



RESEARCH ARTICLE

Characterisation of bacterioplankton communities in the meltwater ponds of Bratina Island, Victoria Land, Antarctica

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Abstract

A unique collection of Antarctic aquatic environments (meltwater ponds) lies in close proximity on the rock and sediment-covered undulating surface of the McMurdo Ice Shelf, near Bratina Island (Victoria Land, Antarctica). During the 2009–10 mid-austral summer, sets of discrete water samples were collected across the vertical geochemical gradients of five meltwater ponds (Egg, P70E, Legin, Salt and Orange) for geochemical and microbial community structure analysis. Bacterial DNA fingerprints (using Automated Ribosomal Intergenic Spacer Analysis) statistically clustered communities within ponds based on ANOSIM ($R = 0.766$, $P = 0.001$); however, one highly stratified pond (Egg) had two distinct depth-related bacterial communities ($R = 0.975$, $P = 0.008$). 454 pyrosequencing at three depths within Egg also identified phylum level shifts and increased diversity with depth, *Bacteroidetes* being the dominant phyla in the surface sample and *Proteobacteria* being dominant in the bottom two depths. BEST analysis, which attempts to link community structure and the geochemistry of a pond, identified conductivity and pH individually, and to a lesser extent Ag^{109} , NO_2 and V^{51} as dominant influences to the microbial community structure in these ponds. Increasing abundances of major halo-tolerant OTUs across the strong conductivity gradient reinforce it as the primary driver of community structure in this study.

Introduction

The Antarctic continent offers access to one of the most physically and chemically demanding environments on earth. Antarctic limnetic ecosystems (lakes and ponds), in particular, experience large fluctuations in temperature and light regimes and are characterised by steep chemical gradients, which greatly impact physiological adaptations and life cycle strategies (Wait *et al.*, 2006). These ecosystems, scattered throughout the Antarctic continent and on the surrounding sea ice, provide an extraordinary and tractable opportunity to examine microbial distribution in distinct and environmentally extreme microenvironments. Of the thousands of lakes and ponds throughout Antarctica, most research has focused primarily on lakes due to their size, stability and influence on the surrounding terrestrial system (Craig *et al.*, 1992; Bell & Laybourn-Parry, 1999; Bowman *et al.*, 2000; Pearce *et al.*, 2003; Taton *et al.*, 2003). However, due to their smaller size,

ponds can be more susceptible to seasonal (and global) climatic changes, while offering easy access to a broad range of unique pond-specific geochemical conditions.

During the peak of the Antarctic summer up to 20% of the McMurdo Ice Shelf (MIS) in southern Victoria Land is covered with meltwater ponds of various sizes, making them a dominant environment in this area (De Mora *et al.*, 1994). The ponds surrounding Bratina Island on the MIS (Fig. 1) provide a cross-section of the many geochemistries found in aquatic systems throughout Antarctica (Cowan & Tow, 2004). Their existence is dependent on the short period, at the peak of summer, when liquid water from local ice melt collects in the depressions of the rugged landscape of the MIS replenishing pre-existing ponds and forming new ones (Howard-Williams & Hawes, 2005). Marine-derived surface sediments and aerosols, as well as seawater trapped in tide cracks across the ice shelf, provide multiple variable inputs of dissolved salts into ponds. The fine balance between water and salt

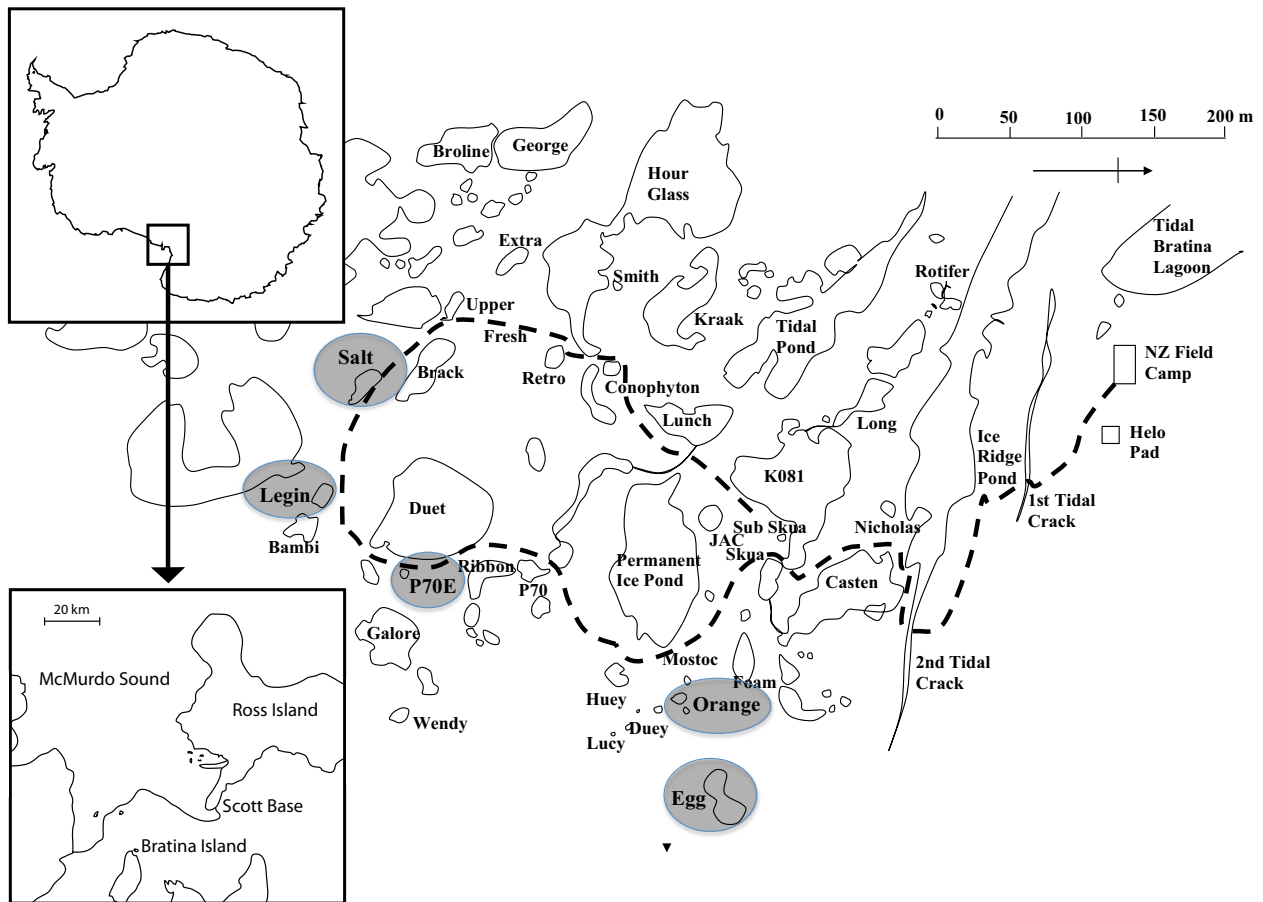


Fig. 1. Map of the MIS Ponds, based on an original map produced by New Zealand Department of Land and Surveys, 1991. Ponds circled were used in this study.

