Understanding globally important biogeochemical processes from a study 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). During sampling, 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). Environmental DNA from size fractionated samples (3.0, 0.8 and 0.1 µm) along the depth profile was sequenced and the taxonomic composition and functional diversity determined. The microbial composition resembles other hypersaline and cold environments indicating a strong selection for species. Eucaryotic phytoflagellates related to *Dunaliella* and *Pseudopedinella* are the dominant photosynthetic organisms.Bacterioplankton throughout the water column was dominated by heterotrophic *Marinobacter*, *Roseovarius* and *Psychroflexus.* Candidate division RF3, *Halomonas* and *Psychromonas* were overrepresented at 6.5 m and associated with high potential for fermentation of particulate matter. The bottom samples were abundant in candidate divisions OD1 and TM7. 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 allowed a rigorous description of the microbial community within a natural environment and sheds light on globally relevant biogeogemical processes such as DMS generation, lithoheterotrophy, chemolithoautotrophy and photoheterotrophy. It shows potential for diverse strategies in may be the rule, not the exception for marine-derived surface water *Gammaproteobacteria* and *Roseobacter* with implications untangling microbial roles in production.

# 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, located 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 from the coast 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 more easily related to abiotic factors. By using molecular techniques, a large proportion of the species diversity and gene content can be covered allowing better inference of the functional roles for 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, which infect eucaryotic algae. The presence of OLV would reduce infective phycodnaviruses leading to increased 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 virophage have a wider ecological role (Yau *et al.*, 2011).

This study extends the previous metagenomic analysis of the Organic Lake surface water 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) as well as polysulfides (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 and potentially the basis for DMS accumulation. 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. 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 Organic Lake salinity is higher than the practical salinity range of 2–42 for which the conductivity to 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 and inorganic N 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. Inorganic N and dissolved nutrients were not included in the PCA analysis as the values were missing for those variables at 4.2 m, but PCA performed excluding 4.2 m sample and including those parameters showed similar separation of samples.

## 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). This software implements hidden markov model based searches to retrieve 12S/16S/18S sequences and trims off regions outside of the SSU gene. 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). QIIME was then used to choose a representative sequence from each OTU and classify the representative set to the genus level using the RDP classifier (Wang *et al*., 2007) trained against SILVA (release 108) sequences (www.arb-silva.de). Assignments were accepted to the highest taxonomic rank with bootstrap value ≥85%. This prevented low confidence matches contributing to counts of high-confidence phylogenetic groups while avoiding grouping all the unclassified taxa together.

To allow comparison of the relative abundance of taxa between samples, the number of SSU matches per sample filter was normalised to the average number of reads (403 577) obtained for each sample filter. Statistical analysis on the relative SSU gene abundances was performed using the PRIMER Version.6 package (Clarke & Gorley, 2006). The SSU gene counts of each sample filter were square root transformed to reduce the contribution of highly abundant taxa. The Bray-Curtis similarity of the community composition from each sample was computed (\*insert ANOSIM test).. To determine if physical and chemical parameters and the patterns in cellular composition were correlated, 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) of the SSU composition

## Analysis of Functional potential

Open reading frames (ORFs) were predicted from quality trimmed metagenomic reads using MetaGene (Noguchi *et al*., 2006). Those ORFs longer than 90 bp were selected for downstream analyses. ORFs were translated into amino acid sequences using the standard bacterial/plastid translation table. Translated ORFs were 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 Orthologs with expectation value below 1e−05 and rank greater than 5. Assignments from each sample to KEGG orthologs that matched to marker enzymes in the carbon, nitrogen and sulfur cycles (\*Table CNS genes) were counted and counts normalized between samples. Frequencies of marker genes from the same pathway were averaged and those from different pathways were summed. Each assignment to a KEGG Ortholog was also given a species assignment based on the species from which the top KEGG GENES match originated from.

Marker genes that were not represented by KEGG Orthologs were retrieved via alternative strategies depending on their representation in sequence databases. 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. These sequences were used to query a BLAST database of the translated ORFs predicted from the Organic Lake metagenomic reads (\*table: functional\_genes). Matches were examined if e-value was <1e−10 and accepted if the sequence identity was within the range for members of the query enzyme family that possess the same function.

