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Planktonic microbial community composition across steep physical/chemical gradients in permanently ice-covered Lake Bonney, Antarctica

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ABSTRACT

Lake Bonney is a chemically stratified, permanently ice-covered Antarctic lake that is unusual because anomalous nutrient concentrations in the east lobe suggest that denitrification occurs in the deep suboxic waters of the west lobe but not the east lobe, resulting in high concentrations of nitrate and nitrite below the east lobe chemocline. Environmental factors that usually control denitrification rates (e.g. organic carbon, nitrate, oxygen) do not appear to explain the nitrate distribution in the east lobe, suggesting that other factors (e.g. trace metals, salts, microbial community structure, etc.) may be involved. In order to explore the potential importance of microbial community composition, samples collected from multiple depths in both lobes were compared on the basis of 16S rRNA gene diversity. 16S rRNA polymerase chain reaction (PCR) clone libraries generated from five depths were subjected to restriction fragment length polymorphism (RFLP), rarefaction, statistical and phylogenetic analyses. Bacterial and archaeal 16S rRNA gene sequences were determined for clones corresponding to unique RFLP patterns. The bacterial community below the chemocline (at 25 m) in the east lobe was the least diverse of the five depths analysed and was compositionally distinct from the communities of the overlying waters. The greatest compositional overlap was observed between 16 and 19 m in the east lobe, while the east lobe at 25 m and the west lobe at 13 and 16 m had relatively distinct communities. Despite very little compositional overlap between the suboxic, hypersaline depths of the east and west lobes (25 m and 16 m, respectively), sequences closely related to the denitrifying *Marinobacter* strain ELB17 previously isolated from the east lobe were found in both libraries. Most of the Lake Bonney sequences are fairly distinct from those reported from other Antarctic environments. Archaeal 16S rRNA genes were only successfully amplified from the two hypersaline depths analysed, with only one identical halophilic sequence type occurring in both libraries, indicating extremely low archaeal diversity. Overall, microbial community composition varies both between lobes and across depths within lobes in Lake Bonney, reflecting the steep gradients in physical/chemical parameters across the chemocline, as well as the anomalous nutrient chemistry of the system.

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INTRODUCTION

The McMurdo Dry Valleys, covering around 4800 km^2 (77°00′S, 162°52′E) and forming the largest relatively ice-free region on the Antarctic continent, are a polar desert characterized by average temperatures of –20 °C and high winds (Priscu & Spigel, 1998). This extreme environment supports an array of microbial habitats, from the cryptoendolithic communities that colonize the pore spaces of exposed rock (de la Torre *et al.*, 2003) to the planktonic communities in the water column of permanently ice-covered lakes in the area (Lizotte & Priscu, 1994; Lizotte *et al.*, 1996; Ward & Priscu, 1997; Voytek *et al.*, 1999; Roberts *et al.*, 2000; Karr *et al.*, 2003; Roberts *et al.*, 2004) to the communities living in the permanent ice cover of those lakes (Olson *et al.*, 1998; Paerl & Priscu, 1998; Priscu *et al.*, 1998; Gordon *et al.*, 2000). Investigators of these Antarctic lake and lake ice communities have suggested that these environments might serve as models for potential life-supporting habitats in other ice environments such as those on Mars and Europa (Priscu *et al.*, 1998).

Lake Bonney (77°43′S, 162°20′E) is a permanently ice-covered lake, approximately 4 km^2 in area, in the Taylor Valley of Antarctica that is included in the ongoing Long-Term Ecological Research (LTER) program in the McMurdo Dry Valleys. The physical and hydrological characteristics of Lake Bonney have been thoroughly characterized by Priscu & Spigel (1998). The lake consists of two lobes, both approximately 40 m deep, which are connected by a narrow and shallow (12–13 m deep) passage. The permanent ice cover (3–5 m) prevents wind-driven advective mixing, so transport in the water column is dominated by molecular diffusion and the lake therefore exhibits permanent chemical stratification. The lake is fed intermittently by glacial melt water during the summer months, which flows into the western end of the west lobe at the terminus of the Taylor Glacier at Blood Falls – a prominent feature of the landscape because of the bright red iron staining that gives the 'falls' its name. Water is lost from the lake through ablation and sublimation of the surface ice.

The depth of sill that separates the lobes is above the chemocline in both lobes. Dramatic increases in conductivity and decreases in oxygen concentration coincide within the chemocline, which lies at 15–17 m in the west lobe (WLB) and at 18–20 m in the east lobe (ELB). The depth of the sill allows communication of the oxygenated surface waters between the two lobes, but there is no exchange of bottom waters due to the stable density stratification of the lake. The bottom waters of both lobes are suboxic to anoxic, have subzero (°C) temperatures, salinities six to 10 times that of seawater, and extremely high bioactive and trace metal concentrations (e.g. Mn, Fe, Co, Ni, Cu, Zn) (Boswell *et al.*, 1967a,b; Weand *et al.*, 1976; Priscu *et al.*, 1993; Priscu, 1995; Ward *et al.*, 2003). Nonetheless, the chemistry of the two lobes is significantly different. Profiles of temperature, O_2, chlorophyll *a*, ammonium, N_2O, nitrite and nitrate concentrations from both lobes have been published by Voytek *et al.* (1999), Ward & Priscu (1997) and Priscu *et al.* (1996). The west lobe exhibits the classical chemical distribution of a stratified lake with oxygenated, nitrogen-depleted surface waters transitioning at depth to anoxic waters with very low nitrate and high ammonium concentrations. These distributions, combined with peaks of nitrate and nitrous oxide near the oxic/anoxic interface, point to the activity of aerobic nitrifying bacteria and anaerobic denitrifying bacteria above and below the chemocline, respectively (Ward & Priscu, 1997; Voytek *et al.*, 1999). In contrast, the suboxic waters below the chemocline in the east lobe contain extremely high levels of nitrate and nitrite, indicating a lack of bulk denitrification. In addition, nitrous oxide occurs at the highest natural concentrations ever reported (~40 μM) below the east lobe chemocline, possibly representing a 'fossil record' of microbial N_2O production (Priscu *et al.*, 1996). No denitrification activity was detected via the acetylene block method at *in situ* temperatures in water samples from 22, 25 and 30 m in the east lobe, while denitrification was detected in water from below the chemocline in the west lobe (Priscu *et al.*, 1996). Interestingly, however, denitrification was recently measured in samples from below the chemocline in the east lobe using the acetylene block method, but only when much longer incubations were performed at 12 °C (~8 °C higher than *in situ* temperatures) (Ward *et al.*, 2005).

The apparent lack of denitrification *in situ* in the east lobe is puzzling considering the proximity and other similarities between the two lobes and could be due to toxicity of suboxic bottom waters, inhibition of the denitrifying microbial community, or the lack of denitrifying organisms (Ward *et al.*, 2003). However, previous cultivation- and immunofluorescence-based studies demonstrated that denitrifying bacteria were present to varying extents in both lobes of Lake Bonney (Ward & Priscu, 1997), and thymidine incorporation measurements indicated that ELB cells are still viable, although the water may be toxic (Ward *et al.*, 2003). This suggests that inhibition of denitrifying activity or differences in denitrifier community composition could be the cause. Although there is limited communication between the two lobes in the present day, there is evidence that a colder and drier climate existed in the recent geological past (~3000 years ago) that led to complete separation of the two lobes, and while freshwater still flowed into the west lobe from the Taylor Glacier, evaporation led to hypersaline conditions in the east lobe (Lyons *et al.*, 2000). Warmer and wetter conditions returned ~1000 years ago, reconnecting the two lobes and giving rise to the present day conditions. The long separation and different evaporation histories of the two lobes are likely responsible for their differing chemical properties. It is reasonable to expect that the deep suboxic waters of the two lobes will have unique microbial populations, reflective of their distinct histories and chemical compositions.

