Geobiology

Geobiology (2012), **10**, 223–235 DOI: 10.1111/j.1472-4669.2012.00317.x

Microbial diversity under extreme euxinia: Mahoney Lake, Canada

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ABSTRACT

Mahoney Lake, British Columbia, Canada, is a stratified, 15-m deep saline lake with a euxinic (anoxic, sulfidic) hypolimnion. A dense plate of phototrophic purple sulfur bacteria is found at the chemocline, but to date the rest of the Mahoney Lake microbial ecosystem has been underexamined. In particular, the microbial community that resides in the aphotic hypolimnion and/or in the lake sediments is unknown, and it is unclear whether the sulfate reducers that supply sulfide for phototrophy live only within, or also below, the plate. Here we profiled distributions of 16S rRNA genes using gene clone libraries and PhyloChip microarrays. Both approaches suggest that microbial diversity is greatest in the hypolimnion (8 m) and sediments. Diversity is lowest in the photosynthetic plate (7 m). Shallower depths (5 m, 7 m) are rich in Actinobacteria, Alphaproteobacteria, and Gammaproteobacteria, while deeper depths (8 m, sediments) are rich in Crenarchaeota, Natronoanaerobium, and Verrucomicrobia. The heterogeneous distribution of Deltaproteobacteria and Epsilonproteobacteria between 7 and 8 m is consistent with metabolisms involving sulfur intermediates in the chemocline, but complete sulfate reduction in the hypolimnion. Overall, the results are consistent with the presence of distinct microbial niches and suggest zonation of sulfur cycle processes in this stratified system.

Received 12 April 2011; accepted 11 January 2012

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INTRODUCTION

Mahoney Lake in British Columbia, Canada, is 15 m deep and has a stable hypolimnion rich in sulfate, sulfide, and polysulfide (Northcote & Hall, 1983). It is a relatively simple system that may be useful as a modern analog for ocean anoxic events (Arthur *et al.*, 1988; Kuypers *et al.*, 2001; Grice *et al.*, 2005; Nabbefeld *et al.*, 2010) or as an analog to sulfidic regions in Proterozoic oceans (Canfield, 1998; Lyons *et al.*, 2009). The physical environment in the epilimnion is oxic, oligotrophic (<0.01 mg L^{-1} phosphorous; ~4 mg L^{-1} nitrogen), alkaline (pH 9), and moderately saline (28 g L^{-1} total dissolved solids, TDS). In the hypolimnion, the lake is eutrophic (~5 mg L^{-1} phosphorous; ~100 mg L^{-1} nitrogen), circumneutral (pH ~7.5), and more saline (~74 g L^{-1} TDS; Hall & Northcote, 1986). There is a corresponding increase in one order of magnitude in total alkalinity across the chemocline (Northcote & Hall, 1983). Temperature in the epilimnion ranges from frozen in winter to ~22 °C in July, while the deep hypolimnion remains isothermal at 14 °C. Density stratification maintains the thermal inversion in winter.

The Mahoney Lake hypolimnion contains one of the highest natural sulfide concentrations observed to date. Soap Lake is the only known more sulfurous lake, having ≥200 mM S^{2-} and 260 mM SO_4^{2-} (Sorokin *et al.*, 2007). Mahoney Lake is in comparison more sulfate-rich (400–500 mM) and sulfide-poor (30–35 mM; Overmann *et al.*, 1996a). Other well-studied, stratified lakes such as Fayetteville Green Lake (1.2 mM S^{2-}, 14–15 mM SO_4^{2-}; Brunskill & Ludlam, 1969), Solar Lake (1 mM S^{2-}, 130 mM SO_4^{2-}; Jorgensen *et al.*, 1979), and Lake Cadagno (~1.2 mM each S^{2-} and SO_4^{2-}; Tonolla *et al.*, 2000) are much less sulfurous. However, in all of these lakes, the S^{2-}:SO_4^{2-} ratios <1 preclude any

thermodynamic inhibition of sulfate reduction, regardless of the presumed organic electron donor (Amend & Shock, 2001). This suggests that all should experience active sulfate reduction in their hypolimnia, unless the absolute concentration of sulfide itself (sulfide toxicity) is inhibitory.

The sulfidic chemocline of Mahoney Lake enables growth of a dense floating layer (or 'plate') of phototrophic bacteria (Overmann *et al.*, 1991), which is stable at 7 m year-round (Overmann *et al.*, 1996b). This layer contains abundant *Chromatiaceae* (purple sulfur bacteria), primarily *Lamprocystis purpurea* (Overmann *et al.*, 1991, 1994; Imhoff, 2001). The *L. purpurea* layer acts as a 'nutrient cap,' limiting the upwelling of nutrients; and beneath it light does not penetrate significantly (Overmann *et al.*, 1994). Enrichment cultures from the plate suggest there are very few *Chlorobiaceae* (green sulfur bacteria). The lake sediments are correspondingly rich in the pigment okenone (Overmann *et al.*, 1993), a biomarker for *Chromatiaceae* (Schaeffer *et al.*, 1997), and they lack isorenieratene, a biomarker for *Chlorobiaceae* (Repeta, 1993; Koopmans *et al.*, 1996). Analogously, okenone diagenetic products in sediments and sedimentary rocks are diagnostic of ancient, highly euxinic systems with penetration of sulfide only to shallow depths within the photic zone (Brocks *et al.*, 2005).