## 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 CD-HIT cluster was used as the representative sequence in a BLASTp query against the NCBI non-redundant (NR) database to retrieve full-length sequenced homologs from bacterial isolates. They were included in phylogenetic analysis along with the Organic Lake representative sequences that resided within a desired conserved region, for example the spectral tuning motif of rhodopsin. Phylogenetic analyses were 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 (Figure 1B). 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. 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. (\*Trophic status. Check how TOC compares to other lakes/ocean). 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 organic 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 (see Table S1 for summary of metagenomic data). 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 S2) indicating Archaea were rare in Organic Lake. (\*Finish pie charts to Figure 1).

{Proportions of SSU genes may not necessarily reflect the number of cells in the environment because of potential SSU copy number, DNA extraction and sequencing biases. In terms of error from copy number, archaeal SSU gene copies only range from 1–4 (Lee *et al.*, 2009) and nearest sequenced relatives of Bacteria present in this study ranges from 1–6 (data not shown). Thus, it is expected Archaea were truly scarce and estimates for bacterial abundance are accurate within this margin of error. (\*mention GAAS here\*recA comparison).}

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) (\*Note that much greater diversity was detected than compared to Bowman).

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 (at least 64% of sequences from that class) which was *Marinobacter*, *Roseovarius* and *Psychroflexus* respectively (Figure 2C). Moderately abundant bacterial classes were *Actinobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and candidate divisions OD1 and RF3. Lower abundance divisions included the *Bacilli*, *Clostridia*, *Spirochaetes*, *Lentisphaera*, TM7, *Opitutae*, *Verrucomicrobia*, Bhi80-139, Bd1-5, SR1 and *Chlamydiae* (Figure 2A). Cyanobacterial sequences were all classified as chloroplasts (Figure 2B), except for three reads that could not be assigned to any lower rank indicating free-living *Cyanobacteria* were absent or extremely rare.

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 S2)*.* Lower abundance Eucarya included *Bacillariophyta* (diatoms), *Dinophyceae* (dinoflagellates), *Fungi* and heterotrophic *Choanoflagellida* (choanoflagellates) and *Ciliophora* (ciliates). *Bacillariophyta* and *Dinophyceae* were related to *Chaetoceros* and *Gymnodinium* respectively (Table S2). Both of these classes would contribute to primary production, however, dinoflagellates are also potentially bacteriovorous. A choanoflagellate described in Organic Lake was the first description of a choanoflagellate in a hypersaline environment (van den Hoff & Franzmann, 1986) and was likely the dominant phagotrophic grazer.

### 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 S2). 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.

Compared to other Antarctic lakes, the Organic Lake 16S composition was most like that of lakes Ekho (Bowman *et al.*, 2000b) and Bonney, (Glatz *et al*., 2006), which are characterized by an abundance of *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* and scarce or absent photolithoautotrophic bacteria as well as Archaea. These lakes are meromictic and of comparable salinity (150–180). This salinity appears to be too high for obligate phototrophic bacteria as Antarctic *Synechococcus* relatives only inhabit lakes of close to marine salinity (Powell *et al.*, 2005) and the upper limit for Antarctic phototrophic sulfur bacteria is 150 (Burke & Burton, 1988). In contrast, Deep Lake in the Vestfold Hills is populated almost solely by haloarchaea and due to high salinity ~350 (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). A similar pattern appears to apply to diatom species assemblages in lakes of the Vestfold Hills (Roberts & McMinn, 1996). 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.

### 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 clearly clustered according to size fraction, and those clusters further separated into mixed and deep zone groups (Figure 3). (\*make rm 2 heat map cluster better?) Furthermore, it identified genera that were differentially distributed with size and depth and those that co-varied (Figure 3). A significant difference in genus-level composition between mixed and deep zone samples was supported by ANOSIM analysis (Rho: 0.53, significance: 0.1%). Differential vertical distribution of taxa is consistent with partitioning of ecological functions in the lake and 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 phototrophic cells 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). They were likely the greatest contributors to photosynthetic primary production. Signatures of algae found at the bottom of the lake are likely due to sedimentation of dead cells or resting cysts as the biflagellated adult cells would be able to control their location in the water column.

