Insight into unusual sulfur biogeochemistry of 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 shallow (6.75 m deep) marine-derived hypersaline lake in the Vestfold Hills, East Antarctica with a high concentration of the cloud-forming gas dimethylsulfide (DMS). The lake was stratified into an aerobic mixed zone and a suboxic deep zone, with a peak of C, S and ammonia below the oxycline (6.5 m). DNA from size fractionated samples (3.0, 0.8 and 0.1 µm) along the depth profile was sequenced and the taxonomic and functional diversity determined. Eucaryotic phytoflagellates related to *Dunaliella* and *Pseudopedinella* are the main photosynthetic organisms.Bacterioplankton was dominated by heterotrophic *Marinobacter*, *Roseovarius* and *Psychroflexus.* Candidate division RF3, *Halomonas* and *Psychromonas* were overrepresented at 6.5 m and associated with fermentation of particulate matter. Genetic potential for nitrogen cycling showed large denitrification potential, limited fixation and no nitrification. Nitrogen limitation was evident in the predominance of ammonia uptake and remineralization pathways. A diverse set of functional genes were assigned to the *Marinobacter* and *Roseovarius* clades including rhodopsin, DMSP lyase (*dddD*, *dddL* and *dddP*),Calvin cycle, anaerobic respiration and CO oxidation genes indicating they are metabolically versatile generalists that may contribute to primary production and the unusual sulfur cycle. This study has described the microbial community within a natural environment and sheds light on strategies of nutrient resourcefulness such as DMSP lysis, lithoheterotrophy, chemolithoautotrophy and photoheterotrophy in globally distributed heterotrophic lineages. It shows potential for diverse strategies in may be key for adaptation to the constraints of saline Antarctic coastal environments.

# 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 from a whole ecosystem perspective. 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, and potentially the basis for 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 to visualize how abiotic factors varied with depth.

## 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 from the 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, primarily NCBI RefSeq. 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 cycling (Table S2) were 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 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. The longest sequence of the cluster was used in a BLASTp query against the NCBI non-redundant (NR) database to retrieve full-length sequenced homologs. Organic Lake representative sequences 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: -2.9, 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 of Organic Lake

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 accumulated 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 S3), 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 compounds such as carbohydrates, lipids and proteins.

The C:N and C:P ratios were high compared to the Redfield ratio (Redfield *et al.*, 1963; \*others?) 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 S4). 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.

## Organic Lake microbial community composition and vertical distribution

### 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. Overall 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 in primer)

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 absent or extremely rare. 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* (dinoflagellates), *Fungi* and heterotrophic *Choanoflagellida* (choanoflagellates) and *Ciliophora* (ciliates) (see Table S4 lower rank assignments of all taxa).

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

Community composition varied with size fraction and depth (Figure 2). 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 in conjunction 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. *Dunaliella* have been previously isolated from Organic Lake and were reported to be the dominant eucaryotic alga (Franzman *et al.*, 1987b). Signatures of algae found 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 long) (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) (reviewed in Wagner-Döbler & Biebl, 2006) and CO oxidation (Moran & Miller, 2007). All of these capabilities appear related to their success in the Organic Lake system (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 iron/manganese-oxidizing facultative chemoautotrophs (Wang *et al.*, 2011) (\*perhaps sulfur oxidizing Swan 2011) or capable of unusual redox cycling (Handley *et al*., 2009). *Marinobacter* isolates from Antarctic Lakes are similarly capable of anaerobic respiration using dimethyl sulfoxide (DMSO) (Matsuzaki *et al*., 2006) or nitrate (Ward & Priscu, 1997). These faculties would allow for the presence of *Marinobacter* throughout the water column in Organic Lake and the possibility of occupying multiple functional roles.

*Saccharospirillum*, like the name suggests degrades polysaccharides (\*ref) and is likely associated with algal blooms (\*ref).

RF3, *Halomonas*, *Psychromonas*, *Bacilli* and *Clostridia* were concentrated on the 6.5 m sample, of which RF3 was the most abundant. RF3 is most likely involved in anaerobic degradation of particulate matter due to its prevalence in the deep zone. Also, the majority of 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 aerobic environments such as surface waters of hypersaline systems (Demergasso *et al*., 2008; Yilmaz *et al.*, 2012), surface of a freshwater lake (Xing *et al.*, 2009) and compost (Partanen *et al.*,2010).

