, unclassified, *Synergistetes*, and *Betaproteobacteria* phyla (figure 4.10). A noteworthy feature was the dominance of a single *Chloroflexi* OTU among all cDNA samples, which accounted for at least 45%, and up to more than 80%, of the total bacterial communities depending on the library (figure 4.10).

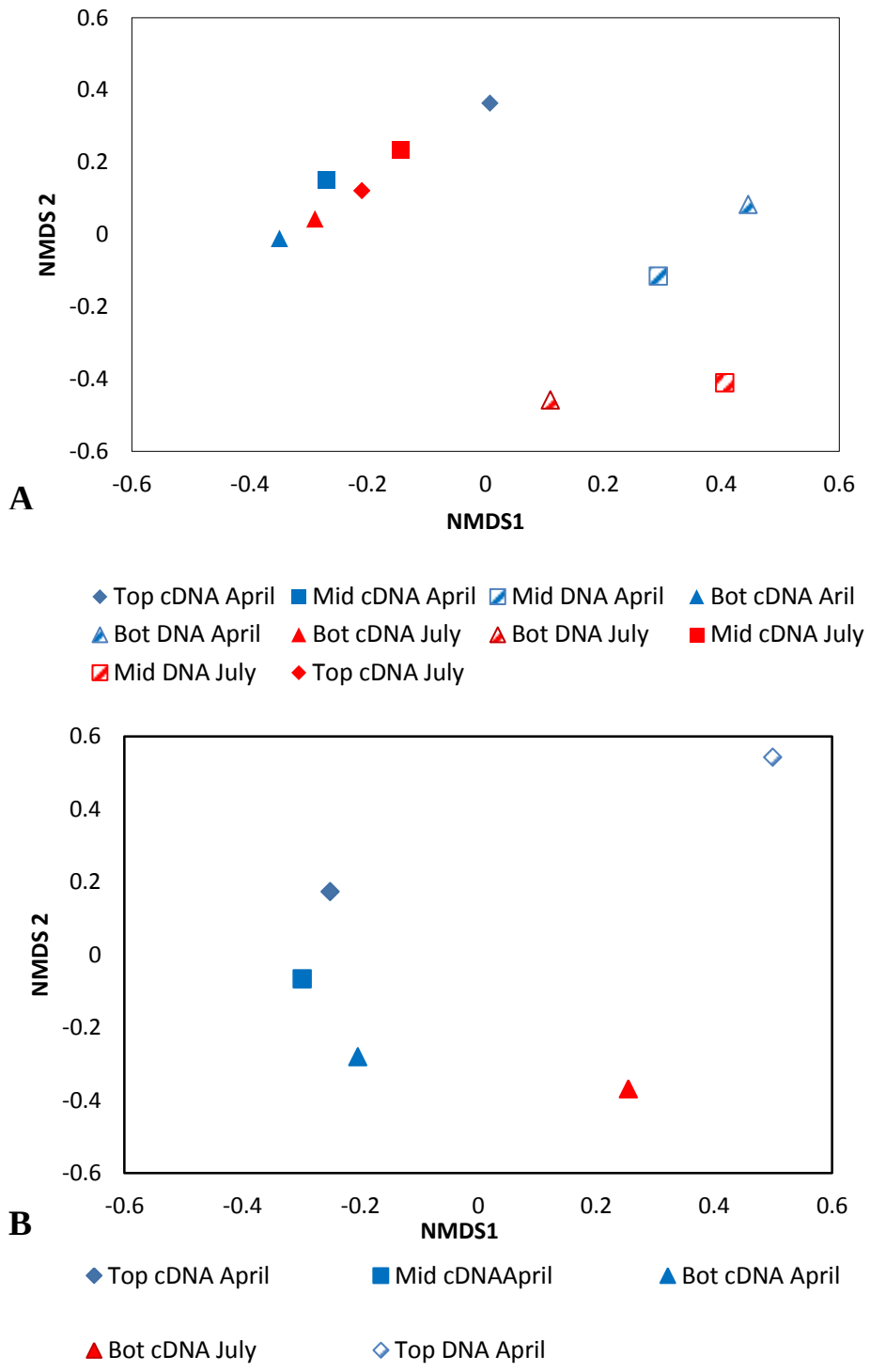


Figure 4.11 NMDS ordinations of Bray-Curtis community dissimilarity of bacterial (A) and archaeal (B) LH 16S rRNA libraries.

4.2.2.2 Bacterial OTUs classification

Table 4.2 shows that the most abundant OTU, which dominated all of the LH cDNA libraries (pale blue *Chloroflexi* OTU on Figure 4.10 A), was related to the unclassified clade T78 of the *Anaerolineales* family based on Greengenes taxonomy. The second most abundant *Chloroflexi* OTU present among the LH cDNA libraries also belonged to an uncultured clade of *Anaerolineales Chloroflexi* (table 4.2). BLAST searches against the NCBI nt database revealed close resemblances of LH *Chloroflexi* to sequences isolated from anaerobic digesters/sludge; both OTUs were however only distantly related to culture representatives, with closest representatives belonging to the non-photosynthetic *Chloroflexi Longillinea arvoryzae* or *Leptolinea tardivitalis* (Table 4.2) (Yamada et al. 2007; Yamada et al. 2006). Consistent with the LH spring anoxic and methane rich nature, these closest BLAST representatives were isolated from environments bearing similar conditions to LH (i.e. mainly anaerobic digesters and/or methanogenic consortia) suggesting that the LH *Chloroflexi* members are most likely anaerobes that may benefit either directly or indirectly from environments rich in methane.

Most *Synergistetes* and Unclassified OTUs were affiliated with clone sequences from anoxic environments rich in hydrogen gas, with closest culture representatives isolated from rice fields, oil beds, or other anoxic environments (Table 4.2). Of special interests was the presence of some Unclassified OTUs which, even though only distantly related, had for closest cultured isolates the salt-tolerant, hydrogenotrophic, sulphate-reducing bacteria *Desulfohalobium retbaense* DSM 5692 strain, isolated from a hypersaline lake, in line with the hypersaline nature of LH (data not shown). LH members of the *Betaproteobacteria* also mainly matched sequences isolated from settings rich in hydrocarbons, although not necessarily anoxic ones (Table 4.2). *Deltaproteobacteria* sequences essentially matched *Syntrophus* or *Desulfovibrio* species of methanogenic and/or hydrocarbon-rich environments (Table 4.2), again, environments bearing similar conditions to the LH spring.

Overall, most dominant cDNA OTUs had cDNA:DNA 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 4.2). These elevated ratios indicate that the dominant LH phylotypes characterised in cDNA libraries appeared relatively active under *in situ* conditions, both during winter and summer, and, across the whole 30 cm sediment layer.

Table 4.2 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 *Delta-proteobacteria*

	Mothur classification (Greengenes database)		Information of closest BLASTn representative (sequences from uncultured strains)			
	Representative sequence[1]	Closest taxonomic ID[2]	Isolation location	E-value	Identities	Acc. Number
Chloroflexi	HXXTI5Q01A5Q18	g: T78 (98)	Full-Scale Anaerobic Digesters	6 E-101	100%	JQ117024.1
	HWU2KQQ01B2NJP	g: WCHB1-05 (90)	Anaerobic sludge	4 E-98	99%	CU924139.1
Unclassified	HWU2KQQ01EL1E5	k: Bacteria (100)	Hydrogen production in a microbial electrolysis cell	2 E-100	100%	GQ152935.1
	H3NC2GB02DSUH6	k: Bacteria (100)	Anaerobic enrichment culture involved in microsystin-LR degradation	1 E-103	100%	AB896655.1
Synergistetes	HWU2KQQ01EEHHK	g: HA73 (100)	Full-Scale Anaerobic Digesters	0.E+00	99%	AB780941.
	HWU2KQQ01CI5CD	g: *Aminobacterium* (100)	*Aminobacterium colombiense* DSM 12261	0.E+00	99%	NR_074624
Beta-proteobacteria	HWU2KQQ01EE2SE	g: *Ralstonia* (100)	South China Sea	0.E+00	98%	GU940717.
	H3NC2GB02EHTQ2	g: *Pelomonas* (95)	Soil around a coal gangue dump	0.E+00	99%	KF506873.1
Delta-proteobacteria	HWU2KQQ01EBPUH	s: *Syntrophus* sp. (100)	Anaerobic digester	4.E-154	100%	JQ167107.1
	HWU2KQQ01D7FVJ	s: *Desulfovibrio aminophilu* (100)	Low temperature oil reservoir fluids	4.E-164	99%	JQ256500.1

N.B. The cDNA:DNA ratios for all above OTUs were as follow for the April and July top, middle, and bottom samples respectively, with rations higher than 1 in bold: HXXTI5Q01A5Q18 (0.8, **2.3**, **4.1**, **3.5**, **4.1**, **7.5**), HWU2KQQ01B2NJP (no DNA, **1.1**, 0.4, no DNA, **1.3**, 0.1), HWU2KQQ01EL1E5 (**2.6**, **2.7**, 0.7, 0.9, **3.5**, 0.9), H3NC2GB02DSUH6 (0, 0.7, 0.1, no DNA, no DNA, no DNA), HWU2KQQ01EEHHK (**6.8**, **3.3**, 0.4, 0.5, no DNA, no DNA), HWU2KQQ01CI5CD (no DNA, 0.3, no DNA, no DNA, no DNA, no DNA), HWU2KQQ01EE2SE (0.5, **16.8**, 0.6, **5.2**, no DNA, **7.8**), H3NC2GB02EHTQ2 (0, no DNA, 1.8, 1, no DNA, no DNA), HWU2KQQ01EBPUH (no DNA, no DNA, 0.13, no DNA, no DNA, no DNA), HWU2KQQ01D7FVJ (0.15, no DNA, no DNA, **1.58**, no DNA, no DNA).

