**Heterotrophic resourcefulness and unusual sulfur biogeochemistry in a hypersaline Antarctic lake**

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# Abstract

**Organic Lake is a shallow marine-derived hypersaline lake in the Vestfold Hills, Antarctica that has the highest reported concentration of dimethylsulfide (DMS) in a natural body of water (Franzmann *et al.*, 1987b). To determine the composition and functional potential of the microbial community and learn about the unusual sulfur chemistry in Organic Lake, shotgun metagenomics (2.4 Gbp titanium 454) was performed on size fractionated samples (3.0, 0.8 and 0.1 µm) collected along a depth profile. Eucaryal phytoflagellates were the main photosynthetic organisms. Bacteria were dominated by the globally distributed heterotrophic lineages *Marinobacter*, *Roseovarius* and *Psychroflexus.* Candidate division RF3 was overrepresented at the oxycline and OD1 in the lake bottom. The dominance of heterotrophic degradation coupled with low fixation potential indicates possible net carbon loss. However, abundant marker genes for aerobic anoxygenic phototrophy, rhodopsins and CO oxidation were also linked to the dominant heterotrophic bacteria and may indicate use of photo and lithoheterotrophy as mechanisms for conserving organic carbon. Similarly, a high genetic potential for the recycling of nitrogen compounds likely functions to retain fixed nitrogen in the lake. Dimethylsulfoniopropionate (DMSP) lyase genes (*dddD, dddL* and *dddP*) were abundant indicating DMSP is a significant carbon and energy source. Unlike marine environments, DMSP demethylases (*dmdA*) were less abundant than DMSP lyases indicating that DMSP cleavage is the likely source of the high DMS concentration. Strategies of nutrient resourcefulness such as DMSP cleavage, photoheterotrophy, lithoheterotrophyand nitrogen remineralization in dominant Organic Lake bacteria are potentially important adaptations to nutrient constraints. This study sheds light on how microbial communities and the functional processes they perform evolve in response to unusual environmental conditions.**

# Introduction

Life in the Antarctic is constrained by low temperature, and water, nutrient and light availability. In the Antarctic frozen desert, ice-free regions containing liquid water in lakes and ponds are rare oases for life (Wilkins *et al.,* 2012). The Vestfold Hills, on the eastern shore of Prydz Bay, East Antarctica (Figure S1), is a unique region where hundreds of lakes are present. The lakes were formed from seawater, trapped less than 10 000 BP when the continental ice-sheet receded and the land rose above sea-level (Zwartz *et al*., 1998; Gibson, 1999). Differing local conditions has led each lake to develop unique physical and chemical properties, and life in the lakes tends to be entirely microbial with low levels of diversity (Bowman *et al.*, 2000b; Wilkins *et al.,* 2012). The Vestfold Hills contains the highest density of meromictic (permanently stratified) water bodies in Antarctica (Gibson, 1999). The strong physico-chemical stratification within a single, largely closed system, provides the opportunity to investigate the ways in which microbial communities and ecosystem processes have evolved in the cold and in response to gradients of nutrients, oxygen, salinity and solar irradiance.

Molecular biology approaches have proven useful for describing the diversity and gene content of microorganisms in Antarctic lakes and for inferring the functional roles of the taxa present (Laybourn-Parry & Pearce, 2007). However to date, only a few large scale shotgun metagenome studies have been performed on the Antarctic continent and in the surrounding Southern Ocean (reviewed in Wilkins *et al.,* 2012). In the Vestfold Hills, metagenomics and metaproteomics have been used to study Ace Lake (64°28′23.2″S, 78°11′20.8″E)) and Organic Lake (68°27′23.4″S, 78° 11′ 22.6″E) (Ng *et al.*, 2010; Lauro *et al.*, 2011; Yau *et al.*, 2011). For Ace Lake, a comprehensive assessment of the community structure, biogeochemical fluxes and responses to resource limitation have been described (Lauro *et al.*, 2011). The metabolism of abundant green sulfur bacteria (Ng *et al.*, 2010) was found to play a central role in nutrient cycling and a mathematical model was developed that showed its dominance was dependent on synchronicity with the polar light cycle leading to absence of phage predation (Lauro *et al*., 2011). For Organic Lake, a member of the virophage virus family was discovered that potentially regulates microbial loop dynamics (Yau *et al*., 2011). The Organic Lake virophage (OLV) likely depends on phycodnaviruses (algal viruses) and it was predicted that OLV would reduce infective phycodnaviruses leading to an increased frequency of algal blooms and thus carbon flux (Yau *et al.*, 2011). OLV-like sequences were also identified in coastal marine, hypersaline and freshwater metagenomes revealing that virophages are likely to play ecologically important roles in many aquatic systems (Yau *et al.*, 2011). These studies on Ace and Organic lakes both used shotgun metagenomics, and the unanticipated nature of the discoveries (e.g. OLV) serve to illustrate the value of adopting a “look and see” metagenomics approach for learning about microbial ecology in Antarctic environments.

Organic Lake is shallow (6.8 m) and has variable surface water temperatures (−14 to +15 °C) while remaining sub-zero throughout most of its depth (Franzmann *et al.,* 1987b; Gibson *et al.,* 1991; Roberts *et al.,* 1993; Gibson, 1999). The lake has a high organic load generated from autochthonous production and input from penguins and terrestrial algae, and nutrient turnover is slow due to the constraints imposed on microbial activity by the lake’s hypersalinity (≈230 g L−1 maximum salinity) and low temperature (Franzmann *et al.,* 1987b; Gibson *et al.,* 1991; Roberts *et al.,* 1993; Gibson, 1999). The salt and marine biota in the lake originate from seawater that was trapped in a basin about ca. 3 000 y B.P. (Zwartz *et al.,* 1988; Bird *et al.,* 1991). The bottom waters of Organic Lake are unusual due to the absence of hydrogen sulfide and the high concentration of the volatile gas dimethylsulfide (DMS) (Deprez *et al*., 1986; Franzmann *et al.*, 1987; Gibson *et al.*, 1991; Roberts & Burton 1993a; Roberts *et al.*, 1993b). Concentrations of DMS as high as 5 000 nM have been recorded in Organic Lake (Gibson *et al*., 1991), 100 times the maximum concentration recorded from seawater in the adjacent Prydz Bay and at least 1000 times that of the open Southern Ocean (Curran & Jones, 1998).

Over forty years ago, atmospheric DMS was proposed to have a regulatory effect on global cloud cover as it forms cloud condensation nuclei (Lovelock & Maggs, 1972; Charlson *et al.*, 1987). However, the first enzymes involved in DMS production were only identified in the last five years (Todd *et al.*, 2007). Rapid progress has been made in this short period and the pathways and organisms involved in DMS transformations have been extensively reviewed (Johnston *et al.*, 2008; Schäfer *et al.*, 2010; Curson *et al.*, 2011b; Reich *et al.*, 2011b; Moran *et al.*, 2012). The main source of DMS in the marine environment is from the breakdown of DMSP. Eucaryal phytoplankton, in particular, diatoms, dinoflagellates and haptophytes produce large quantities of DMSP, which is thought to function principally as an osmolyte. DMSP is released due to cell lysis, grazing or leakage and follows two known fates: DMSP cleavage by DMSP lyases (DddD, -L, -P, -Q, -W and -Y) or demethylation by DMSP demethylase (DmdA). Both pathways are associated with diverse microorganisms that can utilize DMSP as a sole carbon and energy source. However, it is only the cleavage pathway that releases volatile DMS that can lead to sulfur loss through ventilation to the atmosphere.