In this study, we examined the microbial communities at multiple depths in the water column of permanently ice-covered Lake Bonney, Antarctica. The objectives of the study were threefold: (i) to compare the diversity and distribution of microbial communities in the two lobes and to determine the degree of compositional overlap; (ii) to investigate relationships between the diversity and distribution of the communities and the physical/chemical gradients in the water column; and (iii) to compare the Lake Bonney water column assemblages to those previously described in other Antarctic and aquatic environments.

MATERIALS AND METHODS

Sampling and DNA extraction

The water samples analysed in this study were collected as described by Ward *et al.* (2003) from a central station in both lobes of Lake Bonney in December 2000. WLB samples were collected on 7 December 2000, whereas ELB samples were

collected on 11 December 2000. Lake water was pumped from depth using a peristaltic pump and an acid cleaned teflon tube lowered through a hole in the 4 m thick permanent ice cover. Sample depths are reported relative to the free water surface and were measured by a metered line weighted with a Teflon-wrapped lead weight. Approximately 4–10 L of water from each of five depths (13 and 16 m in the west lobe and 16, 19 and 25 m in the east lobe) were filtered onto SpiralCap® capsules and immediately frozen and stored below –70 °C until DNA extraction. DNA was extracted from frozen SpiralCap® filters by coupling a boiling lysis protocol (Voytek and Kirshtein, unpublished) with the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA). Briefly, after the addition of 12 mL of TE/1%SDS to frozen SpiralCap® filters and sealing each end with luer lock caps, samples were boiled (with occasional shaking and venting) for 20 min. The lysate was transferred to centrifuge tubes and placed on ice for 90 min to precipitate the SDS. Following centrifugation for 30 min at 17 000 × *g* to remove cell debris, DNA was precipitated overnight with 1 : 10 volume of 2 M NaCl and two volumes of ice cold 100% ethanol. The DNA was pelleted at 32 000 × *g* for 30 min, resuspended in 300 µL of sterile water, and further purified using the FastDNA SPIN kit for soil (Qbiogene) protocol, according to manufacturer's instructions (except for the omission of the homogenization step).

PCR amplification and cloning

Bacterial 16S rRNA genes were amplified with 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) bacteria-specific oligonucleotide primers (Lane, 1991), using the following protocol: 95 °C for 15 min; 30 cycles consisting of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s; 72 °C for 15 min. Each 50 µL reaction mixture contained 1 µL community DNA as a template, 0.5 µM of each primer, 3.5 mM $MgCl_2$, 0.4 mM of each deoxynucleoside triphosphate, 1.25 U of HotStarTaq™ DNA polymerase (QIAGEN, Valencia, CA, USA), and the 10× polymerase chain reaction (PCR) buffer supplied with the enzyme. After visualization of PCR products via electrophoresis in 1% agarose gels, triplicate PCRs were pooled (to minimize PCR bias), purified, and concentrated using the MinElute PCR Purification kit (QIAGEN).

Archaeal 16S rRNA genes were amplified either directly using the archaea-specific primers A21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and A958R (5′-YCCGGCGTTGAMTCCAATT-3′) (DeLong, 1992), or via a two-step nested PCR in which primers A21F and 1391R (5′-GACGGGCGGTGWGRCA-3′) (Lane, 1991) were used to generate large products, which were subsequently subjected to a second nested amplification using A21F and A958R. Prior to cloning, triplicate PCRs were pooled, gel-purified and concentrated as described above.

PCR products were cloned into the vector pCR®2.1-TOPO® using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA). A total of 222 white colonies from each of the bacterial cloning reactions (WLB13, WLB16, ELB16, ELB19, and ELB25), and 190 positive colonies from each of the archaeal cloning reactions (WLB16A and ELB25A) were transferred to 96-well plates containing Luria–Bertani media with 50 µg mL^{-1} kanamycin, and incubated at 37 °C overnight. Clones were PCR screened in 96-well format directly for the presence of inserts using T7 (5′-AATACGACTCACTATAG-3′) and M13R (5′-AACAGCTATGACCATG-3′) vector primers (QIAGEN). The T7-M13R PCR products were visualized by agarose gel electrophoresis. Selected clones were subjected to RFLP analysis and sequencing.

RFLP analysis

Cloned bacterial 16S rRNA PCR products were digested with the restriction enzyme *Hha*I (Invitrogen), whereas archaeal 16S rRNA gene clones were subjected to a double digest with the enzymes *Hha*I and *Hae*III, for 3 h at 37 °C. RFLP patterns were visualized in 2.5% agarose gels stained with ethidium bromide, using a 50–2000 bp DNA marker (Bio-Rad) as a reference. Gel images were captured and analysed using the Bio-Rad Gel-Doc EQ system. Each unique RFLP pattern was designated as a unique operational taxonomic unit (OTU), and at least two representatives of each unique RFLP pattern were sequenced for phylogenetic identification.

Phylogenetic analysis

Sequencing of both strands of the T7/M13 PCR products was performed using T7 and M13R vector primers and BigDye™ chemistry using ABI 3100 and 3700 capillary sequencers (PE Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared to GenBank database sequences using BLAST (Altschul *et al.*, 1997; Benson *et al.*, 2000). For the bacterial clones, a subset of those sequenced with T7 and M13R primers, with samples corresponding to plastid sequences removed, were then sequenced with the bacteria-specific internal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) (Lane, 1991), giving at least 2× sequence coverage.

Nucleotide sequences were assembled and edited using SEQUENCHER™ 4.2.2 (Gene Codes Corp., Ann Arbor, MI, USA). Initial alignment of amplified bacterial 16S rRNA gene sequences was performed using the automated 16S rRNA sequence aligner of the ARB software package (Ludwig *et al.*, 2004) against the SSU Prokaryote database from the Ribosomal Database Project (RDP), a database of 16 277 complete and partial rRNA sequences. This database was supplemented with relevant environmental sequences from GenBank submitted too recently to be included in the latest RDP database. Ambiguously and incorrectly aligned positions were aligned

manually on the basis of conserved primary sequence and secondary structure. The phylogenetic trees were created based on distance analysis using the neighbour-joining algorithm of the software program PAUP version 4.0b10 with a Jukes–Cantor correction (Swofford, 1999). The phylogenetic associations for the bacterial populations were determined from at least 1218 masked positions and the associations for the archaeal populations from 636 masked positions. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences reported in this study have been deposited in GenBank under accession numbers DQ015764 to DQ015864.

Statistical analysis

To compare the observed 'species' (i.e. OTU) richness within each 16S rRNA gene clone library, rarefaction analysis (Heck *et al.*, 1975) was performed using the ANALYTIC RAREFACTION 1.3 program. For this analysis, OTUs were defined as clones exhibiting distinct RFLP patterns. Nonparametric richness estimators [Abundance-based Coverage Estimators (ACE), Chao1, and Chao2] and classical diversity (Shannon and Simpson's) statistics were computed using EstimateS (http://viceroy.eeb.uconn.edu/EstimateS) (Colwell 1997–2000).

RESULTS AND DISCUSSION

Sampling and generation of clone libraries

In order to examine the microbial community structure across key depths in the water column of Lake Bonney, 16S rRNA PCR clone libraries were generated from community DNA obtained from five depths from both lobes (13 m and 16 m in the west lobe; 16 m, 19 m and 25 m in the east lobe). These depths were chosen as representative of surface waters (WLB13 and ELB16) and waters within the chemocline (WLB16 and ELB19) in both lobes, and deep suboxic water from the east lobe (ELB25). In addition, denitrification activity (at 12 °C) was recently detected in samples collected from four of the five depths (Ward *et al.*, 2005). Bacterial 16S rRNA gene clone libraries were generated from each of these five depths, whereas archaeal 16S rRNA amplicons and clone libraries were only successfully obtained from WLB16 and ELB25. Due to extremely low DNA extraction yields from deep-water samples (>19 m) from the west lobe, no samples from below 16 m were analysed.