[1] The names of the representative sequences correspond to the read ID assigned by the pyrosequencing centre.

[2] 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.

Table 4.2 (*continued*)

	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	Gangxi Oil Bed	4 E-82	95%	GU129077.1
	Aminobacterium colombiense DSM	Anaerobic dairy wastewater lagoon	7 E-105	100%	NR_074624.1
Beta-proteobacteria	*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
Delta-proteobacteria	*Syntrophus* sp.	Methane formation from long-chain alkanes by anaerobic	4.E-119	93%	AJ133795.1
	Desulfovibrio sp. VKM B-2200	Anaerobic microbial community capable of degrading p-	2.E-156	99%	FJ606758.1

4.2.3 Profile and structure stability of LH 2012 archaeal communities

LH archaeal communities exhibited less diversity than bacterial ones, with archaeal samples only comprised of very few different taxonomical classes, all belonging to the *Euryarchaeota* phylum. Aside from the July-upper-sediment-layer sample, of which about 70% was composed of *Methanomicrobia*, *Halobacteria* dominated all of the LH cDNA libraries, with an apparent increase in abundance with sediment depth (Figure 4.9). In contrast, *Halobacteria* only accounted for a small portion of the April upper sediment DNA library and were absent from the July one, both mainly comprised of either *Methanomicrobia* (April) or *Methanobacteria* (July) (Figure 4.9B). 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; despite for a few reads, *Methanobacteria*-related sequences were present only in the July 0-11 cmbs DNA library (Figure 4.9B). Even more pronounced than for the bacterial libraries, the differences between cDNA and DNA samples (at least for the surface samples) point out toward an active archaeal communities at the LH site comprised mainly of *Halobacteria*.

Unlike bacterial samples however, LH archaeal communities appeared to be more stable even at the species level (OTU level of 97% similarities) throughout the surface 30 cm LH sediment layers, at least for the April samples (Figure 4.10B). A decrease in species richness (with respect to the April samples) characterised the July 21-30 cmbs sample, with a marked increase in relative abundance of the major *Halobacteria* OTU that was also present in the April samples (Figure 4.10B). These differences in community structures are further highlighted on Figure 4.11B where April cDNA samples clearly form an outgroup distinct from the July cDNA samples and April DNA ones. Though distinct, the bottom July cDNA sample most closely associate with the bottom April cDNA samples, likely reflecting conserved archaeal community compositions seasonally (Figure 4.11).

Table 4.3 shows that the major *Halobacteria* OTUs are associated to species representatives of *Haloquadratum*, *Halorubrum*, *Halobellus*, and *Halobacterium* of different hypersaline environments. The main *Methanobacteria* OTU present in LH cDNA archaeal libraries was related to *Methanosaeta* species of methanogens 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 account for a major component of the total archaeal LH community, they appear to be significantly less active than their *Halobacteria* counterparts (Table 4.3).

Table 4.3 Taxonomic information of the representative sequences of the ten most abundant archaeal OTUs among all 16S rRNA libraries

Mothur classification (Greengenes database)		**Information of closest BLASTn representative (sequences from uncultured strains)**			
Representative sequence[1]	Closest taxonomic ID[2]	Isolation location	E-value	Identities	Acc. Number
HXXTI5Q02C2RR6	g: *Haloquadratum* (100)	Solar saltern 'Bras del Port'	0	99%	HQ455543.1
HXXTI5Q02DQ7YO	g: *Halorubrum* (54)	Solar saltern 'Bras del Port'	0	99%	HQ455545.1
HXXTI5Q02EA27Y	g: *Halorubrum* (100)	Aran-Bidgol Salt Lake (Hypersaline Playa in Iran)	0	99%	HQ425168.1
H4685KH04JKHA2	g: *Halorubrum* (100)	Aquatic environments of the high altitude Andean Altiplano (northern Chile)	2E-133	99%	EF632687.1
HXXTI5Q02DPDZX	Unclassified	Great salt plains of Oklahoma	7E-138	96%	FJ696261.1
HXXTI5Q02C926P	g: *Halorubrum* (89)	Hypersaline lake	2E-157	96%	JQ033974.1
HXXTI5Q02DXJVA	g: *Methanosaeta* (100)	Hydrocarbon contaminated aquifer	0	99%	JQ087754.1
HXXTI5Q02EUKHQ	g: *Halorubrum* (100)	Yuncheng salt lake, Shanxi, China	0	97%	JN216861.1
HXXTI5Q02DS2T1	g: *Halobacterium* (100)	Salterns of Sfax Tunisia	0	98%	JX982770.1
HXXTI5Q02EH4XY	Unclassified	Hypersaline Environments	1E-111	98%	JN839744.1

Table 4.3 (*continued*)

	Information of closest BLASTn representative (sequences from cultured strains)				
Representative sequence	**Closest culture representative**	**Isolation location**	E-value	Identities	Acc. Number
HXXTI5Q02C2RR6	*Haloquadratum walsbyi*	Solar saltern	0	99%	NR_074200.1
HXXTI5Q02DQ7YO	*Halorubrum* sp. DV427	Ancient halite, Death Valley California	5E-149	91%	FJ492047.1
HXXTI5Q02EA27Y	*Haloarchaeon* CSW1.15.5	Magnesium-rich bittern brine from a Tunisian solar saltern	0	99%	FN994962.1
H4685KH04JKHA2	*Halorubrum arcis strain* AJ201	Saline lake on the Qinghai-Tibet Plateau, China	2E-123	97%	NR_028226.1
HXXTI5Q02DPDZX	*Halobellus salinus*	Marine solar salterns	3E-131	95%	HQ451075.1
HXXTI5Q02C926P	*Halorubrum aquaticum*	Hypersaline lakes	5E-139	93%	AM268115.1
HXXTI5Q02DXJVA	*Methanosaeta concilii* GP6	Anaerobic sludge - municipal sewage treatment plant	0	99%	NR_102903.1
HXXTI5Q02EUKHQ	*Halorubrum* sp. YC-X2	Yuncheng salt lake, Shanxi, China	0	97%	JN216861.1
HXXTI5Q02DS2T1	*Halobacterium salinarum* strain ETD5	Salterns of Sfax Tunisia	0	98%	JX982770.1
HXXTI5Q02EH4XY	*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 0.08.

[1] The names of the representative sequences correspond to the read ID assigned by the pyrosequencing centre.

[2] 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 after the "Closest taxonomic ID" defines the percentage bootstrap confidence level that the classified sequence matches the given taxonomic ID.

4.2.4 Comparison of the 2012 LH 16S rRNA libraries with previous years' community profiles

When comparing the 16S rRNA surveys of the LH-spring surface sediments over the span of 6 years, similarities, but also notable differences, in the LH community profiles can be seen for both archaea and bacteria (Figure 4.12). For example, whereas *Alpha-*, *Beta-*, *Gammaproteobacteria*, and *Firmicutes* accounted for a major portion of the community in each studies, dominant phyla in one survey, such as *Bacteroidetes*, *Verrucomicrobia*, *Synergistetes*, and *Chloroflexi*, were completely absent from another (Figure 4.12). A similar trend also characterised archaeal profiles, with *Methanomicrobia* dominating most libraries, but with a very large portion of *Crenarchaeota*, *Thaumarchaeota*, or Unclassified archaea only present in some surveys and absent from others (it should be reiterated here however that the July 2012 archaeal datasets contained very few reads and that these community profiles are most likely biased) (Figure 4.12). It should also be pointed out that whereas the *Methanomicrobia* members described in Niederberger et al. (2010) study belonged entirely to the ANME-1a clade, *Methanomicrobia* sequences present in the 2010 and 2012 libraries were more related to methanogens than anaerobic methane oxidizers (Lay et al . 2013).