Despite its potential value as an analog for ancient conditions, little information has been reported on other Mahoney Lake micro-organisms. Incubations suggest that in summer, phototrophic activity in the plate may not be sufficient to explain observed sulfide oxidation rates (Overmann *et al.*, 1994). This disparity implies the presence of additional chemoautotrophic sulfide oxidizers, although these remained unidentified (Overmann *et al.*, 1994). Prior to our study, the microbes residing in the aphotic hypolimnion or in the lake sediments also remained unknown, in particular the location and type of active sulfate reducers. (Coolen & Overmann, 1998) suggested that the deep lake might be inactive due to the absence of gradients in the sulfate and sulfide profiles and a general abundance of fragmented DNA in sediments. In support of this argument is the observation that much of the sulfide used as photosynthetic electron donor appears to be supplied by sulfate reduction from within the bacterial plate, rather than from the deeper lake (Overmann *et al.*, 1994, 1996b). These arguments suggest that the plate could be a stable ecosystem in which sulfur species are both the dominant electron donors for photoautotrophy and the dominant electron acceptors for heterotrophic respiration. The latter (sulfate) would be sourced by upwelling, while the former (sulfide) would be produced *in-situ*. Such an arrangement could imply that biological activity in euxinic waters primarily is confined to the photic zone, and the sulfide concentration of deeper waters would be a consequence of plate activity, rather than an enabler of plate growth. In contrast, we hypothesized that each horizon of Mahoney Lake would mediate distinct functions, with sulfate reduction persisting both within the photosynthetic plate and at depth.

To distinguish between these options, it is critical to determine the diversity and activity of the Mahoney Lake microbial community. Here we focused on diversity by profiling distributions of 16S rRNA genes using PhyloChip, a high-density microarray (Brodie *et al.*, 2006; Desantis *et al.*, 2007), and comparing the results to 16S rRNA gene clone libraries. PhyloChip can detect >10 000 different prokaryotic OTUs (operational taxonomic units). Recent applications of PhyloChips reveal their suitability for comparative analysis, including applications to microbial fuel cells (Wrighton *et al.*, 2008), differential characterization of lung bacteria in cases of cystic fibrosis and pneumonia (Flanagan *et al.*, 2007; Klepac-Ceraj *et al.*, 2010), and characterization of uranium and iron-reducing communities (Brodie *et al.*, 2006; Tokunaga *et al.*, 2008). We surveyed the Mahoney Lake mixolimnion (5 m), chemocline (7 m), hypolimnion (8 m), and sediments to yield a semiquantitative picture of all four horizons. Both types of diversity data – PhyloChip and clones – show that all levels of the lake, including the sediments, contain unique microbial assemblages. This suggests that the deep lake does have a biogeochemical role in maintaining the ecosystem.

METHODS

Detailed methods are given in Supporting Information. A brief summary is given below.

DNA samples

Water and sediments were sampled from Mahoney Lake (49°17′N, 119°35′W) in July 2008 (Fig. 1) and were frozen immediately on dry ice (−70 °C). DNA was extracted and purified using a bead-beating protocol (Lysis Matrix B, MP Bio) combined with the AllPrep Qiagen RNA/DNA Isolation Kit (Qiagen, Valencia, CA, USA). DNA yield and purity were assessed by gel electrophoresis and Nanodrop, and 16S rRNA genes were amplified using the bacteria-specific primer set 27F and 1492R (Lane, 1991) as described previously (Klepac-Ceraj *et al.*, 2010). Libraries were cloned using the standard protocol from the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) followed by sequencing using an ABI3700 (Applied Biosystems, Inc., Carlsbad, CA, USA). All sequences shorter than 450 base pairs were removed from further analyses and the remainder grouped into OTUs based on 97% sequence identity. This sequence cutoff was chosen to facilitate comparison with taxa detected by PhyloChip, which uses the same cutoff. Sequences were checked for putative chimeras using Bellerophon version 3 (Huber *et al.*, 2004).

Phylogenetic trees

Aligned sequences were imported into the Greengenes database using the ARB software suite, and maximum likelihood trees were constructed in ARB (Ludwig *et al.*, 2004; Desantis

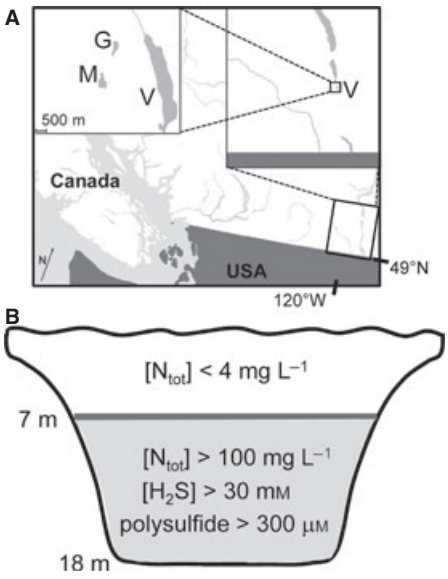

Fig. 1 Location (A) and geochemical cross-section (B) of Mahoney Lake, Canada. Water column data based on Northcote & Hall (1983) and Overmann *et al.* (1996a,b). The purple sulfur bacteria (PSB) plate is located at 7.0 ± 0.1 m and has >10^8 cells mL^{-1} (Overmann *et al.*, 1991). Letters in (A) stand for Vaseux Lake (V), Green Lake (G), and Mahoney Lake (M).

et al., 2006a,b; Stamatakis *et al.*, 2008). Bootstrap values >50 (of 100 replicates) are shown.

PhyloChip

For each sample, we spiked 250 ng of pooled 16S rRNA gene amplicons, corresponding to approximately $\sim 1.5 \times 10^{11}$ rRNA gene copies, with a mix containing a known concentration of amplicons as internal standards (Brodie *et al.*, 2007). The combined mixture was biotin labeled, denatured (99 °C for 5 min), and hybridized to the PhyloChip at 48 °C for 16 h at 60 rpm. Arrays were washed, stained, and scanned using a GeneArray Scanner (Affymetrix) as described previously (Brodie *et al.*, 2006). PhyloChips were processed using the PhyloTrac software package (http://phylotrac.org/Home.html) (Schatz *et al.*, 2010). A taxon is considered to be 'present' in a sample when the number of positive probe pairs divided by the total number of probe pairs in a probe set was equal or >0.9 (Brodie *et al.*, 2006). Hybridization values were converted to gene copy number using a formula derived from a Latin square assay described by Brodie *et al.* (2007).

