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 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* were the main photosynthetic organisms.Bacterioplankton was dominated by heterotrophic *Marinobacter*, *Roseovarius* and *Psychroflexus.* Candidate division RF3, *Halomonas* and *Psychromonas* were overrepresented at the oxycline and associated with fermentation of particulate matter. The dominance of heterotrophic respiration pathways indicates potential for carbon and nitrogen limitation. However, most heterotrophs also possessed genes that may be involved in carbon conservation such as aerobic anoxygenic phototrophy, CO oxidation, rhodopsins, or facultative chemoautotrophy. Potential for nitrogen cycling was limited to assimilation and regeneration that likely serves as a mechanism for retaining nitrogen. Sulfur conversions typical of other aquatic systems was absent or greatly reduced and has likely lead to the accumulation of DMS, likely produced by DMSP lysis. This study 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 and nutrient resourcefulness 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. 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 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

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 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 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 (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 examined 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 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 iron/manganese-oxidizing facultative chemoautotrophs (Wang *et al.*, 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.

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. 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 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), 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;\*other ref). 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 (discussed below). 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 suggesting net negative balance has lead to nutrient limitation. C, N and S conversions typical of aquatic environments were absent or present at very low abundance. This appears to have 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 *Bacteroidetes*, *Actinobacteria* and *Viridiplantae* (Figure S6A). Overall, the respiration potential was much higher than fixation indicating net carbon loss. Potential for carbon fixation was dominated by aerobic fixation (Figure 4A) via the oxygen-tolerant Calvin-Benson-Basham cycle (CBB). This was assessed by the two marker genes ribulose-bisphosphate carboxylase (RuBisCO) and phosphoribulose kinase (PRK) (\*ref for diagnostic). RuBisCO was linked to primarily to *Viridiplantae* (Table 2) and is consistent with the distribution of algae (Figure 2B) supporting their ecological role as the principle photosynthetic organisms. However, a large proportion of CBB cycle potential was also linked to *Gammaproteobacteria* (Table 2, Figure S6A). A minority of the *Gammaproteobacteria* appear to possess RuBisCO (Figure S6A) related to autotrophic sulfur-oxidizing *Thiomicrospira*, which was detected in the SSU analysis (Table S4). A percentage of sulfur oxidation potential was associated with *Gammaproteobacteria* (Figure S6C) indicating chemolithoautotrophy contributed to primary production. The majority of matches were to phosphoribulose kinase (PRK) (\*check other functions of PRK, blast prk, electron dump?) The significance of PRK without RuBisCO is unclear (\*check). The majority of Organic Lake *Gammaproteobacteria* are likely to be facultatively autotrophic (\*ref). This is consistent with an opportunist metabolic strategy whereby autotrophy would only be active in conditions such as carbon limitation (\*ref, lit search).

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**.

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. Similar to the mixed zone, potential for C fixation was much lower than for degradative processes (Figure 4A). Most anaerobic C fixation was represented by reverse tricarboxylic acid cycle (rTCA) and some potential for fixation by the Wood-Ljungdahl (WL) pathway (Figure S6A). ATP citrate lyase, which is diagnostic of rTCA, was linked with sulfur-oxidizing chemolithoautotrophic *Epsilonproteobacteria* (Figure S6A, Table S4) consistent with the genera identified (Figure 2C, Table S4) (\*ref sulfurimonas paper\*what SO pathway?). However, sulfur oxidation potential was not linked to *Epsilonproteobacteria* (Figure S6C) suggesting use of alternative electron donors (discussed below). The majority of rTCA cycle potential was from matches to 2-oxogluterate:ferreoxidin oxidase genes that originated from diverse genera but particularly a 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). These matches could represent true C fixation potential or use of these genes in alternative degradative pathways. 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). This means anaerobic C fixation could be overestimated and C budget is even at a greater deficit in the deep zone.