Where some variations in DNA community structure would be expected from background, dormant, or dead cells, (e.g. from aerial deposition of allochtonous cells), the pronounced differences between 2010 and 2012 cDNA libraries (supposedly representative of active communities) are somewhat surprising. Some of the observed differences can certainly be explained by the difference in techniques used to generate these different libraries, especially between the 2006 and the rest of the datasets (i.e. clone libraries versus 454-pyrosequencing); it should be noted however that the same primer sets were used by Lay et al 2013 and the present study to generate 16S rRNA libraries, which would exclude primer biases to account for the different results between these two studies. But the fact that these differences not only apply to the "rare biosphere" is interesting and raises some questions. They suggest that the LH community is either changing over time (though not too much seasonally according to Figure 4.9), or that each different studies only managed to capture a (slightly) different snapshot of the complete LH sediment community, not only with respect to the rare taxa, but also dominant ones (Figure 4.12).

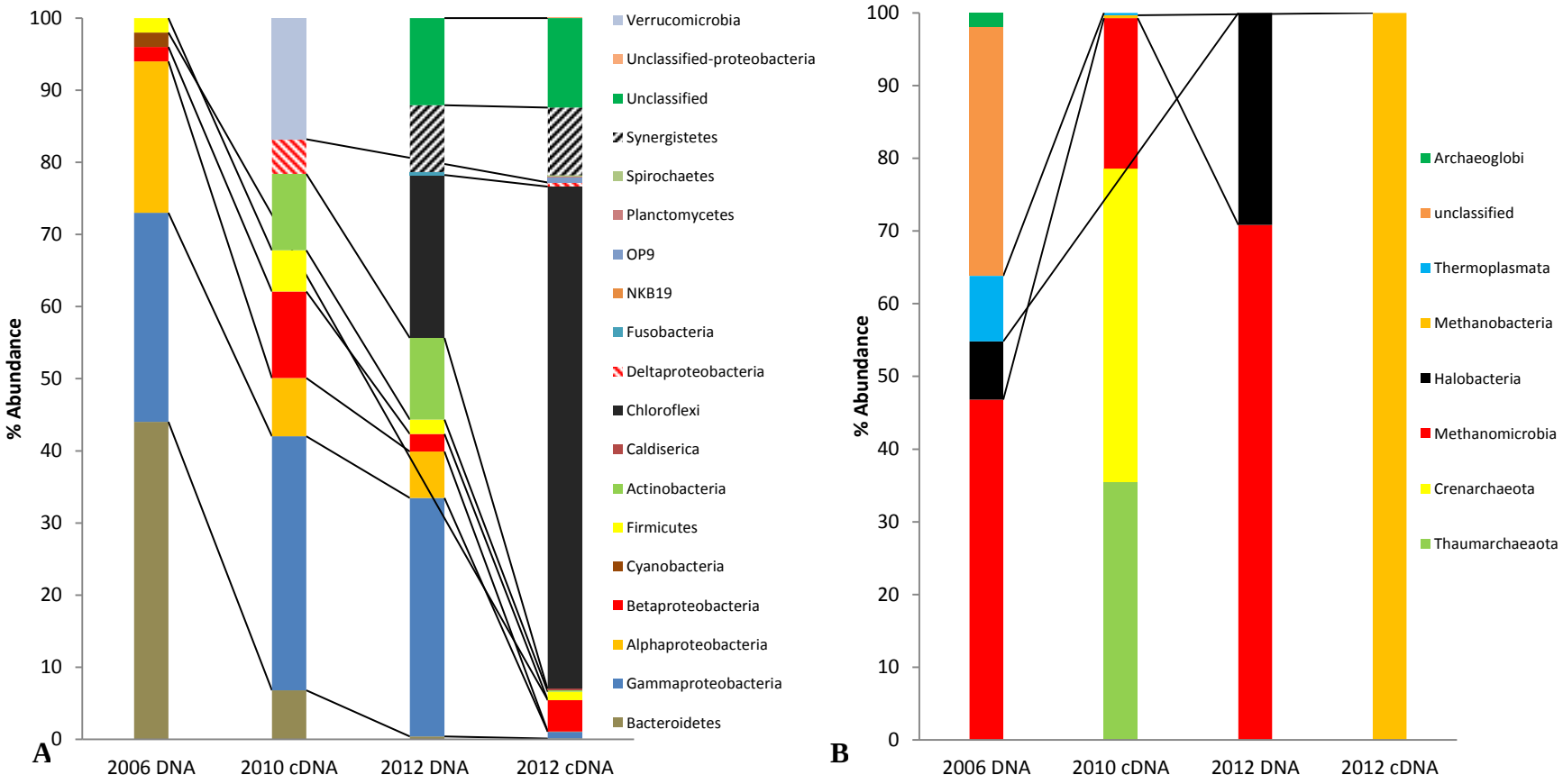


Figure 4.12 Comparison of the different 16S rRNA surveys of the top 5 cm of the LH July sediments over the span of 6 years for both bacterial (A) and archaeal (B) communities. DNA and cDNA labels refer to the type of 16S rRNA sample analysed (i.e. 16S rRNA genes or transcripts). 2006, 2010, and 2012 data were taken from Niederberger et al. (2010), Lay et al. (2013), and the present study respectively.

4.3 Looking for key methanogenesis and methane-oxidation genes in the metagenome of LH spring outlet sediments

Only one gene probably involved in central carbon metabolism in ANME organisms, a putative carbon monoxide dehydrogenase/acetyl coenzyme A (acetyl-CoA) synthase (EC 2.3.1.169) (2 hits; 85% identity), was detected in the LH metagenome (Lay et al. 2013). The presence of the gene encoding the enzyme of the last step of methanogenesis (or the first step of AOM), methyl-coenzyme M reductase (MCR), was not confirmed in the LH metagenome. An additional screening of the metagenome against an MCR target database did identify potential (MCR) homologs, but these sequences most closely matched ABC transporters/ATP-binding proteins when compared to the GenBank nr database. Considering the low frequency of other methanogenesis genes recovered, the absence of *mcr* sequences in the annotated LH data set may result from insufficient sequencing coverage. Similar results have also been found in other metagenomics studies of deep subsurface marine sediments where genes involved in methanogenesis were found but no *mcr* sequences were recovered (Teske and Biddle 2008).

Despite the presence of several reads related to known bacterial clades containing methanotrophic members (i.e., *Gammaproteobacteria* and *Betaproteobacteria*), no gene hits relating to the key enzyme in aerobic methanotrophy, the particulate or soluble methane monooxygenase (i.e., PMO or MMO), were obtained by either MG-RAST or MEGAN annotation (Lay et al. 2013).

4.4 CARD-FISH

Despite several CARD-FISH trials and troubleshooting attempts following Wendeberg et a. (2010) recommendations, no clear CARD-FISH signals could be detected for ANME-1 cells, and no cell count could be accurately performed on samples hybridized with HRP probes due to high background fluorescence. Considering the age of the used sample (i.e. ~ 2 years) and the lack of sample preservation for CARD-FISH analyses (i.e. was not fixed on site in paraformaldehyde nor stored in PBS-ethanol solutions at -20°C), rRNA degradation may have accounted for the failed CARD-FISH attempts. The presence of hydrohalite crystals may have also contributed to the high background fluorescence as reported by Niederberger et al. (2010).

Chapter 5: Discussion

Assessing microbial activity at the LH site

Previous investigations on the Lost Hammer spring, the coldest hypersaline spring surfacing through thick layers of permafrost on Earth, raised the possibility that it is populated by indigenous communities that are metabolically active despite the extreme *in situ* conditions (Niederberger et al. 2010). The present research on LH could not unambiguously confirm the habitability of the LH system, but did provide additional evidence that putatively active cryophilic microorganisms are present *in situ*, and insight regarding their potential metabolism. Considering the LH site's uniqueness, the obtained results, if confirmed, will broaden our understanding of anaerobic microbial metabolism under combined subzero and hypersaline conditions on Earth, and also contribute to better assess what type of microbial metabolism, if any, could be favorable on other analogous environments elsewhere in our solar system. The roles that methane, hydrogen, sulphur/sulphate reduction, and hydrocarbon degradation may play among LH microbial communities are discussed below based on both incubation-dependent and pyrosequencing results.

5.1 Potential methane cycling at the Lost Hammer spring

The high concentration of methane gas that emanates from the LH spring has raised questions as whether or not LH methane could either support, or be derived from, cryophilic microbial consortia under *in situ* conditions. The initial microbial investigations of the LH site hinted at the potential role methane might play in fueling archaeal populations related to known anaerobic methane oxidizers, ANME-1 archaea, detected within the spring sediments (Niederberger et al. 2010). Aerobic methane oxidation by bacterial clades, as well as methanogenesis, also potentially occur at LH based on a second metagenomic and 16S rRNA pyrosequencing study (Lay et al. 2013). The present investigation on the LH outlet sediments, including analyses performed as part of the Lay et al. (2013) metagenomic study, however provided no further evidence that would indicate that LH microbial communities undergo methane-related metabolisms under natural conditions, whether it is methane oxidation, or methanogenesis.

