Heterotrophic resourcefulness and unusual sulfur biogeochemistry in a hypersaline Antarctic lake

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# Potential Aims and Scope

Microbial population and community ecology, Integrated genomics and post-genomics approaches in microbial ecology, **Microbial ecology and functional diversity of natural habitats**. ORIGINAL ARTICLE 5 000 word limit. TITLE: 50 characters.

# Abstract (250 word limit)

Organic Lake is a 6.75 m deep marine-derived hypersaline lake in the Vestfold Hills, East Antarctica that has potentially the highest concentration of the cloud-forming gas dimethylsulfide (DMS) recorded in a natural body of water (Franzmann *et al.*, 1987b). To determine the microbial community and functional processes in Organic Lake, and particularly the basis of the unusual sulfur chemistry, DNA from size fractionated samples (3.0, 0.8 and 0.1 µm) was sequenced along a depth profile. Eucaryotic phytoflagellates *Dunaliella* and *Pseudopedinella* were the main photosynthetic organisms.Bacterioplankton was dominated by heterotrophic *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 serve as a mechanism to conserve carbon. Similarly, there was high genetic potential for regenerated N conversions that likely functions to retain fixed nitrogen. Dimethylsulfoniopropionate (DMSP) lyases (DddD, DddL and DddP) were abundant indicating DMSP is a significant carbon and energy source and DMSP cleavage is the source of the high DMS concentration. DMSP demethylase genes (dmdA) were also present, but unlike marine environments, were less abundant than DMSP lyases. Strategies of nutrient resourcefulness such as DMSP cleavage, lithoheterotrophy, chemolithoautotrophy and photoheterotrophy in globally distributed heterotrophic lineages are potentially important adaptations to nutrient constraints in Organic Lake.

# Introduction

Life in the Antarctic is constrained by extremes of temperature and salinity under a polar light cycle. In the frozen desert, ice-free regions containing liquid water in lakes and ponds are rare oases for life. The Vestfold Hills, on the eastern shore of the Prydz Bay, East Antarctica (Figure S1), is one such region where hundreds of lakes are found. The lakes were formed from seawater, trapped approximately 10 000 BP when the continental ice-sheet receded and the land rose above sea-level (Zwartz *et al*., 1998; Gibson, 1999). Life in these lakes can be entirely microbial and of reduced diversity (Bowman *et al.*, 2000b). Differing local conditions has lead each lake to develop unique physical and chemical properties, making them fitting sites to study microbial ecology, biogeochemistry and evolution. The Vestfold Hills contains the highest density of meromictic (permanently stratified) water bodies in Antarctica (Gibson, 1999). They are advantageous study sites as environmental gradients exist within a single, largely closed system allowing species to be related to abiotic factors. By using molecular techniques, a large proportion of the species diversity and gene content can be covered allowing inference of the functional roles of the taxa present (Laybourn-Parry & Pearce, 2007).

A metagenomic approach, complemented with metaproteomics, has been successfully applied to two lakes in the Vestfold Hills (Ng *et al.*, 2010; Lauro *et al.*,2011; Yau *et al.*, 2011). The first of these was Ace Lake, where a comprehensive description of the community structure, biogeochemical fluxes and responses to resource limitation was achieved (Lauro *et al.*, 2011). The metabolism of the 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). In the surface water of the second lake, Organic Lake, a member of the virophage virus family was discovered that potentially regulates microbial loop dynamics (Yau *et al*., 2011). Virophage require a helper virus to replicate but are detrimental to their helper (La Scola *et al.*, 2008). The Organic Lake virophage (OLV) likely depends on phycodnaviruses (algal viruses). The presence of OLV would reduce infective phycodnaviruses leading to increased frequency of algal blooms and thus carbon flux (Yau *et al.*, 2011). These studies have achieved exceptional insight into Antarctic lakes but are also relevant to other aquatic systems. For example, OLV-like sequences were found in coastal marine, hypersaline and freshwater metagenomes indicating virophages have a wider ecological role (Yau *et al.*, 2011).

This study extends the previous metagenomic analysis of the surface water viriome of Organic Lake (Yau *et al.*, 2011)to examine the entire microbial community along a depth profile. 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). Aerosols derived from atmospheric DMS act as cloud condensation nuclei and are hypothesized to affect climate (Charlson *et al.*, 1987). Although the importance of DMS was proposed forty years ago (Lovelock & Maggs, 1972) the first enzymes involved in DMS production were only identified in the last five years (Todd *et al.*, 2007). Concentrations of DMS as high as 5000 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). This makes it an ideal location to identify the microbes involved in DMS accumulation. This 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 and an integrative understanding of the whole lake ecosystem.

