Heterotrophic resourcefulness and unusual sulfur biogeochemistry in ahypersaline Antarctic lake

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# Abstract

Organic Lake is a shallow marine-derived hypersaline lake in the Vestfold Hills, Antarctica that has potentially the highest concentration of dimethylsulfide (DMS) recorded 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) was performed on size fractionated samples (3.0, 0.8 and 0.1 µm) collected along a depth profile. Eucaryotic 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 associated with fermentation. The dominance of heterotrophic degradation coupled with low fixation potential indicates possible net carbon loss. However, abundant marker genes for aerobic anoxygenic phototrophy, CO oxidation, rhodopsins and facultative chemoautotrophy were also linked to the dominant heterotrophic bacteria and may be indicative of mechanisms for conserving 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 demethylase (*dmdA*)were less abundant than DMSP lyases indicating the DMSP cleavage is the likely source of the high DMS concentration. Strategies of nutrient resourcefulness such as DMSP cleavage and carbon mixotrophy indominant Organic Lakebacteriaare potentiallyimportant adaptations to nutrient constraints. This study sheds light on the factors that may lead to dominance of these pathways in other environments.

# 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). By providing strong physicochemical stratification within a single, largely closed system, these meromictic lakes provide 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 microorganismsin 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 within Antarctica and the surrounding Southern Ocean (reviewed in Wilkins *et al.,* 2012). In the Vestfold Hills, metagenomics and metaproteomics have been used to study Ace Lake (68.4731 S, 78.1891 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.

Organic Lake is shallow (6.8 m) and has variable surface water temperatures (−14 to +15 °C) while remaining subzero throughout most of its depth (Franzmann *et al.,* 1987; 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 nutrients turnover slowly 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.,* 1987; 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 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 regulatory effect on global cloud cover as it forms as 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. Eucaryotic 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 examined the entire microbial community of the lake by analyzing metagenome data from biomass sequentially fractionated 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. By so doing, our study sought to determine the composition and the functional potential of Organic Lake microbiota and, in conjunction with historic and contemporary physico-chemical data, generate an integrative understanding of the whole lake ecosystem.

# Materials and Methods

## Sample collection and preparation

Waterwas collected from Organic Lake on 10 November 2008 (68º27'22.15"S, 78º11'23.95"E) through a 30 cm hole in the 0.8 m thick ice cover abovethe deepest point in the lake. Samples were collected at 1.7, 4.2, 5.7, 6.5 and 6.7 m depths. Lake water was passed through a 20 µm pore size pre-filter,and then microbial biomass was captured by sequential filtration onto 3.0 µm, 0.8 µm and 0.1 µm pore size membrane filters. Between 1–2 L of lake water was sufficient to clog the filters.DNA was extracted from the filters as previously described (Ng *et al.*, 2010; Lauro *et al.*,2011). DNA from all samples was sequenced using the Roche GS-FLX titanium sequencer. Reads were processed to remove low quality bases, assembled and annotated as previously described (Lauro *et al.*, 2011). Water was also collected for microscopic and chemical analysis at the same sample depths and frozen −80ºC.

## Bathymetry and water level measurements

## 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 minimum-maximum mercury thermometer. 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 according to the relation described by Gibson (1999). The adjusted conductivity brings the temperature within the acceptable range to estimate practical salinity by the formula of Fofonoff and Millard (1983). However, salinity was likely underestimated as it is higher than the range (2–42) for which the conductivity–salinity relation holds. 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 Methodsat the Analytical Services, Tasmania.Values for dissolved nutrients were measured after filtration through 0.1 µm pore size membrane. All other nutrients were measured from water collected after filtration through 20 µm pore size membrane. 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.

## Biological diversity analyses

### Cellular diversity

Diversity of cellular life was assessed using ribosomal small subunit (SSU) gene sequences. Metagenomic reads that matched the 16S and 18S rRNA genewere retrieved using Metaxa (Bengtsson*et al.*, 2011). Only sequences longer than 200bp 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 classifiedto the genus levelusing QIIME implementing the 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 SSUcounts 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 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 considering following 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 biclustering dendogram was generated using R and the package ‘seriation’ (Hahsler *et al*., 2008) on the normalized square-root transformed SSU counts.