5.2.1 Anaerobic incubations: No detection of AOM or methanogenesis under mimicked natural conditions

The failure to detect either AOM, or methanogenesis, activity in microcosm experiments could be attributed to several factors. On the one hand, it is possible that experimental errors and/or design may have resulted in the negative results obtained. Considering the strict anaerobic metabolism of the targeted microorganisms (i.e. ANME-1 cells and methanogens), exposure to atmospheric oxygen may have irreversibly affected the collected samples, despite the precautions taken during sampling and incubation set-ups to prevent oxygen from entering into contact with collected sediments (Boetius et al. 2009; John Parkes et al. 2010). Prolonged storage of samples in anoxically packed bags, for example, can increase chances of oxygen penetration, as well as alter community structures, especially methanogen and ANME populations (Lin et al. 2010). Such deleterious effects of sample storage prior to incubation set-ups were hypothesised to have affected putative sulphidogenic LH populations since LH sediments stored for longer periods of time prior to vial inoculations showed decreased rates of sulphide production (see section 4.1.3). It is thus possible that the deleterious effects of storage may have been more pronounced for the putative LH ANMEs/methanogens, especially considering that archaeal populations are thought to account for only a small fraction of overall-LH-sediment microbial communities (consequently resulting in an overall diminution of ANME/methanogen members left unaffected in the sediment samples) (Niederberger et al. 2010). Though experimentally challenging, *in situ* vial incubation set-ups such as whole-core injection methods using radiotracer compounds (e.g. Treude et al. 2003), might have yielded better results and should be considered in future LH investigations.

Methanogenesis from LH sediments incubated with H_2:CO_2 (see sections 3.2.2 and 4.1.2) may also have been prevented by the experimental set-up. Hydrogenotrophic sulphate reducers are known to outcompete methanogens in natural settings (Karhadkar et al. 1987). Despite the use of the sulphate-reduction-inhibitor sodium molybdate in microcosm experiments (see section 3.2.3.1), there is the possibility that the molybdate used was present in lethal concentrations for methanogens considering that high molybdate levels can be to be toxic to some methanogen populations (Karhadkar et al. 1987; Watson and Nedwell 1998). Lower concentrations of molybdate may perhaps have allowed potential LH methanogens to remain active, though the very elevated amount of sulphate present in the LH samples may render low concentrations of

molybdate ineffective to inhibit competitive dissimilatory sulphate reduction (Fleming et al. 2006; Oremland and Capone 1988).

Another explanation regarding the absence of detectable AOM or methanogenesis in laboratory incubations relate to the natural environmental conditions of the LH site that may be inhibitory, or even lethal, to ANME and or methanogen cells detected in LH sediments (e.g. hypersalinity and/or cold temperatures). The high energy costs of life at high salt concentrations, in concert with the low energy yield expected from AOM and some methanogenesis reactions, may prevent such metabolisms in hypersaline environments (Oren 2011). Notwithstanding the above, both ANME-1-mediated AOM with sulphate, as well as methanogenesis, have been reported in environments bearing similar, or even higher, salinities as LH. Methanogenesis, for example, has been reported in endoevaporites (i.e. endolithic microbial communities from evaporitic minerals of hypersaline lagoons or salterns), and recent studies have shown that ANME-1 populations underwent sulphate-dependent AOM in hypersaline cold seeps and evaporitic deposits reaching halite saturation, despite the fact that thermodynamics models still cannot clearly explain the viability of this reaction at these salt concentrations (Maignien et al. 2012; Tazaz et al. 2012; Ziegenbalg et al. 2012). Methanogenesis above about 12% salinity, however, is thought to be limited to methylotrophic clades of *Methanosarcinales* methanogens (Oren 2011). It is therefore surprising that in spite of the high salinity of the LH site, the methanogen sequences most abundant in the present cDNA libraries corresponded to *Methanosaeta* species, a clade of methanogens which almost exclusively grows on acetate (Smith and Ingram-Smith 2007). *Methanosarcinales* sequences, which could potentially correspond to methylotrophic members, were also present in the LH cDNA datasets, though accounted for a small fraction of all archaeal reads (data not shown); the presence of methylated compounds (e.g. methanol, TMA, DMS) at the LH site has however not been reported.

Cold temperatures may also prevent non cold-adapted microbes to be metabolically active below a certain threshold temperature. That being said, psychrophilic methanogenic strains can still divide at 0°C (Wagner et al. 2013) and methanogenesis has been reported in several cryoenvironments such as glacier ice or permafrost at temperatures as low as -16.5°C (Rivkina et al. 2004; Tung et al. 2006; Wagner et al. 2007). Furthermore, most AOM studies have been performed in deep-sea environments, where temperatures average about 4°C (Boetius et al. 2009), although no report of AOM at subzero temperatures have thus far been published.

In summary, it is premature to conclude that the high salt concentration, or the cold temperatures, of the LH outlet were the main causes for failures to detect AOM or methanogenesis in incubations with LH-collected sediments. There is the possibility however that the combined cold and hypersalinity were deleterious to ANME-1 and/or methanogen populations detected at the LH site. If that was the case, it would suggest that the detected populations are allochtonous to the spring outlet, most likely carried up to the surface sediments alongside the LH brine (i.e. originating from upstream of the emerging LH outlet). Aerial deposition, or the surrounding permafrost environment, are possible sources for microbial populations alien to the surface spring system, although it appears unlikely that ANME cells would originate from such locations considering that most characterised ANME populations are found in marine sediments, often associated with methane seeps or hydrates (Adams et al. 2013; Orphan et al. 2002). The fact that the detected LH ANME-1 cells could be allochtonous to the LH outlet is also supported by their absence from transcript surveys of the LH system (Lay et al. 2013 and the present study), but presence in DNA ones (Lay et al. 2013; Niederberger et al. 2010). Environments upstream of the spring outlet would most likely be warmer (due to geothermal warming with depth) and potentially less salty (would the point of origin be located upstream of the buried evaporite layer underlying the LH spring outlet) than surface sediments; incubation experiments under warmer and less salty conditions may perhaps result in positive detections of AOM in LH sediments.

5.2.1 Absence of key genes involved in methane cycling in the LH metagenome

The absence of key genes involved in methane metabolism (i.e. genes of the *mcr, pmo* and *mmo* operons) from the LH metagenome was most likely reflective of low sequencing coverage, especially regarding the *mcr* operon involved in (reverse) methanogenesis, as all molecular surveys of the site have either found 16S rRNA signatures of ANME-1, or methanogen, cells (Figure 4.12; Niederberger et al. 2010); however, identified ABC transporters in the LH metagenome distantly matched ABC2 type transporters found by Meyerdieks et al. (2010) in their meta- genomic/transcriptomic investigations of ANME-1 consortia from a methanotrophic microbial mat in the Black Sea (data not shown). The ABC2 protein ATW, or component A2 of the methylcoenzyme M reductase (MCR) system, is required in the activation of the MCR complex in *Methanobacterium thermoautotrophicum* (Dassa and Bouige 2001;

Kuhner et al. 1993; Rouvière et al. 1985); perhaps more relevant is that the *atw* gene has also been found to link *mcrC* sequences in both ANME-1 and ANME-2 clades (Hallam et al. 2004). As such, the recovered ABC sequences from the LH metagenome most likely represent additional evidences for the presence of ANME-1 cells at the LH site.

Considering the aerobic metabolism of most bacterial methanotrophs and the highly reducing and nearly to completely anoxic LH spring water, the failure to detect *pmo* or *mmo* sequences indicates that the metagenomics reads identified to belong to methanotrophic members (e.g. *Methylococcus* and *Methylibium* (Lay et al. 2013) may have been taxonomically mislabelled by MG-RAST automatic annotation. Alternatively, the very low concentrations of oxygen detected in LH water (Table 1.1) may perhaps be permissive of microaerophilic methanotrophy (van Bodegom et al. 2001). Despite the only moderate salt tolerance of methanotrophic culture representatives (up to 15%), aerobic methanotrophy is exergonic enough to allow growth even at very high salt concentrations and evidence exists of methanotrophy at 33% salt concentrations from sediments of Ukrainian ephemeral hypersaline lake basins (Oren 2011; Sokolov and Trotsenko 1995). The presence of active bacterial methanotrophs at the LH site, if not in the anoxic sediments but in the salt tufa for example, could therefore still be possible given the high levels of methane outgassing from the spring dome. Periodical emptying and re-filling of the salt dome could potentially seed LH sediments with methanotrophic cells, explaining the presence of putative methanotroph sequences in the LH metagenome. Sequences related to bacterial methanotrophs found in the LH sediments could also originate from the surrounding soil environment considering that active methanotrophs are known to inhabit Axel Heiberg Island (AHI) active layer and permafrost soils (Martineau et al. 2014; Martineau et al. 2010).

