RESEARCH ARTICLE

When a habitat freezes solid: microorganisms over-winter within the ice column of a coastal Antarctic lake

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Abstract

A major impediment to understanding the biology of microorganisms inhabiting Antarctic environments is the logistical constraint of conducting field work primarily during the summer season. However, organisms that persist throughout the year encounter severe environmental changes between seasons. In an attempt to bridge this gap, we collected ice core samples from Pony Lake in early November 2004 when the lake was frozen solid to its base, providing an archive for the biological and chemical processes that occurred during winter freezeup. The ice contained bacteria and virus-like particles, while flagellated algae and ciliates over-wintered in the form of inactive cysts and spores. Both bacteria and algae were metabolically active in the ice core melt water. Bacterial production ranged from 1.8 to 37.9 $\mu g\,C\,L^{-1}\,day^{-1}$. Upon encountering favorable growth conditions in the melt water, primary production ranged from 51 to 931 $\mu g\,C\,L^{-1}\,day^{-1}$. Because of the strong H_2S odor and the presence of closely related anaerobic organisms assigned to Pony Lake bacterial 16S rRNA gene clones, we hypothesize that the microbial assemblage was strongly affected by oxygen gradients, which ultimately restricted the majority of phylotypes to distinct strata within the ice column. This study provides evidence that the microbial community over-winters in the ice column of Pony Lake and returns to a highly active metabolic state when spring melt is initiated.

Introduction

Coastal ponds are commonly found on the margins of the Antarctic continent and are particularly abundant in the ice-free areas of the McMurdo Sound region (Armitage & House, 1962; Torii *et al.*, 1988; Broady, 1989). These systems exhibit a wide range of salinities (from freshwater to saline), ionic compositions, and nutrient concentrations (from oligotrophic to eutrophic). A commonly held distinction between ponds and lakes in polar regions is that ponds freeze solid during winter, whereas lakes do not. The process of freezing solid causes considerable stress on organisms. Freezing occurs from the top-down when temperatures decline in the fall. The gradual freezing process attenuates light penetration through the ice cover and leads to a steady concentration of solutes and particulates in the remaining liquid water column (e.g. Schmidt *et al.*, 1991). Conversely, it is still unclear what triggers the initial melt in spring. Whereas Schmidt *et al.* (1991) suggested peripheral melt water draining towards the center of the pond (bottom-up), Hawes *et al.* (1999) reported ice overlain by liquid water in ponds of the McMurdo Ice Shelf (top-down). Regardless, warmer temperatures during the spring initiate melting of the ice cover and subsequent changes in physicochemical parameters over the course of the summer (Healy *et al.*, 2006; Wait *et al.*, 2006). Along with these changes, productivity in Antarctic aquatic ecosystems typically increases throughout the summer following the seasonal cycle of

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increased solar radiation, nutrient availability, and temperature (Goldman *et al.*, 1972). Consequently, organisms that persist throughout the year in these systems must be capable of surviving extreme alterations in environmental factors (e.g. osmotic pressure, pH, temperature) during summer melt and winter freezing.

Previous studies on Antarctic lakes and ponds have focused primarily on plankton community structure or benthic mat communities (e.g. James *et al.*, 1995; Vinocur & Pizarro, 2000; Van Trappen *et al.*, 2002; Jungblut *et al.*, 2005; Glatz *et al.*, 2006); hence, little is known about bacterial communities that over-winter in the ice column of frozen solid ponds or lakes. However, ice is increasingly being recognized as a suitable habitat for life (Priscu *et al.*, 1998) and complex microbial consortia have been found in a wide range of icy systems including glaciers (e.g. Abyzov, 1993; Christner *et al.*, 2000; Zhang *et al.*, 2001), subglacial environments (e.g. Skidmore *et al.*, 2000; Christner *et al.*, 2008; Lanoil *et al.*, 2009; Mikucki *et al.*, 2009), or supercooled cloud droplets (Sattler *et al.*, 2001). Nutrient-enriched microzones embedded within the ice covers of the McMurdo Dry Valley lakes have been shown to sustain communities that are capable of photosynthesis, nitrogen fixation, and decomposition of organic matter (Fritsen & Priscu, 1998; Olson *et al.*, 1998; Paerl & Priscu, 1998; Priscu *et al.*, 1998). Cryoconite holes on dry valley glaciers contain abundant algal and bacterial communities (Porazinska *et al.*, 2004; Foreman *et al.*, 2007).

When shallow ponds refreeze at the end of the summer, cryo-concentration of the major ions may lead to the formation of basal brines (Schmidt *et al.*, 1991; Healy *et al.*, 2006; Wait *et al.*, 2006) by diffusion and convection of the brine at the interface between ice and water. However, salts may also become incorporated into the ice when salts are removed or excluded ineffectively. Ultimately, as ice formation progresses, the incorporation of the residual brine may generate a concentration gradient within the ice column and a salinity stratification in the remaining water (Wait *et al.*, 2006). The highest concentrations of ions are typically found towards the base of the lake and the freezing point temperature of these basal brines can be markedly decreased (Schmidt *et al.*, 1991; Healy *et al.*, 2006; Wait *et al.*, 2006). Further, liquid water enclosures may exist within the ice column of Antarctic lakes (e.g. ice cover of the Dry Valley lakes; Fritsen *et al.*, 1998). Hence, liquid water could be present in Pony Lake for a substantial period of the year. However, when temperatures eventually decline below the freezing point temperature of the basal brine, the planktonic community becomes encapsulated in the ice. Importantly, the 'planktonic' ice community can be contained within the ice for upwards of several years as the area of Pony Lake that becomes ice free during the summer is strongly affected by the local climate and differs quite drastically from year to year (C.M. Foreman, pers. commun.). This entrapment may severely compromise the physiology of the microbial assemblage. Nonetheless, the survival of the microbial community is essential for recolonizing Pony Lake when melt is initiated. The purpose of this study was to investigate the biology of a frozen solid Pony Lake and to provide an insight into the microbial community that over-winters within the ice column.