## Analysis of Functional potential

Open reading frames (ORFs) were predicted from trimmed metagenomic reads using MetaGene (Noguchi *et al*., 2006) accepting those >90 bp.ORFs were translated using the standard bacterial/plastid translation table and comparedto 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).KEGG GENES is a collection of genes from all complete genomes from public resources. 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) groupswith e-value <1e−05 and rank >5.Matches to KO that are functional markersfor carbon, nitrogen and sulfur conversions(Table S2) were normalized to 100 000 reads per sample and counted. Normalized frequencies of markers from the same pathway were averaged and those from different pathways were summed. Marker enzymes wereassigned to taxonomic groupsbased on the species of origin of the best KEGG GENES BLASTp match.

Marker genes not represented in KO were retrieved by alternative strategies. Organic Lake rhodopsin homologs were retrieved 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. The DMSP lyases and demethylases with experimentally confirmed function were retrieved from National Center for Biotechnology Information (NCBI) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) sequence databases (Table S3). These sequences were used to query a BLAST database of translated ORFs predicted from Organic Lake metagenomic reads. Matches were accepted if e-value was <1e−10 and sequence identity was within the range shared by the query enzymes of the same family. The same process was used to determine the frequency of homologs of these genes in Ace Lake and the Global Ocean Sampling (GOS) expedition metagenomes. Counts of *dddD*, *dddL*, *dddP* and *dmdA* was comparable to that obtained by Howard *et al.* (2008) (\*Table S\*\*).

## Comparing functional gene frequencies between metagenomic samples

Frequencies of genes of interest were normalized to 100 Mb. This was to allow comparison between Antarctic Lake and GOS metagenomes, which were sequenced using different sequencing technologies. The number of total base pairs from each GOS sample was estimated by multiplying the number of reads from each sample by the average read length (822 bp) (Rusch *et al.*, 2007). Frequencies of the single copy gene *recA* was retrieved as described for other genes of interest (above) using the *E. coli* K12 (Table S3) homolog as the query. An e-value cut-off of <1e-20 was used as established by Howard *et al.* (2008). The percentage of genomes containing a gene of interest in a sample was estimated by dividing the normalized count of each gene of interest by the *recA* count .

## Phylogenetic analyses

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 NR were 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: −a2.9, gap extension penalty: 0). Neighbor-joining was used to compute the phylogenies with Poisson substitution model, uniform rates of change and complete deletion of alignment gaps. Node support was tested with bootstrap analysis (500 replicates).

# Results and Discussion

## Abiotic properties and water column structure

At the time of sampling, Organic Lake had a maximum depth of 6.8 m and the surface measured 3.87 m above mean sea level.*In situ* physico-chemical profiles (Figure S2) were measured over the deepest point in the lake (Figure S3) to evaluate the water column properties and structure. Two distinct zones were apparent: an upper mixed zone above 5.7 m and a suboxic deep zone below 5.7 m (Figure1A). The separation of these two zones was indicated by a pycnocline starting at 5.7 m. The presence of an oxycline at the same depth indicates depletion of DO due to respiration in the stagnant waters. The pH also decreased with DO, likely due to fermentation products such as acetic, formic and lactic acids that have been previously recorded in the bottom waters (Franzmann *et al*., 1987b; Gibson *et al*., 1994). The deep zone was not completely anoxic, consistent with previous records (Franzmann *et al.*, 1987b; Gibson *et al.*, 1991). 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 (Table1) 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 do 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 short chain fatty acids (SCFA) 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.

The C:N and C:P ratios 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 this depth was related to breakdown of particulate matter and sulfur chemistry.