Data analysis

Rarefaction (the Rarefaction Calculator; http://www2.biology.ualberta.ca/ jbrzusto/rarefact.php), Chao-1 nonparametric species richness estimator (Chao, 1987; Hughes *et al.*, 2001), community richness indices (ACE-1, Shannon and Simpson index), as well as estimation of the taxa shared between the libraries (Bray & Curtis, 1957) were calculated using EstimateS (Colwell, 2009). Clone libraries were compared using Library Compare tool within the Ribosomal Database Project website (Maidak *et al.*, 2001; Cole *et al.*, 2007).

RESULTS AND DISCUSSION

Diversity

We obtained a total of 717 16S rRNA gene clones from three different water depths, 5 m, 7 m and 8 m, as well as lake bottom sediment; representing 63, 62, 110, and 82 unique OTUs, respectively (Table 1). Total sequence diversity as calculated by the Chao-1 nonparametric estimator suggested that the 5-m sample is the least diverse with 119 predicted OTUs (SD ± 26) (Hughes *et al.*, 2001). In contrast, the 7 m, 8 m, and sediment samples ranged from 222 to 300 predicted OTUs (Table 1). Shannon and Simpson indices were higher for both 8 m and sediment when compared with 5 m and 7 m (Table 1). We performed additional rarefaction analyses to estimate how completely the libraries were sampled. None of the four datasets reached an asymptote (Fig. 2). All of the diversity estimators other than Chao-1 clearly divide the ecosystem into two lower-diversity horizons (5 and 7 m) and two higher-diversity horizons (8 m and sediment). The number of bacterial phyla found at each depth reflects these results: 12, 10, 20, and 22 phyla at 5 m, 7 m, and 8 m, and sediments, respectively (Fig. 3A, Table S1).

There also was little overlap of individual OTUs between the depths. The two most similar samples are 8 m and sediments (23% shared OTUs), while the least alike are 7 m vs. sediments (1.7% shared OTUs) and 5 m vs. sediments (1.9% shared OTUs), as calculated using Bray–Curtis similarity distances for pairwise comparisons of sampling depths (Fig. 4) (Bray & Curtis, 1957).

We detected more phyla across the four samples using PhyloChip (44 total phyla) than we did using clone libraries (26 total phyla) (Table S1; Fig. 3B). The number of abundant phyla (those contributing ≥ 0.5% of the 16S hybridization signal) detected by PhyloChip follows a pattern and composition similar to the clone libraries: 13 phyla in the 5 m sample, 9 in the 7 m sample, 14 in the 8 m sample, and 16 in the sediments (Fig. 3). The distribution of OTUs within the PhyloChip analyses was dominated in all cases by a long 'tail' of very minor species (e.g., (Sogin *et al.*, 2006) (Fig. S1), showing that PhyloChip reveals both the abundant and the

Table 1 Statistics for 16S clones, calculated by EstimateS (version 8.2.0), ©R. K. Colwell: http://viceroy.eeb.uconn.edu/EstimateS

Sample	Clones	Uniques	ACE	Chao1	Shannon(H)	Simpson
5 m	167	63	113	119 ± 26	3.65	27.67
7 m	201	62	192	222 ± 89	3.34	14.69
8 m	201	110	282	295 ± 64	4.36	67.22
Sediment	148	83	272	300 ± 97	4.05	43.69

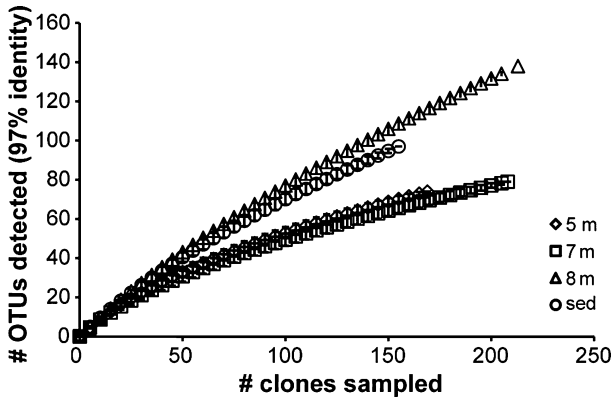

Fig. 2 Rarefaction curves for Mahoney Lake 16S rRNA gene clones grouped at 97% sequence identity.

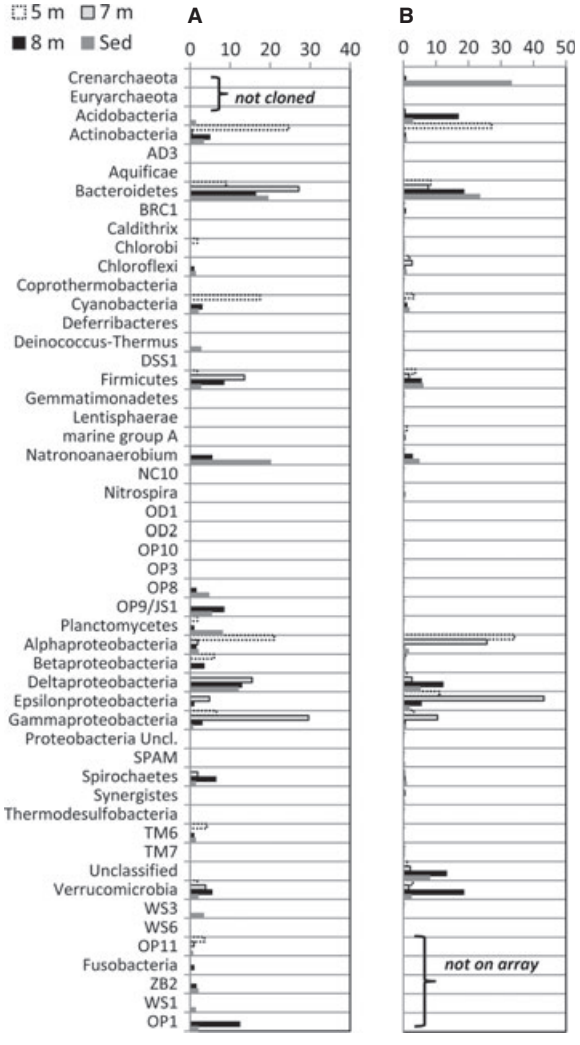

Fig. 3 Phylum distribution of 16S rRNA gene clones (A) and PhyloChip abundance (B) for each depth sampled. All rows represent phyla detected by at least one method: PhyloChip (above experimentally determined detection limits) or clone library.