*Clostridia* was the most abundant member of the *Firmicutes* and principally comprised the genus *Halanaerobium* (Table S4). The type species *H. praevalens* is 2.4 µm long explaining its presence on this size fraction (Ivanova *et al.*, 2011).

*\*Halomonas*, *Psychromonas,Bacilli* and *Clostridia*

#### 0.8–0.1 µm size fraction

There was had a large number of eucaryotic sequences on the 0.1 µm size fraction. The presence of these *Eucarya* 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 consistent with 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). They are known to be small, but their concentration on the 0.1 µm was unusual (\*check). They may be overlooked members of the eucaryotic algal community due 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), consistent with 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 sample 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 OD1 was found to predominate in <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;\*other ref). The distribution of OD1 is consistent with an anaerobic metabolism and potential involvement in sulfur chemistry.

(\*TM7).

## Overall 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. There were differences in the distribution of functional genes according to size fraction and depth (\*ANOSIM test of mixed vs deep functional complement). (\*RELATE of taxa and functional tables). 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. It also generated greater diversity of N and S processes in the deep zone as oxygen sensitive reaction (discussed below) could occur there. Functional potential, taxonomic composition and the physico-chemical data were used to infer the C, N and S cycles in Organic Lake. C and N cycles were characterized by dominance of degradative pathways over fixation indicating net negative balance has lead to nutrient limitation. Similarly, pathways typical of aquatic environments were absent or at very low abundance. This has likely lead to accumulation metabolic end products and also a serves as a strategy for nutrient conservation. Abundant potential for bacterial carbon mixotrophy such as facultative chemoautotrophy, lithoheterotrophy and photoheterotrophy are proposed to be highly relevant adaptations to nutrient constraints in Organic Lake that serve as mechanisms to conserve organic carbon.

## Carbon resourcefulness in dominant heterotrophic bacteria

Genes for respiration were abundant throughout the water column (Figure 4A) and assigned predominantly to *Proteobacteria* (Table 2) in addition to all the major aerobic lineages (Figure S6A). Potential for carbon fixation was dominated by aerobic fixation (Figure 4A) via the oxygen-tolerant Calvin-Benson-Basham cycle (CBB). CBB cycle was linked to *Viridiplantae* (Table 2) and is consistent with the distribution of algae (Figure 2B) further supporting their ecological role as the principle photosynthetic organisms. The abundance of respiration over fixation indicates a potential for net carbon loss, particularly in the mixed zone. However, a large proportion of CBB cycle potential was linked to *Gammaproteobacteria* (Table 2, Figure S6A). Curiously, there were 100 × more matches to gammaproteobacterial phosphoribulose kinase (PRK) than RuBisCO (Figure S6A). Matches to the former were from autotrophic sulfur-oxidizing *Thiomicrospira* while the latter were to *Marinobacter*, of which only one isolated sulfur-oxidizing member is known (Choi *et al.*, 2009). This suggests a minority of the *Gammaproteobacteria* have the complete CBB pathway and most have only a part of the pathway. In the minority with the complete pathway, facultative chemoautotrophy *Gammaproteobacteria* is consistent with an opportunist metabolic strategy that likely functions only when conditions for more energy yielding pathways are not present. Linked sulfur oxidation and CBB genes have been found in *Gammaproteobacteria* in the dark ocean (Swan *et al.*, 2011). C fixation may be advantageous in the lake environment when organic C or oxygen becomes limiting. Alternatively, CBB may also function as an electron dump (\*ref). The significance of PRK without RuBisCO is unclear (\*check).

The 2-oxogluterate:ferredoxin oxidase gene matched to the genera, Maribacter, Alkaliphilus, Mahella, Odoribacter, Brachyspira, Ammonifex, Chintinophaga and Halothermothrix.

In the deep zone, marker genes for anaerobic C fixation, fermentation and CO oxidation were all in greater abundance at 6.5 m (Figure 4A) indicating these processes were involved in the higher biological activity inferred to occur at that depth (see above). Most anaerobic C fixation was represented by reverse citric acid cycle (rTCA) and some potential for fixation by the Wood-Ljungdahl (WL) pathway, linked with *Deltaproteobacteria* (Figure S6A). rTCA cycle genes originated from *Ammonifex* and other organisms known to be anaerobic C fixers. ATP citrate lyase, which is more diagnostic of rTCA was linked with sulfur-oxidizing *Epsilonproteobacteria* (Figure S6A) consistent with what is known of their chemolithoautotrophic metabolism (\*ref sulfurimonas paper).A very small potential for WL-mediated carbon fixation by *Deltaproteobacteria* fits with known genomic potential of sequenced members, some of which can grow autotrophically with hydrogen and sulfate (Strittmatter *et al*., 2009).