Materials and methods

Sampling location

Pony Lake is a small (~120-m-long, 70-m-wide, and 1–2-m-deep), eutrophic lake located at Cape Royds (77°33′S, 166°00′E), Ross Island, Antarctica. Although Pony Lake has been dubbed a lake, it more closely resembles the characteristics of other Antarctic ponds in that it is shallow and, with the exception of a few weeks during mid-summer, the lake is ice covered or frozen solid to its base. The proximity of the lake to McMurdo Sound and the Ross Sea gives the lake its brackish nature (5.5 p.p.t.) (Brown *et al.*, 2004). There is an Adelie penguin rookery on the western shore of the lake. As Pony Lake has no visible inflow, melting of the snowpack that has accumulated on the lake ice replaces water lost by sublimation of surface ice and evaporation in mid-summer. The basin contains no higher plants, but planktonic algae are very abundant (McKnight *et al.*, 1994; Brown *et al.*, 2004).

Sampling

In order to study the organisms entrapped within the ice cover of Pony Lake, we collected ice cores using a hand-operated SIPRE ice auger (10 cm in diameter) when the lake was frozen solid to its base in November, 2004. Eight ice core samples (each ~1.2 m long) were collected in two parallel transects in a 1×2 m area from the center of the lake. The cores reached the bottom of the lake; thus, at this location, the ice was 1.2 m thick. The maximum depth of the lake during the summer time varies between 1 and 2 m depending on the seasonal melt conditions. Samples were stored frozen in darkened coolers and transported (within 4 h) to the Crary laboratory at McMurdo Station, Antarctica. All ice core preparation steps were carried out in a $-20\,^{\circ}$C cold room. Core samples were divided into three sections (top, middle, and bottom), with each segment being ~40 cm long. Ice cores were cleaned mechanically by scraping off approximately 1 cm of the outer surface using sterile blades. Cleaned ice core fragments from each section were pooled, transferred into acid-rinsed and autoclaved Nalgene containers, and allowed to thaw at $4\,^{\circ}$C in the dark (~48 h). Subsequently, all analyses were conducted on the pooled 'ice core' melt water samples (top, middle, and bottom sections).

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Productivity measurements in ice core melt water

Both primary and secondary productivity measurements were used to determine the potential metabolic activity of the organisms within the ice. All productivity measurements were carried out immediately after the ice core sections had been cleaned and completely thawed. Heterotrophic bacterial productivity (BP) was estimated by ^{3}H-thymidine incorporation (20 nM final concentration) into DNA as outlined by Takacs & Priscu (1998). Five ^{3}H-thymidine assays and triplicate formalin-killed controls (5% final concentration, 30 min before ^{3}H-thymidine addition) for each ice core section were incubated with the radioactive compound at 4 °C for 20 h in the dark. Disintegrations were detected in a liquid scintillation counter (Beckman LS 6000). Conversion factors of 2.0×10^{18} cells mol^{-1} TdR (Ducklow & Carlson, 1992) and a cell-to-carbon conversion factor of 11 fg C per cell (Kepner *et al.*, 1998) were used to convert the thymidine incorporation rates into bacterial production rates.

Primary production (PPR) was measured by ^{14}C-carbonate/bicarbonate incorporation (114.4 μCi mL^{-1}, pH∼9.5; ICN/MP Biodmedicals) using the protocol of Lizotte *et al.* (1996) and the McMurdo Dry Valleys LTER group (Priscu & Wolf, 2000). Melt water aliquots of 150 mL with no headspace were dispensed into clear quartz bottles (× 4) for the light assays and into amber bottles (× 2) wrapped with aluminum foil for the dark controls for each core section. Bottles were incubated at 4 °C in a lighted incubator for 24 h (∼80 μmol photons s^{-1} m^{-2}). After incubation, samples were filtered through precombusted 25-mm GF/F filters in the dark. The filters were then transferred into 20-mL scintillation vials, acidified with 500 μL 3 M HCl, and dried before analysis using a liquid scintillation counter (Beckman LS 7200).

Community structure analyses

In conjunction with the productivity measurements, samples were collected in order to enumerate the organisms and determine species composition within the ice core sections.

Abundance

Melt water from each ice core section was preserved with formalin (2% final concentration). Bacteria were filtered onto 25 mm × 0.2 μm black polycarbonate filters with a 0.45 μm nitrocellulose backing filter under gentle vacuum and stained with 500 μL of a 25 × SYBR Gold solution for 15 min as described by Lisle & Priscu (2004). Glassware was baked (450 °C) and solutions were filter sterilized (0.2 μm) before usage to reduce possible contamination. More than 400 cells per sample were counted in randomly selected fields using a Zeiss Axioskop epifluorescence microscope with a final magnification of × 1000.

Sterile 125-mL screw cap flasks were filled with melt water from each core section for the analysis of bacteriophage or virus-like particles (VLP). Samples were flash frozen in liquid nitrogen and stored at − 80 °C until further processing, as recommended by Wen *et al.* (2004). Following this protocol, samples were thawed in the dark at room temperature overnight and then prefiltered through 0.20-μm filters in order to remove bacteria. The filtrate was then filtered through a 0.02-μm filter to collect the VLP, and stained with SYBR Gold as detailed in Lisle & Priscu (2004). VLP were enumerated using an Olympus BX51 epifluorescent microscope.