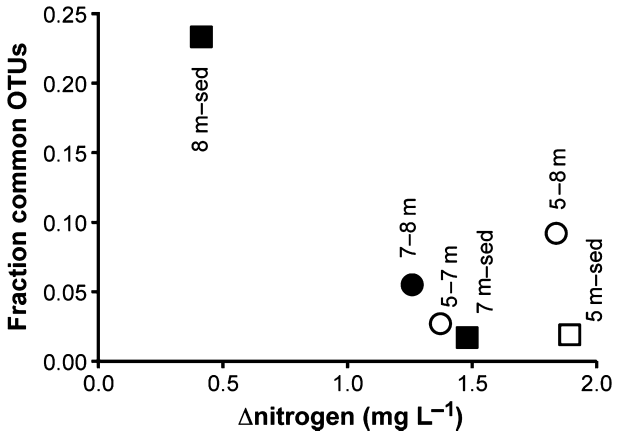

Fig. 4 Bray–Curtis similarity index, or the fractional commonality of species, for all pairwise comparisons of samples as based on 16S rRNA gene clones. Hollow symbols show comparisons between the 5-m (oxic) sample and other samples; solid symbols show comparisons between all anoxic samples; circles show comparisons between water samples; and squares show comparisons between waters and sediment. Nutrient data based on Northcote & Hall (1983).

rare taxa. PhyloChip has a detection range corresponding to nearly five orders of magnitude of DNA copy number abundance (Brodie *et al.*, 2007), which is equivalent to pyrosequencing (http://www.454.com) in the range of 10 000–100 000 sequence reads.

At the taxon level, PhyloChip detected a total of 1100 OTUs across the four samples. Within each layer, the number of phylotypes above detection limit was similar (912, 934, 881, and 862; in 5 m, 7 m, and 8 m, and sediment, respectively). Although the number of phylotypes detected in each sample corresponds inversely to the diversity estimates obtained from the clone libraries, with the sediment appearing slightly less diverse, this apparent relationship is an artifact of the cutoff threshold for total hybridization signal and has no environmental significance. Across the four samples, there was some overlap among the dominant 50 OTUs detected at each depth (Fig. S2). If the system had no overlap between horizons, the total would be 200 OTUs in Fig. S2. Instead, the total is only 132. The greatest number of shared OTUs is observed between the 8 m and sediment samples, in agreement with the results for the clone libraries.

Consistent with our results, a recent study has shown that euxinic deep lakes generally are more taxonomically diverse than surface layers (Barberan & Casamayor, 2011). These authors suggest that permanent stratification may promote the formation of complex, endemic communities. Such stratification is consistent with our assertion that the hypolimnion of Mahoney Lake should contain an actively maintained community. Our results further suggest that the rank order of similarity among the depths may scale with differences in geochemistry: A greater fractional difference in nutrient concentration generally means a greater difference in species composition (Fig. 4). However, at this point, we cannot

exclude that the observed population differences also could result from availability and variability of energy sources, and/or viral or grazing pressures.

The significant taxonomic differences between the depth zones of Mahoney Lake are apparent at the phylum level as well as at the level of individual taxa (Fig. 3, Table S1). For the clone library, the four most represented phyla at 5 m were: Actinobacteria (25%), Alphaproteobacteria (21%), Cyanobacteria (17%), and Bacteroidetes (9.0%). Actinobacteria and Alphaproteobacteria were specific to the 5-m depth as determined by Library compare tool in Ribosomal Database Project (RDP) (both with $P < 0.001$). Most of the sequences at 7 m grouped with Gammaproteobacteria (30%), Bacteroidetes (27%), Deltaproteobacteria (16%), and Firmicutes (14%). Clones from 8 m were dominated by Bacteroidetes (16%), Deltaproteobacteria (13%), the uncultured phylum OP-1 (12%), and Firmicutes (8.5%). The sediment sequences were dominated by Natronoanaerobium (20%), Bacteroidetes (19%), Deltaproteobacteria (12%), and Planctomycetes (8%). For comparison to PhyloChip (below), it is important to note that we did not attempt to clone Archaea.

PhyloChip results agree that some phyla are dominantly found in only one layer (Fig. 3B, Table S1). Actinobacteria (27% of hybridization signal) and Cyanobacteria (3%) appear almost exclusively in the surface waters. Alphaproteobacteria give large signals both in surface waters (34%) and at 7 m (26%). Epsilonproteobacteria (43%) and Gammaproteobacteria (10%), also are abundantly detected at 7 m. Acidobacteria are found maximally at 8 m, as are Deltaproteobacteria, Verrucomicrobia, Nitrospira, and the uncultured phyla BRC1, OP10, and TM7. In the sediment, Crenarchaeota (32%) is the dominant phylum, followed by Bacteroidetes, Firmicutes, and Natronoanaerobium. As with clone libraries, Bacteroidetes and Firmicutes are widespread at all depths.

Both types of data correspond well to expectations for lacustrine environments. Exceptions are the beta-proteobacterial fraction, which is lower (6%) compared with the general average of 20 ± 15% for lacustrine environments, and the alphaproteobacterial contribution, which is higher (21%, compared with an average of 13 ± 5%) (Barberan & Casamayor, 2010). Although our data are still within one standard deviation of reported values and as such are consistent with the global trends in freshwater, the trend of the observed differences is toward a typical 'marine' endmember. This may be influenced by either the saline or the sulfurous conditions of the lake–conflated variables that cannot be decoupled.

The presence of chemoautotrophic sulfide oxidizers in addition to photoautotrophic sulfide oxidizers previously had been implied, but not verified, for Mahoney Lake (Overmann *et al.*, 1994). Such organisms are common in other lakes with oxygen–sulfide interfaces (Tonolla *et al.*, 1999, 2003; Humayoun *et al.*, 2003; Banciu *et al.*, 2004; Sorokin *et al.*, 2007). Our clone libraries and PhyloChip data confirm that such species are overwhelmingly detected at 7 m depth (Fig. 3). The data also suggest more generally that most of the sequences retrieved from 7 m, 8 m, and sediment samples could be associated with taxonomic groups previously found to be common in sulfurous environments. The following discussion examines these organisms and their putative functions in the different stratified zones. Because the detected OTUs often grouped with uncultured phylogenetic clades, in many cases, we can only speculate on the physiological roles of these organisms based on their nearest taxonomic relatives.