input and evaporation leads to both inter and intraseasonal variation of physicochemistry within a pond (Laybourn-Parry *et al.*, 2002) and variable chemistries between ponds. Ponds in close proximity (metres apart) can vary greatly in size (one to several hundred square metres), depth (< 1 m to around 4 m) and age (1 to at least 30 years old) (Gibson *et al.*, 2006), as well as in metal concentrations, salinity (Schmidt *et al.*, 1991; Matsumoto *et al.*, 1992), pH and dissolved oxygen (Koob & Leister, 1972). The water column within some ponds can be highly stratified, with a unique brine geochemistry derived from the freeze concentration of dissolved salts layering the bottom and newer meltwater input layering the surface. Oxygen, derived from *Cyanobacteria* dominated mats lining the ponds, also exhibits a steep gradient increasing with depth, frequently resulting in oxygen supersaturation in the lower depths of certain highly stratified ponds (James *et al.*, 1995; Wait *et al.*, 2006). Photosynthesis, which causes the oxygen supersaturation, increases pH via the removal of CO₂ from the water

reducing carbonic acid (Hendriks *et al.*, 2014), both parameters are constrained by the conductivity density gradients, illustrating the interplay between biotic and abiotic variables along physicochemical gradients that occur with depth in these ponds (Wait *et al.*, 2006).

Past studies of Antarctic ponds, and lakes, have provided some insight into how microbial communities are structured in these extreme aquatic environments. These studies primarily focused on the dense microbial mats and sediments lining the ponds (Fernandez-Valiente *et al.*, 2001; Griffin & Tiedje, 2007) and on the geochemistry of the water column (Matsumoto *et al.*, 1992; De Mora *et al.*, 1994; Wait *et al.*, 2006), with little or no work on the microbiology of the water column itself. Previous research has also been limited by a reliance on microscopic identification of microorganisms, (Vincent & James, 1996; de los Rios *et al.*, 2004; Sabbe *et al.*, 2004), which suggested these pond ecosystems supported low biodiversity, probably due to the presence of cryptic species. However, more recently, the use of culture-independent molecular genetic tools has

allowed microbiologists to identify and investigate a more extensive biodiversity in these systems (Vincent & James, 1996; Brambilla *et al.*, 2001; Sjolting & Cowan, 2003; Hughes *et al.*, 2006; Cary *et al.*, 2010).

This project is the first in-depth study to characterise the microbial communities in the water column of Bratina Island ponds and to describe their relationship to pond geochemistry. Utilising molecular genetic tools (a combination of DNA fingerprinting and high throughput next-gen sequencing) coupled with geochemistry, five ponds were characterised, resulting in the most stratified pond being selected for higher resolution analysis. These data allowed for the identification of possible geochemical drivers, and the specific bacterial groups they control, within a highly stratified water column.

Materials and methods

Field sampling strategy

Water column samples were collected from five separate ponds near Bratina Island 78°01'S latitude, 165°32'E longitude on the MIS (Fig. 1) shortly after most ponds became ice-free at the beginning of the austral summer in December 2009. Sampled pond depths ranged from 0.3 to 0.5 m (Supporting Information, Table S1) and sizes from 3 to 20 m across. Four ponds were ice-free, but a 1-cm surface layer and the middle 25% of Legin pond was still frozen at this time. A customised micromanipulator/sampling apparatus was constructed (Fig. S1) and used to simultaneously collect physicochemical data (*in situ* data used to determine sampling frequency) and water samples from the approximate centre of each pond at 2–10 cm increments (± 1 mm) through a sterilised sampling tube (TYGON[®] R-3603) that was insulated and capable of mild heating with a nicrome wire heating tape controlled via a rheostat. The tube was flushed with two void volumes (at each depth) before each sample was collected. 8–100 mL of sample was immediately filtered through syringe-mounted 0.22- μ m filters (Whatman International Ltd, Kent, UK) until the filter clogged. A small amount of air was pushed through the filter to remove excess water, then gently flooded with a nucleic acid preservative/lysis buffer (CTAB – cetyltrimethylammonium bromide-polyvinylpyrrolidone- β -mecaptoethanol) (Coyne *et al.*, 2001) and frozen for transport back to the laboratory. Thirteen millilitre of the filtrate was also collected in 15-mL falcon tubes and frozen immediately for later geochemical analysis. Oxygen concentration and temperature were measured *in situ* using probes attached near the tip of the sampling tube. Oxygen data were collected using a Fibox 3 LCD trace minisensor oxygen metre with data-logger (PreSens Precision sensing, Regensburg, Germany), and temperature

data were collected using a Hobo temperature data logger (H09-003-08, Onset Computer Corporation). Finally, a 30 mL unfiltered sample was collected in a 50-mL falcon tube for immediate pH, and conductivity measurements performed in the field using a SPER Scientific water quality meter (Sper Scientific, Arizona).

Nutrient and elemental analysis

NH₄, NO₂ and PO₄ measurements on the filtrate from each sample were carried out at the University of Waikato using an Aquakem 200CD following the manufacturer's instructions (Thermo Fisher Scientific, Waltham). Elemental analysis was performed on each sample by inductively coupled plasma mass spectrometry (ICP-MS) using a Mass Spectrometer ELAN[®] DRC II (PerkinElmer Inc., Münster, Germany). To prepare samples for ICP-MS, 0.22 μ m prefiltered pond water was diluted 1 : 50 with Milli-Q water (Millipore, Billerica, MA). Six samples were diluted 1 : 1000 due to excessive salt concentrations. Once diluted, samples were acidified with 2% HNO₃ (Extra pure Nitric Acid, Ajax Finechem, NSW, Australia). Elements analysed were the following: Li⁷, B¹⁰, Na²³, Mg²⁴, Al²⁷, S³⁴, K³⁹, Ca⁴³, V⁵¹, Cr⁵², Fe⁵⁴, Mn⁵⁵, Co⁵⁹, Ni⁶⁰, Cu⁶⁵, Zn⁶⁸, As⁷⁵, Se⁸², Sr⁸⁸, Ag¹⁰⁹, Cd¹¹¹, In¹¹⁵, Ba¹³⁷, Tl²⁰⁵, Pb²⁰⁷, Bi²⁰⁹ and U²³⁸ (results of all measurable variables are in Table S1).

DNA extraction of samples

DNA was extracted from the filtered biomass using a modified CTAB extraction protocol (Campbell *et al.*, 2001). Briefly, the frozen, sealed 0.22- μ m sample filters were first thawed on ice and then connected to a syringe containing 1 mL of fresh CTAB. The exposed filter nipple was parafilm, and the entire assembly incubated in a Ratek Orbital mixer at 150 r.p.m. and 65 °C for 30 min. The filter assembly was allowed to cool then 0.5 mL of the CTAB in the syringe was pushed through the filter assembly to evacuate the lysate into an eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24 : 1) was then added to the lysate and mixed on the orbital mixer at 150 r.p.m. and 65 °C for a further 30 min. The eppendorf tube was then centrifuged for 15 min at 16 900 g, and the aqueous phase transferred to a new tube. Nucleic acids were precipitated by addition of 1 volume of isopropanol and 0.5 volume of 5 M NaCl and then incubated at –80 °C for at least 1 h. The tube was then centrifuged at 26 000 g for 30 min, and the supernatant discarded, and the DNA pellet washed with 0.5 mL of 70% EtOH and then centrifuged at 15 500 r.p.m. for 5 s. The pellet was dried and resuspended in 10–50 μ L of sterile milliQ H₂O. The extracted

DNA was quantified using a Nanodrop ND-1000 at 260 nm (NanoDrop Technologies, Montchanin, DE).