Samples (1 L) for algal and protozoan counts were fixed with Lugol's iodine (10 mL) and concentrated by settling for 1 week. Concentrated samples were transferred into 60-mL amber Nalgene bottles. Subsamples from the concentrated stock were collected and algal and protozoan abundances were enumerated. Counts were conducted in a Sedgewick–Rafter counting chamber using phase microscopy with a magnification of × 320 (Laybourn-Parry & Marshall, 2003).

Environmental DNA extraction and denaturing gradient gel electrophoresis (DGGE)

Pooled melt water samples from each core section (70–100 mL) were collected for DNA extraction and downstream phylogenetic analysis on 47 mm Supor®-200 0.2-μm pore size, sterile membrane filters under low pressure (< 7 psi). Filters were transferred to cryovials containing TES buffer (100 mM Tris, 100 mM EDTA and 2% sodium dodecyl sulfate), flash-frozen in liquid nitrogen, and stored at − 80 °C.

DNA was extracted from the filters using an Ultra Clean Soil DNA Kit (MoBio). Primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 534R (5′-AATACCGCGGCTGCTGG-3′) were used to amplify a portion of the prokaryotic 16S ribosomal gene (Muyzer *et al.*, 1996). A 40-bp GC clamp was added to the 5′ end of the 341F primer (CGCCCG CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG). The amplification protocol included a hot start (94 °C for 4 min) and a touchdown program. The touchdown program began with an initial annealing temperature of 65 °C and decreased by 1 °C every cycle for eight cycles, followed by 17 cycles at 58 °C, and a final elongation step for 10 min at 72 °C. Each 50-μL PCR reaction contained 1.5 μL of environmental DNA extract, $MgCl_2$ buffer (final concentration 1 ×), Taq Master (final concentration 1 ×), PCR nucleotide mix (final concentration 800 μM), and Taq DNA polymerase (final concentration 0.025 U μL^{-1}) (all components from 5 Prime, Eppendorf), upstream and downstream primer

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(final concentration 0.5 μM), and nuclease-free water (Promega). An automated thermal cycler (Mastercycler ep, Eppendorf) was used for PCR amplifications. DGGE was carried out on a BioRad D CodeTM system as outlined by Burr *et al.* (2006). PCR amplicons were loaded onto 8–12% polyacrylamide gels with a 40–70% denaturing gradient. The gels were placed in 1 × TAE buffer at 60 °C and run for 17 h at 60 V. After staining with SYBR Gold (Invitrogen) for 15 min, the gels were imaged using an Alpha Innotech FluorChemTM 8800 system.

16S rRNA gene clone library

The two universal 16S rRNA gene primers 9F (5′-GAGTTT GATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) were used for amplification of the 16S ribosomal gene (Stackebrandt & Liesack, 1993) from the individual ice core sections [top (TC); middle (MC); and bottom (BC)]. PCR products were cloned into pCR®2.1-TOPO vectors (TOPO TA cloning kit, Invitrogen). A total of 70 white colonies for each core section were collected from Luria–Bertani (LB) agar plates containing 50 μg mL^{-1} kanamycin. Clones were screened with DGGE (Burr *et al.*, 2006) using the primers 341F plus a GC clamp and 534R. Based on their DGGE migration pattern, 32 clones from each clone library were selected and sent to Functional Bioscience Inc. on LB agar plates for high-throughput DNA preparation and DNA sequencing using primer M13F (20). Nucleotide sequences were edited using SEQUENCHER 4.5 (Gene Code Corporation). The nucleotide sequences were compared with the NCBI nucleotide database using the BLAST search tool (BLASTN 2.2.21, http://ncbi.nlm.nih.gov/BLAST/, Zhang *et al.*, 2000).

Statistical analyses

Statistical analyses were used to help determine the differences in the microbial diversity between the three ice core sections being studied. The structural diversity of the microbial community in the ice core sections was calculated from the DGGE profiles using the Shannon index. Bands were visually detected and scored based on presence or absence (Gafan *et al.*, 2005; Dieser *et al.*, 2010). Detected bands were ascribed a value of 1, while a value of 0 was assigned to bands that were absent in a sample profile when compared with another DGGE profile. The index was calculated using the following equation:

$$\text{Shannon index } (H') = -\sum_{i=1}^{s} (p_i) \times (\log p_i)$$

where s is the number of bands in the sample and p_i is the proportion of bands i in the sample.

To assess differences in the microbial community structure, monothetic clustering (Kaufman & Rousseeuw, 1990) was used to cluster the Pony Lake clones based on their presence or absence in the three ice core sections. Monothetic clustering is implemented in the R software package cluster (R Development Core Team, 2010) and is designed for clustering binary variables. The responses (clones) were split based on their presence/absence in the different core sections, starting with the section that is most useful in separating the clones. This contrasts with polythetic clustering techniques, which cluster based on all the responses simultaneously. Monothetic clustering provides an insight into the variables that are most useful in separating the finite response patterns as well as summarizing the possible patterns and proportion of responses in each group. The hierarchy in the partitioning provides useful interpretations of the clustering results. A novel modification of a banner plot (Kaufman & Rousseeuw, 1990) was used to display the results in a more insightful manner.

Results and discussion

Productivity measurements and abundance of organisms

The ice core samples profiled the 'water column' of Pony Lake, from its surface to its base, archiving chemical and biological alterations during freezing. Our study provides evidence that bacteria, VLPs, algae, and ciliates over-winter within the lake ice. To the best of our knowledge, this is the first time that microbial processes and community structure analyses were performed in ice core samples from an Antarctic lake that transitions seasonally from open water to completely frozen.