Epilimnion and chemocline

Community stratification is reflected in the taxa unique to or predominantly found only in one horizon (Table S1, Fig. S2). Cyanobacteria were common at 5 m, although there were a few additional instances at 8 m, presumably because of sinking material. The vast majority of cloned sequences are 97–99% similar to the marine cyanobacterium *Synechococcus* sp. WH 8101, which was isolated from a coastal environment and classifies within a halotolerant division of *Synechococcus* spp. (Armbrust *et al.*, 1989). In contrast, the majority of the 'cyanobacterial' signal detected by PhyloChip was from cross-hybridization of eukaryotic chloroplasts, because our samples were not pre-filtered to remove larger (algal and plant) cells (Fig. S2). The aerobic Actinobacteria also appear almost exclusively in the surface waters. Most grouped with *Microbacteriaceae*, a genus recently associated with freshwater zooplankton (Grossart *et al.*, 2009).

The Alphaproteobacteria detected predominantly at 5 m are related to bacteriochlorphyll-*a* containing strains previously isolated from the surface of Mahoney Lake (Yurkova *et al.*, 2002), including the obligately aerobic photoheterotrophs *Porphyrobacter meromictius* and *Roseicyclus mahoneyensis,* which are both salinity- and sulfate-tolerant (Rathgeber *et al.*, 2005, 2007). Alphaproteobacterial (chemosynthetic) sulfur oxidizers also could be detected at 5 m, with some PhyloChip signal observed for the OTU of aerobic *Sulfitobacter* spp., which are capable of oxidizing sulfite (Pukall *et al.*, 1999). Sequences of Gammaproteobacteria detected at 5 m do not group with those from the deeper layers and do not appear to include sulfide-oxidizing photoautotrophs (Fig. 5A). Instead they cluster with sequences associated with other saline environments such as Guerrero Negro hypersaline microbial mat (Ley *et al.*, 2006) or with *Marinobacterium halophilum*, a bacterium isolated from Yellow Sea (Chang *et al.*, 2007). Overall, the environment at 5 m is rich in obligate aerobes and includes some species capable of aerobic oxidation of sulfur cycle intermediates, but this layer otherwise appears to be poor in sulfide-oxidizing phototrophs.

Organisms from multiple phyla with known involvement in the sulfur cycle dominated at 7 m. Fifty-nine of the 76 total gammaproteobacterial sequences obtained from clone libraries were from 7 m and clustered with *L. purpurea* (Fig. 5A) (Imhoff, 2001). This species previously was reported

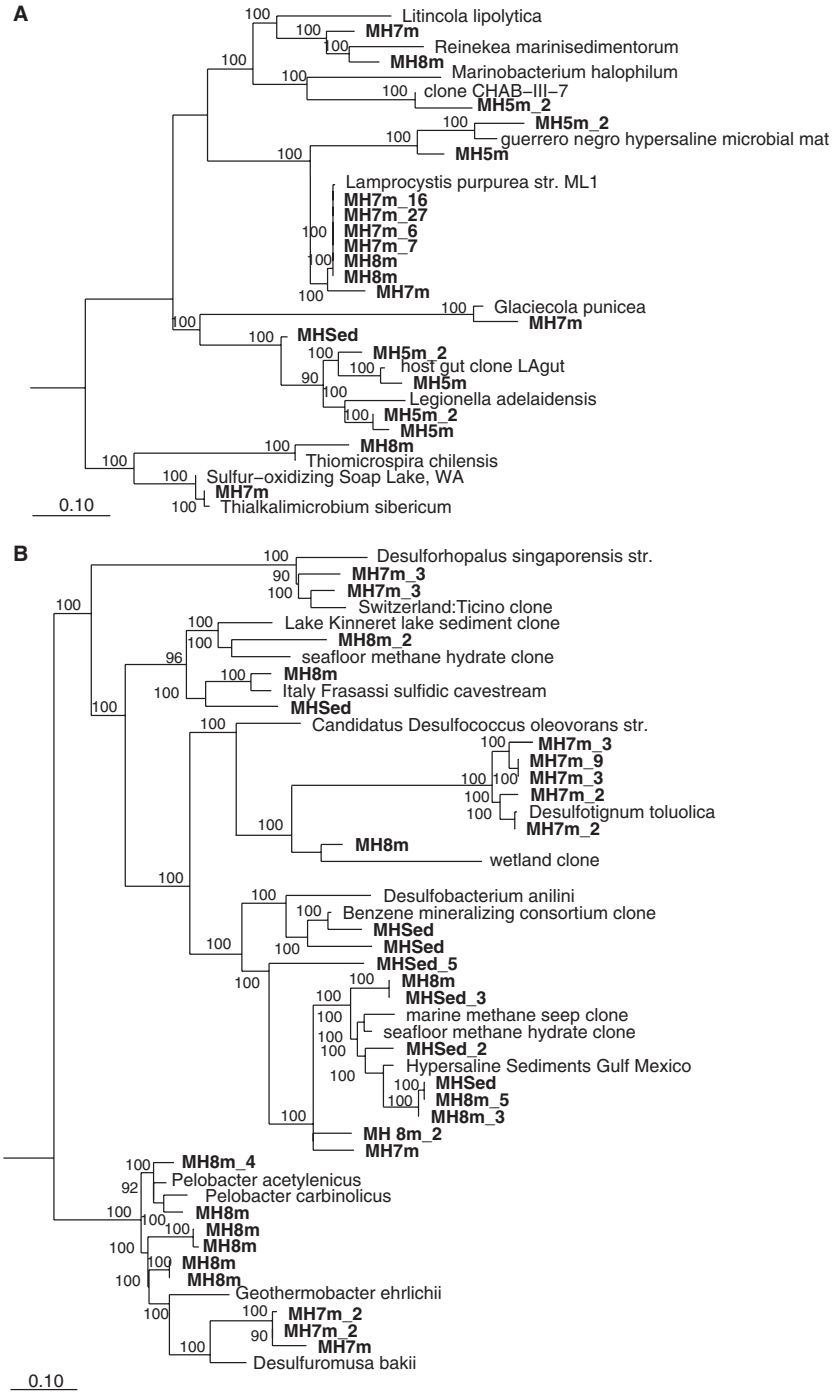

Fig. 5 Phylogenetic trees of 16S rRNA gene clones, coded by depth, for Gammaproteobacteria (A), and Deltaproteobacteria (B).