Automated ribosomal intergenic spacer analysis (ARISA)

ARISA DNA fingerprinting (Fisher & Triplett, 1999) was utilised to resolve bacterial community structure and relative diversity within all ponds, and a comparative subset (Egg, Legin and P70E) was selected to resolve cyanobacterial community structure in Egg pond. From each sample, the bacterial intergenic spacer region (ISR) in the rRNA operon was amplified using PCR. Triplicate 30 μ L reactions [to reduce stochastic PCR bias (Wintzingerode *et al.*, 1997)] were run for each sample. Bacterial primers used were ITSReub-Hex (5'-GCCAAGGCATCCACC-3') and ITSF [5'-GTCGTAACAAGGTAGCCGTA-3'] (Cardinale *et al.*, 2004) and the cyanobacterial primers were CY-ARISA-F (5'-GYCAYRCCCGAAGTCRTTAC-3') and 23S30R (5'-CHTCGCCTCTGTGTGCCWAGGT-3') (Wood *et al.*, 2008). Full PCR components and conditions and quality control procedures are described in Supporting Information.

To visualise, compare and interpret the ARISA fingerprints from different samples, the output was processed with an informatics pipeline (modified from (Abdo *et al.*, 2006) First, all peaks with heights exceeding 250 fluorescence units were accepted as true peaks. The remaining peaks were used to calculate model parameters for a log-normal distribution. Iteratively, peaks with an area exceeding the 99.9% cumulative distribution of the calculated log-normal distribution for noise were accepted as true peaks. Fragment lengths < 100 bp for bacteria and 150 bp for cyanobacteria were removed from analysis as these were considered to be too small to be ITS fragments (Cardinale *et al.*, 2004; Wood *et al.*, 2008). Peaks were binned into ARISA Fragment Lengths (AFLs) with width = 2 nt, and total peak area of each bin was used to calculate relative abundance of each AFL in a given data set. The resulting data matrix was imported into PRIMER 6 for statistical analysis (Clarke & Gorley, 2006).

In PRIMER 6, peaks were converted to presence/absence data for further analysis, and the number of peaks was totalled from each sample to be used as a proxy for relative alpha diversity (the number of species in a sample). These were subjected to various statistical tests (Spearman's coefficient and Tukey's honest significant difference test) to elucidate relationships between communities. Beta diversity (the comparison of diversity between sites) was investigated using a resemblance matrix created based on the Bray Curtis similarity index (Bray & Curtis, 1957). From the resemblance matrix, a hierarchical clustering analysis was performed, producing a relational

dendrogram. Nonmetric multidimensional scaling (MDS) was performed on the resemblance matrix, which displays relative similarities between communities as distance (i.e. the closer two samples are the more similar community). 2-D MDS plots with a stress value of < 0.2 were used as they were considered to have accurate information. Information from the dendrogram of the communities was overlaid onto the 2-D MDS plots, which provide percentage similarity levels at 20, 40, 60 and 80%, assisting in the evaluation of community structure between samples. ANOSIM analyses and Mantel tests were performed on the resemblance matrix to test specific hypotheses formed from interpretation of MDS plots.

The influence of pond geochemistry on the microbial community was determined using a BEST analysis to find the 'best match' in PRIMER 6. Geochemical data were normalised and a Draftsman plot created to view interrelations between the different variables, so the data could be simplified. Spearman's rank correlation coefficients were then calculated, resulting in a probability (*P*) of the community differences being explained by the differences in geochemistry. The combination of geochemical variables whose Euclidean distance matrix gave the highest *P*-value were considered the most likely drivers of community dynamics.

454 pyrosequencing

PCR amplicons containing V5–V6 hypervariable regions of the 16S rRNA gene were utilised to identify specific bacterial community differences in the water column of Egg pond using 454 pyrosequencing. Triplicate 30 μ L reactions were run for each sample, each reaction containing 0.4 μ M of each unadapted primer Tx9F (5'-GGATTAGAWACCCBGGTAGTC-3') and 1391R (5'-GACGGGCRGTGWGTRCA-3') (Ashby *et al.*, 2007), 1x PCR buffer, 0.2 mM dNTPs, 0.02 mg mL⁻¹ BSA, 0.02 U Platinum Taq, 2 mM MgCl₂, 20 ng bp genomic DNA, and the reaction was made up to 30 μ L with milli-Q H₂O. Thermal cycling conditions were the following: 94 °C for 2 min, then 30 cycles of 94 °C for 20 s, 55 °C for 10 s (–0.2 °C per cycle), 72 °C for 20 s and a final extension of 72 °C for 3 min. Once amplified, all triplicate PCRs were combined, run on a 2% TAE agarose gel stained with 'SYBR Safe' (Invitrogen Ltd) at 80 V, the bands excised and DNA retrieved using the MO BIO UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA) as per manufacturer's instructions, but running the final spin step twice. A second round of triplicate PCR was run as above but with only 10 cycles and using 25 ng of the purified DNA from the previous step per reaction (milli-Q H₂O volume adjusted accordingly). The primers used were adapted for one-way reads

according to the Roche GS Junior System Guidelines for Amplicon Experimental Design Manual (August 2010), including unique MID identifiers for each sample [BacX-Tx9F (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-GGATTAGAWACCCBGGTAGTC-3') and BacB-1391R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-GACGGGCR GTGWGTRCA-3')]. A second gel extraction was performed as above. Samples went through a final cleanup step using the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA) as per the manufacturer's instructions. A verification gel was run on a 2% TAE gel to confirm a lack of unwanted bands in the sample. Sample DNA content was quantified using a Qubit Fluorometer (Invitrogen Ltd) and was then diluted to 1×10^9 molecules μL^{-1} as per the Roche Amplicon Library Preparation Method Manual [GS Junior Titanium Series, May 2010 (Rev. June 2010)]. QPCR using a KAPA Library Quantification Kit for Roche 454 Titanium/Universal (Kapa Biosystems, Woburn, MA) was used to check the 1×10^9 dilution and was adjusted accordingly for making the amplicon library. The diluted amplicons were mixed together in the desired proportions to create the 1×10^9 amplicon pool. Sequencing was performed using the GS Junior Titanium emPCR Kit (Lib-L), the GS Junior Titanium Sequencing Kit, PicoTiterPlate Kit and GS Junior System according to the manufacturer's instructions (Roche 454 Life Sciences, Branford, CT).