Water chemistry data and profiles for Lake Bonney have been previously published by Priscu *et al.* (1996), Ward & Priscu (1997), Voytek *et al.* (1999) and Ward *et al.* (2003). The nitrogen, conductivity and temperature profiles measured in December 2000 (corresponding to the collection time of the samples analysed in this study) are illustrated in Fig. 1. The depths from which our clone libraries were derived are briefly characterized as follows: WLB13, oxygen is supersaturated, peaks in chlorophyll *a*, N_2O, and nitrate; WLB16, chemocline depth in the west lobe where oxygen and chlorophyll decrease sharply, salinity increases, nitrite and nitrate concentrations are maximal; ELB16, local minimum in chlorophyll *a* (which peaks at 12–13 m), minimum in all forms of dissolved inorganic nitrogen, supersaturated with respect to oxygen; ELB19, within the chemocline of the east lobe where oxygen starts to decrease while ammonium, N_2O, nitrate, nitrite, and salinity increase; and ELB25, representative of deep ELB water with very high ammonium, N_2O, nitrate and nitrite concentrations, minimum oxygen concentration and salinity roughly 10 times that of seawater (but distinct in chemical composition from seawater). ELB25 is also the depth at which many dissolved trace metals (Mn, Ni, Zn, Cu, Co, Fe, Pb, Mo,

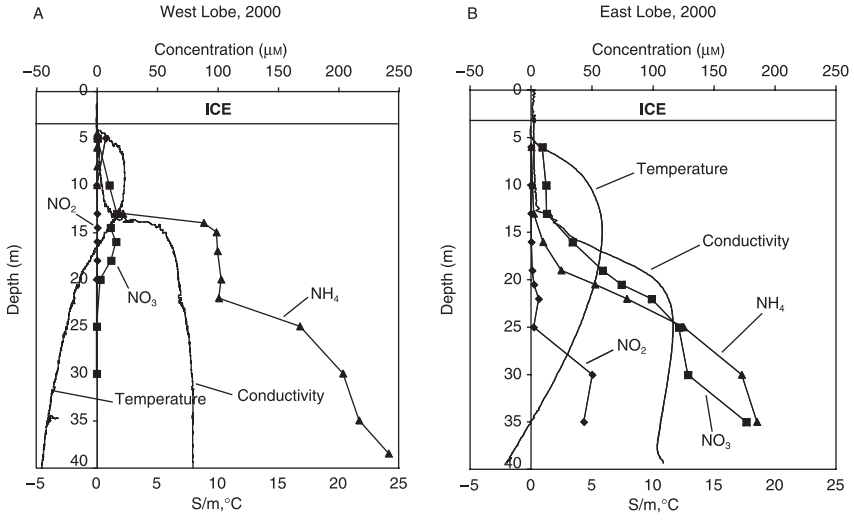

Fig. 1 Nutrient, temperature and conductivity profiles for the (A) west lobe and (B) east lobe of Lake Bonney for December 2000. Ammonium data for the west lobe in 2000 were not available, so the November 1999 data from LTER site (http://huey.colorado.edu/LTER) are plotted instead.

and Cd; in order of decreasing concentration) are at their maximum concentration in the east lobe (Ward *et al.*, 2003).

Although little is known regarding the composition of the prokaryotic communities in the Lake Bonney water column, the composition of phytoplankton communities has been described previously and organisms were shown to occur in highly stratified layers. For example, in ELB, flagellated cryptophyte and chlorophyte algae dominated the shallow assemblages (4–8 m), chrysophytes dominated from 8 to 16 m, and both chrysophytes and chlorophytes constituted the deep chlorophyll maxima (Lizotte *et al.*, 1996; Lizotte & Priscu, 1998; Priscu *et al.*, 1999). While heterotrophic protozoans were long believed to be absent from the water columns of the McMurdo Dry Valley lakes, various rotifers, ciliates, and both heterotrophic and mixotrophic flagellates have now been shown to represent important components of these microbial food webs (Laybourn-Parry *et al.*, 1997; James *et al.*, 1998; Priscu *et al.*, 1999; Roberts & Laybourn-Parry, 1999). In terms of our present study of prokaryotic diversity in Lake Bonney, it is interesting to note that ciliate biomass and diversity have been shown to be much lower in this lake than in Lake Hoare or Lake Fryxell (Priscu *et al.*, 1999). Although not determined for the samples in this study, total bacterial cell numbers for November–December in Lake Bonney have previously been shown to be highest within the chemocline (~15 m) of the west lobe, but curiously highest below the chemocline (between 20 and 30 m) in the east lobe, with maximal reported concentrations ranging from 2 to 8×10^6 mL^{-1} (Ward & Priscu, 1997; Dore & Priscu, 2001).

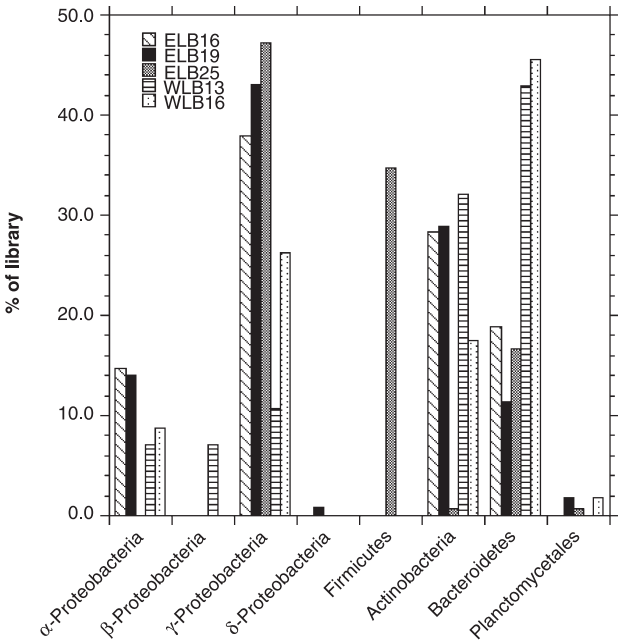

Fig. 2 Distribution of bacterial 16S rRNA gene clone library sequences from five depths within the major phylogenetic groups detected in Lake Bonney. Percentages of each group were determined from RFLP and sequence data.

Analysis of bacterial 16S rRNA gene clone libraries

In total, 222 bacterial 16S rRNA clones from each of five libraries were PCR screened and analysed by RFLP. A significant number of clones in each library corresponded to plastid sequences, but these sequences appeared with much higher frequency in the west lobe libraries (75% of WLB13 and 68% of WLB16, vs. 47% in ELB16, 25% in ELB19, and 4% in ELB25). After patterns corresponding to these plastid sequences were removed, a total of 438 clones from the five bacterial clone libraries (95 from ELB16, 114 from ELB19, 144 from ELB25, 28 from WLB13, and 57 from WLB16) yielded clear RFLP patterns that were suitable for rarefaction and richness analysis, and comparison with sequence data. Representatives of each distinct RFLP pattern were sequenced and aligned with an extensive sequence database from both environmental clones and cultivated organisms. The Lake Bonney sequences corresponded to roughly 55 distinct phylotypes, which encompassed the following bacterial lineages: Bacteroidetes (*Cytophaga–Flexibacter–Bacteroides* or CFB), α-, β-, γ- and δ-Proteobacteria, Gram-positive (Firmicutes and Actinobacteria) and Planctomycetales groups. The relative distribution of the major phylogenetic groups within each clone library is shown in Fig. 2, the phylogenetic relationships among clones are displayed in Figs 3–7, and the relative richness and distribution of phylotypes (i.e. OTUs) among libraries are displayed in Figures 8 and 9, respectively.