## Overall microbial diversity

SSU genes (3 959reads) that were retrieved from the metagenome data (Table S1) 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) indicatingArchaea 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 on all filter sizes at all depths (Figure 2A) and each consisted of one dominant genus, *Marinobacter*, *Roseovarius* and *Psychroflexus*, respectively (Figure 2C). Cyanobacterial sequences were all 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.Moderately abundant bacterial classes were*Actinobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and candidate divisions OD1 and RF3. Lower abundance divisions included *Bacilli*, *Clostridia*, *Spirochaetes*, *Lentisphaera*, TM7, *Opitutae*, *Verrucomicrobia*, Bhi80-139, Bd1-5, SR1 and *Chlamydiae* (Figure 2A). The dominant Eucarya were 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 Eucarya included *Bacillariophyta* (diatoms), *Dinophyceae*,*Fungi* and heterotrophic *Choanoflagellida* and *Ciliophora* (see Table S4 for lower taxonomic rank assignments).

## Variation of microbial compositionaccording 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 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 taken with the physical and chemical data, provided insight into the functional roles of those taxa.

### 20–3.0 µm fraction

The upper mixed zone samples had a relatively high 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 been previously reported to be the dominant alga (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* was overrepresented in the surface and 6.7 m samples. Consistent with enrichment on the 3.0 µm filters, *Psychroflexus gondwanensis* (previously *Flavobacterium*) has been isolated from Organic Lake (Franzmann *et al*., 1987b) and cells are 1.5–11.5 µm in length (Dobson *et al*., 1991).*Flavobacteria* have been associated with phytoplankton blooms in the Southern Ocean (Abell & Bowman 2005a; Abell & Bowman 2005b; Williams *et al.,* 2012\*\*), which is hypothesized to be related to their ability to degrade high molecular weight carbon from algal exudates and detritus (reviewed in Kirchman *et al.*, 2009). It is likely that Organic Lake *Psychroflexus* fills a similar ecological role. In support of this, *Psychroflexus* clusters with *Dunaliella* chloroplasts in the seriation analysis (Figure 3) and *P. gondwanese* abundance in Organic Lake has been correlated with average hours of sunshine per day indicating their population dynamics is related to summer algal blooms (James *et al.*, 1994). Its presence in the deep zone is most likely due to sedimentation as *P. gondwanense* is non-motile and strictly aerobic (Dobson *et al.*, 1991).

*Roseovarius* was enriched at 4.2 mand 6.5 m suggesting different ecotypes may be present in the mixed zonecompared to the deepzone.*Roseovariustolerans*, an isolate from Ekho Lakein Antarcticahas a cell size (1.1–2.2 μm; Labrenz *et al*., 1999) consistent with *Roseovarius* capture on the 3 µm filter.One strain from Ekho Lake is capable of microaerophilic growth (Labrenz *et al.*, 1999). Overrepresentation at 6.5 mmay therefore be indicative of growth at that depth rather than sedimentation to the bottom of the lake.*Roseovarius*is a member of the *Roseobacter* cladewhose diverse metabolic capabilities include DMSP degradation, aerobic anoxygenic phototrophy(AAnP)and CO oxidation (reviewed in Wagner-Döbler & Biebl, 2006). All of these capabilitiesshould facilitate growth in both the mixed and deep zones ofOrganic Lake (see **Carbon resourcefulness in dominant heterotrophic bacteria**below).

### 3–0.8 µm size fraction

On the 0.8 µm filter, *Marinobacter* dominated at all depths except 6.5 m. Their capture on this size fraction is consistent with the cell size of isolates (Gauthier *et al.*, 1992). The genus is extraordinarily metabolically versatile and described as an “opportunitroph” (Singer *et al.*, 2011). Some isolates are capable of interacting with diatoms (Gärdes *et al*., 2010) and dinoflagellates (Green *et al.*, 2006). Others are metal-oxidizing autotrophs (Edwards *et al.*, 2003; Wang *et al.*, 2011)or capable of unusual redox cycling (Handley *et al*., 2009). *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 which of these capabilities were related to its dominance in Organic Lake (see **Carbon resourcefulness in dominant heterotrophic bacteria**below).

RF3 and *Halomonas* were overrepresented at 6.5 m, and RF3 was 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 *Halomonas 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 found 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

A large number of eucaryoticsequenceswere evident in the 0.1 µm size fraction. The upper zone was overrepresented by sequences 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), with subsequent light microscopy analyses observing 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 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 cystsor degraded cellular material.