454 pyrosequencing data processing

454 PCR amplicon pyrosequencing data were first processed using AmpliconNoise v1.0 (Quince *et al.*, 2011). Briefly, raw flowgrams (sff files) with perfectly matching primer and barcode sequences were filtered for a minimum flowgram length of 360 cycles (including primer and barcode sequences) before the first noisy signal (i.e. 0.5–0.7 or no signal in all four nucleotides). All flowgrams were then truncated at 360 bases and clustered to remove sequencing noise using PyroNoise (Quince *et al.*, 2009, 2011). Noise introduced by PCR was removed using SeqNoise (Quince *et al.*, 2011), and PCR chimeras were removed using Perseus (Quince *et al.*, 2011). The resulting de-replicated sequences were processed using Mothur 1.17.0 (Schloss *et al.*, 2009) to create a unique sequence and names file. Pairwise alignments and distance were calculated using Espirit (Sun *et al.*, 2009). Mothur was then used to cluster sequences into operational taxonomic units (OTUs) defined at the furthest neighbour Jukes–Cantor distance of 0.03 (OTU_{0.03}). Rankabundance data were generated for each sample as well as Venn diagrams and Good's nonparametric coverage estimator was used to evaluate sequencing coverage for each sample. For phylogenetic assignments, representative

sequences of all identified OTU_{0.03} were analysed using the Classifier function provided by the Ribosomal Database Project (RDP) Release 10, Update 15 (Wang *et al.*, 2007). Taxonomic assignment threshold was set at 80%. Phylogenetic analysis of sequences was performed with representative sequences of the top 101 OTU_{0.03} (> 0.1% overall abundance in pyrosequencing data sets, representing 12 376 reads). Taxonomic trees were constructed using ARB (Ludwig *et al.*, 2004), with DNADIST and neighbour joining analysis. Sequence length ranged from 244 to 392 bp, with an average of 384.9 bp.

Results

Physicochemical analysis

Inter and intrapond physicochemical properties were found to be extremely variable, with dissolved oxygen concentrations from 4.4 to 27.3 ppm (Fig. 3a), conductivity ranging from 0.43 to 110.9 mS cm^{-1} (Fig. 3b), pH from 7.3 to 10.1 (Fig. 3c), and temperature from -3.5 to 5.1 °C (Fig. 3d). A notable geochemical gradient was identified throughout the water column of most ponds. Oxygen concentration varied through the water column, but generally it was highest at the deepest point (increasing from 9.7 at the top to 17.0 ppm in the bottom of Legin). Conductivity was also found to increase with depth in each pond (from 8.2 mS cm^{-1} at the top to 93.6 mS cm^{-1} at the bottom of Egg). Although there are significant intrapond variations, the geochemistry MDS analysis (Fig. 2) clearly shows that each pond is geochemically distinct, with Egg forming two distinct clusters.

Egg pond exhibits the most extreme physicochemical stratification observed in this study (Fig. 3a–d). The pH was stable between 9.4 and 9.5 in the upper 38 cm, dropping to 8.8 at 40 cm and to 8.1 at the bottom (48 cm). The drop in pH corresponds with an increase in conductivity, which rose modestly from 8.2 mS cm^{-1} at 0 cm to 10.4 mS cm^{-1} at 38 cm, with a steep increase between 40 and 46 cm, up to 93.6 mS cm^{-1} at 48 cm deep. Dissolved oxygen within Egg increased from 11.5 ppm at the surface, to 17.1 ppm at 36 cm, and 27.3 ppm at 44 cm. The temperature profile was relatively stable, decreasing from 1.6 °C at the surface to -1.1 °C at 46 cm, with a notable increase at the bottom (48 cm) to 0.6 °C.

NH_4 was only detected throughout the water column in Egg and Salt pond (which also have the greatest conductivities) and at the top of Orange and at the bottom of P70E. No NH_4 was detected in Legin. NO_2 was found at extremely low concentrations in all ponds, except P70E where it was 10 times higher in the upper region (0.032 ppm compared with 0.002 ppm in most other ponds). ICPMS analysis revealed that most resolved

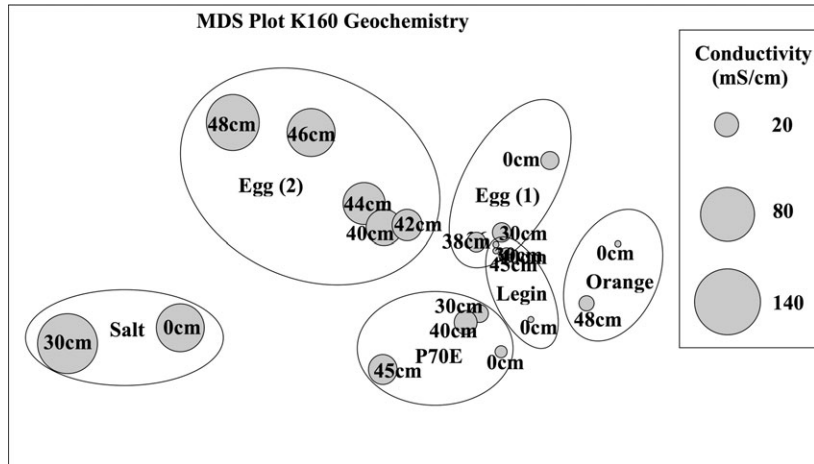


Fig. 2. MDS plot of geochemistry comparing all ponds studied, numbers represent sample depth. The greater the distance between two points, the greater the difference between their geochemistries. Circles surrounding samples represent relative conductivity concentration (the larger the circle the higher the conductivity). MDS plot 2-D stress: 0.08. Outlining circle used to identify pond clusters.