However, there was a much greater potential for fermentation indicating activity in the deep zone is mainly being driven by secondary production. Fermentation potential was linked to *Mollicutes* likely originated from the related candidate division RF3 (Tajima *et al.*, 1999) supporting a fermentative metabolism in this division and their ecological role in Organic Lake. Typically syntrophs, methanogens or sulfate-reducing bacteria comprise the end of the anaerobic food-chain and hydrolyse SCFA (\*ref). Signatures of methanogenesis (Figure 4A) and methanogens were absent and the abundance sulfate-reducing *Deltaproteobacteria* was low (Figure 2A), indicating slow removal rates accounts for the abundant SCFA detected in the bottom waters (Gibson *et al*., 1994).

CO oxidation genes originated from the *Roseobacter* clade of *Alphaproteobacteria* (Table 2; Figure S6A). As C fixation pathways were not associated with *Alphaproteobacteria* (Figure S6A), CO oxidation was likely lithoheterotrophic (Moran *et al*., 2007) or linked to anaplerotic C fixation as proposed for related roseobacters (Moran & Miller, 2007). The concentration of CO oxidation genes at 6.5 m indicates this potential was associated with the deep ecotype of Organic Lake *Roseovarius* (see above) and related to its success at this depth. Organic carbon such as SCFA can be directly assimilated mixotrophically by CO oxidizers limiting oxidative loss of organic carbon (Moran & Miller, 2007) and may be a competitive strategy in a largely closed heterotrophic-dominated system.

\*Why not at 6.7 m? Microaerophilic

### High potential for photoheterotrophy

Aerobic and anaerobic anoxygenic photosynthesis may be occurring and would be mediated by the Roseobacters present such as *Roseovarius*. *R. tolerans* is the type species of the genus and was isolated from Ekho Lake, a meromictic hypersaline lake in the Vestfold Hills (\*Labrenz *et al.*, 1999). It was found to produce bacteriochlorophyll A when grown in the dark, but continuous dim light inhibited production (\*Labrenz *et al.*, 1999). (\*Look for the pufLM genes).

The first rhodopsin found in bacteria, termed proteorhodopsin (PR) acts as a light-driven proton pump and was hypothesized to be used for energy generation (Béjà *et al*., 2000). Metagenomic studies have since shown PR are diverse, widely distributed in the surface ocean (Rusch *et al*., 2007) and associated with diverse bacterial clades including *Alphaproteobacteria* (de la Torre *et al.*, 2003) and *Bacteroidetes* (Venter *et al.*, 2004) as well as *Euryarchaeota* (Frigaard *et al.*, 2006). Actinorhodopsins (Sharma *et al.*, 2008; Sharma *et al.*, 2009) and xanthorhodopsin (Balashov *et al.*, 2005) form a clade related to PR that we will refer to as actino-xanthorhodopsins.

A total of 399 reads matching to rhodopsins were detected in Organic Lake, which formed 124 clusters at 90% amino acid identity. Phylogenetic analysis revealed six well-supported rhodopsin groups: MAR-R, OL-R1, OCT-R, SAL-R, AQU-R and PSY-R (Figure S8). Only the PSY-R clustered with the PRs showing most Organic Lake rhodopsin diversity was within the actino-xanthorhodopsin clade. 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 consistent with the shallow water in Organic Lake and is characteristic of coastal samples (Rusch *et al.*, 2007). MAR-R, PSY-R, OCT-R and AQU-R groups (Figure S8) all clustered with homologs of genera detected in the lake, namely *Marinobacter*, *Psychroflexus*, *Octadecabacter* and “*Candidatus* Aquiluna” (Figure 2C, Table S4). Xanthorhodopsin was described from the sphingomonad *Salinibacter* *ruber* (Balashov *et al.*, 2005), thus SAL-R likely originates from other *Sphingobacteria* (Table S4). However, the most abundant group, OL-R1, had no close homologs from GENBANK. From its high abundance and concentration on the 3.0 µm fraction, OL-R1 group most likely originated from the *Rhodobacterales* including *Roseovarius* (Figure 2).

If there are approximately 3 000 bacterial SSU sequences, assuming PR to be single copy and SSU copy number to range from one to ten, 13–100% of Organic Lake bacteria have a PR. 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%).