Bacteroidetes

The Bacteroidetes sequences account for the major fraction of clones from the west lobe depths (43% of WLB13 and 46% of WLB16), and up to 19% of east lobe depths (Figs 2 and 3). There is some compositional overlap (i.e. closely related sequences) between the five libraries within the Bacteroidetes group. Overlap between the east lobe depths is represented by groups C5, C8, C9 and C11 (Fig. 3). The sequence type represented by C3 is found above the chemocline in both lobes (in ELB16 and WLB13), the C14 sequence type is found only within the chemocline in both lobes (in ELB19 and WLB16) and, interestingly, the C13 sequence type is found in WLB16 and ELB25 but not in any of the overlying depths. Although many of the Lake Bonney Bacteroidetes clones are phylogenetically distinct from database sequences, several of the clones are most similar to Arctic sea ice, Antarctic lake microbial mat, and estuarine sequences.

β- and γ-Proteobacteria

Sequences within the γ-Proteobacteria group comprise the largest fraction of the east lobe libraries (38% of ELB16, 43% of ELB19, and 47% of ELB25), while they account for 10% and 26% of WLB13 and WLB16, respectively (Figs 2 and 4). The *Halomonas*-like sequence type G6 alone makes up 22% of

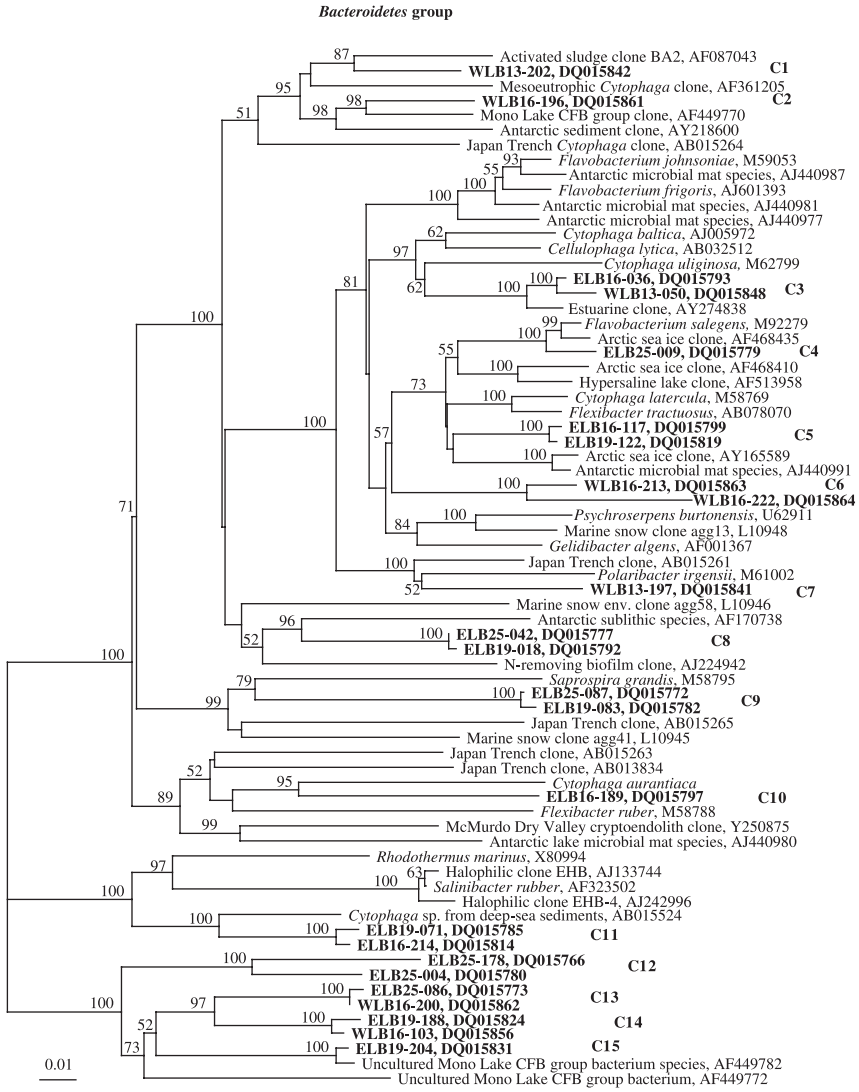

Fig. 3 Unrooted neighbour-joining phylogenetic tree of the Bacteroidetes group (with a Jukes–Cantor correction) using a 1284 nucleotide mask of unambiguously aligned positions. Bootstrap values >50% are shown at the nodes. Scale bar indicates 0.01 substitutions per nucleotide position. GenBank accession numbers are listed after each sequence name. Boldface C1–C15 labels correspond to sequence groups identified by RFLP.

the hypersaline ELB25 library (but only 1% of the ELB19 library), consistent with the extreme salt tolerance and requirements of some *Halomonas* species (Ventosa *et al.*, 1998; Kaye & Baross, 2000). Overall, there appears to be more compositional overlap between the five depths analysed among the γ-Proteobacteria than any other phylogenetic group (Fig. 4). For example, the sequence type represented by G13 (most similar to a widely distributed 'oligotrophic marine' gamma-proteobacterial group) occurs in all five libraries. The G1 sequence type occurs in ELB16 and WLB16, whereas G18 occurs within ELB19, ELB25 and WLB16. Although G14 and G16 cluster with some Arctic sequences, most of the Lake Bonney γ-proteobacterial clones are most similar to various 'nonpolar' marine sequences.

In terms of linking bacterial phylogeny to biogeochemical function, perhaps the most interesting feature of the Lake Bonney γ-Proteobacteria group is the cluster of *Marinobacter*-like sequences in groups G9, G10 and G11. These *Marinobacter*-like sequences account for roughly 10% of ELB25, 2% of ELB16 and ELB19, and 3% of WLB16. Only one of the three *Marinobacter* types (G11) was found above the east lobe chemocline. There is near sequence identity between sequence type G9 and that of the denitrifying *Marinobacter* sp. strain ELB17 isolated from 17 m in the east lobe (Ward & Priscu, 1997). Ward & Priscu (1997) detected ELB17 by immunofluorescence throughout the water column in each lobe, but in much higher abundance below the chemocline than in the overlying waters. The ELB17 isolate comprised up to 2–5% of total cell numbers between 17 and 35 m in the east lobe and up to 2% of total cell numbers at 22 m in the west lobe. This is in excellent agreement with the percentages obtained in this study based on 16S rRNA gene clone libraries. In addition, *Marinobacter*-like nitrite reductase gene (*nir*S) sequences were recently identified as the dominant group in both WLB16 and ELB25 clone libraries (unpublished data), further substantiating the potential importance of this group in the lake.