to dominate the Mahoney Lake chemocline (Overmann *et al.*, 1991, 1994), and it primarily uses sulfide as an electron donor (Imhoff, 2001). PhyloChip revealed the presence of another purple sulfur bacterium at 7 m, an okenone-producing *Thiocapsa litoralis* capable of photolithoautotrophic growth with sulfide, thiosulfate, sulfite, and elemental sulfur as electron donors (Puchkova *et al.*, 2000). Similar species of sulfide-oxidizing phototrophs also are found at the chemocline boundary of other meromictic lakes, for example Lake Cadagno and Fayetteville Green Lake (Tonolla *et al.*, 1999, 2003).

PhyloChip also identified additional organisms associated with sulfur cycling at 7 m that were not detected as abundantly in the clone libraries. Most notably, there was a strong signal for members of the Epsilonproteobacteria,

specifically those most closely related to sulfur-oxidizing *Helicobacteraceae* and sulfur-reducing *Campylobacteriaceae* (*Sulfurospirillum deleyianum*) (clone library signal for Epsilonproteobacteria = 4.8%, while PhyloChip signal = 43%). *Helicobacteraceae* are chemoautotropic sulfide and sulfur oxidizers. *Sulfurospirillum* spp. are notable for their flexible sulfur metabolisms: They can utilize elemental sulfur or thiosulfate, but not sulfate, as electron acceptors and also are capable of lithotrophic growth on sulfide as electron donor (Eisenmann *et al.*, 1995; Sikorski *et al.*, 2010). They can grow syntrophically in association with green sulfur bacteria (Wolfe & Penning, 1977), suggesting an analogous syntrophy with purple sulfur bacteria may occur in Mahoney Lake. PhyloChip also detected *Arcobacter* spp. (>5%; Fig. S2), some of which are autotrophic sulfide oxidizers that produce filamentous sulfur (Wirsen *et al.*, 2002). Other *Arcobacter* spp. include the nitrogen-fixing *Arcobacter nitrofigilis* (Pati *et al.*, 2010), sulfide-oxidizing *Arcobacter sulfidicus* (Sievert *et al.*, 2007), and the sulfide-oxidizing obligate halophile *Arcobacter halophilus* (Donachie *et al.*, 2005). The presence of all of these groups at 7 m could explain the observed excess of sulfide oxidation relative to rates of phototrophy in Mahoney (Overmann *et al.*, 1994). But notably, the commonality of the community at 7 m may be its flexibility to cycle sulfur intermediates, as well as oxidize sulfide.

Both the clone library and microarrays indicate a high diversity of Deltaproteobacteria at and below the chemocline, but the data show that each layer harbors different taxa. Over 60% of deltaproteobacterial clones from 7 m are associated with the genus *Desulfotignum* (Fig. 5B). Other sequences from this depth belong to *Desulforhopalus* and *Desulfuromusa* genera. The localization of *Desulfotignum* to 7 m, i.e., apparently disfavoring 8 m, is intriguing. Known members of this group are capable of oxidizing organic substrates all the way to CO_2 by utilizing sulfate, sulfite, and thiosulfate as terminal electron acceptors (Kuever *et al.*, 2001). Another group found only at 7 m was organisms grouping with *Desulfuromusa bakii*, which can reduce elemental sulfur to sulfide. Again, the community distribution in the chemocline is rich in species with putative metabolisms favoring sulfur cycle intermediates. Processes occurring in the chemocline appear to be dominated by phototrophic sulfide and sulfur oxidation – perhaps coupled syntrophically to organotrophs cycling sulfur and thiosulfate – in addition to conventional sulfate reduction.

Hypolimnion and sediments

Micro-organisms grouping with sulfate-reducing Deltaproteobacteria were more prevalent as a fraction of the total community in the 8-m sample (Figs 3 and S2). How this may translate to quantitative cell numbers or to sulfate-reducing activity cannot be inferred from our data at this time. But when viewed in terms of relative importance within 8 m and sediments, relative to 7 m, it suggests that sulfate reduction potentially is the dominant energy metabolism in the hypolimnion. Rate and activity measurements should be done to test this idea. Taxonomically, in the 8 m and sediment samples, there is a relative absence of OTUs associated with known sulfur intermediate utilizers, but a relative abundance of the strictly anaerobic chemoorganotroph *Desulfobulbus* genus. Members from this genus reduce sulfate completely to H_2S (Widdel & Pfenning, 1982). We suggest these taxa may be involved primarily in complete sulfate reduction, supplying H_2S to be oxidized by other species to sulfur intermediates, which are then recycled in the 7-m layer by the other diverse sulfur cycling microbes detailed above.

Many other Deltaproteobacteria OTUs also were detected by both clone library and PhyloChip. Most do not have any closely related cultured relatives, but do have <1% nucleotide difference from clones recovered from other saline, sulfur-rich, but geographically disparate regions such as marine methane seeps, or seafloor methane hydrate or hypersaline sediments from Gulf of Mexico (Ley *et al.*, 2006; Lloyd *et al.*, 2006). These putatively sulfate-reducing bacteria comprise their own endemic clades, sharing only low similarities with previously cultured Deltaproteobacteria (Fig. S2).