Rhodopsins of marine *Flavobacteria* and *Vibrio* have been associated with light-dependent energy generation, especially under low carbon conditions (Gómez-Consarnau *et al*., 2007; Gómez-Consarnau *et al*., 2010). This is a potential mechanism for conserving carbon for growth or inhabiting low oxygen environments. This is most likely to be the case for Organic Lake *Psychroflexus* as it is related to *Dokdonia* and has a related PR. The ecological function of other rhodopsins are yet to be elucidated and may be involved in diverse roles such as light sensing or as ion pumps (Fuhrman *et al.*, 2008). As rhodopsins were present in all the dominant Organic Lake bacterial lineages and all homologs similar to Organic Lake rhodopsins originated from polar and/or lake species (Gosink *et al.*, 1997;Ward & Priscu, 1997; Bowman *et al.*,1998; Antón *et al.*, 2002;Hahn, 2009; Kang *et al.*, 2012), these data suggest there is strong selection for rhodopsins in the polar coastal environment.

## Predominance of regenerated nitrogen cycling

N cycling potential throughout the lake profile was dominated by assimilation and mineralization/uptake pathways 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 particularly in high ammonium concentrations (\*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 in 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 monophyletic order *Brocardiales*, (Niftrick & Jetten, 2012)which were not present and HAO/HZO was linked instead 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 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 expected to be active in the deep zone. 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 in Organic Lake 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).

## Molecular basis for unusual sulfur chemistry

There was a prevalence of assimilation/mineralization sulfur pathways in Organic Lake while sulfur oxidation genes were undetectable and dissimilatory sulfate reduction (DSR) extremely limited (Figure 4C). Consistent with this, sulfur-oxidizing *Epsilonproteobacteria* and sulfate-reducing *Deltaproteobacteria* were present at very low abundance (Figure 2), despite the presence of sulfate (\*ref). Organic Lake appears to have a reduction in pathways for cycling inorganic S typical in other meromictic Antarctic lakes (Lauro *et al*., 2011, \*others). Lack of these pathways has likely been instrumental in the development of unusually high levels of reduced sulfur compounds, DMS and polysulfides.

To determine the potential source of high DMS in the bottom waters of Organic Lake, the presence of enzymes involved in DMS cycling was investigated. 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). These enzymes catalyse the breakdown of DMSP forming DMS as a by-product and this process is the major source of DMS in the marine environment (reviewed in Curson *et al.*, 2011b).

DddD, was the most abundant of the Organic Lake DMSP lyases (70%) and comprised two main DddDtypes: MAR-dddD and OL-dddD (Figure S7). MAR-dddD grouped with a *Marinobacter* sp. ELB17 homolog and had a distribution consistent with that of *Marinobacter* (Figure S7). OL-dddD had highest identity (~80%) to *ddd*Dfrom *Halomonas* sp. HTNK. The abundance of OL-dddD on the 3.0 µm fraction suggests *Rhodobacteraceae* as the most likely origin (Figure S7) rather than *Halomonas*, which was restricted to the 0.8 µm fraction (Figure 3). Two DddLgroups were detected in Organic Lake: SUL-dddL and a MAR-dddL (Figure S8). SUL-dddL clusters with *Sulfitobacter* sp. EE-36 while MAR-dddL groups with a hypothetical protein from *Marinobacter manganoxydans* MnI7-9. This finding suggests MAR-dddL clade is an unrecognized branch of this enzyme family and is the first report of DddLlinked to *Gammaproteobacteria*. Whether it confers the Ddd phenotype requires further confirmation although in *Sulfitobacter* sp. EE-36 the *dddL* gene alone is sufficient for DMS generation (Curson *et al.*, 2008). Both are predominantly located on the 0.8 µm fraction, which suggests the origin of both Organic Lake DddLtypes are *Gammaproteobacteria*. The MAR-dddL fits the distribution of *Marinobacter* while the abundance of SUL-dddL at 6.5 m indicates this homolog originated from *Psychromonas* or *Halomonas* which are predominant at 6.5 m.

\*dddP \*Check other DMSP demethylation.

In addition, reduction of DMSO to DMS (\*figure) may be a further source of DMS in the deep zone. Potential for DMSO reduction was associated to \*.