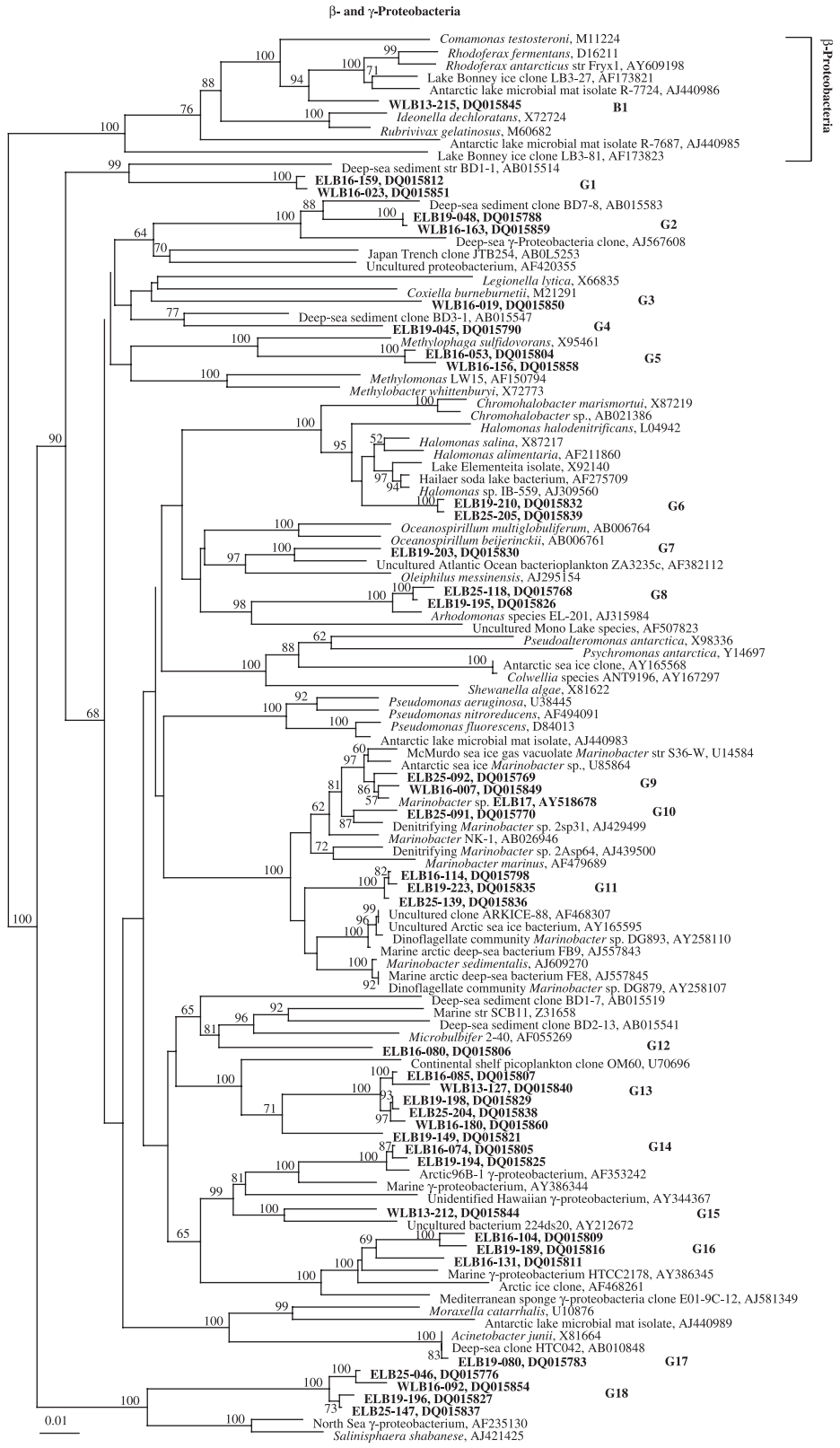

Fig. 4 Unrooted neighbour-joining phylogenetic tree of the β- and γ-Proteobacteria groups (with a Jukes–Cantor correction) using a 1293 nucleotide mask of unambiguously aligned positions. Bootstrap values >50% are shown at the nodes. Scale bar indicates 0.01 substitutions per nucleotide position. GenBank accession numbers are listed after each sequence name. Boldface G1–G19 labels correspond to sequence groups identified by RFLP.

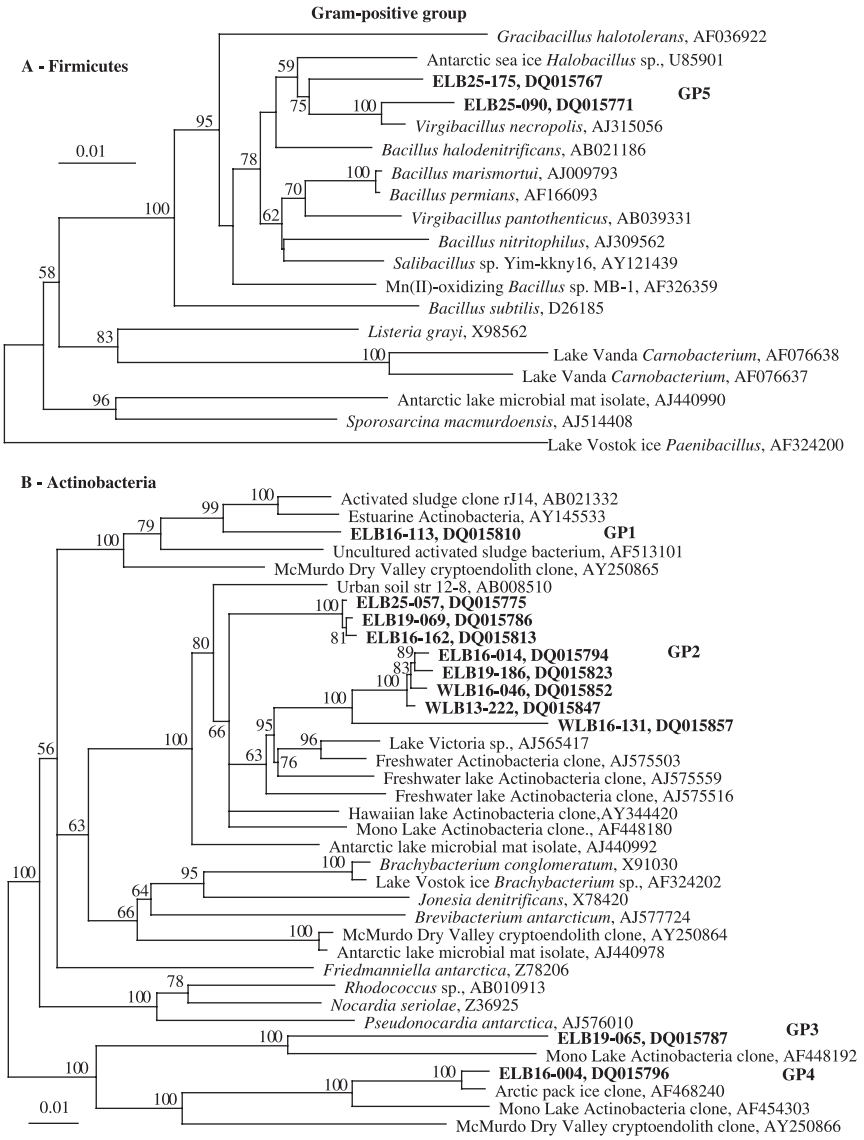

Fig. 5 Unrooted neighbour-joining phylogenetic tree of the Gram-positive group (with a Jukes–Cantor correction) using a 1303 nucleotide mask of unambiguously aligned positions for the (A) Firmicutes, and a 1387 nucleotide mask for the (B) Actinobacteria. Bootstrap values >50% are shown at the nodes. Scale bars indicate 0.01 substitutions per nucleotide position. GenBank accession numbers are listed after each sequence name. Boldface GP1–GP5 labels correspond to sequence groups identified by RFLP.

The only β-Proteobacteria sequence recovered from the Lake Bonney water column was from 13 m in the west lobe. WLB13-215 (B1) is closely related to Lake Bonney ice clone LB3-27 (Gordon *et al.*, 2000), an Antarctic lake microbial mat clone R-7724 (Van Trappen *et al.*, 2002), and *Rhodoferax antarcticus* strain Fryx1, a gas vacuolate planktonic strain from Lake Fryxell. The complete lack of β-proteobacterial clones within the other four depths analysed likely reflects the considerably higher salinities associated with these depths (Fig. 1).

Gram-positives – Actinobacteria and Firmicutes

Sequences belonging to the Gram-positive branches of the bacteria tree can be divided into high G+C (Actinobacteria) and low G+C (Firmicutes) species (Fig. 5). Overall, the Actinobacteria sequences make up ~30% of the ELB16, ELB19 and WLB13 libraries (Fig. 2). Within this group, the most abundant group (GP2) is represented in all five depths and is most similar to various freshwater lake Actinobacteria sequences. While GP2 sequences comprise only a small portion of the ELB25 library (1%), they make up a significant portion of the ELB16 (18%), ELB19 (28%), WLB13 (32%) and WLB16 (14%) libraries. Of the other three sequence types within the Actinobacteria group, which account for less than 10% of any library, GP4 (~99% identical to an Arctic pack ice clone) was found in both WLB16 and ELB16, GP1 was found only in ELB16, and GP3 was found only in ELB19.