*Psychroflexus* were enriched on the 3.0 µm samples, although they were also present on the smaller filter sizes. *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 adaptation to aerobic and microaerophilic conditions. *R. tolerans*, an isolate from the meromictic Ekho Lake, also in the Vestfold Hills and a close relative of Organic Lake *Roseovarius*,has a large cell size (1.1–2.2 μm long) (Labrenz *et al*., 1999) accounting for accumulation on this size fraction. The abundance of *Roseovarius* in the deep zone is somewhat unexpected as, *Roseovarius* isolates are strictly aerobic. However one strain from Ekho Lake is capable of microaerophilic growth (Labrenz *et al.*, 1999). This is consistent with their overrepresentation at 6.5 m rather than at the lake bottom. *Roseovarius* is a member of the *Roseobacter* clade whose diverse metabolic capabilities. These include DMSP degradation, aerobic anoxygenic phototrophy (AAnP) and symbiotic relationships with dinoflagellates (reviewed in Wagner-Döbler & Biebl, 2006), all of which could be highly relevant processes in Organic Lake.

(\*check bacteriochlorophyll A from Roseovarius. likely none is being produced expressed in this sample. Look for the pufLM genes which form part of the reaction centre, not the harvesting complex. Some Roseobacters have pufLM but no Bchl expressed as Bchl expression is dependent upon environmental conditions).

#### 3–0.8 µm size fraction

*Marinobacter* dominated the 0.8 µm size fraction at all depths except 6.5 m. Their abundance on this size fraction is consistent with the cell size of planktonic isolates (Gauthier *et al.*, 1992). They are known as aerobic heterotrophs with a general preference for labile carbon sources such as sugars, amino acids and organic acids (\*ref) as well as hydrocarbons (Gauthier *et al.*, 1992). The genus is extraordinarily metabolically versatile and described as a “generalist” or “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 such as switching from arsenate oxidation to dissimilatory arsenate reduction (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). Either faculty 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* were concentrated on the 6.5 m sample and are the most likely involved in degradation of particulate matter. RF3 very likely has an anaerobic lifestyle as most 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).

*\*Halomonas*, *Psychromonas*

*Bacilli* and *Clostridia* were also exclusively present

#### 0.8–0.1 µm size fraction

There was had a large number of eucaryotic SSU sequences on the 0.1 µm size fraction, specifically from fungi, *Dictyochophyceae*, *Dinophyceae* and choanoflagellates. These taxa were all found on larger size fractions, except fungi which were uniquely present in the 1.7 m 0.1 µm sample and were classified as *Cordyceps* and *Ascomycota*. 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 potentially degraded cellular material.

The mixed zone was dominated by *Pedinellales* that co-varied with unclassified chloroplasts. This is consistent with active cells localizing to surface light. *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 again their concentration on the 0.1 µm was unusual (\*check).

The 0.1 µm deep samples were distinguished by the presence of candidate divisions OD1 and TM7 which were concentrated on the lake bottom. The prevalence of these two divisions almost exclusively on the smallest size fraction is consistent with small cell size. OD1 has also been found to predominate in <0.2 µm fraction of ground water plankton (Miyoshi *et al.*, 2005) supporting small cell size in this division. OD1 has a wide distribution and has been consistently associated with anoxic environments implying an anaerobic physiology (Harris *et al*., 2004). In the marine environments, it has also been associated with reduced environments with high sulfur such as sulfate and sulfides (Harris *et al*., 2004; Elshahed *et al.*,2005;\*other ref). Genomic fragments of a member of OD1 from Zodletone Spring, Oklahoma showed oxygen sensitive enzymes related to anaerobic or facultative anaerobic bacteria (Elshahed *et al.*,2005). Thus, the distribution of OD1 is consistent with an anaerobic metabolism and potential involvement in the unusual sulfur chemistry.

(\*TM7 sizes).

“*Candidatus* Aquiluna”, in the Luna-1 cluster of *Actinobacteria* (Hahn *et al.*, 2004; Hahn *et al.*, 2009) was most abundant on the 0.1 µm size fraction at 1.7 m depth, however it was also present in the deep zone of the 0.1 and 3.0 µm size fractions. The genus has small cells, <1.2 µm in length (Hahn *et al.*, 2009), consistent with their concentration on the smallest size fraction. Although originally described in freshwater lakes, the same cluster 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. Isolates are aerobic chemoheterotrophs (Hahn *et al.*, 2009) and in the Ace Lake mixolimnion, they were associated with utilization of labile dissolved carbon and nitrogen (Lauro *et al.*, 2010). Their presence of this clade in both the mixed and deep zones implies it is facultatively anaerobic or present in the bottom due to sedimentation.