DMSP degradation appears to be the main source of DMS in Organic Lake and was mediated by *Roseobacter-*cladeand *Gammaproteobacteria* such as *Marinobacter*, *Psychromonas* and *Halomonas*. Concentration of DMSP cleavage potential in the bottom (Figure 4C) where the DMS concentration is highest (\*ref) is consistent with production of DMS in the deep zone. The likely origin of DMSP is the breakdown of algal cells. Other sources are DMSO reduction or a yet undefined pathway of anaerobic production from cysteine (\*ref). Usually methanogenic Archaea or sulfate-reducing bacteria break down DMS in anoxic conditions (\*ref). Since only a very low abundance of sulfate-reducing bacteria and DSR genes were detected and methanogens are absent, DMS can accumulate in Organic Lake as a metabolic end-product.

## Discussion points

What are the sulfate reducing Deltaproteobacteria doing if not sulfate reduction?? Could they make DMS anaerobically? Can sulfur go to DMS? Amino acids to DMS?

{\*intro: The bottom waters of Organic Lake were found to be anoxic, but not sulfidic or methanogenic (Franzmann *et al.*, 1987b; Gibson *et al.*, 1991). Although sulfates and organic acids have been recorded (Franzmann *et al.*, 1987b; Gibson *et al.*, 1994), the cold and salinity six times that of seawater, appears to preclude the establishment of sulfate-reducing bacteria and phototrophic sulfur bacteria (Burke & Burton, 1988) indicating other microbes are involved in the unusual sulfur chemistry. }

### {\* Selection for psychrophilic and halophilic *Eucarya* and *Bacteria*

Cultured relatives of taxa detected in Organic Lake are known to be halophilic and/or psychrophilic (Gauthier *et al.*, 1992; Dobson *et al.*, 1991; Labrenz *et al*., 1999;\*\*\*\* Aquiluna\*Kang *et al*., 2012). Similarly, uncultured taxa have highest identity to sequences from saline and/or cold environments (\*OD1 Mosier *et al*., 2007; RF3 Demergasso *et al.*, 2010;\*\*) (Table S4). The consistent association with phylotypes from similar environments indicates a strong selection for species by common environmental factors. This is further supported by the persistence of the same taxa in Organic Lake over time, such as *Dunaliella*, *Chaetoceros, Psychroflexus*,(Franzmann *et al*., 1987b)  *Marinobacter*, *Halomonas* and *Roseovarius* (Bowman *et al*., 2000b), which indicates they are particularly adapted to the lake conditions.

selection for cold species: The Organic Lake 16S composition was most like that of meromictic hypersaline Antarctic lakes Ekho and Bonney. They are characterized by an abundance of *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* and scarce or absent photolithoautotrophic bacteria as well as haloarchaea (Bowman *et al.*, 2000b; Glatz *et al*., 2006). The salinity in these lakes (150–180) appears to be too high for planktonic photosynthetic bacteria such as *Synechococcus* relatives (Powell *et al.*, 2005) and anoxygenic sulfur bacteria (Burke & Burton, 1988). In contrast, it appears too low for haloarchaea found to dominate Deep Lake (Bowman *et al.*, 2000b). These findings correspond well to a study of solar salterns along a salinity gradient that showed Cyanobacteria were confined to salinity <65 and haloarchaea at salinity >190 (Ghai *et al.*, 2011). Salinity is therefore a crucial constraining factor for Organic Lake species composition; a factor that is inter-related to other variables such as freezing point and thus ice-cover and available light. This has lead to species diversity that is reduced to the point that entire divisions present in other Antarctic lakes are excluded in Organic Lake. }

{\**functional:*The majority of the genetic potential was restricted to the 0.8 and 3.0 µm size fractions evident in the higher percentage of ORFs with matches to KEGG genes ( average 55%) compared to 0.1 µm fraction (average 28%) (Table S1). The lack of ascribed functional genes in the 0.1 µm filter reflects the paucity of cellular life in that fraction and possibly the high representation of candidate divisions, with poorer representation of functional sequences.}

**{Net loss:** A net loss in essential elements implies that a there may be an influx of exogenous nutrients occurs to sustain the lake system. However, external input, such as from glacial melt-water, could only occur in the summer months when the lake is ice-free. Furthermore, the water column structure is characteristic of a negative water balance (\*Gibson) indicating the Organic Lake system has been largely closed in the recent past. Thus, if external inputs occur, they are episodic and would necessitate interim strategies for C, N and S conservation.}

{Methane oxidation enzymes that were detected are related to alkane hydroxylases and therefore most likely involved in hydrolysis of compounds such as phenol, which has been previously detected in the bottom waters of Organic Lake (Roberts & Burton 1993a; Roberts *et al.*, 1993b).}

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