5.3 Biogenic sulphidogenesis in LH sediments: Does the LH spring host active cryophilic and halophilic sulphate or sulphur reducing populations?

Sections 4.1.1 and 4.1.3 investigated putative biological releases of H_2S from LH sediments. Some inconsistencies and unexpected observations regarding the recorded H_2S releases, such as the abnormally low to absent background sulphide levels in formalin-treated samples, as well as the hyperthermophilic sulphide releases from LH sediments, raised some questions regarding the biogenicity of these reactions. A possible explanation could be that the

detected H_2S was solely derived from the degassing of dissolved sulphides – of which the most probable source would be the introduced reducing agent sodium sulphide ($Na_2S•9H_2O$) – and that the increasing rates with temperature of released sulphides are only reflective of abiotic chemistry. The addition of killing agents (e.g. formalin) to the anoxic slurries may have potentially introduced some oxygen (e.g. dissolved in the killing agent), which would have been reduced by the $Na_2S•9H_2O$, and therefore prevent such reducing agent from being released as H_2S in the killed controls (Figure 4.3). Formaldehyde, though commonly used as a killing agent in negative controls of microbiology studies, including those assaying SR (e.g. Zhang et al. (2009)), is known to react with H_2S to form a complex mixture of mercapto derivatives, and therefore most likely should be avoided in future studies (Aleev et al. 2002; Rafikov et al. 1982). On the other hand, several control experiments, such as the use of an alternative reducing agent (i.e. cysteine-HCl) at 5°C and 80°C that resulted in similar sulphide releases, as well as the absence of H_2S detection in vials incubated under a N_2:CO_2 atmosphere, provide evidence that the recorded H_2S emissions from LH sediment were biogenically genuine.

The following sections will discuss the observed H_2S emissions from a biogenic perspective, assuming that microbially-derived H_2S from LH sediments is possible. For convenience and because the electron acceptor compound used in sulphide production processes from LH-sediments has not been confirmed, the term "sulphur-reduction" (SR) will be used to describe dissimilatory reduction processes of all sulphur-containing-compound, such as sulphate, elemental sulphur, thiosulphate, etc., unless otherwise stated.

5.3.1 *In situ* SR by LH communities

Several lines of evidence support the idea that hydrogenotrophic SR can occur under natural conditions at the LH site. The extremely high concentrations of sulphate present in the spring water and sediments, the fact that hydrogen amounts for an important fraction (~ 1%) of the gas exsolving out of the LH outlet, as well as the detectable levels of dissolved sulphides in the LH spring water, all support the idea that hydrogenotrophic SR populations could be present and active *in situ* (Niederberger et al. 2010). The relatively high abundance of sequences distantly related to hydrogenotrophic and halophilic SRBs in the 2012 cDNA libraries (Table 4.2), as well as the presence of *Desulfovibrio*-related reads in these libraries, are further indicative of putative SRB populations being active at the LH site, and supportive of the

sulphidogenic incubation experiments performed on LH sediments (section 4.1.3). Also in accordance with these findings, 16S rRNA *Desulfovibrionales* sequences, as well as reads matching sulfite reductase genes, the key gene in dissimilatory sulphate reduction, were found in the metagenome investigations of LH-sediment performed by Lay et al. (2013). Furthermore, sulphate reduction occurring above salinities of about 15% is normally restricted to autotrophic and hydrogenotrophic processes as other types of SR, such as those based on acetate oxidation, are thought to yield too little energy to support halophilic metabolisms (Oren 2011). Lastly, it should be pointed out that abiotic sulphate reduction has never be shown experimentally bellow temperatures of about 100-140°C (Elsgaard et al. 1994; Trudinger et al. 1985; Worden et al. 1995); thermochemical sulphate-reduction below 100-140°C is considered thermodynamically possible but the reaction rates are thought to be so low as to be insignificant even over geological time scales (Machel 2001).

Alternative to sulphate reduction, sulphide releases from LH sediments could be attributed to sulphur and/or thiosulfate reducing microorganisms. Members of the family *Dethiosulfovibrionaceae* essentially accounted for all *Synergistes* present in the 2012 LH 16S rRNA libraries (Figures 4.9, 4.10 and Table 4.2). Several members of the *Dethiosulfovibrionaceae* are known sulphur reducers and have also been found in hydrocarbon-rich, hypersaline, or cold sea sediments (Godon et al. 2005; Magot et al. 2000; Magot et al. 1997; Sorokin and Muyzer 2010; Surkov et al. 2001). Lastly, H_2S production from LH sediments could, at least partly, be derived from *Halobacteria* activity. Even though *Halobacteria* typically grows aerobically, some strains are capable of anaerobic growth via nitrate, DMSO, or TMAO reduction, as well as arginine fermentation, and are known to produce sulphide from cysteine or thiosulphate metabolism (Oren et al. 1997; Ozcan et al. 2006).

5.3.2 Comparison of the measured sulphide releases at cold temperatures with other psychrophilic and/or halophilic sulphate-reducing communities

5.3.2.1 Cold sulphidic spring environments

Investigations on other (hyper)saline spring systems present on AHI in the Canadian high Arctic revealed that salt-tolerant sulphate reducers were active under cold (5°C) and saline (7.5% salinity) conditions in microcosm experiments with lactate; hydrogen was, however, not tested as a potential SR energy source in that study (Perreault et al. 2008). Despite the relatively close

proximity and geochemical resemblances between those springs and the LH system (~ 40 km apart, cold, elevated salinities and sulphate content), the SRM phylotypes detected in LH 16S rRNA libraries (most closely related to *Desulfohalobium* or *Desulfovibrio* sp.; Table 4.2) differed from those detected by Perreault et al. (2007) (which included *Desulfuromusa*, *Desulfuromonas*, *Desulfobulbus*, and *Desulfobacula* sequences). Biological sulphate reduction was also hypothesised to account for sulphide releases from a supraglacial sulfur spring located on Ellesmere Island in the Canadian high Arctic, and which surfaces at about 1-2°C (Grasby et al. 2003); whether sulphate reducers were active in the colder surface water or only at warmer depths was however not determined.

5.3.2.2 Sulphur reduction rates in cold sediments

Regarding sulphur reduction rates (SRR), the SRR recorded for the LH-sediment consortia incubated between -20°C and 5°C were comparable to those measured in other cold environments such as permanently 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) . Moreover, the calculated activation energy (Ea) for the hydrogenotrophic LH SR (i.e. 40.9 kJ mol^{-1}), also fell within the lower range of Ea obtained for psychrophilic SR populations of Svalbard Arctic sediments (Robador et al. 2009). It should be noted however that the different techniques used to calculate SRR between the present study (i.e. measurements of gaseous sulphide over time) and other studies on cold SR communities (i.e. using radiotracer amendments), as well as the difference in the range of incubation temperatures, may introduce biases in comparisons of both SRR and Ea.

As pointed out by Robador et al. (2009), the concept of activation energy, when applied to whole microbial communities as opposed to isolated chemical reactions or even specific metabolic processes in pure cultures, does not necessarily translate into "the cooperative process between structural elements of an enzyme or a rate-limiting chemical step", but is more reflective of "an ecological response of the whole SRB community to temperature changes". That is, in an ecological sense, Ea is dependent of a myriad of temperature-dependent factors such as changes in nutrient availability, physiological adaptions within one specific species, as well as changes of community structure and composition (e.g. replacement of one species by a better adapted one) (Abdollahi and Nedwell 1979). Higher Ea are normally reflective of

populations showing reduced activity rates under a given range of incubation temperatures (Westrich and Berner 1988). Robador et al. (2009) also showed that mesophilic SRB populations tended to have a higher Ea than psychrophilic ones when incubated at cold temperatures. The relatively low Ea recorded for SRR in LH sediments, in concert with the sustained relatively high SRR at subzero temperatures, are both suggestive of cold-adapted SR populations, and reflect the metabolic plasticity of LH cryophilic SR to cold (and salinity), with temperature unlikely to be a limiting factor for activity in the perennially subzero LH system. It should also be highlighted that the temperatures permissive of SR in LH sediments (i.e. down to -20°C), represent, to the best of my knowledge, the coldest temperatures ever recorded for SR, which was never shown before below ~ -4°C (Tarpgaard et al. 2006).