## Organic Lake functional gene complement and links to taxonomic groups

To determine the functional capacity in Organic Lake, functional molecular markers for key C, N and S conversions (Figure 4), as well markers for other processes of interest were retrieved from metagenomic reads. Like taxonomic composition, there were differences in the distribution of functional genes according to size fraction and depth indicating a strong link between taxa and function (\*ANOSIM test of mixed vs deep functional complement). 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 from these sizes ( 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, which would have fewer homologs in sequence databases. The consistency between marker gene and taxonomic distributions, the phylogenetic assignments of the marker genes to taxa present in the lake (\*table) and the imputed metabolic capabilites of those taxa provides a solid link between taxa and ecological functions.

(\*relate of species matrix and functional matrix).

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 had the effect of generating a much greater diversity of functions involved in N and S conversions in the deep zone (outlined below). Some of these are known to be oxygen sensitive processes such as anammox, N fixation and dissimilatory nitrate and sulfate reduction consistent with a strong correlation between these parameters and vertical differences.

## Reduced potential for nitrogen cycling related to N limitation

Potential for nitrogen conversions was greatly reduced in Organic Lake. Low abundance of nitrogenase genes and of diazotrophs indicates a low capacity for nitrogen fixation in Organic Lake and is a likely contributor to N limitation. N2 fixation was confined to the deep zone (Figure 2B) and principally linked to *Epsilonproteobacteria* such as *Arcobacter*, as well as *Deltaproteobacteria* and *Clostridia* (\*functional table), which is consistent with their restriction to the lake bottom. Genes for aerobic ammonia oxidation (*amo*ABCD) were not detected, nor were ammonia oxidizing bacteria or archaea present supporting a lack of nitrification potential in Organic Lake. AMO was similarly absent in nearby Ace Lake (Lauro *et al*., 2011) indicating some factor may be limiting nitrification in the lakes in the Vestfold Hills. Similarly, anaerobic ammonia oxidation (anammox) potential, indicated by the hydroxylamine oxidase (HAO) gene related to the HZO gene central to anammox, was in low abundance. Known anammox organisms were not present (\*check) and HAO was linked instead to sulfate reducing bacteria (\*functional table) and may in fact be involved in other N pathways (\*check). This indicates an inability for nitrification to occur in the mixed zone and a very limited potential for ammonia loss.

Fixed N in Organic Lake was largely confined to assimilation and mineralization/uptake pathways. Assimilation potential was indicated by the GS-GOGAT enzymes and was linked to dominant microbes throughout the lake profile. Assimilatory nitrite reductase was not abundant indicating a predominance of reduced N uptake in the community. Potential for mineralization to ammonia, indicated by glutamate dehydrogenase (*gdh*A) genes was similarly present throughout the whole lake and associated abundant taxonomic groups. *gdh*A may function in reverse as an ammonium uptake mechanism, particularly in high ammonium concentrations (\*ref). The genetic potential for N metabolism indicates a shift away from oxidized N forms and increased cycling between organic N and ammonia. The higher ammonia concentration in the deep zone, particularly at 6.5 m would result from a higher rate of mineralization than assimilation. Ammonia would also be produced from dissimilatory nitrate reduction to ammonia (DNRA), associated mainly to *Sphingobacteria* and other anaerobic bacteria, (\*Table) in addition to Stickland fermentation (\*figure) by *Clostridia*. Higher rates of organic N catabolism in the deep zone is consistent with the higher potential for degradative processes such as fermentation (\*see C cycle) and fermenting organisms such as *Clostridia* and candidate division RF3 (Figure 2A) (Figure 1).

Denitrification genes and were present throughout the water column (Figure 4B) and was linked to numerous denitrifiying organisms (\*table). Low nitrate and nitrite in the deep zone (Figure 1B, Table1) is indicative of depletion having occurred by denitrification or DNRA and a potential for net N loss from the system. This may have contributed to the establishement of N limitation in the lake. Denitrification enzymes are usually induced by low oxygen or oxidized N (\*ref) and thus expected to only be active in the deep zone. However, denitrification may not be active for other reasons. A natural example of this is Lake Bonney, Antarctica where denitrification occura in the west lobe, but is inhibited by an unknown factor in the east lobe of the same lake despite the presence of denitrifying *Marinobacter* species (Ward & Priscu, 1997; Ward *et al*., 2005). Known inhibitors of denitrication includes sulfides (\*ref). In Organic Lake, abundant reduced sulfur compounds such as DMS may have a similar effect. Furthermore, in the absence of nitrification, denitrification would be limited by the lack of re-formed oxidized N. The preponderance of assimilation/mineralization pathways would function as means to “short circuit” the typical N cycle and conserve nitrogen in a closed system, similar to what was proposed to occur in Ace Lake (Lauro *et al*., 2011). Denitrification appears to be the only major pathway for N loss, thus *in situ* rate measurements would be necessary to determine if it is a major factor in the Organic Lake N budget.