The high potential for fermentation indicates this is driving biological activity in the deep zone. Fermentation potential, linked to *Mollicutes* (Table 2), most likely originated from the related candidate division RF3 (Tajima *et al.*, 1999). This supports a fermentative metabolism and a crucial ecological role of this candidate division in Organic Lake. Typically methanogens or sulfate-reducing bacteria comprise the end of the anaerobic food-chain and hydrolyse SCFA. 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 *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). It may also be linked to some anaplerotic C fixation (Moran *et al*., 2007). Organic carbon can be assimilated directed towards biosynthesis rather than respiration (Moran & Miller, 2007). The concentration of CO oxidation genes at 6.5 m was thus associated with the deep-zone ecotype of Organic Lake *Roseovarius* (see above) and appears to contribute to metabolic success at this depth. The likely source of organic carbon at that depth would be SCFA indicating assimilation of fermentation products may play a significant role in Organic Lake rather than complete anaerobic oxidation.

### 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 to be abundant in the ocean and related to diverse *Proteobacteria* (Béjà *et al.*, 2002).Similarly, metagenomic studies have shown 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 or as ion pumps (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 S8, 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 its abundance and distribution on the 3.0 µm fraction suggests it originates from *Roseobacter* clade (Figure 2A).

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 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. 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 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 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

Sulfur cycling was dominated by assimilation/mineralization pathways in Organic Lake (Figure 4C). Dissimilatory sulfur cycling potential was extremely limited. Sulfur oxidation, by the Sox multienzyme system, was linked to *Roseobacter-*clade *Alphaproteobacteria* (Table 2) and restricted to the mixed zone consistent with oxygen being 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-oxidizing were also present in the deep zone at similarly low abundance(Figure 2A, 2C). All deep-sea 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 roseobacters while PSR genes were not detected. Likely S oxidation cannot proceed in the deep zone as the known terminal electron acceptors oxygen and nitrate are depleted. This suggests Organic Lake *Epsilonproteobacteria* make use of alternate electron donors such as SCFA or hydrogen (\*check). Overall, Organic Lake differs from other meromictic Antarctic lakes (Ng *et al.*, 2010;Lauro *et al*., 2011, \*others) in the limited potential for dissimilatory sulfur cycling in the deep zone. Lack of these pathways has likely been instrumental in development of unusually high levels of the reduced sulfur compounds, DMSP, 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). (\*recA normalized) 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 S8). OL-dddD had highest identity (~80%) to *ddd*Dfrom *Halomonas* sp. HTNK. The abundance of OL-dddD on the 3.0 µm fraction suggests *Roseobacter­*-clade as the most likely origin (Figure S8) rather than *Halomonas*, which was restricted to the 0.8 µm fraction (Figure 3) (\*what about phytoplankton or Bacteroidetes?). 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 linked 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 \*Why do DMSP lysis? energy? sulfur? carbon?

DMSP can also be demethylated by the DMSP demethylase (DmdA) to methylmercaptopropionate (MMPA) and finally to methanethiol (MT). This pathway limits sulfur loss as MT can be assimilated (Curson *et al.*, 2011b). This is estimated to be the dominant pathway of DMSP degradation in the ocean (\*ref from Howard 2006) and has been associated with diverse *Proteobacteria* clades, but principally *Roseobacter* (\*Ref). DmdA was found in Organic Lake (Figure 4C) related to roseobacter homologs (Table 2)(\*which clades?). Distribution of DmdA was followed that of Organic Lake *Roseovarius* (Figure 3) showing peaks at 4.5 and 6.5 m. DMSP demethylation potential was lower than DMSP lysis (Figure 4C). Even though the regulation of each pathway is not well understood, dominance of lysis potential over demethylation differs from open ocean estimates (\*ref). This provides a clue as to what conditions may lead to different fates of DMSP and is relevant for climate models.

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 *Clostridia*.

DMSP cleavage appears to be a significant source of DMS in Organic Lake and was mediated by *Gammaproteobacteria* and likely *Roseobacter-*clade. 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. Production of DMSP varies among algae, the highest known DMSP producing algae detected in Organic Lake were *Dinophyceae* (\*ref). (\*check if dunaliella makes DMSP). Other sources of DMS are DMSO reduction, which was not apparent at high abundance, 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 the stagnant waters would preclude loss by ventilation, this allows DMS likely accumulate in the Organic Lake deep zone as a metabolic enp-product.

## Discussion points

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.}

{A total of 399 reads matching to rhodopsins were detected in Organic Lake, which formed 124 clusters at 90% amino acid identity.}

**{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).}

{ 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 a strong selection for rhodopsins in the polar coastal environment.}

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