“*Candidatus* Aquiluna”, in the Luna-1 cluster of *Actinobacteria* (Hahn *et al.*, 2004; Hahn *et al.*, 2009) was 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 probably perform similar functions in Organic Lake surface waters. 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 candidate divisions OD1 and TM7.OD1 was more abundant, and its prevalence on this size fraction is consistent with similar findings for size fractionation ofground water (Miyoshi *et al.*, 2005).OD1 is consistently associated with anoxic environments (Harris *et al*., 2004). Genomic analyses identified OD1 in the anoxic zone of Ace Lake (Lauro *et al.,* 2011), and OD1 from Zodletone Spring, Oklahoma was reported to possess oxygen sensitive enzymes related to those from anaerobic bacteria (Elshahed *et al.*,2005). In the marine environmentOD1 has been associated with reduced conditions with high sulfur (Harris *et al*., 2004; Elshahed *et al.*,2005). The distribution of OD1 in Organic Lake is consistent with an anaerobic metabolism and potential involvement in sulfur chemistry.

(\*TM7).

## Organic Lake functional potential

To determine the functional processes occuring in Organic Lake, genemarkers for C, N and S conversions (Figure 4), and marker genes involved in photoheterotrophy 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,and functions to allow 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. The majority of phosphoribulose kinase (PRK) genes were from *Gammaproteobacteria*, predominantly *Marinobacter* (Table 2, Figure S6A). RuBisCO was only associated with a small proportion of *Gammaproteobacteria* (Figure S6A), principally from sulfur-oxidizing *Thiomicrospira*, indicating some *Gammaproteobacteria* are *bona fide* autotrophs. However, all genomes reported for *Marinobacter* have PRK but lack RuBisCO, and only one of these *Marinobacter* is known to oxidize manganese and is thus possibly capable of autotrophy (Wang *et al.*, 2011) andiron-oxidizing autotrophic members of the genus have been isolated (Edwards *et al.*, 2003). It is therefore possible that PRK is functioning as part of the CBB cycle in association with an unknown functional analogue of RuBisCO, or serving another phosphotransferase function that is not linked to carbon fixation. The majority of respiration potential in Organic Lake was linked to *Proteobacteria* (Table 2), specifically to *Marinobacter*, indicating any autotrophic potential would likely be facultative. (\*possibleelectron dump?)

Anaerobic C fixation was represented by some potential for the Wood-Ljungdahl (WL) pathway, but mostly by the reverse tricarboxylic acid (rTCA) cycle (Figure S6A). WL-mediated carbon fixation was linked to *Deltaproteobacteria* that are known to grow autotrophically using this pathway (Hügler & Sievert, 2011). ATP citrate lyase, which is the most definitive marker for rTCA, was linked to sulfur-oxidizing chemolithoautotrophic *Epsilonproteobacteria* (Figure S6A, Table S4) indicating some anaerobic C fixation does proceed by this pathway. However, the majority of rTCA cycle potential was assigned to *Clostridia* (Figure S6A) including the genera *Ammonifex*, *Chitinophaga*, *Halothermothrix* and *Thermoanaerobacter* due to the presence of 2-oxogluterate:ferreoxidin oxidase genes linked to these organisms*.* Some of these genera are known to fix carbon anaerobically by an unknown mechanism that involves the rTCA cycle (Hügler & Sievert, 2011).

In the deep zone, potential for fermentation and CO oxidation was greatest at 6.5 m (Figure 4A) and likely the main biological activity that was occurring at that depth. Fermentation marker genes were linked to *Mollicutes* (Table 2), but as no *Mollicutes* were detected, they most likely 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** above). This would be the first data indicating 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 as typically the end of the anaerobic food chain involves methanogens or sulfate-reducing bacteria; the former were absent and the latter were present in low abundance (Figure 2A, 2C).CO oxidation is a lithoheterotrophic process involving the oxidation of CO to generate energy and metabolism of organic carbon for growth (Moran & Miller, 2007), although CO oxidation may also be involved in anaplerotic C fixation (Moran *et al*., 2007). CO oxidation genes matched to *Alphaproteobacteria* (Table 2), predominantly the *Roseovarius* genus (Figure 2C).The CO oxidation capacity at 6.5 m is therefore associated with the deep-zone ecotype of Organic Lake *Roseovarius*. CO oxidation may allow SCFA to be directly assimilated rather than oxidized, and for some CO2 to be fixed in the deep zone (Figure 4A) thereby addressing the overall carbon shortfall in the lake.