# Materials and Methods

## Sample collection and preparation

Water was 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 above the 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.

## 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 Methods at 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 gene 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 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 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 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). 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) groups with e-value <1e−05 and rank >5. Matches to KO that are functional markers for 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 were assigned to taxonomic groups based 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.

## 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

During sampling on November 2008, Organic Lake had a maximum depth of 6.75 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: a mixed zone above 5.7 m and a suboxic deep zone below (Figure 1A). 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 as has been recorded in the past (Franzmann *et al.*, 1987b; Gibson *et al.*, 1991) indicating DO had entered the bottom waters in the last 13 years. Oxygen may be episodically introduced with currents of cold dense water that are generated during ice-formation in the lake shallows (Ferris *et al*., 1999). Samples were collected from the mixed (1.7, 4.2 and 5.7 m) and deep (6.5 m and 6.7 m) zones to examine their nutrient content and microbiology.

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 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 at 6.5 m suggest increased degradation of particulate matter by the microbial community via processes enriched in the microaerophilic environment. This is supported by the high concentrations of dissolved short chain fatty acids (SCFA) and free amino acids found in the deep zone (Gibson *et al.*, 1994) indicative of 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 increased activity at this depth was related to breakdown of particulate matter and sulfur chemistry.

## Overall microbial diversity

Metagenomic sequences were obtained from size fractionated (3.0 µm, 0.8 µm and 0.1 µm) microbial biomass from each sample depth (Table S1). To determine the microbial composition, a total of 3 959 reads matching to the SSU gene were retrieved from the metagenomic sequences. These grouped into 983 OTUs. Bacteria were numerically dominant comprising 76.2% of all SSU sequences. 16.3% of sequences were assigned as Eucarya and 7.5% of SSU sequences could not be classified. Only 2 reads, assigned to a deep sea hydrothermal clade of *Halobacteriales*, were classified as Archaea (Table S4) indicating Archaea were rare in Organic Lake. Microbial diversity was fairly low, with 15 bacterial phyla and 6 eucaryotic superkingdom divisions in total. Of these, only 7 bacterial phyla and 4 eucaryotic phyla were predominant. (\*diversity indices)

Three bacterial classes, *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteria*, were the most abundant and were found on all filter sizes at all depths (Figure 2A). Each of these three classes consisted of one dominant genus, which was *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 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 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 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 were likely the main source of primary production in Organic Lake and have been previously reported to be the dominant eucaryotic alga (Franzman *et al.*, 1987b). Signatures at the bottom of the lake are likely due to sedimentation of dead cells or resting cysts.

*P*. *gondwanensis* (previously *Flavobacterium*) has been isolated from Organic Lake (Franzmann *et al*., 1987b) and ranges in length from 1.5–11.5 µm (Dobson *et al*., 1991) consistent with enrichment on the 3.0 µm filter. *Psychroflexus* was overrepresented in the surface and the 6.7 m sample. *Flavobacteria* have been associated with phytoplankton blooms in the Southern Ocean (Abell & Bowman 2005a; Abell & Bowman 2005b), 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). Likely, 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 (James *et al.*, 1994). Its presence in the deep zone was most likely due to sedimentation as *P. gondwanense* is non-motile and strictly aerobic (Dobson *et al.*, 1991).

*Roseovarius* was enriched at 4.2 m and 6.5 m suggesting mixed zone and deep ecotypes. *R. tolerans*, an isolate from the Antarctic Ekho Lake has a large cell size (1.1–2.2 μm) (Labrenz *et al*., 1999) accounting for accumulation of *Roseovarius* on this size fraction. One strain from Ekho Lake is capable of microaerophilic growth (Labrenz *et al.*, 1999). Overrepresentation at 6.5 m is therefore consistent with growth at that depth rather than sedimentation, which would present as accumulation at the lake bottom. *Roseovarius* is a member of the *Roseobacter* clade whose diverse metabolic capabilities include DMSP degradation, aerobic anoxygenic phototrophy (AAnP) and CO oxidation (reviewed in Wagner-Döbler & Biebl, 2006). All of these capabilities appear related to their success in the Organic Lake system and influence their colonization of both mixed and deep zones (see below).

### 3–0.8 µm size fraction

*Marinobacter* dominated at all depths except 6.5 m. Their abundance 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* (see below) revealed which of these capabilities were related to its dominance in Organic Lake.