Bacterial cell numbers and VLP enumerated via epifluorescence microscopy (Fig. 1) and microbial activity measurements detected via the incorporation of ^{3}H-thymidine are summarized in Table 1. Abundances increased with ice core depth for both bacteria and VLP. Bacterial numbers ranged from 1.10×10^6 to 2.97×10^7 cells mL^{-1}. With the exception of the mid core section, the VLP numbers were lower than the bacterial counts and ranged between 2.05×10^4 and 1.89×10^6 cells mL^{-1}. VLP to bacteria ratios ranged between 0.01 and 0.14. Bacterial production rates ranged from 1.8 to 37.9 μg C L^{-1} day^{-1} and were the highest in the mid core section. However, it is important to note that the activity rates were obtained from the bacterial community suspended in melt water. Rather than representing true *in situ* metabolic rates, these results demonstrate that microorganisms entrapped within the ice retained the capacity to return to metabolic activity when environmental conditions are more favorable. Similar BP rates expressed in the top ice core melt water in this study were reported in ice core melt water

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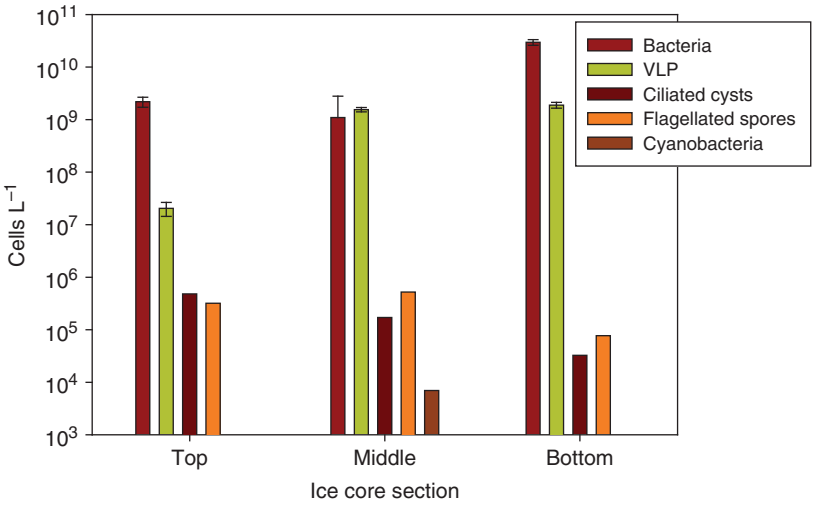

Fig. 1. Abundance of organisms in Pony Lake ice core sections collected in November 2004. The ice column of Pony Lake is colonized by bacteria, VLPs, and to a small extent by cyanobacteria. Inactive forms of flagellates and ciliates were also identified.

Table 1. Activity measurements of bacteria (BP) and primary producers (PPR) in 40-cm ice core sections (top, middle, and bottom) from Pony Lake on November 15, 2004

	Top	Middle	Bottom
BP ($\mu g\,C\,L^{-1}\,day^{-1}$)	6.69 ± 0.20	37.9 ± 5.29	1.80 ± 1.18
PPR ($\mu g\,C\,L^{-1}\,day^{-1}$)	931 ± 564	308 ± 99	51 ± 27

samples from the perennial ice cover of the Dry Valley lakes (Priscu *et al.*, 1998) and in the ice melt water of cryoconites in the Taylor Valley (Foreman *et al.*, 2007).

The low BP in the bottom core melt water is intriguing because the highest cell numbers and the greatest diversity were enumerated in this layer. It is noteworthy that Pony Lake bottom ice smelled strongly of H_2S, indicating the presence of reduced conditions (Schmidt *et al.*, 1991; Wait *et al.*, 2006). Analogous to other coastal ponds (Wait *et al.*, 2006), Pony Lake surface ice was glass like and colorless, whereas the ice towards the bottom showed intense yellow-brownish coloration, a typical signature of the incorporation of brine solutions into the ice (Wait *et al.*, 2006). The colored bottom ice also had a high ionic content (e.g. $Na > 10\,000\,mg\,L^{-1}$; $Cl > 6200\,mg\,L^{-1}$; $SO_4^{2-} > 4600\,mg\,L^{-1}$; C.M. Foreman *et al.*, unpublished data). Thus, organisms living in a basal brine would have been exposed to a substantial increase in salt concentrations as well as extremely low temperatures (Schmidt *et al.*, 1991; Healy *et al.*, 2006; Wait *et al.*, 2006), environmental stressors that are even more pronounced after complete freezeup, when microorganisms may be concentrated together with salts, minerals, and gases into highly saline, liquid-filled veins (Mader *et al.*, 2006). Such extremes impose challenges to the survival of microorganisms and can cause severe cellular damage to organisms over-wintering in the bottom section of Pony Lake. On the other hand, thawing of the bottom ice would have increased the organisms' exposure to oxygen. Assuming a micro-oxic or an anoxic environment in the bottom ice (H_2S odor), the predominantly anaerobic, bacterial community would have faced limiting and damaging growth conditions in the aerated melt water; hence, BP would have been highly restricted in the chemically altered bottom ice core melt water.

Algae and ciliates were present in all ice core sections (Fig. 1); however, active forms were not observed and algae and ciliates appeared to over-winter in the ice in the form of spores or cysts. These encapsulated forms were found in the entire ice core, with average values of 2.28×10^5 cells L^{-1} for ciliated cysts and 3.06×10^5 cells L^{-1} for flagellated spores. Higher abundances were enumerated in the top and mid core sections than in the bottom section. The formation of spores or cysts can be a typical stress response to freezing, darkness (for algae), desiccation, osmotic pressure, and/or anoxia (Mataloni *et al.*, 1998; Bell & Laybourn-Parry, 1999; Roberts *et al.*, 2000). However, the PPR rates imply that the wintering spores from phototrophic flagellates germinated in response to our experimental manipulations (melt water and light exposure) (Table 1). The production rates (potential activity, not *in situ* activity, as the cores were melted before analysis) ranged from $931\,\mu g\,C\,L^{-1}\,day^{-1}$ in the top core section to $51\,\mu g\,C\,L^{-1}\,day^{-1}$ in the bottom ice.

