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

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Received 11 May 2010; revised 21 January 2011; accepted 25 January 2011. Final version published online 2 March 2011.

DOI:10.1111/j.1574-6941.2011.01061.x

Editor: Riks Laanbroek

Keywords
Antarctica; lake ice; microorganisms.

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 μg C L⁻¹ day⁻¹. Upon encountering favorable growth conditions in the melt water, primary production ranged from 51 to 931 μg C L⁻¹ day⁻¹. Because of the strong H₂S 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
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; Junghblut 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 super-cooled 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 colonizing 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 °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 °C in the dark (∼48 h). Subsequently, all analyses were conducted on the pooled ‘ice core’ melt water samples (top, middle, and bottom sections).
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 \(\times 10^{18}\) 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 \(\mu\)Ci 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 (\(\times 4\)) for the light assays and into amber bottles (\(\times 2\)) 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 \(\mu\)mol 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 \(\mu\)L 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 \(\times 0.2\) \(\mu\)m black polycarbonate filters with a 0.45 \(\mu\)m nitrocellulose backing filter under gentle vacuum and stained with 500 \(\mu\)L of a 25 \(\times\) SYBR Gold solution for 15 min as described by Lisle & Priscu (2004). Glassware was baked (450°C) and solutions were filter sterilized (0.2 \(\mu\)m) 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 \(\times 1000\).

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 \(-80^\circ\) 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-\(\mu\)m filters in order to remove bacteria. The filtrate was then filtered through a 0.02-\(\mu\)m 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 \(\times\) 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\(^{R}\)-200 0.2-\(\mu\)m 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 \(-80^\circ\)C.

DNA was extracted from the filters using an Ultra Clean Soil DNA Kit (MoBio). Primers 341F (5’- CCTACGCGGAGG-CAGCAG-3’) and 534R (5’- AATACCGGGGCTGCTGG-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 CGCGCGCGGGCGGGCGGGGGGCGACGGG). 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-\(\mu\)L PCR reaction contained 1.5 \(\mu\)L of environmental DNA extract, MgCl\(_2\) buffer (final concentration 1 \(\times\)), Taq Master (final concentration 1 \(\times\)), PCR nucleotide mix (final concentration 800 \(\mu\)M), and Taq DNA polymerase (final concentration 0.025 U \(\mu\)L\(^{-1}\)) (all components from 5 Prime, Eppendorf), upstream and downstream primer.
TACGACTT-3

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absence (Gafan were visually detected and scored based on 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 & Rousseauw, 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 were simultaneously measured in the lake ice. 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 obtained via the incorporation of 3H-thymidine are summarized in Table 1. Abundances increased with ice core depth for both bacteria and VLP. Bacterial numbers ranged from 1.10 × 10⁶ to 2.97 × 10⁷ 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 × 10⁴ and 1.89 × 10⁶ 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.

16S rRNA gene clone library
The two universal 16S rRNA gene primers 9F (5′-GAGTTT GATCCTGGCCTAG-3′) and 1492R (5′-GGTTACCTTGT TACGACT-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 SEQUENCER 4.5 (Gene Code Corporation). The nucleotide sequences were compared with the NCBI nucleotide database using the BLAST search tool (BLAST 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:

\[ H' = - \sum_{i=1}^{s} (p_i) \times (\log p_i) \]

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 & Rousseauw, 1990) was used to cluster the Pony Lake clones based on their presence or absence in the three ice core sections. Monothetic clustering 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.
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 H₂S, 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 production rates (potential activity, not in situ activity, as the cores were melted before analysis) ranged from 931 ± 564 10⁵ cells L⁻¹ day⁻¹ in the top ciliated cysts and 3.06 × 10⁵ 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) ranged from 931 ± 564 10⁵ cells L⁻¹ day⁻¹ in the top core section to 51 ± 27.6200 mg L⁻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).

### 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

<table>
<thead>
<tr>
<th>Ice core section</th>
<th>Top</th>
<th>Middle</th>
<th>Bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (µg C L⁻¹ day⁻¹)</td>
<td>6.69 ± 0.20</td>
<td>37.9 ± 5.29</td>
<td>1.80 ± 1.18</td>
</tr>
<tr>
<td>PPR (µg C L⁻¹ day⁻¹)</td>
<td>931 ± 564</td>
<td>308 ± 99</td>
<td>51 ± 27</td>
</tr>
</tbody>
</table>

### 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. The colored bottom ice also had a high ionic content of brine solutions into the ice (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). Antagonistic 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). 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).
**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 $\sim 140 \, \text{mg L}^{-1}$ 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 $\text{H}_2\text{S}$ 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.

**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 sections.

![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.](image-url)
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, Delta-proteobacteria, 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 Desulfurodomonas 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 H2S 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 droppings 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
<table>
<thead>
<tr>
<th>Taxonomic phylum</th>
<th>Taxonomic class</th>
<th>16S rRNA gene identification (closest neighbor)</th>
<th>GenBank no.</th>
<th>Core section</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td><strong>Flavobacteria</strong></td>
<td><em>Cellulophaga algicola</em> S3-1, AY771718</td>
<td>HM192934</td>
<td>MC77(^1) 98</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Flavobacterium frigidarium</em>, AF162266</td>
<td>HM192935</td>
<td>TC15(^*) 98</td>
</tr>
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*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\_’.
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 core 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 into an oxygenated upper section and a micro-oxic or anoxic bottom section. Clones related to sulfate-reducing bacteria, *Clostridium*, *Verrucomicrobia*, 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
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.

Acknowledgements

Logistical support was made available by Raytheon Polar Services and Petroleum Helicopters Incorporated. We are grateful to J. Guerard, K. Cawley, R. Fimmen, and the RPSC volunteer field assistants who aided our field work and the invaluable service provided by the PHI helicopter pilots. Funding for this project came from NSF OPP-0338260 to Y.-P.C., OPP-0338299 to D.M.M., OPP-0338121 to P.L.M., and OPP-0338342 to C.M.F. Any opinions, findings, or conclusions stated in this paper are solely those of the authors and do not necessarily reflect the views of the National Science Foundation.

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