5.3.3 Potential for hyperthermophily at the LH site: SR at 80°C

Though somewhat unusual, reports exist that describe viable thermophilic consortia recovered from permanently cold environments such as Arctic-sea sediments, especially concerning sulphate reducing populations. Thermophilic spore-forming sulphate reducing *Firmicutes* of the genus *Desulfotomaculum* were recently shown to populate Arctic-sea sediments off the coast of the Svalbard archipelago, isolated from any warm environments (Hubert et al. 2010; Hubert et al. 2009). The detection of related phylotypes 3000 km away in the Aarhus Bay supported the idea that these thermophilic organisms are most likely dispersed passively in the ocean from thermal sources as endospores (de Rezende et al. 2013; Isaksen et al. 1994). Cold sediments overlaying hydrothermal vents (and underlying *Beggiatoa* mats) in the Guaymas Basin have also been found to host SRB populations capable of sulphate reduction from 3°C to 90°C (Elsgaard et al. 1994).

Notwithstanding that findings of thermophilic *Desulfotomaculum* populations in cold ocean environments set precedents for the observed sulphide releases at 80°C from the collected subzero LH sediments (Figure 4.6), notable differences prevent drawing direct parallels between these findings and the present results. First, none of the LH *Firmicutes* sequences (in either DNA or cDNA libraries) closely matched spore-forming *Desulfotomaculum*. Second, even though the source of the LH water is unknown, marine dispersion of thermophilic SRBs appears unlikely to seed the LH hydrological system. The very cold discharges from the LH outlet argue for a relatively shallow water source and evaporite layer (shallow groundwater would not be warmed

as much as deeper groundwater by geothermal gradient), perhaps even fed by subglacial water, as has been hypothesised for other cold springs located on AHI (Andersen et al. 2002; Heldmann et al. 2005). These conditions (cold, relatively shallow) appear unlikely to favour the presence of thermophilic cells in LH samples as they imply that the LH hydrological system is somewhat isolated in relatively cold settings. Even if the LH water is marine in origin, the persistence of non-spore forming thermophiles originating from distant oceanic thermal sources and randomly transported to the LH site also appears as an unlikely event.

There still remains however the possibility for a warmer subsurface underlying the LH spring. The thermogenic signature of the hydrocarbon gases exsolving from the LH outlet have been proposed to derive from potential clathrates of coal bed gas (or methane hydrates) trapped deep in the permafrost (Niederberger et al. 2010), but could alternatively be reflective of hydrothermal conditions (Welhan 1988). A hydrothermal origin for the spring water has been mostly excluded for other AHI spring, but remains a viable possibility regarding the Lost Hammer system (Battler et al. 2013; Pollard et al. 1999; Pollard 2005). If such a hydrothermal system underlies the LH outlet, it seems plausible that thermophilic cells could remain viable as they are being transported upward to the spring surface. In line with a potential warm subsurface source, all molecular surveys of the LH site reported sequences related to (hyper)thermophilic members. Niederberger et al. (2010) reported sequences related to the hyperthermophilic sulphate-reducing archaea *Archaeoglobi*, nearly half of the archaeal 16S rRNA library in Lay et al. 2013 was composed of thermophilic *Crenarchaeota*, including sequences related to sulphidogenic *Desulfurococcus* strains, and the present 16S rRNA archaeal pyrosequencing libraries also contained *Thermoplasmata* representatives (Figures 9, 10). Although such 16S rRNA sequences may represent biological artifacts (e.g. reports of thermophiles preserved in permafrost environments do exist (Steven et al. 2009)), or correspond to putative novel cold-adapted or mesophilic members of normally thermophile clades (e.g. *Thermoplasmata* have been reported in several non-hydrothermal hypersaline systems (Benlloch et al. 2002; Eder et al. 1999; Orphan et al. 2008; Sørensen et al. 2005)), their genuine thermophilic nature should not be disregarded. Furthermore, the SRR from the LH sediments incubated at 80°C falls within the range of SRR reported for other hyperthermophilic consortia, supporting their possible biogenicity (Elsgaard et al. 1994). On the other hand, the very rapid SRR at 80°C with

essentially no lag period (Figure 4.6) do argue against their biological origin and could only reflect chemical sulphide formation.

5.4 16S rRNA surveys of LH sediment communities

5.4.1 Spatial and temporal stability of LH microbial communities

Microbial diversity analyses of very similar communities normally require relatively deep sample sequencing, often in the order of tens of thousands of reads, in order to reliably capture variations amongst the different analysed samples (Lemos et al. 2011). As such, the incomplete sequencing coverage of the studied LH samples rendered comparison analyses somewhat challenging, limited to the most abundant phylotypes present in each respective 16S rRNA libraries (see section 4.2.1). 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 (Table 1.1). The overall similarities between the 2012 April and 2012 July cDNA libraries were to be expected, and similar clades of microorganisms did populate all of the different samples based on cDNA sequencing (Figure 4.9), often down to the species level (>97% sequence similarity), especially in the archaeal April samples (Figure 4.10).

Differences were nonetheless observed at the species level among bacterial libraries. Such differences were most notable for the April surface and July shallow (11-20 cmbs) cDNA samples, which exhibited higher richness than the other libraries, with a higher degree of OTUs unique to these samples (Figure 4.10 A).This phylogenetic profile of the LH sediment samples indicate that LH communities most likely share functional similarities across the top 30 cm of sediments, but that niche specialisation with depth most likely account for more subtle genetic differences between related populations (e.g. regarding O_2 sensitivity/requirement). It could be hypothesised, for example, that the higher biodiversity observed in the April surface, and July shallow, sediment-layer bacterial communities (Table 4.1 and Figure 4.10A) 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.

The somewhat subtle differences observed at the OTU level between the 2012 April and July samples could also be attributed to site patchiness, as only one core was sampled during each sampling seasons and compared. It would be interesting to see if the patterns portrayed here would still hold during subsequent analyses of the LH site, or if sample heterogeneity is really at cause. Similarly and because of the poor sequencing coverage obtained during the present investigation, it is hard to assess how much of the differences in microbial communities observed between the different LH studies were caused by true community turnovers, or sampling efficiency (Figure 4.12). In order to better address these issues however, an optimized nucleic acid extraction protocol should be designed for working with LH sediment samples. The very low nucleotide-extract yields obtained during the present investigation of LH sediments, as well as in the two previous studies which had to rely on multiple-displacement amplification prior to sample sequencing (an additional source of amplification bias), indeed underline the importance of improving protocols for nucleotide extraction from LH samples (Lay et al. 2013; Niederberger et al. 2010; Yergeau et al. 2010). Though low biomass may be a cause behind the inefficient extraction methods (Niederberger et al. 2010), the conflicting high cell counts reported in the present investigation (section 4.1.3.2) argue that the high salt contents, or other sample contaminants, may account for the main factor preventing efficient extractions.

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

The dual sequencing of 16S rRNA genes and reversed transcribed rRNA transcripts had for main objective to better define and survey potentially active LH populations. Considering that a species abundance do not always correlate with a species importance in biogeochemical flux within a (microbial) community, directly comparing OTUs between cDNA and DNA libraries aimed at better identifying actively metabolizing members among the surveyed communities, such as the involvement of rarer phylotypes in the overall activity of LH communities (Campbell et al. 2011; Röling 2007). Even though 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 most likely "diluted out" by the high amount of dead or dormant cells buried within 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 (Figure 4.9A). Such phyla are indeed known to contain a high proportion of spore-former representatives, and are also common to several permafrost environments on Earth (Steven et al. 2009).

Interestingly, the present 16S rRNA profiles of LH sediments depicted a somewhat opposite picture of LH archaeal and bacterial communities than the one presented in Niederberger et al. 2010. That is, 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 DNA survey (Niederberger et al. 2010), cDNA sequencing here revealed archaeal communities dominated by halophiles (Figure 4.9B and Table 4.3) and bacterial ones mostly related to clades either directly or indirectly involved in hydrocarbon and/or hydrogen metabolism (Table 4.2). 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 sulphate-methane transition zones where ANME-1-mediated AOM was 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 sulphur-reducing LH hydrogenotrophs (see section 4.1.3), 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 4.2). Most

cultivated *Synergistes* strains are known to degrade amino acids and it has been proposed that *Synergistes* functions in natural settings may well center around amino acids turnover (Godon et al. 2005). The exact ecological role of *Synergistes* in anaerobic consortia however often remains speculative yet has been hypothesised to mainly relate to either hydrogenotrophic thiosulphate or elemental sulphur reduction, or, alternatively, syntrophic associations with hydrogenotrophic methanogens (Godon et al. 2005). Similar methanogen syntrophic associations are also known to occur with *Deltaproteobacteria Syntrophus* species; 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). Interestingly, the most abundant methanogen members present in these hexadecane-degrading consortia (though apparently not involved in syntrophic hexadecane degradation) were closely related to *Methanosaeta* species (Cheng et al. 2013; Embree et al. 2013); *Methanosaeta* accounted for the most abundant methanogen clade in the present 16S rRNA archaeal libraries (Table 4.3, Figure 4.10B). The fact that H_2 oxidation appears to play a central role in the metabolic processes undergone in the very cold LH spring sediments is also consistent with thermodynamics findings that showed that H_2-consuming reactions become more exergonic with decreasing temperatures (Conrad and Wetter 1990).