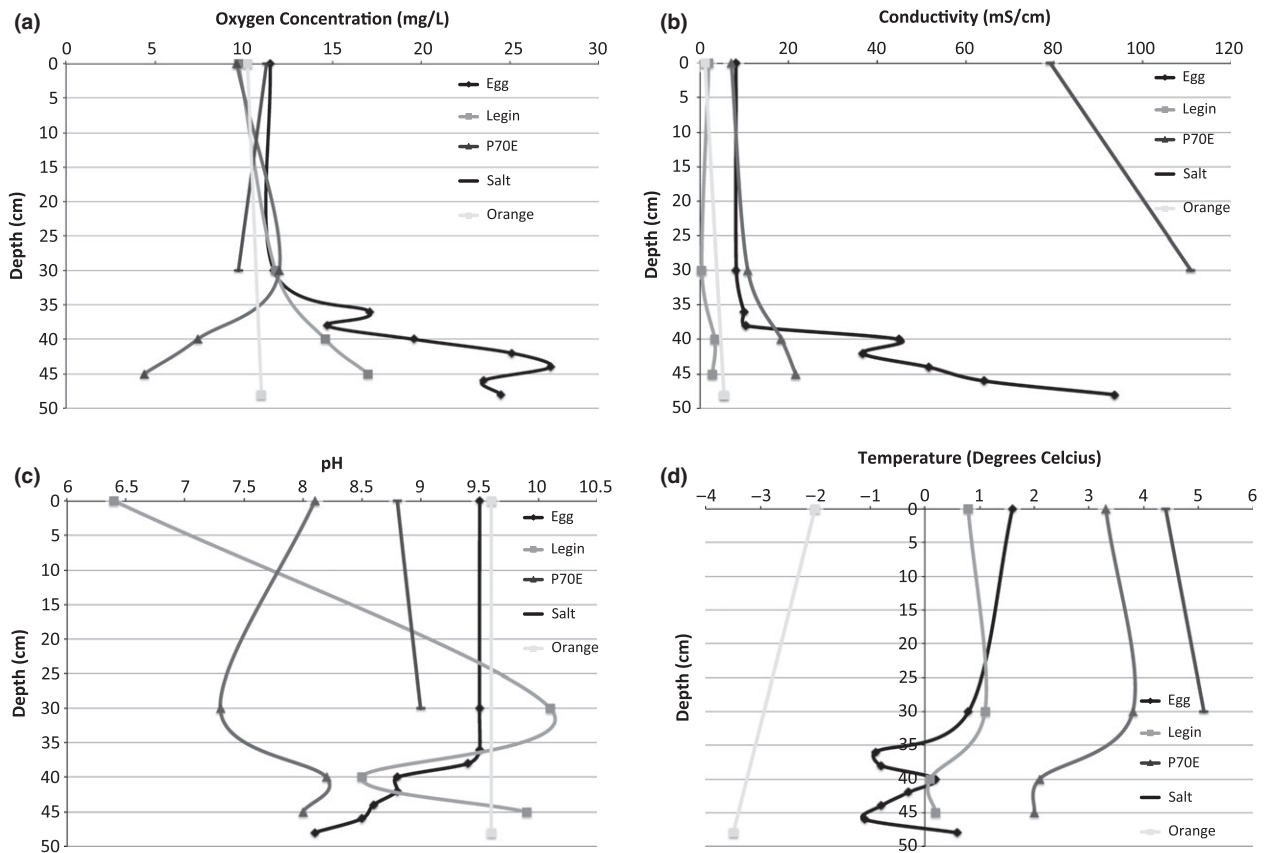


Fig. 3. Geochemistry depth profile showing changes in oxygen concentration (ppm) (a) conductivity (mS cm^{-1}) (b), pH (c) and temperature ($^{\circ}\text{C}$) (d).

elements increased along conductivity gradients, notable observations were that Co^{59} was only detected in the bottom of Egg (46–48 cm) and throughout Salt pond. Ni^{60} was below the detection limit in Legin and Orange and the upper 38 cm of Egg. Legin also had a significantly reduced amount of Cu^{65} and Mn^{55} compared with other ponds, and Orange had a reduced level of Mn^{55} .

DNA fingerprinting

The number of AFLs was used as a proxy for alpha diversity (Fig. S2). There are no significant differences between alpha diversity of *Bacteria* from different ponds; however, P70E has significantly higher *Cyanobacteria* diversity (29–39 AFLs per sample compared with a mean of 16.6 over

all ponds) than Legin and Egg according to Tukey's Honest Significant Difference Test ($P \leq 0.001$ for Egg and $P \leq 0.001$ for Legin). A Spearman's rank correlation coefficient showed no significant correlations between alpha diversity and depth for *Bacteria* or *Cyanobacteria*. Lowest diversity occurred in Salt pond (12–25 AFLs) containing the highest overall conductivity of the sample set; however, when removed as an outlier, there was a positive correlation between average conductivity and average bacterial ARISA AFLs but with only a marginal significance ($P = 0.04$).

Beta diversity comparisons were visualised using a 2-D MDS plot for bacterial communities from five different ponds (Fig. 4a) and cyanobacterial communities from three ponds (Fig. 4b). Microbial communities were identified as significantly dissimilar between ponds at 40%

similarity: cyanobacterial ANOSIM $R = 0.8605$, $P = 0.001$; bacterial ANOSIM $R = 0.766$, $P = 0.001$ (Fig. 4 a and b). P70E and Egg ponds were exceptions to this general observation. In P70E, the bacterial community observed at 0 cm depth differs from the bacterial communities observed at all other depths (40% similarity). A Mantel test confirmed that bacterial communities at similar depths in Egg pond were more comparable than those further apart ($R = 0.8934$, $P = 0.002$). Bacterial communities from Egg pond formed two major clusters based on depth; Egg (1), the upper 0–38 cm and Egg (2), the lower depths, 40–48 cm (Fig. 4a), while a similar clustering structure is apparent in cyanobacterial communities at 60% similarity (Fig. 4b). These observations were reflected in an ANOSIM test for bacterial ($R = 0.975$, $P = 0.008$) and cyanobacterial communities ($R = 0.4438$, $P = 0.026$). This combined data resulted in the selection of three samples from Egg pond, which represented the greatest variation in community structure down the stratified water column. Bacterial communities from Salt pond stood out as being unique from all other ponds (20% similarity).

BEST analysis was used to correlate bacterial and cyanobacterial community structure to differences in geochemical profiles (Table 1), with both appearing to be well correlated to conductivity as a major contributing factor. Conductivity and Ag^{109} were the best explanatory variables for cyanobacterial communities (Spearman's $\rho = 0.662$), and temperature, conductivity and V^{51} were the major explanatory variables for bacterial communities (Spearman's $\rho = 0.698$). When Egg was analysed on its own, higher probabilities were achieved for community

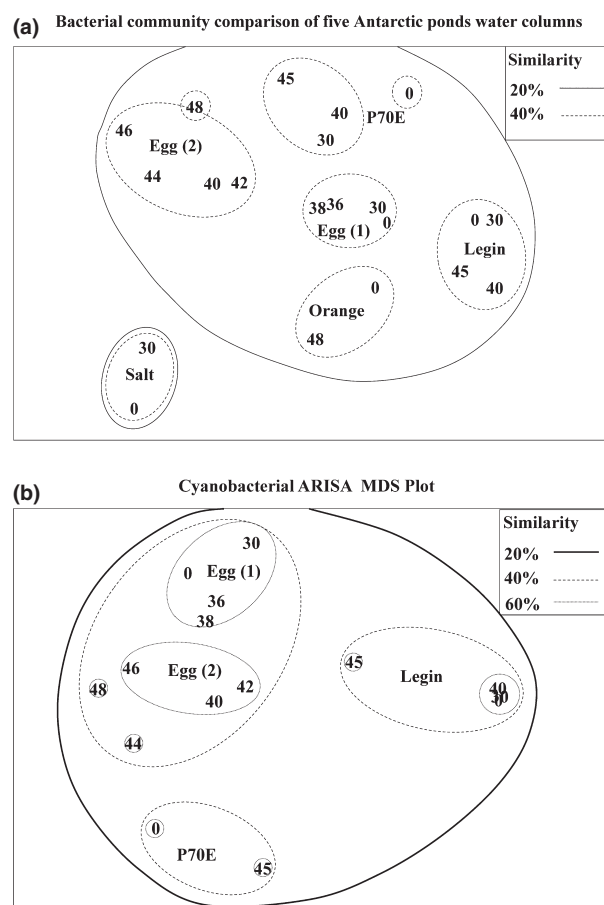


Fig. 4. Bacterial and Cyanobacterial ARISA MDS Plots showing percentage similarities of pond communities (a) bacterial ARISA, data normalised, converted to presence or absence points, and subjected to a Bray Curtis similarity matrix, MDS plot 2-D stress 0.15. (b) cyanobacterial ARISA, data normalised, converted to presence or absence points, and subjected to a Bray Curtis similarity matrix, MDS plot 2D stress 0.09.