The only Firmicutes sequence type (GP5) recovered out of all five libraries was from ELB25, and comprises 35% of that library (Fig. 2). These sequences fall within a group of halophilic and halotolerant *Bacillus* species, and are most closely related to *Virgibacillus necropolis*, the denitrifier

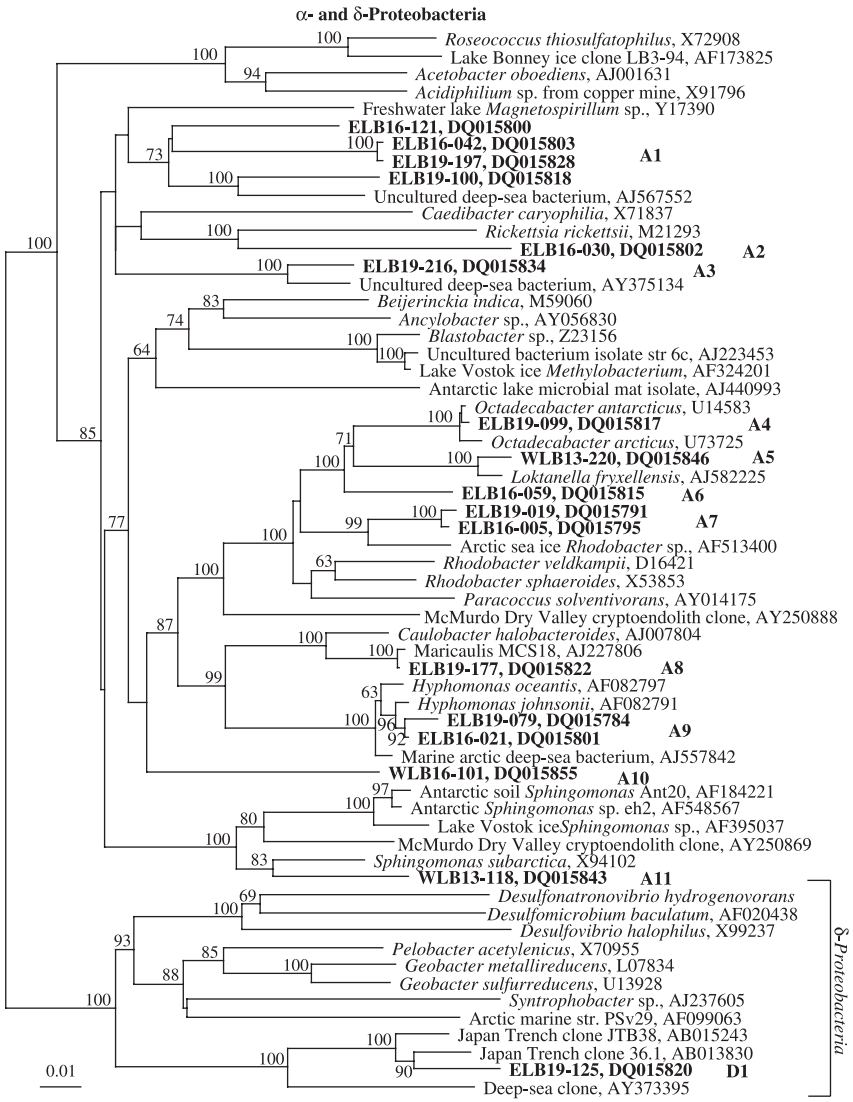

Fig. 6 Unrooted neighbour-joining phylogenetic tree of the α- and δ-Proteobacteria groups (with a Jukes–Cantor correction) using a 1283 nucleotide mask of unambiguously aligned positions. Bootstrap values >50% are shown at the nodes. Scale bar indicates 0.01 substitutions per nucleotide position. GenBank accession numbers are listed after each sequence name. Boldface labels correspond to sequence groups identified by RFLP.

Bacillus halodenitrificans and *Halobacillus* sp. strain MB6-08, a halophile isolated from Antarctic sea ice (Bowman *et al.*, 1997). Despite high concentrations of manganese in Lake Bonney, no sequences were found to be close to the low G+C Mn-reducing *Carnobacterium* species found in Lake Vanda, or the similar *Carnobacterium* sequences found in Ace Lake and the accretion ice of Lake Vostok (Franzmann *et al.*, 1991; Bratina *et al.*, 1998; Christner *et al.*, 2001).

α- and δ-Proteobacteria

The α- and δ-Proteobacteria groups are shown together in Fig. 6. No sequence type makes up more than 10% of a library and, in total, the α- and δ-Proteobacteria make up less than 15% of any one library (Fig. 2). Three sequence types (A1, A7 and A9) show a high degree of overlap (i.e. >99% identity) between ELB16 and ELB19, while all the other sequence types are unique to a single library. No α- or δ-Proteobacteria sequences were recovered in ELB25, A10 is the only α-Proteobacteria sequence type in WLB16, and ELB19 is the only library in which a δ-Proteobacteria clone was found. Consistent with the 'suboxic' but not 'anoxic' state of the subchemocline waters (Lee *et al.*, 2004), no δ-proteobacterial sequences closely related to dissimilatory sulphate- or metal-reducing bacteria were recovered in our clone libraries. Many of the Lake Bonney α-proteobacterial sequences cluster with those from other cold and salty environments (e.g. Arctic sea ice, deep sea). In fact, several clones were quite closely related (98–99% identity) to sequences from cultivated Antarctic strains, including *Octadecabacter antarcticus* (a psychrophilic gas vacuolate bacteria from sea ice) and *Loktanella fryxellensis* (a novel *Rhodobacter*-like isolate from a Lake Fryxell microbial mat). Many Lake Vostok ice clones (Christner *et al.*, 2001), Antarctic lake microbial mat sequences (Van Trappen *et al.*, 2002) and McMurdo Dry Valley cryptoendolith clones (de la

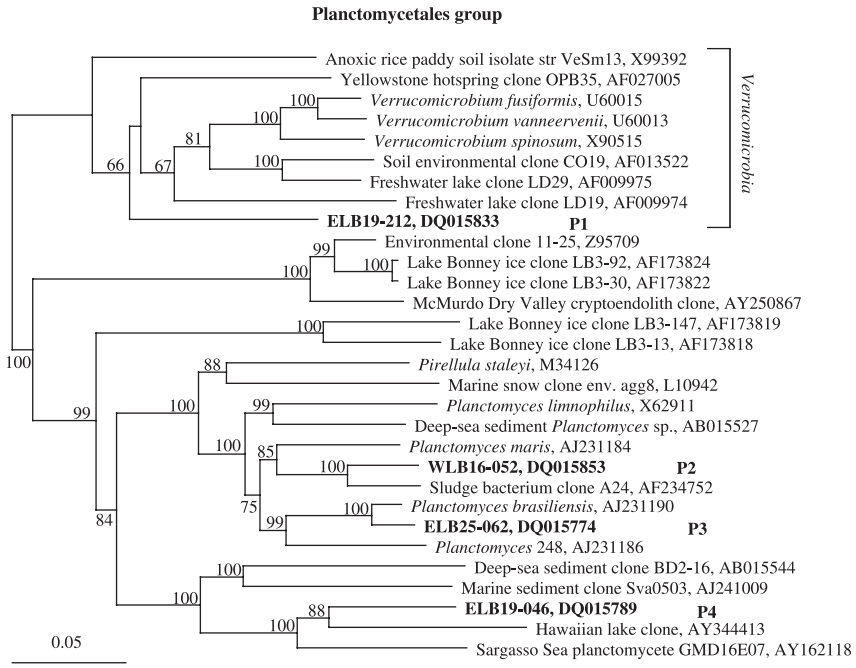

Fig. 7 Unrooted neighbour-joining phylogenetic tree of the Planctomycetales group (with a Jukes–Cantor correction) using a 1249 nucleotide mask of unambiguously aligned positions. Bootstrap values >50% are shown at the nodes. Scale bar indicates 0.05 substitutions per nucleotide position. GenBank accession numbers are listed after each sequence name. Boldface P1–P4 labels correspond to sequence groups identified by RFLP.