RF3 and *Halomonas* were overrepresented on the 6.5 m, of which RF3 was more abundant. Their dominance 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 (Bowman *et al.*, 2000b; Humayoun *et al*., 2003; Schmidtova *et al*., 2009). 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 stict anaerobes. Several *Halomonas* strains have been isolated from Organic Lake including two described species *H. subglaciescola* and *H. meridiana*, both which generally grow as short rods that lead to accumulation on this size fraction (Franzmann *et al.*, 1987a; James *et al.*, 1990). Despite being aerobic, *Halomonas* has been previously found to overrepresented at the oxycline (James *et al.*, 1994) supporting ecological role in the suboxic niche. This may be linked to observed growth stimulation in the presence of free amino acids and organic acids (Franzmann *et al.*, 1987a).

### 0.8–0.1 µm size fraction

There was had a large number of eucaryotic sequences on the 0.1 µm size fraction. Their presence on the smallest filter may due to sampling particular stages in their life history, such as a cysts or spores, or degraded cellular material. The mixed zone was dominated by *Pedinellales* that co-varied with chloroplasts indicating active photosynthetic cells. *Pedinellales* have only been previously detected in Antarctic lakes from molecular studies (Unrein *et al.*, 2005; Lauro *et al.*, 2011; Yau *et al.*, 2011). *Pedinellales* observed by light micrscopy were 5–8 µm (Unrein *et al.*, 2005). Potentially, smaller free-living members of this eucaryotic class exist that have not been identified in microscopy-based surveys to their small size.

“*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.*, 2010) and surface Artic seawater (Kang *et al*., 2012) demonstrating they are relevant to polar saline systems. In Ace Lake surface water they were associated with utilization of labile C and N substrates (Lauro *et al.*, 2010) suggesting a similar ecological function in Organic Lake surface waters. 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 most abundant and its prevalence in this fraction due to small cell size is supported by other studies where it predominated in the <0.2 µm fraction of ground water (Miyoshi *et al.*, 2005). OD1 has been consistently associated with anoxic environments (Harris *et al*., 2004) and genomic fragments from Zodletone Spring, Oklahoma showed oxygen sensitive enzymes related to anaerobic bacteria (Elshahed *et al.*,2005). In the marine environment, it has also been associated with reduced conditions with high sulfur (Harris *et al*., 2004; Elshahed *et al.*,2005). The distribution of OD1 is consistent with an anaerobic metabolism and potential involvement in sulfur chemistry.

(\*TM7).

## Organic Lake functional potential

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

## Carbon resourcefulness in dominant heterotrophic bacteria

Typically methanogens or sulfate-reducing bacteria comprise the end of the anaerobic food-chain and hydrolyse SCFA. However, signatures of methanogenesis (Figure 4A) and methanogens were absent and the abundance sulfate-reducing *Deltaproteobacteria* was low (Figure 2A).

In both the 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 carbon fixation was dominated by aerobic fixation (Figure 4A) via the oxygen-tolerant Calvin-Benson-Basham (CBB) cycle. This was assessed by the presence of ribulose-bisphosphate carboxylase (RuBisCO) and phosphoribulokinase (PRK), molecular markers of the CBB cycle (Hügler & Sievert, 2011). RuBisCO was linked to primarily to *Viridiplantae* (Table 2, Figure S6A) supporting their ecological role as the principle photosynthetic organisms. However, the majority of CBB potential was linked to *Gammaproteobacteria* phosphoribulose kinase (PRK) (Table 2, Figure S6A), predominantly from *Marinobacter*. A small proportion of *Gammaproteobacteria* RuBisCO was detected (Figure S6A), principally from sulfur-oxidizing *Thiomicrospira*, indicating some *Gammaproteobacteria* are *bona fide* autotrophs. All *Marinobacter* genomes to date have PRK but lack RuBisCO. Only one sequenced representative is known to be a chemolithoautotrophic manganese oxidizer (Wang *et al.*, 2011) (\*Are Mn oxidizers necessarily autotrophic?), which raises the question of whether PRK is functioning as part of the CBB cycle. Photolithotrophic C fixation can occur in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* even when RuBisCO genes are deleted if reduced sulfur compounds are present as electron donors (\*Wang *et al.*, 1993) establishing a possibility that CBB-like pathway may occur in the absence of RuBisCO. In accordance with an opportunitroph nutritional strategy, autotrophy in Organic Lake *Marinobacter* would likely be facultative. (\*electron dump?)