The very high levels of DMS in Organic Lake make it an ideal system for identifying the microorganisms and the processes they perform that lead to high levels of DMS accumulation. The previous Organic Lake metagenome study examined viruses from the 0.1 µm fraction of surface water that was collected from Organic Lake in December 2006, and November and December 2008 (Yau *et al.*, 2011). In the present study we focused on the cellular population rather than viruses, and examined the microbial community throughout the entire lake. Metagenomic analyses were performed on biomass captured by sequential filtration through a 20 µm pre-filter onto 3.0, 0.8, 0.1 µm filters, from a depth profile (1.7, 4.2, 5.7, 6.5 and 6.7 m) taken in November 2008 from the deepest point in the lake. This filtration and shotgun sequencing approach was originally adopted by the Global Ocean Sampling (GOS) expedition (Rusch *et al*., 2007) and has proven to be a powerful approach for studying Antarctic aquatic microbial communities (Ng *et al*., 2010; Lauro *et al*., 2011; Yau *et al*., 2011; Brown *et al*., 2012; Williams *et al*., 2012b; Wilkins *et al.,* 2012ab). By taking this approach our study determined the composition and functional potential of Organic Lake microbiota and, in conjunction with historic and contemporary physico-chemical data, generated an integrative understanding of the whole lake ecosystem.

# Materials and Methods

## *Characteristics of the lake and samples taken*

The water level of Organic Lake was measured by surveying as +1.886 m relative to the survey mark (NMV / S / 53) located at S 6827’28.3” E07811’20.9”. Water was collected from Organic Lake on 10 November 2008 through a 30 cm hole in the 0.8 m thick ice cover above the deepest point in the lake. The sampling hole (S 6827’22.2” E 7811’23.9”) was established following bathymetry measurements constructed on a metric grid (Figure \*\*\*\*). Samples were collected for metagenomics, microscopy and chemical analyses at 1.7, 4.2, 5.7, 6.5 and 6.7 m depths (maximum depth 6.8 m). For metagenomics, lake water was passed through a 20 µm pore size pre-filter, and microbial biomass captured by sequential filtration onto 3.0 µm, 0.8 µm and 0.1 µm pore size 293 mm polyethersulfone membrane filters, and samples immediately preserved in buffer and cryogenically frozen in liquid nitrogen, as described previously (Ng *et al.*, 2010; Lauro *et al.*, 2011). Between 1–2 L of lake water was sufficient to saturate the holding capacity of the filters. DNA was extracted from the filters, samples sequenced using the Roche GS-FLX titanium sequencer, and reads processed to remove low quality bases, assembled and annotated, as previously described (Ng *et al.*, 2010; Lauro *et al.*, 2011).

## *Physical and chemical analyses*

An *in situ* profile of pH, conductivity, turbidity, dissolved oxygen (DO) and pressure was measured using a submersible probe (YSI sonde model V6600). A temperature profile was measured using a maximum-minimum mercury thermometer (the YSI probe did not have a capacity to record temperature below −10ºC). The 5.7 m sample corresponded to the turbidity maximum and the 6.5 m sample to the turbidity minimum. Conductivity at *in situ* temperature was converted to conductivity at 15ºC as described previously (Gibson, 1999). The adjusted conductivity brings the temperature to within a range suitable for estimating practical salinity using the formula of Fofonoff and Millard (1983). Salinity was likely to have been underestimated as it is higher than the range (2–42) for which the conductivity–salinity relation holds. However, the relative difference in salinity between the samples would be accurate. Density was calculated from the *in situ* conductivity and temperature using the equations described by Gibson *et al.* (1990) and expressed at temperature T as:

σT = (1000 − density) kg/m3

Ammonia, nitrate, nitrite, total nitrogen (TN), total dissolved nitrogen (TDN), dissolved reactive phosphorus (DRP), total phosphorus (TP), total dissolved phosphorus (TDP), total organic carbon (TOC), total dissolved carbon (DOC), total sulfur (TS) and total dissolved sulfur (TDS) were determined by American Public Health Associations Standard Methods at the Analytical Services, Tasmania. Values for dissolved nutrients were measured after filtration through a 0.1 µm pore size membrane filter. All other nutrients were measured from water collected after filtration through the on-site 20 µm pore size pre-filter. Ammonia, nitrate, nitrite, DRP, TN, TDN, TP and TDP were measured in a Flow Injection Analyser (Lachat Instruments, Colorado, USA). TOC and DOC were determined in the San++ Segmented Flow Analyser (Skalar, Breda, Netherlands). TS and TDS were analyzed in the 730ES Inductively Coupled Plasma–Atomic Emission Spectrometer (Agilent Technologies, California, USA). Principal Component Analysis (PCA) was performed using the PRIMER Version 6 statistical package (Clarke & Gorley, 2006) on the normalized physical and chemical parameters.

## *Epifluorescence microscopy*

Water samples collected for microscopy were preserved in formaldehyde (1% v/v). Cells and virus-like particles (VLPs) were vacuum filtered onto 25 mm polycarbonate 0.015 µm pore-size membrane filters (Nuclepore Track-etched, Whatman, GE Healthcare, USA) with a 0.45 µm pore-size backing filter. The 0.015 µm filter was mounted onto a glass slide with ProLong® Gold anti fade reagent (Invitrogen, Life Technologies, NY, USA) and 2 µl (25 × dilution in sterile filtered milliQ water <0.015 µm) SYBR® Gold nucleic acid stain (Invitrogen, Life Technologies, NY, USA). Prepared slides were visualized in an epifluorescence microscope (Olympus BX61, Hamburg, Germany) under excitation with blue light (460–495 nm, emission 510–550 nm). Cell and VLP counts were performed on the same filter over 30 random fields of view.

### *Cellular diversity analyses*

Diversity of *Bacteria, Archaea* and *Eucarya* was assessed using ribosomal small subunit (SSU) gene sequences. Metagenomic reads that matched the 16S and 18S rRNA genes were retrieved using Metaxa (Bengtsson *et al.*, 2011). Only sequences longer than 200 bp were accepted for downstream analysis. The Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.4.0) (Caporaso *et al*., 2010) implementing UCLUST, was used to group SSU sequences into operational taxonomic units (OTUs) at 97% percent identity against the SILVA SSU reference database (release 108). SSU sequences that did not cluster with sequences from SILVA were allowed to form new OTUs (no suppression). A representative sequence from each OTU was chosen and classified to the genus level using QIIME implementing the RDP classifier (Wang *et al*., 2007) trained against SILVA (release 108) sequences (www.arb-silva.de). Assignments were accepted to the lowest taxonomic rank with bootstrap value ≥85%. To allow comparison of the relative abundance of taxa, the number of SSU matches per sample filter was normalized to the average number of reads (403 577). Statistical analysis on the relative SSU abundances was performed using the PRIMER Version 6 package (Clarke & Gorley, 2006). The SSU counts of each sample filter were aggregated to the genus level and square root transformed to reduce the contribution of highly abundant taxa. A resemblance matrix was computed using Bray-Curtis similarity. The upper mixed zone (1.7, 4.2 and 5.7 m) and deep zone (6.5 and 6.7 m) samples were designated as separate groups and an analysis of similarity (ANOSIM) performed to test for difference between the two groups. BEST analysis was performed with the abiotic variables: conductivity, temperature, turbidity, DO, pH, TOC, TN, TP, TS, total C:N, total C:P, total N:P, cell counts and VLP counts. The Bio-Env procedure in BEST looks at all the abiotic variables in combination and finds a subset sufficient to best explain the biotic structure. A heat map with bi-clustering dendogram was generated using R and the package ‘seriation’ (Hahsler *et al*., 2008) on the normalized square-root transformed SSU counts.