## Carbon cycling

**Primary production by photo and chemoautotrophy**

Oxygenic photosynthesis was presumably mediated out by phytoflagellates and diatoms (Figure 2). Rubisco, a marker gene for the Calvin carbon fixation cycle, was primarily assigned to *Chlorophyta*, consistent with this assertion. However, phosphoribulose kinase (*prk*A), another diagnostic gene of the Calvin cycle, was assigned to *Marinobacter*. This implies the *Gammaproteobacteria*, even the surface heterotrophic lineages, have the capacity for autotrophic carbon fixation. Most likely they have chemolithoautotrophic capacity such as metal oxidation. Aerobic carbon fixation which appears to be linked primarily to *Marinobacter* or Alteromondales in general and so is most abundant in the mixed zone and the very bottom sample. This is consistent with *Marinobacter* having a generalist metabolic strategy and thus many members possessing carbon fixation genes, likely involved in chemolithoautotrophic iron or manganese oxidation. Genes for respiration were abundant throughout the water column and assigned to the major heterotrophic bacterial lineages.

**High biological activity at 6.5 m**Anaerobic carbon fixation and fermentation were processes associated with the increased biological activity at 6.5 m. (\*why not at 6.7 m too?)

**Absence of C cycle genes**: Genes for methanogenesis were absent (Figure 4A). This was expected as methanogenesis usually only occurs when alternate electron acceptors are depleted, but Organic Lake sulfate concentrations in the deep zone are high (Franzman *et al.*, 1987). (\*ref paper SRB vs methanogens) Moreover, methane oxidation enzymes that were detected are not indicative of active methane production. They 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).

**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.

### Potential for lithoheterotrophy

CO is an indirect green house gas as it contributes to methane and nitrous oxide concentrations (\*refMoran). It is formed in aquatic environments during photochemical degradation of organic molecules (\*ref). Purely carboxydotrophic bacteria oxidize CO to CO2 using the enzyme CO dehydrogenase and fix a proportion of the CO2 with ribulose 1,5-bisphosphate carboxylase (RuBISCO). Alternatively, it has been proposed that roseobacters may fix CO2 by anaplerotic mechanisms (\*ref Moran 2007). Many roseobacters such as *R. pomeroyi* only possess CO dehydrogenase and are able to oxidize CO at low concentrations as an energy source and assimilate organic carbon for growth thus limiting organic carbon oxidation. (\*link CO oxidation to taxon).

### Diverse proteorhodopsin homologs are linked to most bacterial lineages

The first rhodopsin found in bacteria, termed proteorhodopsin (PR) because of its *Gammaproteobacteria* origin, 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). Related to PRs is a clade of rhodopsins linked to non-marine or coastal aquatic environments (Sharma *et al.*, 2008). Within this clade are actinorhodopsins, associated with *Actinobacteria* (Sharma *et al.*, 2008; Sharma *et al.*, 2009) and xanthorhodopsin, characterized from the sphingomonad *Salinibacter ruber* (\*ref), which 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 named for their taxonomic affiliation: MAR, OL-1, OCT, SAL, AQU and PSY (Figure S8). Only the PSY 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). All sequenced homologs that clustered with 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).