The relatively high abundance of bacterial clades related to known hydrocarbon degraders, or syntrophic partners of known hydrocarbon degraders, suggests that hydrocarbon degradation, perhaps not only restricted to methane oxidation, may play an important role in fueling the LH microbial consortia. In addition to methane, heavier gaseous alkanes (e.g. ethane, propane, etc.) have been detected on site (Niederberger et al. 2010). Anaerobic oxidation of heavier alkanes was long thought to not be thermodynamically favourable (Onstott et al. 2010), yet recent studies have confirmed that short-chain alkanes indeed play an important role in both carbon and sulfur cycling, often occurring under sulphate-reducing conditions (Adams et al. 2013). 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 characterised oligotrophic LH spring (Niederberger et al. 2010). Furthermore, it might be interesting to assess for the presence of other hydrocarbons at LH, such as long-chain alkanes (e.g. hexadecane) or chlorinated hydrocarbons, and verify their potential roles in LH microbial metabolism

considering that microbial consortia known to utilised such hydrocarbons are very similar in composition to the described clades present in the current 2012 LH 16S rRNA libraries (i.e. *Syntrophus*, *Methanosaeta* and *Desulfovibrio* species) (Cheng et al. 2013; Zengler et al. 1999).

Other than hydrocarbon and hydrogen metabolism, a central role in sulphur cycling, not restricted to sulphidogenic processes, is most likely played by LH consortia as highlighted by previous research on the site and considering the high concentrations of sulphur compounds present at LH (Lay et al. 2013). The high proportion of relatively active *Chloroflexi* and *Betaproteobacteria* sequences among the different LH samples is consistent with 16S rRNA surveys of other sulphur-rich cryoenvironments. For example, a study on alpine tundra wet meadow soil revealed that *Chloroflexi* accounted for a major portion of the soil microbial community; these *Chloroflexi* appeared active at near 0°C temperatures under likely anoxic conditions in a sulphate 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 sulphide oxidation. The most abundant and apparently active LH *Betaproteobacteria* OTU closely matched members of the *Ralstonia* genus (Table 4.2). Despite the fact that *Ralstonia* species are typically associated with oxic environments, some *Ralstonia* strains, such as *Ralstonia eutropha* H16, are capable of hydrogenotophic lithoautotrophic metabolism under anoxic conditions (Pohlmann et al. 2006); *R. eutropha* H16 has also recently been shown to possess sulphur oxidation (*sox*) genes (Cramm 2009). More relevant to the present study was the report of *Ralstonia* phylotypes dominating the 16S rRNA clone libraries of a supraglacial sulphur spring in the Canadian high Arctic and their possible role in sulphur cycling (Gleeson et al. 2011).

5.4.3 LH genetic pool: indigenity of nucleotide sequences and concerns in extrapolating microbial activity from rRNA surveys

The rationales behind inferring microbial activity using rRNA transcripts (in the present case referred to as cDNA) over simple rRNA genes mainly lie in the intrinsic instability of the RNA molecule (when compared to DNA), as well as the relatively rapid degradation of RNA by internal RNAses during times of lower cell activity, or external RNAses in the case of naked RNA (Deutscher 2003). Caution should nonetheless be considered when inferring activity from rRNA surveys; a recent review by Blazewicz et al. (2013) exposed how directly inferring activity

from rRNA can be misleading and potentially falsely portray microbial assemblages. Among other, they underlined the knowledge gap between the fate of rRNA in pure cultures (from which most of the assumptions regarding rRNA stem) and in natural communities, such as the often high concentration of rRNA in dormant cells, as well as some inconsistencies between rRNA concentrations and growth rates in different microbial taxa or among different members of the same populations within an environmental community. The potential for nucleic acid preservation in the absence of active nucleases should also be considered in studies performed on certain environments/samples less prone to nucleotide degradation. DNA molecules, for example, are now recognised to potentially be preserved for long periods of time in cold environments and studies are lacking that would show significant differences regarding rRNA molecules (Ah Tow and Cowan 2005; Charvet et al. 2012; Willerslev et al. 2004). Hypersaline conditions are also known to not only preserve DNA, but also to protect tRNA from thermal degradation (Borin et al. 2008; Danovaro et al. 2005; Tehei et al. 2002). Recent studies have even raised the possibility that eutectic solutions in ice may provide stable enough environments for the emergence of ribozymes on Earth (Vlassov et al. 2005).

Considering the subzero and hypersaline nature of the LH spring, cautions should be taken in interpreting the 16S rRNA findings. The surfacing LH water is most likely warmer at depths, and it could be possible that some of the labelled “active” LH populations were in fact active only deeper in the brine conduit and then transported upward to the spring surface. The relative disproportion of RNA to DNA molecules in the 16S rRNA surveys, however, remain good lines of evidence of truly active populations at the LH site but the possibility of a foreign origin from the spring outlet should not be discarded. There is little doubt as to whether most of the previously identified clades of LH organisms identified using cDNA sequencing are true active members of the LH hydrological system based on the logical associations of implied metabolisms with the environmental conditions of the LH site (e.g. sulphur and/or hydrocarbon related metabolisms and sulphur and hydrocarbon rich environment). Though whether all implied active populations really are indigenous the surface subzero LH outlet should be confirmed with additional experiments such as microcosm incubations, radiotracer assays, or stable isotope probing (SIP).

Chapter 6: Conclusion

Significance of research findings and possible follow-up studies

The present investigation on the Lost Hammer spring intended to identify active microbial populations under *in situ* conditions. Anaerobic methane oxidation and chemolitho(auto)trophy were hypothesised to play important roles among the spring's communities based on a previous molecular and geochemical investigation of the site (Niederberger et al. 2010). Even though incubation experiments could not confirm that LH consortia undergo methane metabolism, they did provide indications that the reduction of sulphur compounds (SR) with hydrogen, most likely sulphate-reduction, is carried out by cryophilic clades of LH microorganisms under subzero and hypersaline conditions. The evolution of H_2S from LH sediments at high temperatures also raised the possibility of the presence of hyperthermophilic clades of microorganisms in the recovered samples, though such releases may alternatively represent artifacts of abiotic chemical reactions. Lastly, 16S rRNA pyrosequencing of LH sediment communities revealed that relatively stable microbial communities appear to populate the perennially subzero and hypersaline spring outlet. Notable differences between the present 16S rRNA survey and previous ones however raise questions as whether the observed community snapshots presented here truly depict complete community profiles of the LH-outlet sediments, or are only artifacts of the limited sampling replicates and the methods used. Still, the obtained 16S rRNA results illustrated the potential for hydrocarbon metabolism, other than methane, among LH populations (e.g. among the *Synergistetes* and T78 *Chloroflexi* LH clades) based on sequence similarities between LH communities and those of hydrocarbon-degrading consortia (e.g. Cheng et al. (2013), Embree et al. (2013), and Harrison et al. (2009)). The importance of anaerobic hydrocarbon degradations should therefore be considered in future studies of the LH site.

The biogenicity of the detected SR remains to be confirmed but its potential genuineness bears significance that should be underlined. First, 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 important roles in other cryoenvironments. Very few cryophilic strains have been genome-sequenced and most of those are heterotrophic aerobes (Goordial et al. 2013); further characterisation of the enriched SR consortia in LH microcosms (e.g. via strain isolation

or single-cell sequencing from enrichment cultures) would certainly allow to characterise novel adaptations of anaerobic and lithotrophic microbial life living under combined subzero and hypersaline conditions, adaptations which are currently understudied. Of particular interest would be to confirm the autotrophy of the putative LH sulphur/sulphate reducers considering the potential for hydrocarbon metabolism previously described.