The 2-oxogluterate:ferredoxin oxidase gene (K00174)matched to the genera, Maribacter, **Alkaliphilus**, Mahella, Odoribacter, Brachyspira, **Ammonifex**, **Chintinophaga** and Halothermothrix **Thermochitinophaga**. K00175 **Clostridium, Bacillus, Halothemothrix, Thermoanaerobacter**.

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 with this pathway (Hügler & Sievert, 2011). ATP citrate lyase, enzyme that is most diagnostic of rTCA, was linked with sulfur-oxidizing chemolithoautotrophic *Epsilonproteobacteria* (Figure S6A, Table S4) indicating some anaerobic C fixation by this pathway. However, the majority of rTCA cycle potential was from matches to 2-oxogluterate:ferreoxidin oxidase genes that originated from diverse genera but particularly from members of the class *Clostridia* including *Ammonifex*, *Chitinophaga*, *Clostridium*, *Halothermothrix* and *Thermoanaerobacter.* Some of these genera are known to fix carbon anaerobically but the exact mechanism is not understood (\*ref, Hügler & Sievert, 2011). As for *Marinobacter* PRK, these matches could represent true C fixation potential or use of these genes in alternative pathways and indicating overall C fixation potential could be lower than estimated.

In the deep zone, potential for anaerobic C fixation, fermentation and CO oxidation was greatest at 6.5 m (Figure 4A) indicating these processes were involved in the higher biological activity at that depth. As fermentation potential was high, it appears to be the main driver of biological activity in the deep zone through degradation of high molecular weight compounds to SCFA that other organisms can utilize. Fermentation was linked to *Mollicutes* (Table 2) but most likely originated from the related candidate division RF3 (Tajima *et al.*, 1999). This establishes a fermentative metabolism for this candidate division and suggests it has a crucial ecological role in Organic Lake. 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 is occupied by methanogens or sulfate-reducing bacteria; the former were absent and the latter were present at low abundance (Figure 2A, 2C).

CO oxidation is a lithoheterotrophic process in whereby CO is oxidized to generate energy but organic carbon is required for growth (Moran & Miller, 2007), although it may also be linked to anaplerotic C fixation (Moran *et al*., 2007). CO oxidation genes originated from *Alphaproteobacteria* (Table 2) that predominantly comprised *Roseovarius* relatives (Figure 2C). The concentration of CO oxidation genes at 6.5 m was thus associated with the deep-zone ecotype of Organic Lake *Roseovarius*.

CO oxidation genes originated from *Alphaproteobacteria* (Table 2) and were of the *Roseobacter* clade. CO oxidation is a lithoheterotrophic process in roseobacters whereby CO is oxidized to generate energy but organic carbon is required for growth (Moran & Miller, 2007). Organic carbon can be assimilated directed towards biosynthesis rather than respiration (Moran & Miller, 2007). and appears to contribute to metabolic success at this depth. The likely source of organic carbon at that depth would be SCFA indicating.

### photoheterotrophy

Photoheterotrophy is a microbial process where light is used to generate energy but organic carbon is still required to for growth. Two bacterial photoheterotrophic processes are known: 1) aerobic anoxygenic phototrophy (AAnP) mediated by bacteriochlorophyll A (BchlA) and associated photosynthesis reaction centers and 2) rhodopsin mediated phototrophy (Moran & Miller, 2007). Metagenomic analysis has found AAnP genes are abundant in the ocean and related to diverse *Proteobacteria* (Béjà *et al.*, 2002).Similarly, proteorhodopsins (PR), the first bacterial rhodopsin identified,are widely distributed in the surface ocean (Rusch *et al*., 2007) and associated with diverse bacterial clades (de la Torre *et al.*, 2003; Venter *et al.*, 2004). The ecological function of rhodopsins is yet to be fully elucidated as they may be involved in alternative roles such as light sensing (Fuhrman *et al.*, 2008). However, PRs of marine *Flavobacteria* and *Vibrio* have been associated with 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). This is consistent with the known metabolic potential of roseobacter isolates such as *R. tolerans* from Ekho Lake, Antarctica 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 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). Xanthorhodopsin originates from the sphingomonad *Salinibacter* *ruber* (Balashov *et al.*, 2005), thus other *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 *Roseobacter* clade (\*link to scaffolds to show phylogeny).