The phylogeny of most Organic Lake rhodopsins was consistent with the bacterial groups present. MAR, PSY, OCT and AQU groups clustered with homologs from *Marinobacter*, *Psychroflexus*, *Octadecabacter* and “*Candidatus* Aquiluna rubra” respectively (Figure S8), all of which are genera detected in the lake (Table S2). Xanthorhodopsin was described from the sphingomonad *Salinibacter* *ruber* (\*ref), thus the Organic Lake SAL group likely originates from *Sphingobacteria*, such as *Lewinella* or the environmental clade E6ac02 (Table S2). The abundant MAR and PSY rhodopsins has a distribution that agrees with the distribution of *Marinobacter* and *Psychroflexus* (\*Figure) further supporting to their phylogenetic origins. However, the most abundant group, OL-1, had no close homologs from GENBANK. From its high abundance and concentration on the 3.0 µm fraction, OL-1 group most likely originated from the *Rhodobacterales* including *Roseovarius* (\*Figure). Although it is possible that OL-1 was encoded by *Flavobacteria* as they are similarly abundant in the 3.0 µm fraction, all known *Flavobacteria* only possess PRs, not actino-xanthorhodopsins.In contrast, diverse rhodopsins occur in roseobacters: *Octadecabacter articus* and *O. antarcticus* have actino-xanthorhodopsins and *Alphaproteobacteria* HTCC2255 has a PR (\*ref, Moran 2007). However the occurrence of rhodopsins is highly variable with only two of the 42 sequenced roseobacter genomes encoding a rhodopsin. As most of the sequenced roseobacters are marine, apart from *Octadecabacter* it stands to reason that there is selection for rhodopsins in certain polar or coastal bacterial lineages. For example, only *Marinobacter* sp. ELB17, which is an Antarctic lake isolate possesses a rhodopsin gene. It is not present in current genomic sequences for or oil degrading *M. hydrocarbonoclasticus*, dinoflagellate symbiont *M. algicola* or particle associated *M. adhaerens* which are from the ocean surface. However, it is also not present in the Canada Basin, Artic isolate *Marinobacter* sp. BSs20148.

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 or radA, as Sharma *et al.*, 2008 which saw Punta Cormorant has 36% vs open ocean 63%).

\*Proton pump activity positions

Recently, proteorhodopsins of marine Flavobacteria and *Vibrio* have been associated with light-dependent energy generation (Gomez-Consarnau *et al*., 2007), especially under low carbon conditions (\*ref). This is a potential mechanism for conserving carbon for growth and may contribute to the success of PR bearing lineages Organic Lake. Certainly this is likely to be the case for Organic Lake *Psychroflexus* as it is both taxonomically related to *Dokdonia* and has a PR of the same phylotype. This is less clear for the otherrhodopsin groupsthey do not have well characterized relatives.If these proteorhodopsin homologs in Organic Lake add to energy generation, this would indicate mixotrophy is a common strategy in all the dominant bacterial lineages present. This may also allow them to occupy low oxygen environments.

### Aerobic Anoxygenic Photosynthesis

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).

## Molecular basis for unusual sulfur chemistry

### Absence of typical S cycle

Although Organic Lake is not sulfidic, it is possible for ‘cryptic’ sulfur cycling to occur where there is no chemical signature. Sulfate reduction can be tightly coupled to sulfide oxidation such that there is no detectable sulfide (\*Canfield *et al.*, 2010), or sulfate (Ng *et al.*, 2010; Lauro *et al.*, 2011). However, in Organic Lake, sulfur oxidation genes were undetectable and dissimilatory sulfate reduction extremely limited (Figure 4C). Consistent with this, sulfur-oxidizing *Epsilonproteobacteria* and sulfate-reducing *Deltaproteobacteria* were present at very low abundance (Figure 2 and Table S2). Despite the presence of sulfate, sulfate-reducers appear to be limited (\*which) and sulfur cycling typical in other stratified water bodies is absent. Several reasons have been suggested such as high salinity (check other saline lakes\*), oxidizing environment (\*check), cold? In the absence of sulfide, sulfur-oxidizers would be limited to utilizing other substrates (\*double check the genes for sulfur cycles\*check what else they can use).

Assimilation and mineralization are the major processes present. As described for the N cycle, Organic Lake appears to have a reduction pathways for sulfur cycling between inorganic oxidized S.

assimilatory sulfate reduction is lowest at 6.5 m, perhaps because sulfur can be assimilated from DMSP/DMS breakdown (see below).