Overall, the sulfate reducers in the hypolimnion and sediments may participate in total community metabolism by acting as hydrogen sinks for fermentors. Among these may be the Firmicutes and Acidobacteria identified predominantly at 8 m. Although Acidobacteria is a newly characterized phylum with few cultivated isolates (Thrash & Coates, 2011), it appears to be a metabolically and phylogenetically diverse group abundant in natural ecosystems (Quaiser *et al.*, 2003). For example, isolates belonging to genera *Geothrix* and *Halophaga* are anaerobic and capable of fermenting a number of different compounds, with *Halophaga* having strictly anaerobic metabolism that is obligatively fermentative (Liesack *et al.*, 1994; Coates *et al.*, 1999). Our results are consistent with other reports (c.f., Barberan & Casamayor, 2011) and suggest a considerable affinity of some Acidobacteria for anoxic environments. Anaerobic clades of Acidobacteria are thought to be the major source of environmentally diagnostic bacterial lipids found abundantly in other anoxic systems such as peat bogs (Weijers *et al.*, 2006; Damste *et al.*, 2011). Another group dominant at 8 m is Natronoanaerobium, which, although classified as a phylum by PhyloChip, sometimes is classified as a genus within the Firmicutes (class, Clostridia); fermentation is the dominant metabolism of Clostridia. Interestingly, Clostridia are the other major taxonomic group also known to produce the same type of distinctive lipids (Jung *et al.*, 1994). Natronoanaerobium spp. have been characterized as halophilic, strictly anaerobic, and thermophilic, although new mesophilic members have been also discovered (Sorokin & Muyzer, 2010).

For both the 8 m and sediment sample, there also was a strong signal (between 6 and 20% of total microbial community) for genera within the order Bacteroidales, specifically

those reported previously to be associated with sulfur-rich mucus secretions of the hydrothermal vent polychaete *Paralvinella palmiformis* (Alain *et al.*, 2002). This contrasts with genera within the order Flavobacteriales and Cytophaga – including sequences grouping closely with sequences isolated from the surface waters (2 m) of Mono Lake (Humayoun *et al.*, 2003) – which are more prevalent in the 5- and 7-m samples. This contrast shows that the apparent ubiquity of Bacteroidetes masks a depth-stratified diversity among members of this phylum.

Finally, the 8-m PhyloChips also revealed a strong signal for *Verrucomicrobia* (>18% total relative abundance), with related phylotypes mostly being identified from anoxic or marine environments. On the basis of culture-independent surveys, it has become apparent that Verrucomicrobia are ubiquitous across a wide range of marine and terrestrial systems, including sulfide-rich waters and sediments as well as the hypolimnion of Mono Lake (Freitag & Prosser, 2003; Humayoun *et al.*, 2003; Schlesner *et al.*, 2006), but their role in anaerobic environments and/or their metabolic interdependencies with the sulfur cycle remain unknown.

Finally, although we examined Archaea only by PhyloChip, this domain was nearly exclusive to the sediments and was dominated by Crenarchaeota (including OTUs now reclassified as Thaumarchaeota; (Brochier-Armanet *et al.*, 2008). The dominant PhyloChip classification of the detected Crenarchaeota OTUs is Marine Group 1a. Group 1a Crenarchaeota (Thaumarchaeota) are ubiquitous components of marine phytoplankton (Delong, 1992; Fuhrman *et al.*, 1992), and these species generally are regarded – based on presence of archaeal ammonia monooxygenase (*amoA*) genes and experiments on pure cultures – to be ammonia oxidizers (Venter & Al, 2004; Konneke *et al.*, 2005; Treusch *et al.*, 2005). However, to date, all archaeal ammonia oxidation has been shown to be aerobic or microaerophilic (Francis *et al.*, 2007). Crenarchaeota from other clusters, such as Miscellaneous Crenarchaeota Group, have been found in anoxic sulfurous lakes (Lliros *et al.*, 2008), but this group is not well-represented on the PhyloChip. The closest hit is <85% identical based on the comparison of their 16S rRNA genes. It is also possible that at the 97% OTU taxonomic level of PhyloChip, the sequences detected in Mahoney sediments actually derive from another major sedimentary group of Crenarchaeota – e.g., Marine Benthic Group A (MBG-A) (Teske & Sorensen, 2008) – which to date has unknown physiology. Whether these Crenarchaeota are involved in the sulfur cycle in Mahoney Lake sediments remain to be investigated.

Comparison of PhyloChip results to clone libraries

For both approaches, we used the same DNA extracts, enabling comparison of the two techniques. By PhyloChip, Cyanobacteria were detected as a lesser proportion of the total community, while Epsilonproteobacteria and Alphaproteobacteria were both detected in greater proportion. PhyloChips also show more Acidobacteria and Verrucomicrobia compared with the clone libraries, but give approximately equal abundances of Deltaproteobacteria, Bacteroidetes, and Firmicutes. The microarrays from the sediment sample are difficult to compare with clone library results, as we did not sequence Crenarchaeota in the clone libraries. Although it is clear that each method must be showing some selective biases and that the results therefore are semiquantitative, there is a general consistency of the results between the clone library and the microarray data. In addition, correspondence analysis of the PhyloChip data (Fig. 6) confirms that the two most similar communities are in the 8 m and the sediment samples. Correspondence analysis of the clone library data resulted in a similar degree of spatial separation between the locations, with the 5- and 7-m samples located distantly from the more similar 8 m and sediment samples (not shown).