Table 1. Geochemistry linked to community structure using BEST analysis for cyanobacterial and bacterial DNA fingerprints (ARISA). A Spearman's rank correlation of 1.0 would indicate a 100% linkage between the variable/s selected and the differences in communities

	Cyanobacterial ARISA		Bacterial ARISA	
	Correlation	Variable	Correlation	Variable
All ponds	0.662	4, 11	0.698	2, 4, 10
	0.639	2, 4, 11	0.697	2, 4
	0.635	4, 7, 11	0.675	4, 10
	0.623	2, 4, 7, 11	0.654	4
	0.591	4, 5, 11	0.652	2–4, 10
Only egg	0.734	3, 11	0.927	5
	0.732	5	0.927	5, 7
	0.732	5, 7	0.926	4
	0.730	4, 11	0.926	4, 7
	0.714	3, 5	0.925	4, 5

Variable designation: 1 = depth, 2 = temperature ($^{\circ}\text{C} + 10$), 3 = dissolved oxygen (ppm), 4 = conductivity (mS cm^{-1}), 5 = pH, 6 = total phosphate (ppm), 7 = NO_2 (ppm), 8 = NH_4 (ppm), 9 = Al^{27} , 10 = V^{51} , 11 = Ag^{109} .

linkages to geochemical variables within Egg. Cyanobacterial communities of Egg appear moderately influenced by pH, which had the highest probability as a single driver of diversity (Spearman's ρ of 0.732). Both pH and conductivity were determined to be strongly correlated to bacterial diversity differences within Egg (Spearman's $\rho = 0.927$ for pH and $\rho = 0.926$ for conductivity).

454 pyrosequencing

454 pyrosequencing of three representative samples from Egg pond created a data set of 12 995 reads that passed quality scoring, which clustered into 583 OTUs at a 97% sequence identity (OTU_{0.03}; Fig. 5). Good's nonparametric coverage estimator indicates that sampling coverage was 98.6% for 0 cm, 97.4% for 40 cm and 93.5% for 48 cm. Each sample contained a high number of unique OTUs, the number increasing with depth; however, the percentage of reads represented by sample-specific unique OTUs was < 7% in the surface and middle (40 cm) depths and 14.2% in the bottom (48 cm) depth. A majority of reads present in the surface (93.1%) were represented by OTUs present at all depths. A majority of reads in the middle and bottom depths (83.6% and 84.2%, respectively) were represented by OTUs shared between those depths, but absent in the surface.

Phylum/class level investigation revealed dramatic shifts in the bacterial communities with depth through the

water column of Egg (Fig. 6). At the surface (0 cm), the community was least diverse, with six phyla well represented. This sample was dominated by *Bacteroidetes* (68%), with one OTU contributing 64.8% [the sequence of this OTU has 100% identity with *Algoriphagus yeomjeoni*, isolated from a marine solar saltern (Yoon *et al.*, 2005)], and β -*Proteobacteria* (18.9%), with one OTU contributing 18.6% [which has 100% sequence identity to *Hydrogenophaga taeniospiralis* isolated in the Arctic, and to a number of Antarctic lake isolates (VanTrappen *et al.*, 2002)]. At 40 cm, community diversity increased, with 12 phyla including the six seen at the surface. At this depth, the community had also shifted to a γ -*Proteobacteria* (66.1%) dominated community with the reads from one OTU contributing 60.8% [this OTU has 99% identity to *Psychromonas* sp. isolated from Arctic sea ice (Auman *et al.*, 2006)], the *Bacteroidetes* were again a major phyla but only 10.9% of the community. In the deepest sample (48 cm), diversity increased dramatically, with 18 phyla including all those seen in the 40 cm sample. Again the γ -*Proteobacteria* were the dominant phyla (50.8%), with one OTU contributing 46.1% (the same OTU dominant at 40 cm), the only other major group were uncharacterised, although this contained only one OTU (16.8%) with a [96–98% identity to environmental sequences from a hypersaline microbial mat (Isenbarger *et al.*, 2008)] The *Bacteroidetes* (5.6%) were the third most represented phyla.

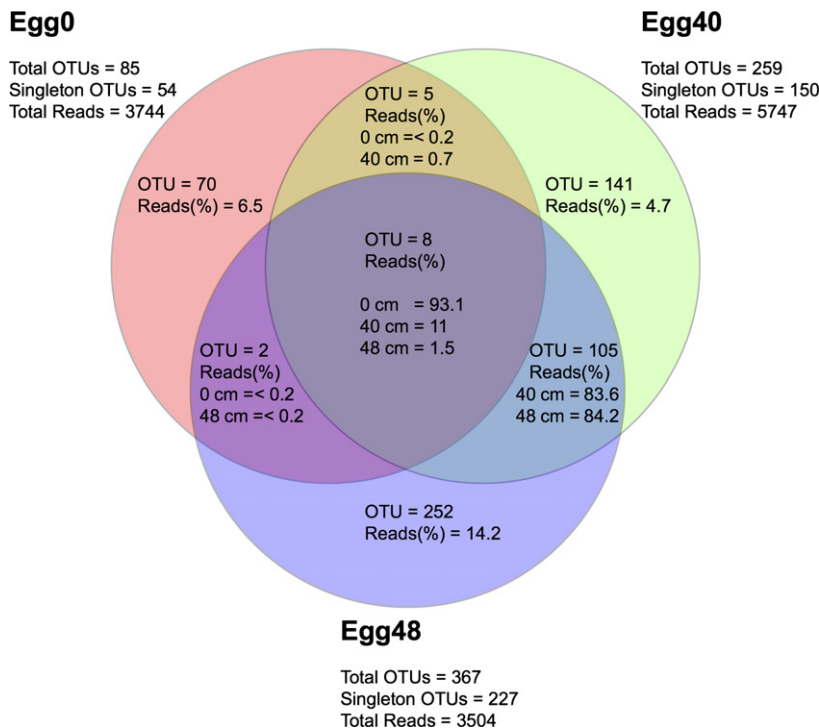


Fig. 5. Summary of 454 sequencing data (at a distance of 0.03), and similarities between depths of Egg pond. The total OTU richness of all the groups is 583, and total read number is 12 995.