If there are approximately 3 000 bacterial SSU sequences, assuming rhodopsins to be single copy and SSU copy number to range from one to ten, 13–100% of Organic Lake bacteria have a rhodopsin. This is comparable to the Mediterranean Sea estimates of 13% (Sabehi *et al.*, 2005).(\*use recA, as Sharma *et al.*, 2008 which saw Punta Cormorant has 36% vs open ocean 63%). (\*and AAnP genes).

The abundance of photoheterotrophic potential in dominant Organic 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 these genes are under regulatory control which is largely unknown. For example, there was no difference in abundance of AAnP and PR containing bacteria between winter and summer in the Artic (\*Cottrell *et al.*, 2009). In *R. tolerans* BchlA is only expressed when grown in the dark, but is inhibited by continuous dim light (Labrenz *et al.*, 1999). However, the apparent negative balance in the Organic Lake carbon conversion potential could be moderated by photoheterotrophy. This is most likely to be the case for Organic Lake *Psychroflexus* as it has a PR related to *Dokdonia* that was shown to function under C limitation (Gómez-Consarnau *et al*., 2007.

## Predominance of regenerated nitrogen cycling

N cycling potential 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 indicating a predominance of reduced N uptake (Figure S6B). Potential for mineralization to ammonia, indicated by glutamate dehydrogenase, may function in reverse as an ammonium uptake mechanism (\*ref). The high ammonia concentration in the deep zone would result from a higher rate of mineralization than assimilation, dissimilatory nitrate reduction to ammonia (DNRA), associated mainly with *Sphingobacteria* and other anaerobic bacteria, (Table 2, Figure S6B) in addition to Stickland fermentation (\*figure) by *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 supporting a lack of nitrification potential in Organic Lake. This was also the case in nearby Ace Lake (Lauro *et al*., 2011) suggesting some limiting factor in the lakes of the Vestfold Hills. Similarly, anaerobic ammonia oxidation (anammox) potential, indicated by hydroxylamine/hydrazine oxidase-like proteins (HAO/HZO), was extremely low. All known anammox organisms are from the order *Brocardiales*, (Niftrick & Jetten, 2012)which were not present. Instead, HAO/HZO was linked to sulfate-reducing *Deltaproteobacteria* (Table 2, Figure S6). HAO/HZO genes have been noted in non-ammonia oxidizing bacteria and proposed to be related to NrfA heme cytochrome C nitrite reductase used in DNRA (Bergmann *et al*., 2005). This indicates an inability for nitrification to occur in the mixed zone and likely no potential for ammonia loss in the deep zone.

Denitrification genes and were present throughout the water column (Figure 4B) and was 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 enzymes 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; this is the case in Lake Bonney, Antarctica where denitrification occurs in the west lobe, but not in the east lobe of the same lake despite the anoxia, available nitrate and presence of 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 reflects a “short circuit” of the typical N cycle that would conserve N in a largely closed system, similar to what was proposed to occur in Ace Lake (Lauro *et al*., 2011). Hence, the predominant N source is regenerated fixed N. (\*DMSP can inhibit N2O reductase Magalhaes *et al.*, 2012)

## Molecular basis for unusual sulfur chemistry

Sulfur cycling was dominated by assimilation/mineralization pathways in Organic Lake (Figure 4C). Dissimilatory sulfur cycling potential was extremely limited. In the mixed zone, sulfur oxidation by the Sox multienzyme system, was linked to *Roseobacter-*clade *Alphaproteobacteria* (Table 2) indicating oxygen would be used as the terminal electron acceptor (\*S oxidzing roseobacter info). In the deep zone, dissimilatory sulfate reduction (DSR) potential was low (Figure 4C) as was abundance of sulfate-reducing *Deltaproteobacteria* (Figure 2A, 2C). *Epsilonproteobacteria* known to be sulfur-oxidizers were also present in the deep zone at similarly low abundance(Figure 2A, 2C). All deep-sea (\*general non-deep sea reference) sulfur-oxidizing *Epsilonproteobacteria* are known to possess the Sox multienzyme system and hydrogen oxidizing sulfur respiration pathway mediated by polysulfide reductase (PSR) (Yamamoto & Takai, 2011). However, as mentioned previously, Sox genes were associated with aerobic *Alphaproteobacteria* and PSR genes were not detected. Likely S oxidation cannot proceed in the deep zone as the known terminal electron acceptors oxygen and nitrate were depleted. This suggests Organic Lake *Epsilonproteobacteria* make use of alternate electron donors such as SCFA or hydrogen (\*check). Organic Lake differs from other meromictic Antarctic lakes (Ng *et al.*, 2010;Lauro *et al*., 2011, \*others) in the low potential for dissimilatory sulfur cycling. The reason for the limited DSR potential is unclear (\*). Some possibilities are the high salinity, transient oxygenation or positive electropotential. Lack of dissimilatory sulfur cycling has likely contributed to the abundance of reduced sulfur compounds in Organic Lake.