Cyanobacteria were sparse within Pony Lake ice and were only detected in the mid section of the ice column. Also, benthic microbial mats, with cyanobacteria commonly found as the dominant form of vegetation (e.g. Vincent & Howard-Williams, 1986; Hawes *et al.*, 1993; Jungblut *et al.*, 2005), were not recovered from the lake bottom with the core sampler. Nutrient-enriched Antarctic lakes, such as Pony Lake, typically lack phytobenthic communities due to limited light penetration during summer (Hawes, 1990).

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DGGE fingerprinting analysis

We used DGGE as a molecular fingerprinting tool to characterize the microbial community structure found in the different ice core sections. The number of bands increased from the top to the bottom of the ice column (Fig. 2). Calculated diversity indices (H') support this finding and successively increased towards the base of the ice column (top: $H' = 0.67$; middle: $H' = 0.96$; and bottom core section: $H' = 1.35$). It is important to stress that the diversity and distribution of the microbial ice community corresponds largely to processes that occurred during freezing such as changes in the freezing rates and in the residual water column chemistry. Considering the partial incorporation and more importantly the rejection of microorganisms at the freezing front, microorganisms will systematically accumulate in the remaining liquid water column, thus becoming most concentrated in the bottom waters before complete freezeup. Besides the accretion of bacteria as indicated by the highest cell numbers found in the bottom ice (Fig. 1), the formation of a basal brine, typically observed in shallow Antarctic ponds or lakes during the final stage of freezing (Schmidt *et al.*, 1991; Healy *et al.*, 2006; Wait *et al.*, 2006; C.M. Foreman *et al.*, unpublished data), may also contribute to the increase in diversity in the bottom water and ultimately ice core section. Insulated by the overlying ice, the residual water will remain liquid until the temperature declines below the freezing point temperature of the basal brine solution, and basal brine temperatures as low as $-20\,^{\circ}C$ have been reported (Healy *et al.*, 2006; Wait *et al.*, 2006). Although hypersaline in nature, a liquid basal brine could potentially extend the growth season. It is noteworthy that the bottom ice of Pony Lake was highly enriched with dissolved organic matter (DOM). C.M. Foreman *et al.* (unpublished data) detected $\sim 140\,mg\,L^{-1}$ of DOM in the melt waters from the bottom ice core sections of Pony Lake. Extensive utilization of the organic matter pool could deplete oxygen levels, driving a shift in the microbial community from aerobic to anaerobic members. In particular, the strong odor of H_2S supports this assumption. Thus, in addition to freeze concentration and the partial incorporation and rejection of organisms at the freezing front, the physicochemical environment of the bottom waters (e.g. severe osmotic, pH, temperature, and redox conditions; Schmidt *et al.*, 1991) may be a driving force for alterations in the microbial community composition.

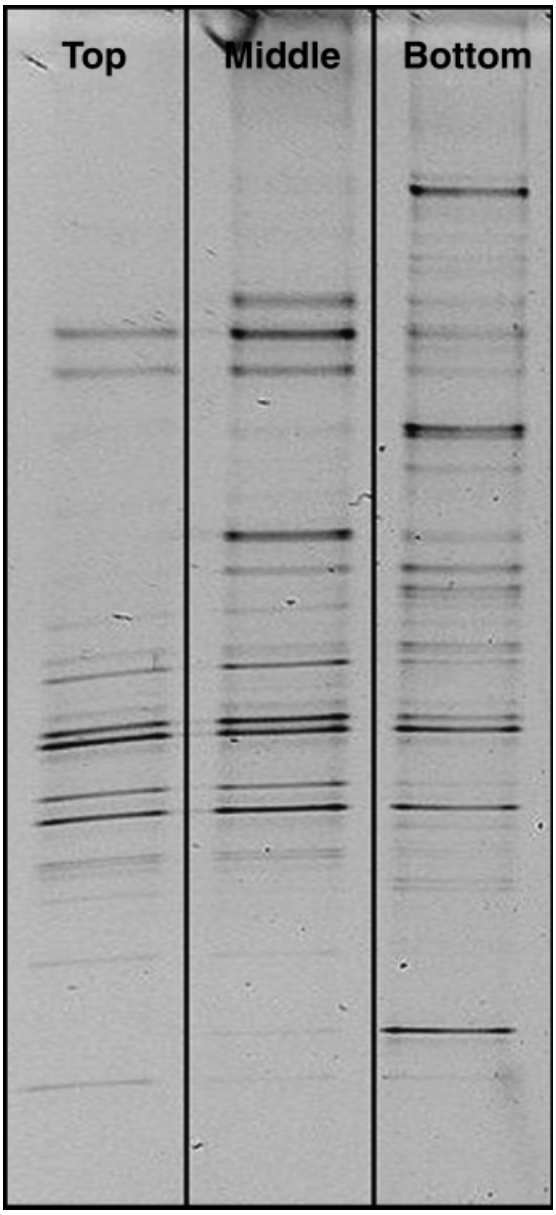

Fig. 2. Differences in DGGE banding patterns between microbial communities in ice core sections from Pony Lake in November 2004 based on the amplification of the 16S rRNA gene. Profiles from left to right: top, middle, and bottom 40-cm sections. Image colors were inverted on the camera, but not manipulated.