## *Analysis of Organic Lake functional potential*

The relative abundance and taxonomic origin of functional marker genes was used to determine the potential for carbon, nitrogen and sulfur conversions in Organic Lake. Open reading frames (ORFs) were predicted from trimmed metagenomic reads using MetaGene (Noguchi *et al*., 2006) accepting those >90 bp in length. ORFs were translated using the standard bacterial/plastid translation table and compared to protein sequences from the Kyoto Encyclopedia of Genes and Genomes (KEGG) GENES database (release 58) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). The BLAST output was processed using KEGG Orthology Based Annotation System (KOBAS) version 2.0 (Xie *et al.*, 2011) accepting assignments to KEGG Orthology (KO) groups with e-value <1e−05 and rank >5. KO groups used as functional markers in this study are listed in Table S1. Marker enzymes were assigned to taxonomic groups based on the species of origin of the best KEGG GENES BLASTp match. Marker genes not represented by a KO group were assessed by alternative strategies. Homologs of bacterial rhodopsins were retrieved from Organic Lake reads if they had a top BLAST match to any in a list of 139 entries in the KEGG GENES database affiliated with bacteriorhodopsin, xanthorhodopsin, halorhodopsin or proteorhodopsin. TranslatedDMSP lyase and DMSP demethylase sequenceswith experimentally confirmed function (Table S2) were used to query a BLAST database of translated ORFs predicted from metagenomic reads. Matches were accepted if the e-value was <1e−10 and sequence identity was within the range shared by homologs of the query sequence(s) (Table S2). Mmarker genes

The same process was used to determine the frequency of homologs of these genes in metagenome data from Ace Lake, the Southern Ocean and the GOS expedition. .

Marker gene sequences for phylogenetic analysis were clustered using the CD-HIT web server (Huang *et al*., 2010) at 90% global amino acid identity. A representative sequence from the clusters that resided within a desired conserved region and homologs from isolated strainswere used in phylogenetic analyses performed in MEGA 5.05 (Tamura *et al.* 2011). Sequences were aligned with MUSCLE (Robert, 2004) using default parameters (gap opening penalty: −2.9, gap extension penalty: 0). Neighbor-joining was used to compute the phylogenies with a Poisson substitution model, uniform rates of change and complete deletion of alignment gaps. Node support was tested with bootstrap analysis (500 replicates).

*Analysis of the distribution of DMSP catabolism and photoheterotrophy potential*

Potential for DMSP catabolism and photoheterotrophy was assessed in Organic Lake, Ace Lake (Lauro *et al.*, 2011), Southern Ocean surface water samples (Wilkins *et al.*, 2012b) and GOS metagenomes (Rusch *et al.*, 2007). Translated DMSP lyase, DMSP demethylase, rhodopsin, photosynthetic reaction center gene sequences and the single copy *recA* gene with verified function (Table S2) were used as BLAST queries against translated ORFs predicted from metagenomic reads. For GOS samples, the peptide sequences were retrieved from CAMERA (camera.calit2.net). Matches to the query sequences were counted if e-value was <1e-10 and sequence identity was above that shared by homologs of the query sequence(s), except for

*recA* where a lower e-value cut-off of <1e-20 was applied (as established by Howard *et al.* 2008). Frequencies of genes of interest were expressed per 100 Mb in order to allow comparison between metagenomes sequenced using different DNA sequencing technologies. This required tallying the total number of high quality base pairs from each metagenome. For the GOS samples, this was estimated by multiplying the number of reads from each sample by the average read length (822 bp) (Rusch *et al.*, 2007). The percentage of genomes containing a marker gene of interest in a sample was estimated by dividing the normalized count of each marker gene by the *recA* count.

# Results and discussion

## *Abiotic properties and water column structure*

*In situ* physico-chemical profiles (Figure S2) measured over the deepest point in the lake (Figure S3) determined the existence of two zones: an upper mixed zone above 5.7 m and a suboxic deep zone below 5.7 m (Figure 1A). The separation of the two zones was indicated by a pycnocline and oxycline starting at 5.7 m. The pH also decreased with DO, likely due to fermentation products such as acetic, formic and lactic acids that have previously been recorded in the bottom waters (Franzmann *et al*., 1987b; Gibson *et al*., 1994). The deep zone was not completely anoxic. Oxygen may be episodically introduced to bottom waters as a result of currents of cold dense water sinking during surface ice-formation (Ferris *et al*., 1999). In comparison to meromictic lakes such as Ace Lake which have strong pycnoclines and a steep salt gradient in the anoxic zone, Organic Lake is shallow and has relatively weak stratification (Gibson, 1999). Samples were collected from the upper mixed (1.7, 4.2 and 5.7 m) and deep (6.5 m and 6.7 m) zones.

All nutrients, except for nitrate and nitrite reached maximum concentrations at 6.5 m (Table 1) suggestive of a layer of high biological activity above the lake bottom. Consistent with this, cell and VLP counts were highest at 6.5 m. However, turbidity was lowest at this depth demonstrating turbidity was not principally determined by cell density (Figure 1B). Microscopy images did not show a shift in cell morphology that could account for the large drop in turbidity (\*Figure S4), which suggests particulate matter primarily contributed to turbidity readings. The low turbidity and peak in cell counts and nutrients in the microaerophilic zone at 6.5 m may be caused by an active microbial community degrading particulate matter. This inference is supported by the report of high concentrations of dissolved organic acids and free amino acids in the deep zone (Gibson *et al.*, 1994) as these nutrients are indicative of the breakdown of high molecular weight carbohydrates, lipids and proteins. Furthermore, the C:N and C:P ratios throughout the lake were high compared to the Redfield ratio (Redfield *et al.*, 1963) except at 6.5 m indicating this was the only depth where dissolved N and P were not relatively limited (Table 1). PCA analysis of physico-chemical parameters showed all samples, except the 6.5 m sample, separated with depth along the PC1 axis (Figure S5). Accordingly, turbidity, TS and cell density were the strongest explanatory variables for the separation of the 6.5 m sample from the other deep sample, indicating that increased activity at 6.5 m was related to breakdown of particulate matter and sulfur chemistry.