To determine the source of high DMS in the bottom waters of Organic Lake, the presence of enzymes involved in DMS formation was investigated. 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 dimethylsulfonioproprionate (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 be lead to sulfur loss through ventilation to the atmosphere.

Genes for DMSP lyases DddD,DddL andDddP, 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) and comprised two main DddDtypes: MAR-dddD and OL-dddD (Figure S8). Both of these DddD were part of a clade including homologs with demonstrated activity supporting their function as DMSP lyases. MAR-dddD grouped with a *Marinobacter* sp. ELB17 homolog indicating its *Marinobacter* origins (Figure S8). OL-dddD did not cluster with homologs from cultured bacteria. The abundance of OL-dddD on the 3.0 µm fraction suggests it was linked to *Roseobacter­*-clade, *Bacteroidetes* or *Dunaliella* (\*Figure S) (\*link ddd gene to scaffolds!!!!!). Two DddLgroups 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 supporting the same functional role for SUL-dddL. Apart from a carboxy-terminal cupin pocket, DddL has no similarity any other known enzyme families or domains (Curson *et al.*, 2008) and thus no other functions for DddL-like proteins are known. This suggests MAR-dddL clade is an unrecognized branch of this enzyme family. Whether it confers the Ddd phenotype requires confirmation, although in *Sulfitobacter* sp. EE-36 the *dddL* gene alone is sufficient for DMS generation (Curson *et al.*, 2008). DddP was also detected and was the least abundant of the DMSP lyases (\*table). Phylogenetic analysis showed Organic Lake DddP likely originates from *Roseovarius* (Figure S10) and was part of the clade that included the functionally verified *Roseovarius nibinhibens* DddP (Todd *et al.*, 2009).

A single DmdA type was found, which allied with *Roseobacter*-clade dmdA (Figure S11), and corresponds to the marine clade A (Howard *et al.*,2006). This clade includes functionally verified *R. pomeroyi* DSS-3 DmdA indicating the Organic Lake DmdA were true DMSP demethylases and not related glycine cleavage T proteins or aminomethyltransferases (Howard *et al.*, 2006).

DMSP cleavage potential was more than twice that of DMSP demethylation potential (Figure 4C, Table\*). This differs from estimates from the marine environment that place demethylation potential as up to two orders of magnitude greater than cleavage (Howard *et al.*, 2008; Todd *et al.*, 2009; Todd *et al.*, 2011b; Reisch *et al.*, 2011). Moran *et al.* (2012) proposed 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. Prevalence of cleavage over demethylation may be the rule in non-marine saline systems eg. Punta Cormorant (Todd *et al.*, 2009). It certainly shows the known Ddd enzymes can be highly ecologically relevant. (\*test bias for particle attached bacteria). 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. (\*maybe compare to Ace Lake).

DMSP cleavage appears to be a significant source of DMS in Organic Lake. DMSP likely originates from *Bacillariophyta* or *Dinoflagellida* as Organic Lake *Dunaliella* do not produce DMSP in culture (Franzmann *et al.*, 1987b). In fact, marine *Dunaliella tertiolecta* is known to cleave DMSP extracellularly (\*Seymour *et al.*, 2010) so *Dunaliella* may in fact break down DMSP.DMSP cleavage potential was highest 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). The first of these was not a major pathway of DMS generation in Organic Lake (Figure 4C). Pathways for the second two processes have not been established making it difficult to assess if they occur. *Halomonas* isolates from Organic Lake can produce DMS from cysteine in culture (\*ref) and provides some evidence these processes in the deep zone. In the mixed zone, DMS can be oxidized as a carbon and energy source or utilized as an electron donor by sulfur-oxidzing autotrophs (Schäfer *et al.*, 2010). In anoxic environments, methanogenic Archaea or sulfate-reducing bacteria break down DMS (\*Scholten *et al.*, 2003 or Schäfer *et al.*, 2008). Since sulfate-reducing bacteria were not abundant, and the stagnant waters would preclude loss of DMS by ventilation, this allows DMS to accumulate in the Organic Lake deep zone.

## Conclusion

## Acknowledgements

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