### DMSP and DMS metabolism

Homologs of DMSP lyase genes *ddd*D, *ddd*L and *ddd*P, which catalyse the breakdown of DMSP forming DMS as a by-product, were detected in Organic Lake at levels comparable to other dominant processes such as respiration and fermentation (\*figure:DMS\_cycle). DMSP lyases are from completely unrelated enzyme families and confer the Ddd (DMSP-dependent DMS) phenotype (Curson *et al.*, 2011). The most abundant in Organic Lake, *ddd*D, comprised approximately 70% of the DMSP lyase genes. Organic Lake *ddd*D clustered with homologs with confirmed DMSP lyase activity (Figure S6) which supports their putative function as DMSP lyases. They were divided into two main *ddd*Dtypes: MAR-dddD and OL-dddD. MAR-dddD grouped with a *Marinobacter* sp. ELB17 homolog and was enriched on the 0.8 µm fraction, consistent with the distribution of *Gammaproteobacteria* including *Marinobacter* (Figure S6). OL-dddD did not cluster with good support with sequenced *ddd*D, but had highest identity (~80%) to *ddd*Dfrom *Halomonas* sp. HTNK. However, the distribution of OL-dddD did not reflect the location of *Halomonas*, which is concentrated on the 0.8 µm fraction. It comprised the majority (75%) of *ddd*Dhomologs and was restricted to the 3.0 µm fraction which suggests Alphaproteobacteria such as, *Roseovarius*, *Loktanella*, *Albimonas* and other unclassified Rhodobacterales as most likely origin.

*ddd*Lencodes a small polypeptide with unknown functional domains apart from a C-terminal cupin metal-binding pocket. To date, it has been found only in *Alphaproteobacteria*, predominantly from the *Roseobacter* clade (Curson *et al.*, 2011). Two *ddd*Lgroups were detected in Organic Lake: SUL-dddL and a MAR-dddL (Figure S7). SUL-dddL clusters with *Sulfitobacter* sp. EE-36 and other *Rhodobacteraceae* (\*check if they are from different Roseobacter clades). In *Sulfitobacter* sp. EE-36, the *dddL* gene aloneis sufficient for the Ddd phenotype (Curson *et al*., 2008). MAR-dddL forms a separate clade from the known *dddL* homologs and includes a hypothetical protein from *Marinobacter manganoxydans* MnI7-9, a deep-sea manganese oxidizer. This finding suggests MAR-dddL clade is an unrecognized branch of this enzyme family and is the first report of *ddd*Llinked to *Gammaproteobacteria*. Whether it confers the Ddd phenotype requires further confirmation. The SUL-dddL is most abundant at 6.5 m depth while MAR-dddL is most abundant at 5.7 m. Both are predominantly located on the 0.8 µm fraction, which suggests the origin of both Organic Lake *ddd*Ltypes 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

These data suggest *ddd*Dwould mediate the majority of DMSP degradation, followed by *ddd*Land *ddd*P leading to the high concentration of DMS that has been detected in bottom waters (Deprez *et al*., 1986;Franzmann *et al.*, 1987; Gibson *et al.*, 1991; Roberts & Burton 1993a; Roberts *et al.*, 1993b). This function was most likely performed by *Rhodobacterales* and *Gammaproteobacteria* such as *Marinobacter*, unclassified *Alteromonadales* and potentially *Psychromonas* and *Halomonas*. The most abundant DMSP lyase, OL-1 *ddd*D, was concentrated in the deep zone which is where the highest DMS concentration has been consistently detected. 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*A genes were detected and methanogens are absent, faster rates of DMSP production than DMS degradation would account for the high concentration in the deep zone. In addition, reduction of DMSO to DMS (\*figure) would also contribute to DMS in the deep zone. Cultures of *Halomonas* have been obtained that form DMS anaerobically from cysteine (\*ref).The enzymes for these pathways have not been defined so the presence of this pathway could not be determined. However, based on the abundance and distribution of *Halomonas*, this could be also provide a source of DMS production in the bottom waters. Degradaed algal detritus from the lake bottom is the likely source of DMSP. DMS therefore accumulates in Organic Lake as a metabolic end-product that accumulates in the absence of removal.

## Discussion points

OD1 might be involved in sulfur cycling as they are normally found in reduced environments high in sulfur compounds.

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?

Most of the bacterial lineages with cultured relatives are known to be heterotrophic aerobes (Dobson *et al.*, 1991; Gauthier *et al.*, 1992; Labrenz *et al*., 1999; Hahn *et al.*, 2004; \*). Their predominance implies the suboxic environment precludes the establishment of high numbers of strictly anaerobic bacteria. Only low numbers of strictly anaerobic bacteria were detected including *Clostridia* (primarily *Halanaerobium*) and sulfate-reducing *Deltaproteobacteria*. Known facultative anaerobes included sulfur oxidizing *Epsilonproteobacteria* that may be chemolithoautotrophic. Clearly, if the deep zone of Organic Lake is episodically oxygenated, anaerobes must have some degree of aerotolerance or form spores to endure these events. (\*check amino acid utilization).

## Acknowledgements

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