Photoheterotrophy generates energy from light (organic carbon is still required for growth) by two bacterial processes: aerobic anoxygenic phototrophy (AAnP) mediated by bacteriochlorophyll A (BchlA) and associated photosynthesis reaction centers, or rhodopsin mediated phototrophy (Moran& Miller, 2007). AAnP genes are abundant in the ocean and related to diverse *Proteobacteria* (Béjà *et al.*, 2002), and proteorhodopsins (PR) are widely distributed in the surface ocean (Rusch *et al*., 2007) in diverse bacterial clades (de la Torre *et al.*, 2003;Venter *et al.*, 2004). The ecological function of rhodopsins may be diverse and are hypothesized to include light or depth sensing (Fuhrman *et al.*, 2008). However, PRs of marine *Flavobacteria* and *Vibrio* have been linked to light-dependent energy generation, particularly during C limitation (Gómez-Consarnau *et al*., 2007; Gómez-Consarnau *et al*., 2010).

Both AAnP and rhodopsin genes were abundant in Organic Lake (Figure 4A). AAnP was linked to *Roseobacter* clade *Alphaproteobacteria* (Table 2), consistent with the known metabolic potential of *R. tolerans* from Ekho Lake that produces BchlA (Labrenz *et al.*, 1999). Organic Lake rhodopsins 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 corresponding to position 105 in the SAR86 PR denoting tuning to surface green light (Man *et al.*, 2003; Gomez-Consarnau *et al.*, 2007), which is 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 “*Candidatus* Aquiluna” (Figure S7, Table S4). In the SAL-R group, Xanthorhodopsin originates from the sphingomonad *Salinibacterruber* (Balashov *et al.*, 2005). It is therefore likely that*Sphingobacteria*(Table S4) are the likely origin of the SAL-R 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 member of the*Roseobacter*clade (\*link to scaffoldsto establish taxonomic origin definitively).

(Table comparing frequencies of rhodopsin, AAnP, DMSP lyases and Dmd genes to other marine environments).

The abundance of photoheterotrophic potential in abundantOrganic Lake bacteria suggests an important role for light-driven energy generation. The contribution of photoheterotrophic processes to the carbon budget is difficult to infer from genetic potential alone as little is known about how expression of these genes is regulated. In this context it was noted that the abundance of AAnP and PR containing bacteria was the same between winter and summer in the Artic (\*Cottrell *et al.*, 2009), and in *R. tolerans*, BchlA was expressed when grown in the dark but inhibited by continuous dim light (Labrenz *et al.*, 1999).It is possible that in Organic Lake the apparent negative balance in carbon conversion potentialcould be augmented by photoheterotrophy. In particular, the Organic Lake *Psychroflexus* could play a particular role as it has a PR related to *Dokdonia* that was shown to function under C-limitation (Gómez-Consarnau *et al*., 2007.

## Predominance of regeneration in the nitrogen cycling

N cyclingpotential throughout the lake profile was dominated by assimilation and mineralization/uptake pathways (Figure 4B) linked to *Proteobacteria* (Table 2, Figure S6). Assimilatory nitrite reductase was not abundant but genes involved in reduced N uptake were (Figure S6B). The presence of glutamate dehydrogenase genes indicated the potential for mineralization to ammonia,and also, by functioning in reverse,amechanism for ammonium uptake (\*ref). The high ammonia concentration in the deep zone would result from a higher rate of mineralization than assimilation (Table 2,Figure S6B). In addition, a capacity for dissimilatory nitrate reduction to ammonia (DNRA) waslinked to*Sphingobacteria* and other anaerobic bacteria (Table 2,Figure S6B),and Stickland fermentation (\*figure) to *Clostridia*.