## *Overall microbial diversity*

SSU genes (3 959 reads) that were retrieved from the metagenome data (Table S3) grouped into 983 OTUs. OTUs for *Bacteria* comprised 76.2%, *Eucarya* 16.3% and 7.5% of SSU sequences could not be classified. Only 2 reads, assigned to a deep sea hydrothermal clade of *Halobacteriales* (Table S4) indicating *Archaea* were rare in Organic Lake. Microbial diversity was low, consisting of 15 bacterial phyla and 6 eucaryal superkingdom divisions. Of these, only 7 bacterial phyla and 4 eucaryal phyla were predominant. (\*diversity indices)

The most abundant bacterial classes, *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteria*, were represented by OTUs on all filter sizes at all depths (Figure 2A) and each consisted of one dominant genus, *Marinobacter*, *Roseovarius* and *Psychroflexus*, respectively (Figure 2C). Essentially all OTUs for *Cyanobacteria*/chloroplasts were classified as chloroplasts (Figure 2A), except for three reads that could not be assigned to any lower rank (Table S4) indicating free-living *Cyanobacteria* were rare or absent. OTUs for moderately abundant bacterial classes were *Actinobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and candidate divisions OD1 and RF3. Lower abundance divisions included OTUs for *Bacilli*, *Clostridia*, *Spirochaetes*, *Lentisphaera*, TM7, *Opitutae*, *Verrucomicrobia*, Bhi80-139, Bd1-5, SR1 and *Chlamydiae* (Figure 2A). The dominant eucaryal OTUs were for photosynthetic *Chlorophyta* (green algae) and *Dictyochophyceae* (silicoflagellate algae) (Figure 2B) principally assigned to the genus *Dunaliella* and the order *Pedinellales*, respectively (Table S4)*.* Lower abundance eucaryal OTUs included *Bacillariophyta* (diatoms), *Dinophyceae*, *Fungi* and heterotrophic *Choanoflagellida* and *Ciliophora* (see Table S4 for lower taxonomic rank assignments).

## *Variation of microbial composition according to size and depth*

Community composition varied with size fraction and depth. This was supported by seriation analysis that showed samples clustered according to size fraction, and those clusters further separated into upper mixed and deep zone groups (Figure 3). A significant difference in genus-level composition between mixed and deep zone samples was supported by ANOSIM test (Rho: 0.53, significance: 0.1%). Differential vertical distribution of taxa is consistent with partitioning of ecological functions in the lake and in association with the physical and chemical data, described functional roles of those taxa.

### *20–3.0 µm fraction community composition*

The upper mixed zone samples had a relatively high OTU abundance of *Dunaliella* chloroplasts and chlorophyte algae consistent with large active photosynthetic organisms concentrating near surface light. They are likely the main source of primary production in Organic Lake and have previously been reported to be the dominant algae (Franzman *et al.*, 1987b). The SSU sequences for these algae at the bottom of the lake are likely to be due to sedimentation of dead cells or resting cysts.

*Psychroflexus* OTUs were overrepresented in the surface and 6.7 m samples. Consistent with enrichment on the 3.0 µm filters, *Psychroflexus* (formerly *Flavobacterium*) *gondwanensis* (Bowman *et al.*, 1998) isolated from Organic Lake (Franzmann *et al*., 1987b) had cells 1.5–11.5 µm in length (Dobson *et al*., 1991). *Flavobacteria* associate with phytoplankton blooms in the Southern Ocean (Abell & Bowman 2005a; Abell & Bowman 2005b; Williams *et al.,* 2012), and have specialized abilities to degrade polymeric substances from algal exudates and detritus (reviewed in Kirchman *et al.*, 2009; Williams *et al.,* 2012). It is likely that Organic Lake *Psychroflexus* fills a similar ecological role. In support of this, *Psychroflexus* OTUs cluster with *Dunaliella* chloroplasts in the seriation analysis (Figure 3) and *P. gondwanensis* abundance in Organic Lake has been correlated with average hours of sunshine per day indicating population dynamics that is related to summer algal blooms (James *et al.*, 1994). The *Psychroflexus* OTUs in the deep zone are most likely due to sedimentation as *P. gondwanensis* is non-motile and strictly aerobic (Dobson *et al.*, 1991).

*Roseovarius* OTUs were enriched at 4.2 m and 6.5 m suggesting different ecotypes may be present in the upper mixed zone compared to the deep zone. *Roseovarius tolerans*, an isolate from Ekho Lake in the Vestfold Hills, Antarctica has a cell size (1.1–2.2 μm; Labrenz *et al*., 1999) consistent with *Roseovarius* capture on the 3 µm filter. A strain from Ekho Lake is capable of microaerophilic growth (Labrenz *et al.*, 1999). Overrepresentation at 6.5 m may therefore be indicative of growth at that depth rather than sedimentation as sinking cells would be more abundant close to the lake bottom at 6.7 m. *Roseovarius* OTUs cluster with *Dunaliella* chloroplast and *Psychroflexus* OTUs in the seriation analysis (Figure 3), suggesting that Organic Lake *Roseovarius* may be utilizing compounds released from algal-derived particulate matter, or made available by processing of complex organic matter by *Psychroflexus*. *Roseovarius* is a member of the *Roseobacter* clade, which is inferred to have an opportunistic ecology frequently associated with nutrient-replete plankton aggregates, including by-products of flavobacterial exoenxymatic attack (Moran *et al*., 2007; Teeling *et al*., 2012). Additionally, the diverse metabolic capabilities of the *Roseobacter* clade include DMSP degradation, aerobic anoxygenic phototrophy (AAnP) and CO oxidation (reviewed in Wagner-Döbler & Biebl, 2006). All of these capabilities should facilitate growth in both the upper mixed and deep zones of Organic Lake (see *Carbon resourcefulness in dominant heterotrophic bacteria*below).

### *3–0.8 µm size fraction community composition*

On the 0.8 µm filter, OTUs for *Marinobacter* dominated at all depths except 6.5 m. Their capture on this size fraction is consistent with the cell size of isolates (1.2–3 µm) (Gauthier *et al.*, 1992). *Marinobacter* is metabolically versatile, which likely permits it to occupy the entire water column. The genus is heterotrophic and includes hydrocarbon-degrading strains (e.g., Gauthier *et al*., 1992; Huu *et al*., 1999), although deep-sea metal-oxidizing autotrophs have been affiliated with this genus (Edwards *et al.*, 2003). Some isolates are capable of interacting with diatoms (Gärdes *et al*., 2010) and dinoflagellates (Green *et al.*, 2006). *Marinobacter* isolates from Antarctic lakes are capable of anaerobic respiration using dimethyl sulfoxide (DMSO) (Matsuzaki *et al*., 2006) or nitrate (Ward & Priscu, 1997). Analysis of functional potential linked to *Marinobacter* revealed additional metabolic capabilities potentially related to its dominance in Organic Lake (see *Carbon resourcefulness in dominant heterotrophic bacteria*and *Molecular basis for unusual sulfur chemistry* below).