Torre *et al.*, 2003) also fall within the α-Proteobacteria lineage, but these are not closely related to the Lake Bonney clones.

Planctomycetales

The smallest contributors to the overall bacterial species composition, as represented by the clone libraries, were members of the Planctomycetales group. Only a few sequences from ELB19, ELB25 and WLB16 fall within this group (Fig. 7). These sequences make up less than 2% of the each library, and there is no overlap of sequences between libraries (Figs 2 and 7). Sequences corresponding to clones from a study of microbes living in the permanent ice cover of Lake Bonney (Gordon *et al.*, 2000) and from a study of the cryptoendolith communities in the McMurdo Dry Valleys (de la Torre *et al.*, 2003) also fall within the Planctomycetales group, but these sequences are not closely related to the Lake Bonney water column clones. The only *Verrucomicrobium*-like sequence recovered from Lake Bonney was the clone ELB19-212, which is quite divergent from other Verrucomicrobia sequences in the database.

Analysis of archaeal clone libraries

RFLP analysis of 190 insert-containing clones from each of the two archaeal 16S rRNA gene libraries (designated WLB16A and ELB25A) generated by nested PCR revealed only one dominant pattern, comprising >90% of the clones. Multiple representatives of this unique pattern were sequenced, and the resulting sequences corresponded to only one unique phylotype found in both WLB16A and ELB25A. This sequence falls into the Euryarchaeota lineage of the domain archaea, which contains extreme halophiles, sulphur and sulphate reducers, methanogens, and the uncultured marine 'Group 2' archaea (Woese *et al.*, 1990; DeLong, 1992; Olsen, 1994). Consistent with the hypersaline depths from which the sequences were amplified, the Lake Bonney archaeal 16S rRNA gene sequence is most closely related (95–96% identity) to environmental clones obtained from various hypersaline environments, including anoxic hypersaline pond sediments (Moune *et al.*, 2003), salt water from a slag heap of a former potassium mine (Ochsenreiter *et al.*, 2002), and Alpine Permo-Triassic rock salt (Radax *et al.*, 2001). Because contamination and other PCR-related artifacts can be easily introduced during re-amplification of PCR products during nested PCR, singly amplified archaeal 16S rDNA from ELB25 was also cloned and unique RFLP patterns sequenced for comparison. These clones were identical to the halophilic archaeal sequence type recovered from the nested ELB25A and WLB16A PCR clone libraries.

The recovery of only one sequence type using the commonly used archaeal-specific primers, 21F and 958R, suggests that the archaeal diversity in Lake Bonney is extremely low; and based on the difficulty of amplifying any archaeal DNA from the depths analysed in this study, the overall abundance of archaea is also likely to be extremely low. This lack of archaea is somewhat surprising given the ubiquity of archaea in the Earth's oceans (DeLong, 1992; Stein & Simon, 1996; Massana *et al.*, 2000), including cold Arctic and Antarctic waters (DeLong *et al.*, 1994; Bano *et al.*, 2004), and the fact that halophilic archaea often dominate under conditions of extremely high salinity (Olsen, 1994). However, our findings are consistent with several other Antarctic studies in which no

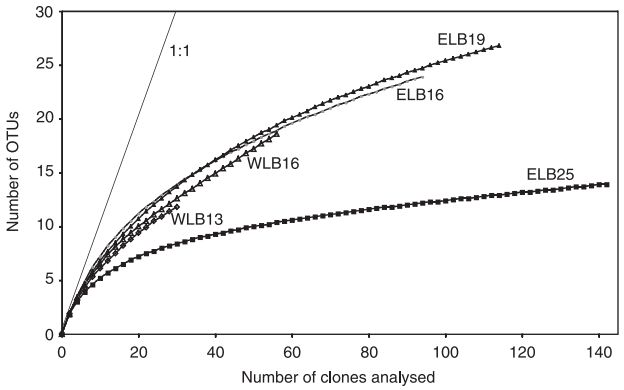

Fig. 8 Rarefaction curves indicating relative richness within bacterial 16S rRNA gene clone libraries in which a unique OTU is defined as a distinct RFLP pattern.

archaeal DNA could be amplified from the McMurdo Dry Valleys cryptoendolithic communities or the accretion ice over Lake Vostok (Christner *et al.*, 2001; de la Torre *et al.*, 2003). The apparently low abundance and diversity of archaea in these systems remains a mystery.

Statistical analysis of bacterial diversity

To compare the observed bacterial taxon richness among the five clone libraries, rarefaction analysis was performed based on the number of unique RFLP patterns (i.e. OTUs) observed relative to the number of clones screened (Fig. 8). From 438 clones, a total of 55 OTUs across all five libraries were identified through the RFLP analysis, the distribution of which is shown in Fig. 9. The rarefaction analysis indicates that the overall bacterial richness in Lake Bonney is quite low, especially compared to other less 'extreme' aquatic systems (e.g. temperate lakes), which often exhibit extremely steep (approaching 1 : 1) rarefaction curves (Hughes *et al.*, 2001). The lowest observed richness clearly occurs in the deep waters of the east lobe (ELB25) where conditions are the most extreme in terms of salinity, nitrate, and dissolved metals, while the remaining four depths appear to have fairly similar levels of richness, all considerably greater than that of ELB25. The rarefaction curves for the west lobe samples are much shorter than those for the east lobe depths, due to the much greater abundance of plastid sequences in the west lobe clone libraries.

Additional measures of relative diversity and richness can be obtained with the statistical richness estimators and diversity indices shown in Table 1, calculated using the EstimateS software package. ACE, Chao1, and Chao2 are nonparametric estimators, which are used to predict the total number of species (or OTUs) in a community based in part on either the distribution of singletons and doubletons (for Chao), or the distribution of abundant (>10) and rare species (for ACE). ACE and Chao1 are often regarded as a lower bound on 'species' richness (Hughes *et al.*, 2001; Bohannan & Hughes, 2003), whereas Chao2 represents more of an upper bound. In the case of the classical ecological diversity indices, Shannon and Simpson's, a higher number represents greater diversity. As with the rarefaction curves, the richness and diversity estimates for WLB16 and especially WLB13 are probably skewed due to the small sample size (as a result of the large fraction of plastid sequences) and, thus, should not be emphasized. However, it is clear from these analyses that the ELB25 library has the lowest diversity and richness of the five depths analysed, consistent with the rarefaction analysis.