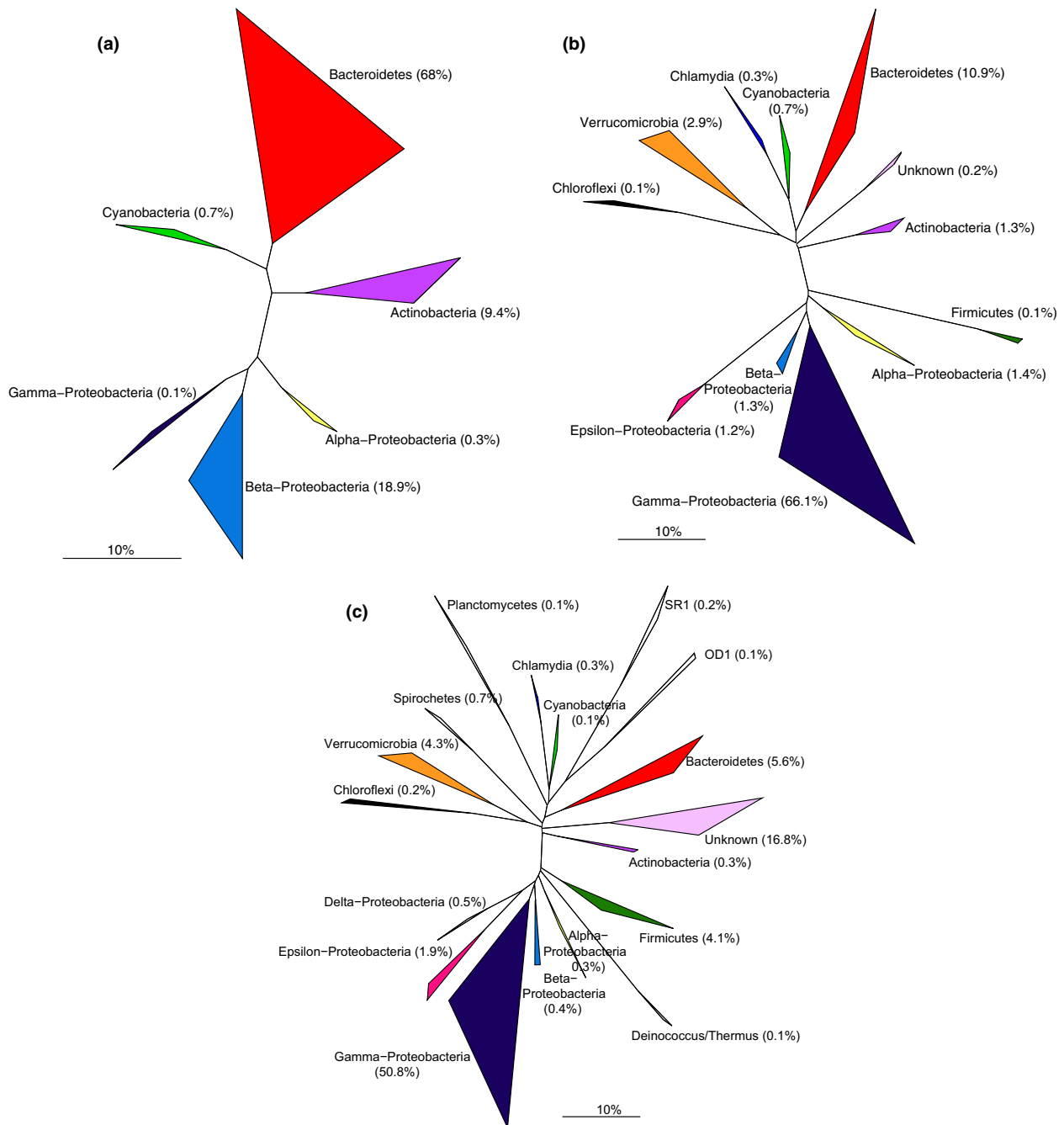


Fig. 6. Phylum-level diversity of bacterial 16S rRNA gene sequences from different depths of Egg pond. Percentage abundance of phyla within each site are shown (in parentheses) for the (a) surface sample, (b) 40 cm deep sample and (c) 48 cm deep sample. 454 pyrosequencing reads were denoised and clustered at 97% (farthest neighbour) to obtain 583 OTU_{S0.03} in total, of which 120 OTU_{S0.03} were shared. The top 101 OTU_{S0.03} with > 0.1% abundance in any sample are used in this analysis.

Discussion

The focus of this study was to determine whether the unique geochemistry previously reported in the water columns of Bratina ponds supports unique bacterioplankton

communities (Matsumoto *et al.*, 1992; Wait *et al.*, 2006; Webster-Brown *et al.*, 2012). Early studies of pond sediments from Bratina meltwater ponds have identified highly diverse (Sjoling & Cowan, 2003) and variable microbial communities between ponds (Kemp & Aller,

2004); however; the environmental factors driving these differences were not determined, and overlying waters were never investigated. Analysis of ARISA fragments in this study has revealed a statistically significant difference (ANOSIM) between pond microbial communities for *Bacteria* ($R = 0.766$, $P = 0.001$) and *Cyanobacteria* ($R = 0.8605$, $P = 0.001$). The highly geochemically stratified Egg pond formed two distinct bacterial communities ($R = 0.975$, $P = 0.008$), with BEST analysis indicating pH and conductivity as major contributors to community differences along this steep gradient. Pyrosequencing of representative samples from Egg pond identified a diverse community of 583 unique OTUs, which exhibit notably different community structures with depth reinforcing the conclusions based on ARISA results. Although sequencing coverage was not 100% in any sample for this investigation, this would not affect conclusions formed from this data, only resulting in a slight underestimation of diversity.

The relative beta diversity of *Cyanobacteria* and *Bacteria* (ARISA) was generally pond-specific (visualised using a MDS plot, confirmed statistically by ANOSIM). This observation is consistent with microscopic analysis of planktonic communities (James *et al.*, 1995), which classified three groups of ponds based on size, conductivity, and the presence and abundance of various planktonic groups. Each of the three groups included ponds sampled in this study (P70E, Legin and Salt). This suggests that these ponds consistently harbour different geochemical environments and biological communities. The exception to this observation was Egg pond, which formed two depth-related microbial groupings (Fig. 4a, confirmed by ANOSIM) consistent with a steep geochemical gradient of conductivity and pH, both found to be drivers of community shifts in other studies (Sabbe *et al.*, 2004; de Figueiredo *et al.*, 2010).

BEST analysis has previously been successfully utilised to link geochemical variability to variation in biological community profiles from fingerprinting data (Wood *et al.*, 2008; Soo *et al.*, 2009; Smith *et al.*, 2010). In this study, BEST analysis indicated a strong correlation between differences in conductivity contributing to cyanobacterial and bacterial community structure for all five ponds studied. In addition, independent analysis of Egg indicated that both bacterial and cyanobacterial community structure was significantly correlated to differences in pH and conductivity. The pH and conductivity in Egg show an inverse relationship to one another, so neither can be singled out as the primary variable responsible for community structure changes; however, both have previously been identified as drivers of community structure in aquatic environments (James *et al.*, 1995; Sabbe *et al.*, 2004; Kaartokallio *et al.*, 2005; de Figueiredo *et al.*, 2010;

Jiang *et al.*, 2010; Laque *et al.*, 2010). Examination of major OTU abundances between depths reinforces conductivity as the dominant driver to community structure. The Genus *Hydrogenophaga* was identified as a major OTU in the surface sample, decreasing significantly with depth along the increasing conductivity gradient. Several species of this genus have been previously isolated from a number of Arctic and Antarctic environments (Newdell & Rutter, 1994; VanTrappen *et al.*, 2002) and found to have a maximum NaCl tolerance of 1% (Yoon *et al.*, 2008) explaining this observation. *Algoriphagus yeomjeoni*, previously identified in a cold marine environment and in Antarctic lake mats (Bowman *et al.*, 2003; Van Trappen *et al.*, 2004), has a broader salt tolerance of up to 9% NaCl and an optimum of 1% (Yoon *et al.*, 2005). Its abundance is highest at the surface then decreases significantly with depth; however, it remains at higher concentrations than *Hydrogenophaga* in the 40 and 48 cm samples likely due to its greater salt tolerance. The genus *Psychromonas*, previously isolated from pond sediments at Bratina Island (Mountfort *et al.*, 1998) and Arctic ice (Auman *et al.*, 2006), was dominant at both 40 and 48 cm and was barely detected at 0 cm, reflective of its ability to grow well between 1 and 10% NaCl (Auman *et al.*, 2006). The second most abundant OTU at 48 cm further reinforces halophilic dominance with depth, sharing a high identity to sequences gained from a hypersaline solar saltern (Isenbarger *et al.*, 2008).