OTUs for RF3 and *Halomonas* were overrepresented at 6.5 m, and RF3 sequences were more abundant (Figure 2 and 3). Their relative abundance in the deep zone indicates a role in microaerophilic processes. The majority of RF3 sequences to date are from anaerobic environments including mammalian gut (Tajima *et al.*, 1999; Ley *et al*., 2006; Samsudin *et al*., 2011), sediment (Yanagibayashi *et al.*, 1999; Röske *et al.*, 2012), municipal waste leachate (Huang *et al.*, 2005), anaerobic sludge (Chouari *et al.*, 2005; Goberna *et al*., 2009; Rivière *et al.*, 2009; Tang *et al*., 2011), a subsurface oil well head (Yamane *et al.*, 2011), and the anaerobic zone of saline lakes (Humayoun *et al*., 2003; Schmidtova *et al*., 2009) including an Antarctic lake (Bowman *et al.*, 2000b). However, some members have been found in surface waters (Demergasso *et al*., 2008; Xing *et al.*, 2009; Yilmaz *et al.*, 2012) suggesting not all members are strict anaerobes. Several *Halomonas* isolates have been sourced from Organic Lake including two described species *Halomonas subglaciescola* and *H. meridiana*, both of which grow as rods with dimensions consistent with capture on this size fraction (Franzmann *et al.*, 1987a; James *et al.*, 1990). Despite these isolates being aerobic, *Halomonas* has been reported to be enriched at the oxycline in Organic Lake (James *et al.*, 1994) indicating *Halomonas* in the lake play an ecological role in the suboxic zone. This capacity may be linked to the ability of free amino acids and organic acids (which are abundant in the deep zone) to stimulate the growth of isolates (Franzmann *et al.*, 1987a).

### *0.8–0.1 µm size fraction community composition*

A large number of eucaryal sequences were evident in the 0.1 µm size fraction. The upper zone was overrepresented by OTUs for *Pedinellales* (silicoflagellate algae) that co-varied with chloroplasts (Figure 2 and 3). *Pedinellales* have only been detected in Antarctic lakes from molecular studies (Unrein *et al.*, 2005; Lauro *et al.*, 2011) including Organic Lake (Yau *et al.*, 2011). Subsequent light microscopy analyses in freshwater lakes of the Antarctic Peninsular reported cells resembling *Pseudopedinella* that were 5–8 µm in diameter (Unrein *et al.*, 2005). It is possible that in Organic Lake small (0.8–0.1 µm) free-living members or chloroplast containing cyst forms (Thomsen, 1988) of this eucaryal class exist. However, without evidence to support this (*e.g.* microscopy-based) it seems more likely that the lake sustains a relatively small number of active photosynthetic cells and the sequences detected arise from cysts or degraded cellular material.

OTUs for *Candidatus* “Aquiluna”, in the Luna-1 cluster of *Actinobacteria* (Hahn *et al.*, 2004; Hahn *et al.*, 2009) were most abundant at 1.7 m. The genus has small cells (<1.2 µm; Hahn *et al.*, 2009), accounting for their concentration on this size fraction. Although originally described in freshwater lakes, the same clade was detected in abundance in Ace Lake (Lauro *et al.*, 2011) and surface Artic seawater (Kang *et al*., 2012) demonstrating they play ecological roles in polar saline systems. In Ace Lake surface waters they were associated with utilization of labile C and N substrates (Lauro *et al.*, 2011), and in Organic Lake surface waters, probably perform similar functions. The presence of this clade in the deep zone implies a facultative anaerobic lifestyle or sedimented cells.

The bottom of the water column was distinguished by the presence of OTUs for candidate divisions OD1 and TM7. OD1 was more abundant, and its prevalence on this size fraction is consistent with similar findings for size fractionation of ground water (Miyoshi *et al.*, 2005). OD1 is consistently associated with reduced, sulfur-rich, anoxic environments (Harris *et al*., 2004; Elshahed *et al.*, 2005). OD1 from Zodletone Spring, Oklahoma was reported to possess enzymes related to those from anaerobic microorganisms (Elshahed *et al.*, 2005). Genomic analyses identified OTUs for OD1 in the anoxic zone of Ace Lake (Lauro *et al.,* 2011). The distribution of OD1 in Organic Lake is consistent with an anaerobic metabolism and potential involvement in sulfur chemistry.

## *Organic Lake functional potential*

To determine the functional processes occurring in Organic Lake, gene markers for C, N and S conversions (Figure 4) were retrieved from metagenomic reads. BEST analysis showed that variation in the population structure was significantly correlated (Rho: 0.519, significance: 0.3%) with the abiotic parameters, DO, temperature, TS and TN. The DO gradient has an obvious effect of separating aerobic from anaerobic taxa, allows oxygen sensitive N and S processes to occur in the deep zone. Functional potential, taxonomic composition and the physico-chemical data were integrated to infer the C, N and S cycles in Organic Lake.

## *Carbon resourcefulness in dominant heterotrophic bacteria*

In both the upper mixed and deep zones, potential for C fixation was much lower than for degradative processes, indicating potential for net C loss (Figure 4A). Potential for aerobic carbon fixation (Figure 4A) via the oxygen-tolerant Calvin-Benson-Basham (CBB) cycle was assessed by presence of the marker genes ribulose-bisphosphate carboxylase (RuBisCO) and phosphoribulokinase (PRK) (Hügler & Sievert, 2011). The majority of RuBisCO homologs were related to *Chlorophyta* (Table 2, Figure S6A) supporting the ecological role of green algae as the principle photosynthetic organisms. RuBisCO was associated with a small proportion of *Gammaproteobacteria* (Figure S6A), principally from sulfur-oxidizing *Thiomicrospira*, indicating some *Gammaproteobacteria* are autotrophs. (Table 2, Figure S6A)Although deep-sea, iron-oxidizing autotrophic members of *Marinobacter* have been isolated (Edwards *et al.*, 2003), all genomes reported for *Marinobacter* have PRK but lack RuBisCO, Across *Marinobacter* genomes the PRK homolog gene is adjacent to a gene for a putative phosphodiesterase, suggesting that the enzymes expressed by these genes may be involved in a pathway involved in pentose phosphate metabolism unrelated to C fixation, This decoupling of PRK from RuBisCO involved in C fixation (forms I and II), also observed in *Ammonifex* (Hügler & Sievert, 2011), undermines the utility of PRK as a marker gene for the CBB cycle. Thus, there is no evidence for autotrophy in Organic Lake mediated by *Marinobacter* and PRK was not used in estimates of C-fixation potential (Figure 4A).

Evidence for C fixation via the reverse tricarboxylic acid (rTCA) cycle was also indicated, with genes for ATP citrate lyase linked to sulfur-oxidizing *Epsilonproteobacteria* (Figure S6A). In general, the rTCA cycle is restricted to anaerobic and microaerophilic bacteria (Hügler & Sievert, 2011), which is consistent with the detection of *Epsilonproteobacteria* in the lake bottom where oxygen is lowest, and the microaerophilic/anaerobic metabolisms characteristic of the group (Campbell *et al*., 2006). Anaerobic C fixation was represented by potential for the Wood-Ljungdahl (WL; or reductive acetyl-CoA) pathway(Figure S6A). WL-mediated C fixation, for which CO dehydrogenase/acetyl-CoA synthase is the key enzyme, was linked to *Clostridia* and *Deltaproteobacteria* that are known to grow autotrophically using this pathway (Hügler & Sievert, 2011).

*Alphaproteobacteria*, predominantly *Roseovarius* (Figure 2C), were implicated in CO oxidation (Table 2; Figure S6A), which is used to generate energy for lithoheterotrophic growth (Moran & Miller, 2007), although CO oxidation may also be involved in anaplerotic C fixation (Moran *et al*., 2007). The CO oxidation capacity was at a maximum at 6.5 m (Figure 4A), and therefore associated with the deep-zone *Roseovarius* ecotype of Organic Lake*.* CO oxidation can function as a strategy to limit oxidation of organic carbon for energy so that a greater proportion can be directed towards biosynthesis (Moran & Miller, 2007).