All 16S rRNA gene-based techniques have biases, so a combined approach offers distinct advantages. Clone libraries offer continuous long reads of sequences, which are essential when sampling unique and extreme environments such as Mahoney Lake where novel micro-organisms are certain to be present. The OTUs of such organisms are unlikely to be found among the species included on a PhyloChip microarray, which by definition can include only taxa that have been recognized previously. Furthermore, although cloning bias sometimes overlooks dominant species (e.g., Polz *et al.*, 1999), this approach is usually successful in identifying most of the dominant members of mixed communities and is broadly consistent with amplification-independent approaches (Rappe & Giovannoni, 2003; Venter & Al, 2004; Acinas *et al.*, 2005; Cottrell *et al.*, 2005; Delong & Karl, 2005; Moeseneder

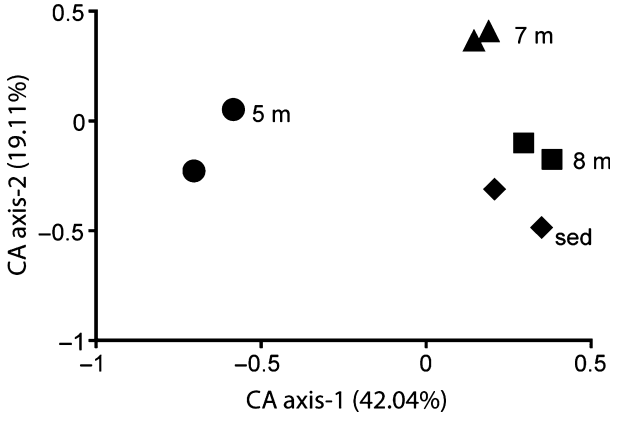

Fig. 6 Correspondence analysis (CA) of the Mahoney Lake microbial community at the four analyzed depths. Separate analyses of two PhyloChip replicates are shown and are labeled by sample depth. The percentages of variation described by the correspondence analysis coordinates are shown in parentheses.

et al., 2005; Not *et al.*, 2009; Feingersch *et al.*, 2010). It is therefore generally accepted that the major limitation of the clone library approach is the small amount of data that are generated. Clone libraries consistently underpredict total diversity because of the relatively small number of sequences for statistical metrics (Sogin *et al.*, 2006). In contrast, PhyloChip identifies presence of an organism by the threshold of hybridization to combinations of oligonucleotides representing >10 000 species (at the 97% threshold). By avoiding cloning bias and having a calibrated fluorescence signal, this method is more quantitative than a clone library, with amplification bias being the primary concern. When we compare the results from the PhyloChips with the results from the clone libraries, the overall outcomes are quite similar (Fig. 3). Similar discrepancies between these two methods have been reported elsewhere (Lemon *et al.*, 2010). A combination of amplification bias, cloning bias, and/or presence/absence of OTUs on the PhyloChip can explain our cases of apparent under-detection (e.g., Chromatiales) or over-detection (e.g., Epsilonproteobacteria) of certain groups by PhyloChip.

A different artifact could result from possible cross-hybridization of sequences to PhyloChip probes or lack of a perfect match. One of the biggest surprises in our data was that the 7-m microarray did not yield the highest relative abundance signal for *L. purpurea*. *L. purpurea* was the most abundant clone and is visibly abundant based on its pigment composition (Overmann *et al.*, 1994, 1996a,b). Instead, *Xanthomonadaceae* was the most abundant family of Gammaproteobacteria detected at 7 m. It is possible that this signal actually stems from *L. purpurea*. Ten of 13 probes on the array that map to the *Xanthomonadaceae* OTU for *Dyemonas todaii* str. XD10 also have a perfect match to the *L. purpurea* sequence. The three non-matching probes have only a single nucleotide mismatch (C to A). Thus, it is possible that the target sequence of *L. purpurea* successfully hybridized array probes designated as *D. todaii*. To confuse matters further, the *L. purpurea* target sequences on the microarray were derived from *L. purpurea* strain ML-1, which was originally isolated from Mahoney Lake. However, to our surprise, the *L. purpurea* ML-1 sequence does not match our most dominant *L. purpurea* sequence from the 7-m clone libraries. We found no errors upon manual inspection of our sequencing chromatograms. Therefore, it is likely that the cultivated *L. purpurea* ML-1 isolate was either a minor member of the *Lamprocystis* spp. assemblage residing at Mahoney Lake or that the original sequence contained errors that were then propagated to the microarray probes.

CONCLUSIONS: IMPLICATIONS FOR SULFUR CYCLE

Microbial community analysis of euxinic Mahoney Lake suggests the entire lake participates in maintaining an active sulfur cycle, with different metabolisms supported at each depth studied:

1 Microbial diversity increases with depth in the lake, with the hypolimnion and sediments showing greater diversity than the chemocline and epilimnion.

2 Community composition at each depth is unique, but there are many taxa affiliated with known sulfur cycling species throughout every zone of Mahoney Lake.

3 Taxonomic distinctions among Deltaproteobacteria detected in each layer may point to different putative functions associated with these groups. The results may suggest that complete sulfate reduction dominates at depth, while cycling of sulfur intermediates may be favored at the chemocline.

4 Clone library and PhyloChip analyses yielded similar results in this diversity study. However, the PhyloChip results were limited by absence of or sequence dissimilarity between Mahoney Lake species and species printed on the existing microarray.

This work highlights the challenge of determining links between biological diversity, functional diversity, and biogeochemical complexity. In the future, this challenge may be approached by new techniques that allow simultaneous detection of metabolic activity and molecular identification of micro-organisms (Jehmlich *et al.*, 2008; Dekas *et al.*, 2009). Such data may provide insights into how specific environmental factors select for the presence of one population over another. It also will be important to carry out comparative, laboratory-based as well as environmental studies, perhaps combined with genomics and proteomics, to elucidate how the community maintains the stability of the ecosystem and its geochemical cycles.

ACKNOWLEDGMENTS

We thank Chris Reinhard, Noah Planavsky, Ben Gill and Anthony Chappaz for their help with sample collection and other aspects of the fieldwork; Eoin Brodie and Yvette Piceno for their help with the PhyloChip setup, and Harvard University's LS100r students for their help with the data analyses. We thank the editors at Geobiology and three excellent reviewers for their feedback. This work was supported by The David and Lucille Packard Foundation and by NSF Chemical Oceanography (AP) and the NASA Exobiology Program and the ACS Petroleum Research Fund (WG and TL).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Comparison of Clone library and PhyloChip relative abundances.

Fig. S1 Rank order abundance of the top 50-most abundant OTUs as detected by PhyloChip for each sample; in each case a few dominant OTUs are detected, with minor species forming a long tail.

Fig. S2 Phylogenetic identification of the 50-most abundant OTUs for each sample, showing that the most abundant OTU at a given depth generally is not among the most abundant OTUs at adjacent depths.

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