Potential for nitrogen conversions typically found in other aquatic environments was greatly reduced in Organic Lake. There as 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). Potential for aerobic ammonia oxidation was not detected, nor were ammonia-oxidizing bacteria or archaea. Organic Lake microorganisms therefore have a very low capacity to perform nitrification. Anaerobic ammonia oxidation (anammox) potential, indicated by hydroxylamine/hydrazine oxidase-like proteins (HAO/HZO), was also very lowand the small number of genes were linked to sulfate-reducing *Deltaproteobacteria* (Table 2, Figure S6). All known anammox organisms are from the order *Brocardiales* (Niftrick & Jetten, 2012),which were not present in the lake. HAO/HZO genes have been noted in non-ammonia oxidizing bacteria and proposed to be related to NrfA heme cytochrome C nitrite reductase that functions in DNRA (Bergmann *et al*., 2005). 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 S6). Low nitrate and nitrite in the deep zone (Figure 1B, Table1) indicates depletion by dissimilatory reduction has contributed to the establishment of N-limitation in the lake. 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, denitrificationmay be inhibited even if conditions appear appropriate. For example, inLake Bonney, Antarcticadenitrification 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-formoxidized N. The preponderance of assimilation/mineralization pathways geared towards reduced N appears to reflect a“short circuit” of the typical N cyclethat would conserve N in alargely closed system. Hence, the predominant N source is regenerated fixed N. Similar findings were also madefor 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).

In OrganicLake it is possible that the nitrogen 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 contribute substantial nitrogen. 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 haveacted as a sump for organic material. Marine basins tend to be very productive, with phytoplankton blooms and benthic mat production, and organic matter is 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).(\*DMSP can inhibit N2O reductase Magalhaes *et al.*, 2012)

## 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). These genes were most abundant in the upper mixed zone indicating that sulfur oxidation was restricted to where terminal electron acceptors, most likely oxygen, were available. Although sulfur-oxidizing *Epsilonproteobacteria* (Figure 2A, 2C) were present in the deep zone, no potential for sulfur oxidation was linked to them (Figure S6C) and neither was polysulfide reductase (PSR) present; both genes are known to be possessed by deep-sea sulfur-oxidizing *Epsilonproteobacteria* (Yamamoto & Takai, 2011). appreciable 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 possibile that the high salinity, transient oxygenation or positive electropotential inhibit microorganisms from performing DSR, and hence colonizing the deep zone of the lake. 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 *Dinoroseobacteria shibae* DFL 12 and *Ruegeria pomeroyi* DSS-3 (Todd *et al.*, 2011) or carnitine coenzyme A transferase outgroups, thereby providing support for their proposed role as functional DMSP lyases. MAR-dddD grouped with a *Marinobacter* sp. ELB17 homolog indicating it derives from *Marinobacter* (Figure S8). OL-dddD did not have close relative from cultured bacteria making its taxonomic origins uncertain. The abundanceof OL-dddD on the 3.0 µm fraction suggests it originates from*Alphaproteobacteria*, *Bacteroidetes* or *Dunaliella*(\*link ddd gene to scaffoldsto determine taxonomic orgin).

Two *dddL* groups were detected in Organic Lake: SUL-dddL and MAR-dddL (Figure S9). The former clusters with *Sulfitobacter* sp. EE-36 and the latter with *Marinobacter manganoxydans* MnI7-9 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 functional 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 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) and marine *Dunaliella tertiolecta* is known to cleave DMSP extracellularly (\*Seymour *et al.*, 2010). 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). The frequencies DMSP lyase genes far exceeded those of the GOS or from nearby Ace Lake with (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 sampledor 3) that DMSP cleavage is not performed principally by bacteria(Moran *et al.,*2012). Prevalence of cleavage over demethylation may bethe rule in non-marine saline systemseg. Punta Cormorant (Todd *et al.*, 2009). (\*check for bias to 0.1 fraction). This provides come insight into conditions may favor different fates of DMSP. It appears hypersaline coastal environments may favor a “messy-eater” strategy of sulfur compound utilization as sulfur is in excess. (\*ref).

## Conclusion

## Acknowledgements

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