Photosynthesis reaction center genes *pufL* and *pufM*, involved in photoheterotrophy via aerobic anoxygenic phototrophy (AAnP), were abundant in Organic Lake. These were linked to the *Roseobacter* clade of *Alphaproteobacteria* (Table 2), major contributors to AAnP in ocean surface waters (Béjà *et al.*, 2002; Moran et al., 2007). This is consistent with the known metabolic potential of bacteriochlorophyll A (BchlA) producing *Roseovarius tolerans* from Ekho Lake (Labrenz *et al.*, 1999). Photoheterotrophy can also be rhodopsin-dependent, with proteorhodopsins (PRs) of marine *Flavobacteria* and *Vibrio* previously linked to light-dependent energy generation to supplement heterotrophic growth, particularly during C limitation (Gómez-Consarnau *et al*., 2007; Gómez-Consarnau *et al*., 2010). However, the function(s) of rhodopsins are diverse, and PRs are also hypothesized to be involved in light or depth sensing (Fuhrman *et al.*, 2008).

Rhodopsin genes were associated with all the dominant Organic Lake aerobic heterotrophic lineages. Phylogenetic analysis revealed six well-supported Organic Lake rhodopsin groups (Figure S7). All groups had an L or M residue at position 105 (*vs* the SAR86 PR), denoting tuning to surface green light (Man *et al.*, 2003; Gomez-Consarnau *et al.*, 2007), and is a characteristic of oceanic coastal samples (Rusch *et al.*, 2007). Four of the groups clustered with homologs of genera detected in the lake, namely *Marinobacter*, *Psychroflexus*, *Octadecabacter* and “*Ca.* Aquiluna” (Figure S7, Table S4). Another group (SAL-R group) originates from the sphingobacterium *Salinibacter ruber*, which produces xanthorhodopsin(Balashov *et al.*, 2005); it is therefore likely that Organic Lake *Sphingobacteria* (Table S4) was the origin of this rhodopsin group. The most abundant group (OL-R1; Figure S7) had no close homologs from GENBANK, but it was abundant on the 3.0 µm fraction and has a distribution suggesting it originates from Organic Lake members of the *Roseobacter* clade (Figure 4A). All ORFs adjacent to OL-R1 rhodopsin containing contigs were related to *Octadecabacter* further supporting their *Roseobacter* clade provenance (Figure S\*\*\*) . Genes downstream of OL-R1 were involved in carotenoid synthesis, indicating OL-R1 is a xanthorhodopsin, which occur as a retinal protein/carotenoid complex (Balashov *et al.*, 2005).

Photoheterotrophic potential of Organic Lake was compared with other aquatic environments including the nearby Ace Lake, the Southern Ocean and GOS expedition samples. Rhodopsin genes were detected in all sites, but lower rhodopsin abundances occurred in the 0.1 µm fraction of non-marine sites (Table 3) consistent with the trend observed by Sharma *et al*. (2008). The Organic Lake 0.1 µm fraction showed the lowest percentage of rhodopsin containing cells of all environments surveyed. However, in the larger size fractions, Organic Lake rhodopsin abundance was similar to Ace Lake mixolimnion suggesting rhodopsin containing cells in Organic Lake were large or particle attached. The paucity of rhodopsins in the Organic Lake 0.1 µm fraction is likely due to the lack of SAR11 clade from which is the expected origin of the rhodopsin genes in Ace Lake and marine samples. Therefore, although still lower than for the Southern Ocean, cells with rhodopsins were as abundant in Organic Lake as Ace Lake. This demonstrates the value examining larger or particle attached cells as they may be functionally relevant members of the population.

In the Organic Lake 0.1 µm size fraction, counts for *pufLM* genesin the 4.2 m and deep samples was higher than all other environments except for Punta Cormorant hypersaline lagoon. In constrast, potential for AAnP in all other environments was generally lower than Organic Lake. As for rhodopsins containing cells, the percentage of AAnP bacteria in Organic Lake was higher in the larger size fractions indicating that, AAnP bacteria in Organic Lake are larger or particle associated. This suggests AAnP provides an adaptive advantage in hypersaline aquatic environments.

relative & Kirchman, .Furthermore, regulation of pigment synthesis is complex; for exampleexpression occurs is , which (\*NB compare to Koh sea ice and Ross Sea water).

In the deep zone, potential for fermentation was greatest at 6.5 m (Figure 4A) and likely the main biological activity that was occurring at that depth. Fermentation was indicated by the marker genes for lactate dehydrogenase (*ldh*) and pyruvate:ferredoxin oxidoreductase (*porA, porB*), the two enzymes that compete for pyruvate at the acetate-lactate branch point. These genes were linked to *Mollicutes* (Table 2); but as no *Mollicutes* OTUs were detected, one possibility is that they originated from the related candidate division RF3 (Tajima *et al.*, 1999) which has relatively high abundance in this zone (see *0.8–3.0 µm size fraction* *community composition* above). Thus, there is circumstantial evidence that RF3 possesses fermentative metabolism and may play an important ecological role in Organic Lake by degrading high molecular weight compounds to SCFA that other organisms could utilize. Assimilation of fermentation products may play a greater role in Organic Lake rather than complete anaerobic oxidation involving methanogens or sulfate-reducing bacteria; the former were absent and the latter were present in low abundance (Figure 2A, 2C).

## *Regenerated nitrogen is predominant in the nitrogen cycle*

N cycling potential throughout the lake was dominated by assimilation and mineralization pathways (Figure 4B) linked to *Proteobacteria* (Table 2, Figure S6). Assimilatory nitrite reductase was not abundant but genes involved in ammonia assimilation were (Figure S6B). Glutamate dehydrogenase (GDH) genes were abundant, and linked predominantly to *Proteobacteria* and to a lesser extent *Bacteroidetes* (Figure S6B). However, the significance of the readily reversible GDH depends on its origin; *Bacteroidetes* are likely to use GDH in the oxidative direction for glutamate catabolism (Takahashi *et al*., 2000; Williams *et al*., 2012b), whereas the use of GDH in the oxidative or reductive directions by *Proteobacteria* is likely to depend upon the source of reduced N (ammonia *vs* amino acids). Glutamine synthetase and glutamate synthase genes, which were predominantly linked to *Proteobacteria*, indicate high-affinity ammonia assimilation by this group in Organic Lake. The high ammonia concentration in the deep zone would result from a higher rate of mineralization (ammonification) than assimilation (Table 2, Figure S6B). This is consistent with abundant OTUs of the bacteroidetan *Psychroflexus* in this zone, and due to either turnover of organic matter or lysis of bacteroidetan cells after sedimentation in anoxic water. In addition, the gene for ammonia-generating nitrite reductase (*nrfA*) was linked to *Bacteroidetes* and *Planctomycetes*; this offers another potential avenue for ammonia production by these putative aerobic heterotrophs. Overall, the data suggest that ammonia is actively assimilated in the aerobic upper mixed zone, but is permitted to accumulate in the anaerobic deep zone.