16S rRNA gene sequences

In the Pony Lake ice core clone libraries, 36 unique phylotypes were identified in the Pony Lake ice core clone libraries, including the following bacterial lineages: *Bacteroidetes*, *Firmicutes*, *Beta-*, *Gamma-*, *Delta-*, *Epsilonproteobacteria*, *Spirochaetes*, and *Verrucomicrobia*. The sequences were submitted to GenBank, with all Pony Lake clones having the prefix ANTPL_. Accession numbers for the Pony Lake clones are from HM192934 to HM193003. Figure 3 shows the relative distribution of the groups within the clone libraries from the three ice core sections. The phylogenetic relationship of Pony Lake clones with their closest neighbors is summarized in Table 2. The number of different bacterial groups, as well as the number of phylotypes present in each group, varied considerably between the three ice core

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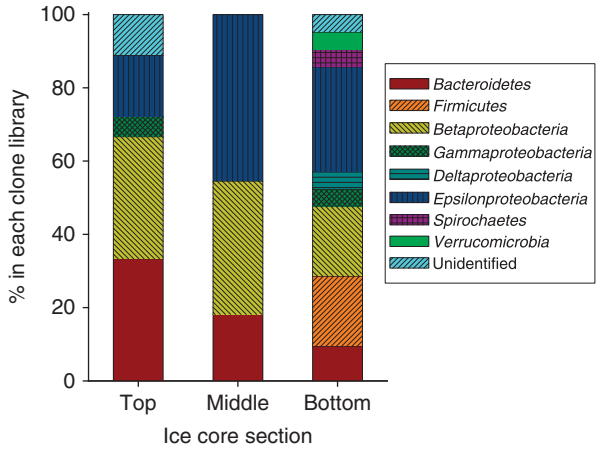

Fig. 3. Distribution of taxonomic classes within clone libraries from Pony Lake ice core sections. *Bacteroidetes*, *Beta-*, and *Epsilonproteobacteria* were the dominant classes identified. Differences occur in the number of taxonomic groups found within each ice core section.

sections. The top core section contained 18 phylotypes. These clones belonged to four major phyla, and included *Betaproteobacteria* (33.3%), *Bacteroidetes* (33.3%), *Epsilonproteobacteria* (16.7%), and *Gammaproteobacteria* (5.6%). In the mid ice core section, 11 different phylotypes were identified, representing three bacterial phyla: *Epsilonproteobacteria* (45.5%), *Betaproteobacteria* (36.4%), and *Bacteroidetes* (18.2%). The bottom core section showed the highest degree of diversity; 21 phylotypes were identified associated with eight different phyla. The dominant phyla came from the *Epsilonproteobacteria* (28.6%), *Betaproteobacteria* (19%), *Firmicutes* (19%), and *Bacteroidetes* (9.5%) lineages. All other phyla in the bottom core section were described by only one sequence type. Members of the *Firmicutes*, *Deltaproteobacteria*, *Spirochaetes*, and *Verrucomicrobia* lineages were exclusively identified in the bottom core section.

In an attempt to link the phylogenetic data to Pony Lake biogeochemistry, the most informative relationships can be found within the *Delta-* and *Epsilonproteobacteria*. The Pony Lake bottom core clone ANTPL_BC19 was most closely related to *Desulfuromonas svalbardensis* 60, an Fe(III)-reducing bacteria isolated from Arctic sediments, Svalbard (Vandieken *et al.*, 2006), and belonging to a group of sulfur-reducing bacteria. Pony Lake bottom ice had a strong H_2S odor, and the presence of such a phylotype indicates that *in situ* sulfur reduction may be occurring at the base of Pony Lake during winter. ANTPL_TC07, found within the top of the ice column, showed close sequence identity with *Sulfurimonas denitrificans* DSM 1251, a sulfur-oxidizing, chemolithoautotroph *Epsilonproteobacterium* (Sievert *et al.*, 2008). *Sulfurimonas denitrificans* is involved in the transformation of sulfur through the process of sulfur oxidation, and converts nitrate to dinitrogen gas via denitrification. The process of denitrification would decrease nitrate concentrations. Thus, the two phylotypes may play an important role in the sulfur cycle and the latter in the nitrogen cycle of the lake ecosystem.

Overall, ~64% of the phylotypes were related to bacteria reported from other Antarctic lake and marine environments. However, many of these nearest phylogenetic neighbors were reported to be uncultured bacterial strains and clones. Therefore, in order to provide a more informative level of identification, we assigned isolated bacterial strains from non-Antarctic environments to the Pony Lake clones as the nearest neighbors in Table 2. The uncultured Antarctic bacterial strains and clones were related to the members of *Bacteroidetes* (Prabagaran *et al.*, 2007), *Betaproteobacteria* (Van Trappen *et al.*, 2002; Pearce *et al.*, 2005) and *Epsilonproteobacteria* (Bowman & McCuaig, 2003), uncultured bacterial clones from penguin dropping sediments, and uncultured bacteria from Heywood Lake (Pearce *et al.*, 2005). The uncultured *Verrucomicrobia* strain was found in anoxic sediment from marine and coastal meromictic lakes in the Vestfold Hills, Antarctica (Bowman *et al.*, 2000). Cultured *Bacteroidetes* clones were isolated from Antarctic sea ice habitats and marine sediments (Bowman *et al.*, 1997; Humphry *et al.*, 2001). *Rhodoferax* was associated with purple nonsulfur bacteria isolated from microbial mats from Lake Fryxell (Jung *et al.*, 2004). The close similarity of Pony Lake clones to the bacterial strains described in many different Antarctic environments implies a high degree of phylogenetic and ecophysiological consensus. One might see Antarctica as an ideal place to find evolutionary, endemic species; however, the close relationship of the same Pony Lake clones with bacteria from temperate environments suggests that bacteria found in Antarctica have adapted to the cold and harsh conditions rather than being restricted to the continent. Nonetheless, the occurrence of numerous Antarctic bacterial strains and clones related to Pony Lake clones suggests a certain level of geographic speciation in Antarctica.