The combined anaerobic, chemolithotrophic, cryophilic, and halophilic nature of the detected putative SR also is highly relevant for astrobiology. Future space-exploratory missions intend to look for signs of life on the very cold Mars, Europa, and Enceladus, all showing evidence for both past and present liquid brines on their surface (Des Marais et al. 2008). Life capable of surviving or living on these planetary bodies would arguably need to be cold-adapted but also most likely capable of lithotrophic and anaerobic metabolism (due to the scarcity of molecular oxygen outside the Earth and considering that complex organics are unlikely to represent significant carbon pools on other planets). Recent discoveries of sulphate 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 sulphate reduction as a possible anaerobic metabolism for potential extraterrestrial microorganisms (Gendrin et al. 2005; McCord et al. 2001). The recorded putative sulphate reduction from LH sediments are also significant with respect to Snowball Earth events, events in Earth history of global cold on the Earth's surface and that are still poorly understood. Evidence suggests that sulphate reduction played an important role in overall biogeochemical cycling during these time periods (Hurtgen et al. 2002). Increasing our knowledge regarding such process under subzero temperatures is therefore expected to help us understand the involvement of microbial life (e.g. SR) in biogeochemical feedbacks to climate and global nutrient cycling that occurred during these epochs. Overall, the obtained findings provided additional evidence that the LH system host active communities of microorganisms despite the extreme conditions *in situ*. The present research strengthens the view that cryoenvironments represent real habitats (as opposed to sole reservoirs) for microorganisms on Earth, and that the cryosphere truly represents an active component of the Earth's biosphere.

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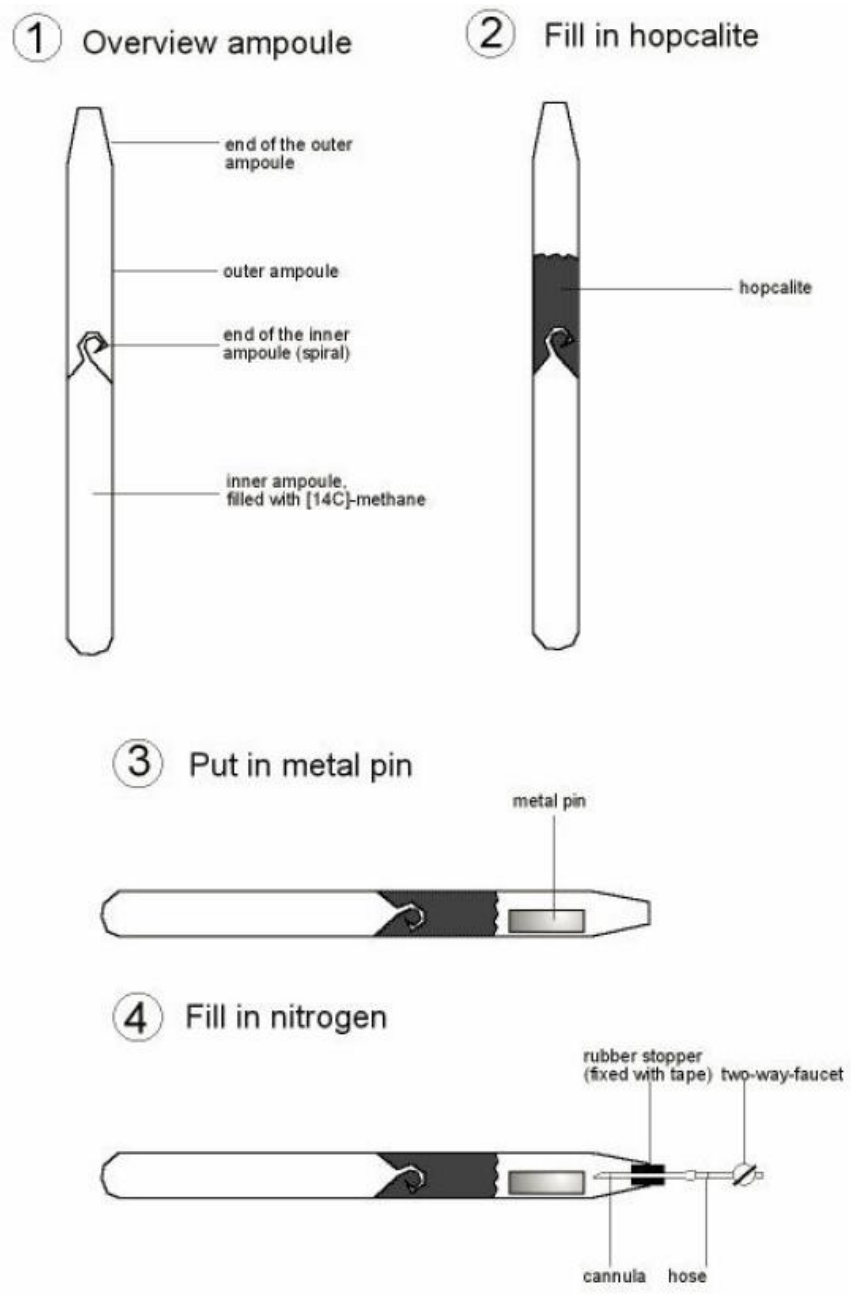
Appendices

Appendix A: Extraction of $^{14}CH_4$ from a sealed glass ampoule

The $^{14}CH_4$ was purchased from American Radiolabelled Chemicals inc. (ARC, St-Louis, MO, USA) and came in a sealed glass ampoule (50 mCi/mmol, 1 mCi/tube). The methane was extracted from the ampoule following a modified protocol described in Iversen and Blackburn (1981) provided by Dr. Treude (personal communication, 2010). Below is a re-written version of Dr. Treude's protocol:

To remove any $^{14}CO_2$ and ^{14}CO contaminants potentially present in the methane sample, the connected outer tube (see Appendix Figure 1) was filled with hopcalite powder (commercial name, Monoxycon, Lawrence Factor, Miami Lakes, Fl, USA), previously crushed with a mortar and pestle, to about ¼ of the outer ampoule volume. A small magnetic stir-rod was also carefully inserted so as to not break the lower sealed ampoule. The outer ampoule was then sealed with a custom-made ground-glass joint fitted with a blue butyl stopper and taped into place. The ampoule was kept horizontally to prevent breakage of the lower ampoule seal. The upper ampoule was then sequentially flushed with N_2 gas and evacuated using a vacuum pump three times. 2 mL of CO_2 was injected into the evacuated ampoule in order to rarefy any leftover $^{14}CO_2$. The lower ampoule was then broken by vigorous shaking. The broken ampoule was left undisturbed for 24 hours to allow removal of carbon monoxide contaminants by reaction with the hopcalite powder. 50% NaOH was then added to the ampoule using a 10 mL gastight syringe free of trapped air bubble. The $^{14}CH_4$ was transferred to 6 mL serum vials filled with 50% NaOH (free of any headspace) using gastight syringes. An equal amount of 50% NaOH than the amount of extracted $^{14}CH_4$ was simultaneously added to the ampoule when sampling to avoid partial vacuum formation; the same procedure was performed in reverse when injecting the $^{14}CH_4$ into the 6 mL serum vials filled with 50% NaOH. Serum vials containing 50% NaOH were stored upside down in the dark at 5°C.



Appendix Figure 1 Overview of the procedure to extract $^{14}CH_4$ from the sealed ampoule. The image was provided by Dr. Tina Treude via personal communication.

Appendix B: Summary of control experiments regarding sulphidogenesis from LH sediments

Appendix Table 1 Summary of the incubation set-ups assaying SR from LH sediments using either Na2S•9H2O, cysteine-HCl, or no reducing agent under both cold and hot temperatures. Unless stated otherwise, the headspace of microcosms was H2:CO2 (80:20).

Treatments		Reducing agent used/H_2S release				
		$Na_2S•9H_2O$ (5 mM)		Cysteine-HCl (0.05%)		No reducing agent
		Cold (5°C)	Hot (80°C)	Cold (5°C)	Hot (80°C)	Hot (80°C)
Only sediments	H_2:CO_2	++	++	++	++	-
	N_2:CO_2	-	N.P.	N.P.	N.P.	N.P.
	Distilled water + sediments	N.P.	++	N.P.	++	N.P.
	Filtered LH water + sediments	N.P.	++	N.P.	++	N.P.
Killed controls	Formaldehyde (2-5%)	-	-	N.P.	N.P.	N.P.
	NaOH (3%)	N.P.	-	N.P.	N.P.	N.P.
	Bleach (10%)	N.P.	-	N.P.	N.P.	N.P.
	Sodium Molybdate (20 mM)	-	-	N.P.	N.P.	N.P.
	HCl (2 N)	N.P.	N.P.	N.P.	N.P.	-
	HCl (2N) N_2:CO_2	N.P.	N.P.	N.P.	N.P.	-
No sediment controls	LH water only	N.P.	+	N.P.	-	N.P.
	Filtered LH water only	±	+	-	-	N.P.
	FeS (in distilled water)	N.P.	-	N.P.	N.P.	N.P.
	Distilled water only	N.P.	+	N.P.	-	N.P.
	FeS (in distilled water)	N.P.	-	N.P.	N.P.	N.P.

The symbols -, ±, +, and ++, describe the levels of gaseous H_2S released in ascending order, with "-" corresponding to no sulphide detection.

N.P. Analysis not performed