Combinations of pH and conductivity with temperature, V, NO₂, Ag and dissolved oxygen are all highly correlated to microbial community structure. Each identified variable has previously reported to have some influence on planktonic microorganisms. Vanadium can interfere with iron uptake (Baysse *et al.*, 2000); Ag has well-known antimicrobial properties (Throback *et al.*, 2007); nitrogen limitation alters primary productivity in phytoplankton population in these ponds (Sorrell *et al.*, 2013); hyper-variation in oxygen concentration with depth selects for zones of aerobic, anaerobic (Bell & Laybourn-Parry, 1999) and hyperoxic tolerant microorganisms (Fridovich, 1998) and due to variable optimum growth temperatures for microorganisms minor variations in temperature, especially at near-freezing temperatures can dramatically influence community structure (Mountfort *et al.*, 2003). Regardless of biological mechanism, the extent of differences in V, Ag and NO₂ concentration are well below those found to have biological significance (Yamanaka *et al.*, 2005; Sorrell *et al.*, 2013), and their low correlation scores individually indicate that it is unlikely that any of these variables will strongly influence microbial community structure compared with pH and conductivity. DO and temperature are capable of influencing community structure; however, they too provide weak correlations

individually. Interestingly although PO_4 is an essential nutrient, it was not identified as a driver of community structure in this analysis. It is likely the interplay between these, and other unknown factors, produces the net effects in the microbial communities.

Pyrosequencing data indicated an increase in diversity with depth in Egg, inconsistent with bacterial ARISA, which showed no significant correlation between diversity and depth (Spearman's rank correlation coefficient). This could be due to the dominance of a few major groups at the surface (86.9% for the top two abundant groups compared with 66.8% at 48 cm), while the large number of minor groups seen at the bottom in 454 data could have been below the detection limit for ARISA. The dominance of a few taxa in bacterial communities is not uncommon (Massana *et al.*, 2000; Tyson *et al.*, 2004; Zeng *et al.*, 2013; Laas *et al.*, 2014; Lee *et al.*, 2014) however rarely to this extent. The annual extreme freeze thaw process this pond goes through could skew the community towards bacteria that remain metabolically active over winter or those that could rapidly re-emerge from their inactive states (Foreman *et al.*, 2010). The fact that the dominant OTU at each depth had increasing salt tolerances along the conductivity gradient shows the biological response to increasing selective pressures. A large proportion of reads were shared between the middle and bottom depths but not with the surface sample. The middle depth geochemically represented the beginning of the brine layer, which due to its density would impede direct mixing of the surface waters with the lower layers of the pond (Wait *et al.*, 2006); however, the large proportion of shared OTUs indicates there is mixing within this brine layer or that certain taxa share broader tolerances to transcend these extreme gradients. Due to the dynamic nature of these ponds, over the course of a season many groups are likely to emerge from the rare biosphere to become dominant within a community, only to be out-competed once again when conditions change.

Both ARISA and 454 data separate the surface (whose dominant phyla is *Bacteroidetes*), from the 40 cm and 48 cm samples (where the dominant phyla is γ -*Proteobacteria*). The increase in γ -*Proteobacteria* and decrease in *Bacteroidetes*, with depth correlates to the increase in conductivity and decrease in pH down the water column. These differences in dominant phyla agree with the BEST analysis, which indicated these two factors were the major cause for community shifts. Although many other minerals associated with the conductivity also differed, none of those elements revealed through ICPMS analysis appeared to be significant drivers of community diversity.

An important consideration in any study of meltwater ponds is that the time of sampling can dramatically influence geochemistry (Matsumoto *et al.*, 1992; James *et al.*,

1995; Hawes *et al.*, 1999; Wait *et al.*, 2006), which will in turn influence the resident microbiology. Samples for this study were collected early in the summer (early December 2009), so ponds were not at their maximal biological activity compared with those previously collected in January (James *et al.*, 1995; Wait *et al.*, 2006), when ponds would have been melted for longer, were probably warmer and may have undergone greater mixing due to the lack of surface ice and significant summer winds. As the ponds are meltwater fed, the amount of ice and snow input each year is important to the overall soluble ion concentration of the ponds. In 2009, ponds were significantly shallower than in other years, for example, P70E was 0.85 m deep in January 1992 (James *et al.*, 1995), at least 0.8 m deep in October 2003 and at least 1 m deep in January 2004 (Wait *et al.*, 2006), compared with a significantly shallower 0.45 m in this study. Although differences in meltwater input can rapidly alter geochemical variables during and between seasons (Schmidt *et al.*, 1991; Hitzfeld *et al.*, 2000; Jungblut *et al.*, 2005), comparison with previous work reveals that for a given pond, certain geochemical parameters can remain stable over considerable time scales (De Mora *et al.*, 1994; James *et al.*, 1995; Wait *et al.*, 2006).

Conclusions

The water column of five Bratina Island meltwater ponds was examined to understand how local geochemistry effects the structure of the resident microbial community, using fine scale biological and geochemical sampling. Biological samples were genotyped using ARISA, which allowed systematic comparison of bacterial and cyanobacterial communities supported in each pond through the entire depth profile. Significant differences between and within pond geochemistry and microbial diversity were detected. Specifically, when examining Egg pond separately from other ponds, clear links between the bacterial and cyanobacterial communities with pH and conductivity were identified as major variables that appear to influence microbial diversity and community structure. These results were verified by utilising 454 pyrosequencing to identify specific phylum-level differences throughout the water column. This reinforces the belief that geochemistry can be a driver of community structure. However, the microbial communities of a single Bratina Island pond may be found to have individual drivers, different to those of other ponds. This makes these ponds extremely valuable as a scientific resource, as they can be considered to be completely isolated extreme aquatic ecosystems sharing the same climate, but representative of the geochemical diversity of aquatic ecosystems (both ponds and lakes) scattered across the Antarctic continent.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Diagram showing equipment used to obtain central pond water column samples.

Fig. S2. Summary of bacterial and Cyanobacterial ARISA fragment lengths (AFL) number from each pond sample.

Table S1. Summary geochemistry from Bratina Island ponds December 2009.

Data S1. Automated Ribosomal Intergenic Spacer Analysis (ARISA).