Both the DGGE profiles and the clone libraries reflect differences in microbial community structure between the three ice core sections. Besides the differences in the total number of phylotypes (TC: 18; MC: 11; and BC: 21) and the number of phyla (TC: 4; MC: 3; and BC: 8) present in each core fraction, the fraction of sequence types that were present in only one of the three core segments was high. 56% of the phylotypes in the top core, 36% in the mid core, and 62% of the bottom core sequence types were restricted to only that specific core section. Five sequence types related to *Betaproteobacteria* and *Epsilonproteobacteria* were found in all three core sections. Monothetic clustering underscored the small degree of overlap between ice core sections and confirmed the pronounced variation between the top, middle, and bottom core clone libraries (Fig. 4). Exploring the

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Table 2. Affiliation of 16S rRNA gene phylotypes in Pony Lake ice core sections

				Core section					
Taxonomic phylum	Taxonomic class	16S rRNA gene identification (closest neighbor)	GenBank no.	Top clone	% id.	Middle clone	% id.	Bottom clone	% id.
Bacteroidetes	*Flavobacteria*	*Cellulophaga algicola* S3-1, AY771718	HM192934			MC77†	98		
		Flavobacterium frigidarium, AF162266	HM192935	TC15*	98				
		Gelidibacter algens ACAM 536T, U62914	HM192936	TC02*	99				
		Gelidibacter algens ACAM 551T, U62916	HM192937	TC75†	98				
	Sphingobacteria	*Algoriphagus* sp. ZS3-3, FJ196000	HM192938			MC48*	95		
	Uncultured	Uncultured *Cytophaga* sp. JTB220, AB015266	HM192939	TC57	99				
		Uncultured *Bacteroidetes* bacterium MT054, AM157468	HM192940	TC19	97				
		Uncultured *Bacteroidetes* bacterium F4C06, AY794184	HM192941	TC21*	98				
		Uncultured *Bacteroidetes* bacterium 1D5, AJ627991	HM192942					BC10	98
		Uncultured *Bacteroidetes* bacterium CF61, AY274859	HM192943					BC73	97
Firmicutes	*Clostridia*	*Clostridiales* bacterium 12-2A, EU523731	HM192944					BC06†	95
		Clostridium sp. D3RC-3r, FJ527031	HM192945					BC67	97
	Uncultured	Uncultured *Clostridiales* bacterium LEO_13, EU158818	HM192946					BC50	93
		Uncultured *Firmicutes* bacterium D25_36, EU266909	HM192947					BC04	96
Proteobacteria	*Betaproteobacteria*	*Bordetella trematum* DSM 11334 (T), AJ277798	HM192948	TC04†	99	MC39†	99	BC49†	98
		Hydrogenophaga atypica BSB 41.8T, AJ585992	HM192949	TC01†	97	MC07†	97	BC42†	97
		Hydrogenophaga taeniospiralis SE57, AY771764	HM192950	TC03†	99	MC06†	99	BC30†	99
		Hydrogenophaga taeniospiralis, AF078768	HM192951	TC35†	97				
		Rhodoferax antarcticus Fryx1, AY609198	HM192952	TC46*	99			BC26*	99
	Uncultured	Uncultured *Achromobacter* sp. 2SN, EU887771	HM192953	TC12†	97	MC03†	98		
	Gammaproteobacteria	*Glaciecola polaris* LMG 21857, AJ293820	HM192954	TC14†	97			BC51†	97
	Deltaproteobacteria	*Desulfuromonas svalbardensis* 60, AY835390	HM192955					BC19	99
	Epsilonproteobacteria	*Arcobacter* sp., R-28214, AM084124	HM192956			MC02	98	BC34	98
		Arcobacter cibarius LMG 21997, AJ607392	HM192957			MC12	98		
		Arcobacter sp. BSs20195, DQ514311	HM192958					BC02†	95
		Sulfurimonas denitrificans DSM 1251, CP000153	HM192959	TC07	98				
	Uncultured	Uncultured epsilonproteobacterium MERTZ_2CM_162, AF424297	HM192960	TC06*	97	MC11*	97	BC03*	97
		Uncultured *Campylobacterales* bacterium DS057, DQ234141	HM192951	TC20†	98	MC01†	98	BC05†	98
		Uncultured epsilonproteobacterium 131631, AY922199	HM192962			MC51	97		
			HM192963					BC33	96
		Uncultured epsilonproteobacterium D004025D06, EU721824	HM192964					BC46	96
Spirochaetes	*Spirochaetales*	*Sphaerochaeta* sp. TQ1, DQ833400	HM192965					BC14	97
Verruco-microbia	*Verrucomicrobiae*	Uncultured bacterium CLEAR-26, AF146249	HM192966					BC45*	97
Unidentified	Unidentified	Uncultured bacterium KD3-68, AY218614	HM192967					BC75*	98
		Uncultured bacterium KD6-47, AY218761	HM192968	TC70*	95				
		Uncultured bacterium CARB_ER2_7, AY239579	HM192969	TC63	97				

*Pony Lake clones with relatives reported from other Antarctic environments. Many of these close neighbors from Antarctic environments were unidentified bacteria or clones.

†To provide a higher level of identification well-described relatives from non-Antarctic habitats were selected. All Pony Lake clone sequences have been deposited in GenBank with the prefix 'ANTPL_'.