Potential for nitrogen conversions typically found in other aquatic environments was greatly reduced in Organic Lake. There was a very low potential for N fixation that was confined to the deep zone (Figure 2B) and principally linked to anaerobic *Epsilonproteobacteria* (Table 2, Figure S6B). This diazotrophic potential may not be realized by N-fixing *Epsilonproteobacteria,* given the high ammonia concentration present in the deep zone. The potential for ammonia oxidation was the hydroxylamine/hydrazine oxidase-like proteins (*hao*) genes, which was low abundance and linked to *Deltaproteobacteria* (Table 2, Figure S6). *hao* genes have been documented in non-ammonia-oxidising bacteria (Bergmann *et al*., 2005) and inspection of the genes in Organic Lake reveal they belong to a family of multiheme cytochromes c in sulfate-reducing *Deltaproteobacteria* that have no proven role in ammonia oxidation. In the genomes of sulfate-reducing *Deltaproteobacteria* this *hao* gene is invariably situated adjacent to a gene for a NapC/NirT protein, which suggests a role in nitrogen cycling. Collectively these data indicate an inability for nitrification to occur in the upper mixed zone and likely no potential for ammonia loss in the deep zone.

Denitrification genes were present throughout the water column (Figure 4B) and were linked primarily to *Gammaproteobacteria* (Table 2, Figure S6B). Low nitrate and nitrite in the deep zone (Figure 1B, Table 1) indicates oxidized N has been depleted by dissimilatory reduction. Denitrification genes are phylogenetically widespread and usually induced by low oxygen or oxidized N species (Kraft *et al*., 2011) and thus expected to be active in the deep zone or oxycline. However, denitrification may be inhibited even if conditions appear appropriate. For example, in Lake Bonney, Antarctica, denitrification occurs in the west lobe, but not in the east lobe of the lake despite the presence of anoxia, nitrate and denitrifying *Marinobacter* species (Ward & Priscu, 1997; Ward *et al*., 2005). Moreover, in the absence of nitrification, denitrification would be limited by the lack of potential to re-form oxidized N. The preponderance of assimilation/mineralization pathways geared towards reduced N appears to reflect a “short circuit” of the typical N cycle that would conserve N in a largely closed system. Hence, the predominant N source is regenerated fixed N. Similar findings were also made for Ace Lake, although in this system the presence of a dense layer of green sulfur bacteria with the potential to fix nitrogen augments the N cycle (Lauro *et al*., 2011).

## *Molecular basis for unusual sulfur chemistry*

Organic Lake differs markedly from other meromictic Antarctic lakes (Ng *et al.*, 2010; Lauro *et al*., 2011, \*others) in possessing a low potential for dissimilatory sulfur cycling (Figure 4C). Sulfur oxidation by the Sox multienzyme system was linked to *Alphaproteobacteria* (Table 2), with *sox* genes most abundant in the aerobic waters of the upper mixed zone, which is consistent with the use of thiosulfate as the sulfur source. Although sulfur-oxidizing *Epsilonproteobacteria* (Figure 2A, 2C) were present in the deep zone, no potential for sulfur oxidation was linked to them, including via the Sox system. Also not detected was the hydrogen-oxidizing sulfur respiration pathway using polysulfide reductase (Psr), which, like the Sox system, has been documented in deep-sea *Epsilonproteobacteria* (Yamamoto & Takai, 2011).

and polysulfide reductase (PSR) genes were not detected; both genes are known to be possessed by deep-sea sulfur-oxidizing *Epsilonproteobacteria* (Yamamoto & Takai, 2011). This suggests that rather than S, Organic Lake *Epsilonproteobacteria* make use of alternate electron donors such as SCFA or hydrogen (\*check). Importantly, it is therefore likely that appreciable S oxidation cannot occur in the deep zone as the known terminal electron acceptors, oxygen and nitrate are deplete.

In the deep zone, dissimilatory sulfate reduction (DSR) potential was extremely low (Figure 4C) as was the abundance of sulfate-reducing *Deltaproteobacteria* (Figure 2A, 2C). The reason for the limited DSR potential is unclear, although it is possible that the high salinity, transient oxygenation or positive electro-potential inhibits microorganisms from performing DSR, and hence colonizing the deep zone of the lake. (\*\*\*\*ref paper) It is also likely that the lack of dissimilatory sulfur cycling contributes to the accumulation of DMS and DMSP in Organic Lake in the deep zone. In the upper mixed zone, DMS could potentially be oxidized as a carbon and energy source or utilized as an electron donor by sulfur-oxidizing autotrophs (Schäfer *et al.*, 2010). In anoxic zones, methanogenic *Archaea* or sulfate-reducing bacteria are the main organisms known to break down DMS (\*Scholten *et al.*, 2003 or Schäfer *et al.*, 2008). However, the very low dissimilatory sulfur conversion potential in the deep zone coupled with the relatively stagnant waters would likely minimize DMS oxidation and loss by ventilation. DMS would therefore be expected to accumulate in the deep zone.

To determine the source of high DMS in the bottom waters of Organic Lake, the genes involved in DMS formation were surveyed. Genes for DMSP lyases *dddD*, *dddL* and *dddP*, were detected in Organic Lake at levels comparable to other dominant processes such as respiration and fermentation (Figure 4C) indicating DMSP is an important carbon and energy source in Organic Lake. *dddD* was the most abundant of the Organic Lake DMSP lyases (\*Table 3) and comprised two main types: MAR-dddD and OL-dddD (Figure S8). Neither of these types clustered with the non-functional *Dinoroseobacter shibae* DFL 12 and *Ruegeria pomeroyi* DSS-3 *dddD* homologs (Todd *et al.*, 2011) or carnitine coenzyme A transferase outgroups, thereby providing support for their proposed role as functional DMSP lyases. The MAR-dddD type includes the *Marinobacter* sp. ELB17 *dddD* homolog, and MAR-dddD sequences were most abundant on the 0.8 µm fraction where *Marinobacter* OTUs were also more abundant, indicating MAR-dddD derives from Organic Lake *Marinobacter* (Figure S8). OL-dddD did not have a close relative from cultured bacteria making its taxonomic origins uncertain. The abundance of OL-dddD on the 3.0 µm fraction suggests it originates from *Alphaproteobacteria*,. OL-dddD containing contigs carried genes of mixed *Alpha-* and *Gammaproteobacteria* origin consistent with the “pick ‘n mix” arrangement of genes found beside sequenced *dddD* regions (Johnston *et al.*, 2008). Upstream of OL-dddD was *dddT*(Figure S\*\*\*), a betaine, choline, carnitine transporter (BCCT) family protein that likely functions in substrate import, indicating the OL-dddD forms an operon structure like that of *Halomonas* sp. HTNK1(Todd *et al*. 2010).