CONCLUSIONS

While the overall microbial diversity of Lake Bonney is quite low (especially the archaeal diversity, which is extremely low), the phylogenetic analysis and the statistical diversity/richness estimation techniques both point to the lowest microbial diversity below the chemocline in the east lobe. Two distinctive phylogenetic groups dominated the ELB25 clone library: halophilic *Bacillus*-like sequences and *Halomonas* sequences, consistent with the hypersalinity and generally harsh physical and chemical conditions below the chemocline of the east lobe. As can be seen in Fig. 9, the greatest compositional overlap among clone libraries occurred between ELB16 and

Table 1 Richness and diversity estimates for bacterial 16S rRNA gene clone libraries from five depths in Lake Bonney

Sample	No. of clones*	OTUs†	ACE‡	Chao1§	Chao2§	Shannon¶	Simpson††
ELB16	95	24	40	32	312	2.7	12.4
ELB19	114	27	40	38	392	2.6	8.73
ELB25	144	14	20	27	112	2.0	5.2
WLB13	28	12	24	15	84	2.1	6.8
WLB16	57	19	52	104	200	2.4	8.4

*Number of nonplastid 16S rRNA gene clones in each clone library analysed by RFLP

†Operational Taxonomic Units based on unique RFLP patterns

‡Abundance Coverage Estimator – nonparametric statistical prediction of total richness of different OTUs based on distribution of abundant (>10) and rare (≤10) OTUs

§Nonparametric statistical predictions of total richness of OTUs
based on distribution of singletons and doubletons

¶Shannon diversity index – higher number represents more diversity

††Simpson's diversity index – higher number represents more diversity

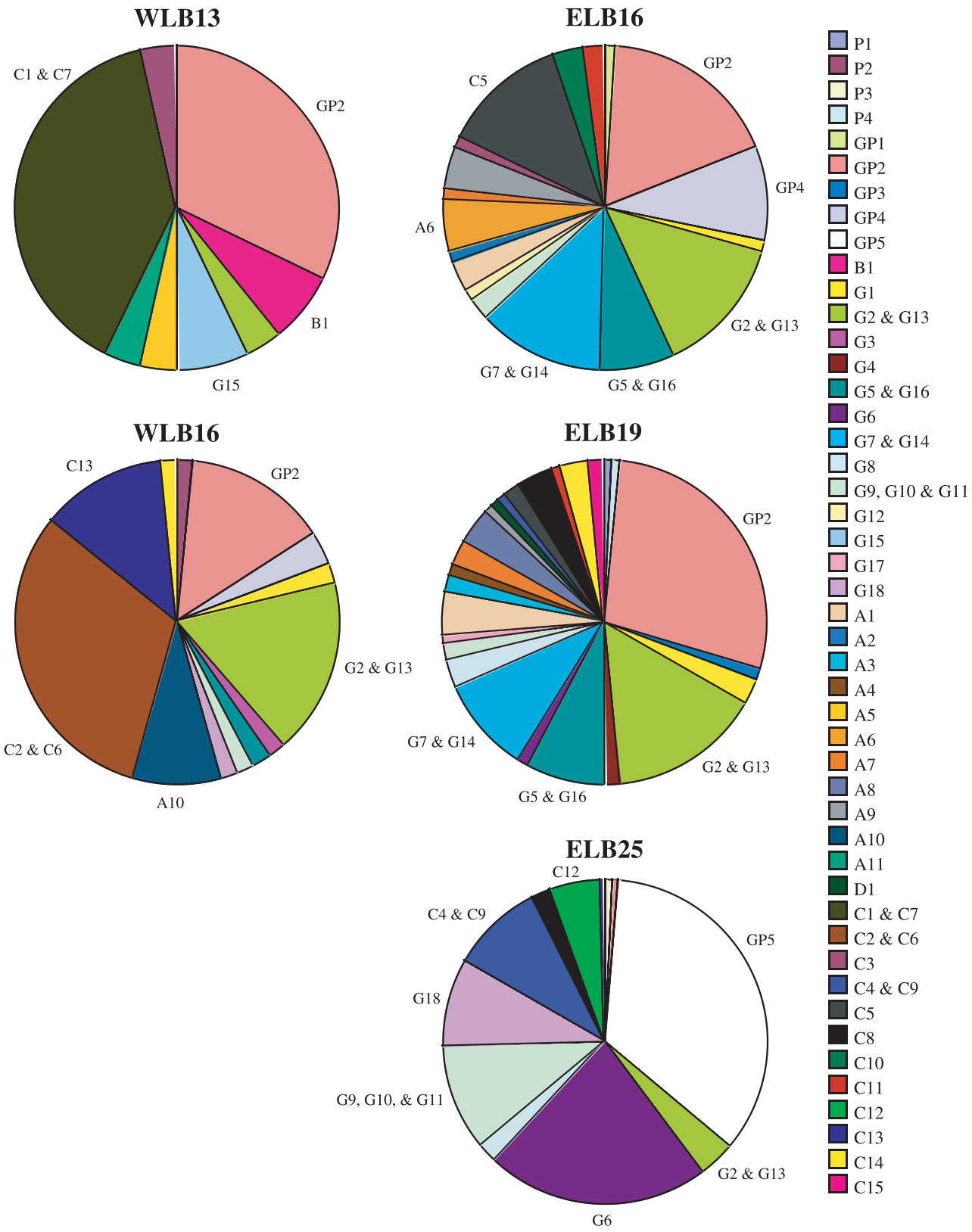

Fig. 9 OTU distribution in the bacterial 16S rRNA gene clone libraries. Each coloured slice of 'pie' represents a unique OTU (i.e. RFLP pattern) and the names (e.g. p1), correspond to the sequence groups highlighted in Figures 3–7.

ELB19, while ELB25, WLB13 and WLB16 appear to have relatively distinct communities. ELB25 has the most distinct and least diverse community, with the large percentage of *Halobacillus*-like sequences (35%) that do not appear in any other library, while ELB16 and ELB19 appear quite similar.

16S rRNA PCR clone library analysis revealed the presence of denitrifying *Marinobacter*-like sequences in the suboxic 'denitrification zones' of each lobe (WLB16 and ELB25), as well as another distinctive *Marinobacter* sequence type also present in ELB16 and ELB19, consistent with the previous enumeration of *Marinobacter* isolates in the lake (Ward & Priscu, 1997). Several sequences showed near identity with the denitrifying *Marinobacter* isolate ELB17, originally isolated from 17 m in the east lobe but found throughout the water column in both lobes (Ward & Priscu, 1997). Thus, the apparent lack of *in situ* denitrification below the chemocline in the east lobe is unlikely to be due to a lack of denitrifying bacteria. This was confirmed in a recent study by Ward *et al.* (2005) in which denitrification was detected below the chemocline in the east lobe at 25 m via acetylene block experiments performed at 12 °C.

The 55 phylotypes into which the Lake Bonney sequences fall represent only a fraction of the known bacterial realm, likely reflecting the limited range of organisms capable of living under such extreme conditions. Interestingly, Bacteroidetes sequences dominate the west lobe depths, while γ-Proteobacteria sequences dominate the east lobe. Many of the Lake Bonney 16S rRNA gene clones were most closely related to environmental clones or isolates from cold (e.g. sea ice, deep-sea sediments) and/or salty (e.g. hypersaline, marine, soda lake) environments. While a number of Lake Bonney clones were similar to Arctic sea ice clones, only one sequence clustered with a sequence from the overlying ice community. The Lake Bonney sequences were not notably similar to the Lake Vostok accretion ice sequences (Christner *et al.*, 2001), McMurdo Dry Valley cryptoendolith communities (de la Torre *et al.*, 2003), Antarctic lake microbial mat communities (Van Trappen *et al.*, 2002), or Antarctic sea ice communities (Bowman *et al.*, 1997), although these and the Lake Bonney ice/sediment clones (Gordon *et al.*, 2000) did fall into more broadly related phylogenetic groups. There may be greater compositional overlap between the surface waters of the lake (5–7 m), which were not analysed in this study, and the surrounding environment, than was detected in this investigation of the deeper communities. The differences we observed may reflect differential survival of components from a common inoculum (derived from both the modern environment and the historical inputs during ice free stages), modified by interactions with the unique chemistry of the lobes.

Although we cannot conclusively infer metabolic function of most lake microbes from a study of 16S rRNA gene diversity alone, the sequence assemblages do reflect the physical and chemical differences between the two lobes. The community compositional differences between each lobe are consistent with their different geological histories and current physical separation. Likewise, the assemblages detected at subchemocline depths (at least in the east lobe where deepwater populations were analysed) are relatively distinct from the overlying waters, reflecting the steep chemical gradients and density stratification of the water column. Although the west lobe biogeochemistry has a clear signature of biological activity, abiotic factors appear to dominate the geochemistry of the east lobe, despite the presence of microbial community that likely includes members capable of denitrification.

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