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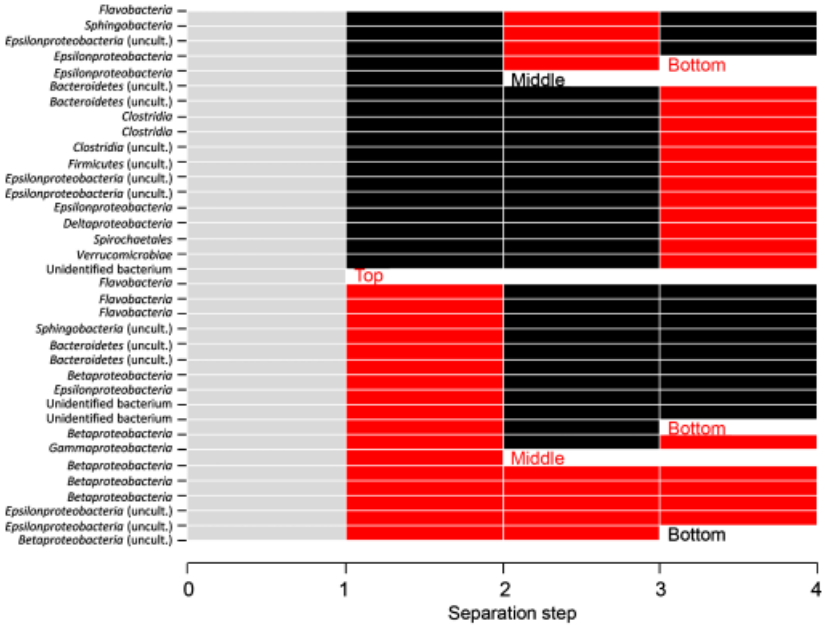

Fig. 4. Banner plot of the monothetic cluster analysis of closely related taxonomic classes and phyla to distinct clones found within Pony Lake ice core sections (top, middle, and bottom). The plot is read from left to right as the clones are separated based on the variable indicated in the white cells in each column. The color of the bar indicates the presence/absence in a particular ice level, with red denoting presence, black absence, and gray indicating the initial cluster before any divisions. White cells indicate the transition between presence/absence in the named ice core section, with the color of the text indicating the presence or absence of the clone at the bottom of a cell. This plot was modified from the typical version to illustrate the results more clearly. The order of variables (top, middle, and then bottom) used in the splits was chosen by the clustering algorithm to provide optimal divisions of responses. It is a coincidence that the variables used coincide with the depth of the cores.

clustering results in more detail, it becomes obvious that the majority of clones present in the top core were absent in the other ice core sections and vice versa. This zonation of microorganisms may involve several factors and could be, for instance, linked to the nature of the summer melt water, whether the lake was fully mixed during high wind events or stratified. We observed circulation of the water column during summer melt later in 2004 and January 2005. However, it is possible that chemical stratification of the lake occurred before our sampling year and cannot be ruled out. Retention of residual basal summer brine is not uncommon for shallow Antarctic ponds (Healy *et al.*, 2006; Wait *et al.*, 2006). Further, once an ice cover has been established, density gradients as well as the incorporation or rejection of microorganisms at the freezing front of the growing ice column may account for the zonation of the microbial assemblage. Based on the phylogenetic information, we hypothesize that oxygen levels were a key regulator in the zonation process, dividing the residual water column before complete freezeup and the ice column into an oxygenated upper section and a micro-oxic or anoxic bottom section. Clones related to sulfate-reducing bacteria, *Clostridium*, *Verrucomicrobiae*, and *Spirochaetales* were found exclusively in the bottom of the ice core. These closest relatives are obligate anaerobes or anaerobes and provide supportive evidence for anoxic conditions at the bottom of Pony Lake.

Summary

The present study provides an insight into the microbial community that over-winters in the ice column of an Antarctic lake. While the harsh Antarctic winter slowly turns Pony Lake into a solid block of ice, the entrained microbial community faces extreme physical and chemical environmental changes. Progressive freezing ineffectively excludes, and partially incorporates, salts into the ice matrix, creating density gradients at the freezing front and in the residual water column that eventually concentrates salts into a basal brine solution. These changes in ionic concentrations alter the lake and ultimately the ice chemistry (Schmidt *et al.*, 1991; Healy *et al.*, 2006; Wait *et al.*, 2006; C.M. Foreman, unpublished data) and increase the physiological stresses on microorganisms towards the base of the lake significantly. Consequently, organisms that persist throughout the year in Pony Lake not only have to endure periods of a frozen entrapment but also must be capable of surviving drastic alterations in their environmental milieu (e.g. osmotic pressure, pH, temperature, oxygen levels) between summer melt and winter freezing. We have demonstrated that the entrapped microbial assemblage was able to withstand these extreme environmental changes and to over-winter within the ice column. When released from the ice into melt water, the bacteria became highly metabolically active. Although only inactive algal spores were found in the ice, the PPR assays clearly indicated that high potential activity is possible when exposed to more favorable growth conditions in the melt water. The majority of phylotypes were restricted to distinct strata within the ice column of Pony Lake (top, middle, and bottom ice core sections). Based on the identification of phylogenetic neighbors to Pony Lake ice clones, we hypothesize that an oxygen gradient within the water column before complete freezeup as well as in the ice column was a driving force for this stratification. Closely

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related obligate anaerobic or anaerobic phylotypes assigned to Pony Lake 16S rRNA gene clones, which were only present in the bottom ice core section, support this assumption. Attempts to link the phylogenetic data to Pony Lake biogeochemistry indicate that this anaerobic community could have a substantial impact on the sulfur and nitrogen cycle of the lake ecosystem. It is noteworthy that typically for Pony Lake, early peripheral melting occurs along its western shore. Progressive melt causes the entire ice front to retreat, releasing its entrapped microbial assemblage. A comparative study of the Pony Lake summer water DGGE profiles (M. Dieser *et al.*, unpublished data) to the DGGE ice core profiles presented herein showed that the bacteria in the top ice core sections were similar to the early melt lake water community. As the season progressed, the in-lake microbial community evolved, the waters became well mixed, and the DGGE profiles changed over the course of the summer.

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