Two *dddL* groups were detected in Organic Lake: SUL-dddL and MAR-dddL (Figure S9). The former includes the *Sulfitobacter* sp. EE-36 *dddL* and the latter the *Marinobacter manganoxydans* MnI7-9 homolog indicating they originate from *Roseobacter*-clade and *Gammaproteobacteria*, respectively. *Sulfitobacter* sp. EE-36 has demonstrated DMSP lyase activity and the *dddL* gene alone is sufficient for DMS generation (Curson *et al.*, 2008). These data indicate that the Organic Lake members of the SUL-dddL group perform the same functional role. The MAR-dddL clade appears to be a new branch of the *dddL* family. *dddP* was detected as the least abundant of the DMSP lyases (Table 3). Phylogenetic analyses showed Organic Lake *dddP* likely originate from *Roseovarius* (Figure S10). The Organic Lake sequences formed a clade with the functionally verified *Roseovarius nibinhibens* ISM *dddP* (Todd *et al.*, 2009).

A single type of DMSP demethylase, *dmdA* was identified. It clustered with *Roseobacter*-clade *dmdA* (Figure S11), corresponding to the marine clade A (Howard *et al.*, 2006), and includes the functionally verified *R. pomeroyi* DSS-3 homolog. These data indicate that the Organic Lake sequences correspond to true DMSP demethylases and not related glycine cleavage T proteins or aminomethyltransferases (Howard *et al.*, 2006).

DMSP cleavage appears to be a significant source of DMS in Organic Lake. DMSP likely originates from *Bacillariophyta* or *Dinoflagellida* as Organic Lake *Dunaliella* have been reported not to produce DMSP in culture (Franzmann *et al.*, 1987b). Based on the abundance of marker genes, DMSP cleavage is predicted to occur at highest levels in the deep zone (Figure 4C) where the DMS concentration has been measured to be highest (Deprez *et al*., 1986; Franzmann *et al.*, 1987; Gibson *et al.*, 1991; Roberts & Burton 1993a; Roberts *et al.*, 1993b). DMS can also be produced in anoxic environments from the reduction of DMSO, degradation of sulfur containing amino acids, and sulfide methylation (Schäfer *et al.*, 2010). Our data indicates that reduction of DMSO was not a major pathway (Figure 4C; \*figure S), and the potential for the other DMS yielding processes could not be determined because the pathways have not been established. When cultivated, *Halomonas* isolates from Organic Lake produced DMS from cysteine (Franzmann *et al.*, 1987b) providing some evidence that DMS production from anaerobic degradation of amino acids can occur.

The potential for DMSP cleavage to occur is more than twice that of DMSP demethylation (Figure 4C, Table 3). This ratio differs from estimates from the marine environment that place demethylation potential as up to two orders of magnitude higher than cleavage (Howard *et al.*, 2008; Todd *et al.*, 2009; Todd *et al.*, 2011b; Reisch *et al.*, 2011), and also applies to Ace Lake and the Southern Ocean (Table 3). The frequency of DMSP lyase genes in Organic Lake far exceeded those of the GOS expedition or from nearby Ace Lake (Table 3).

It has been proposed that the cleavage pathway may be underrepresented in the ocean environment because 1) ecologically relevant Ddd enzymes may not have been discovered 2) larger or particle-attached bacteria have not been sampled or 3) that DMSP cleavage is not performed principally by bacteria (Moran *et al.,* 2012). Organic Lake is a marine-derived system where known DMSP lyase genes are highly abundant and linked to close relatives of marine bacteria. These genes were more abundant on the larger size fraction (Table 3) indicating some of the DMSP lysis potential may be under-sampled in the marine metagenomes due to bias against large or particle attached communities. However, the DMSP lyase genes were still clearly abundant in the 0.1 µm fraction in Organic Lake suggesting the dominance of DMSP lysis potential in Organic Lake is related to the high density of DMSP degrading bacteria. There is evidence that the prevalence of DMSP cleavage may be the rule in hypersaline systems, as a higher proportion of *ddd* genes were detected in the Punta Cormorant hypersaline lagoon (Todd *et al.*, 2009) and saltern ponds (Raina *et al*., 2010) than *dmdA*. This suggests organisms from hypersaline systems may favor the relatively wasteful lysis pathway, where both sulfur and carbon is lost to the organism performing the DMSP lysis, over the more ‘thrifty’ demethylation pathway; particularly in the *Roseobacter* lineages that can also perform either process. One possibility that has been proposed is that when sulfur is in excess and the organism can easily assimilate alternative sulfur sources, the lysis pathway may be competitive (Johnston *et al.,* 2008). This is in contrast to SAR11 for which a dependence on assimilation of reduced sulfur would favour demethylation.

## Conclusion

Through the use of shotgun metagenomics and size partioning of samples, we discovered that the lake system is dominated by remineralization and not net C and N fixation. The microbial community is characterized by organic material degradation, nutrient uptake and heterotrophy that occurs greatly in excess of fixation. However, the most active layer in the lake exists below the pycnocline/oxycline where cell and VLP numbers and nutrients are highest, turbidity is lowest due to microbial degradation of particulate matter, and processes occur, such as CO oxidation that may lead to carbon conservation. While the upper mixed zone is characterized by *Dunaliella* and chlorophyte algae performing primary production and polymeric algal material is remineralized by *Psychroflexus*, the deep zone is characterized by facultative anaerobic autotrophy and CO oxidationby *Marinobacter* and *Roseovarius*. In the deep zone, particularly the active layer, *Marinobacter* and *Roseovarius* play key roles in DMS formation by synthesizing DMSP lyases to catalyse DMSP cleavage of the DMSP generated by upper mixed zone phototrophic algae. The low potential for dissimilatory sulfur cycling (both S oxidation and DSR) and relatively stable waters of the deep zone, combined with the generation of DMS from DMSP, facilitate the accumulation of a high level of DMS in the lake.

In addition to being able to answer targeted questions about the biology of the unusual lake sulfur chemistry, the shotgun metagenomics approach provided insight into possible functional capacities and ecological importance of poorly understood classes of bacteria (*e.g.* RF3, *Ca.* “Aquiluna” *Actinobacteria*, OD1 and TM7), and the potential importance of poorly understood microbial processes occurring in the lake performed by a broad range of types of lake bacteria (*e.g.* photoheterotrophy by *Alphaproteobacteria*).

In view of the organic richness, including high levels of DMS in Organic Lake, we did not anticipate the extent to which the lake microbial community is orientated towards a net negative C and N balance. In contemplating this we examined what input the lake may have received throughout its relatively brief ~3 000 year history. The volume of the lake is relatively small (\*\*\*\*\* ). It is possible that the C and N balance is sporadically readdressed by exogenous input from guano deposited in a small penguin rookery nearby the lake, through Giant Petrel or Skua grazing and defecation, and/or by decaying animal carcasses such as elephant seals which can weigh on the order of 1 ton and therefore contribute substantial organic material. It is also possible that during isolation from the ocean, the base of the water column in the marine basin that formed the lake may have acted as a sump for organic material. Phytoplankton blooms and benthic mats tend to make coastal marine basins very productive, and organic matter that sediments out of the surface waters will become trapped in the denser bottom layers (REF). Retention of captured organic matter in the lake may also have been facilitated by Organic Lake having become highly saline quickly (REF). Studies in the future experimentally determining exogenous input and historical lake dynamics (*e.g.* stable isotope and biomarker analyses of lake sediment), and metaproteogenomic analyses of interannual community composition and function, will provide improved knowledge of the unusual biogeochemistry of Organic Lake and better enable predictions to be made about how the lake may be affected by ecosystem changes.

## Acknowledgements

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