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ORIGINAL ARTICLE

Metatranscriptomic analysis of prokaryotic communities active in sulfur and arsenic cycling in Mono Lake, California, USA

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This study evaluates the transcriptionally active, dissimilatory sulfur- and arsenic-cycling components of the microbial community in alkaline, hypersaline Mono Lake, CA, USA. We sampled five depths spanning the redox gradient (10, 15, 18, 25 and 31 m) during maximum thermal stratification. We used custom databases to identify transcripts of genes encoding complex iron-sulfur molybdoenzyme (CISM) proteins, with a focus on arsenic (*arrA*, *aioA* and *arxA*) and sulfur cycling (*dsrA*, *aprA* and *soxB*), and assigned them to taxonomic bins. We also report on the distribution of transcripts related to the *ars* arsenic detoxification pathway. Transcripts from detoxification pathways were not abundant in oxic surface waters (10 m). Arsenic cycling in the suboxic and microaerophilic zones of the water column (15 and 18 m) was dominated by arsenite-oxidizing members of the Gammaproteobacteria most closely affiliated with *Thioalkalivibrio* and *Halomonas*, transcribing *arxA*. We observed a transition to arsenate-reducing bacteria belonging to the Deltaproteobacteria and Firmicutes transcribing arsenate reductase (*arrA*) in anoxic bottom waters of the lake (25 and 31 m). Sulfur cycling at 15 and 18 m was dominated by Gammaproteobacteria (*Thioalkalivibrio* and *Thioalkalimicrobium*) oxidizing reduced S species, with a transition to sulfate-reducing Deltaproteobacteria at 25 and 31 m. Genes related to arsenic and sulfur oxidation from *Thioalkalivibrio* were more highly transcribed at 15 m relative to other depths. Our data highlight the importance of *Thioalkalivibrio* to arsenic and sulfur biogeochemistry in Mono Lake and identify new taxa that appear capable of transforming arsenic.
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Introduction

Redox reactions mediated by microbes are important to the geochemical cycles of both sulfur and arsenic. Historically, sulfur and arsenic have been studied together due to their co-occurrence in the minerals arsenopyrite, orpiment and realgar (Lengke *et al.*, 2009). Soluble arsenic-sulfur oxyanions, known as thioarsenic compounds, have been identified in the environment where they play an important role in arsenic geochemistry, especially in sulfidic and alkaline waters (Hollibaugh *et al.*, 2005; Planer-Friedrich *et al.*, 2007; Fisher *et al.*, 2008b). Thioarsenic compounds have been shown to decompose rapidly in oxic environments (Hollibaugh *et al.*, 2005; Planer-Friedrich *et al.*, 2009), and laboratory experiments (Fisher *et al.*, 2008b; Edwardson *et al.*, 2014) suggest that bacteria capable of oxidizing reduced sulfur species (sulfide, thiosulfate and so on) are involved.

The diversity, ecology and physiology of microorganisms catalyzing the transformation of sulfur compounds such as sulfide, elemental sulfur, sulfate and thiosulfate are well known (Kelly *et al.*, 1997; Muyzer and Stams, 2008), and the role of bacteria in arsenic transformations is also well established. Both oxidative (Oremland *et al.*, 2002; Hamamura *et al.*, 2009) and reductive (Saltikov and Newman, 2003; Hoeft *et al.*, 2004) pathways have been characterized (Oremland and Stolz, 2003; Silver and Phung, 2005). These energy-yielding redox transformations occur in a few lineages of prokaryotes (mainly Firmicutes and Proteobacteria) in contrast to arsenic detoxification transformations that are ubiquitous in both prokaryotes and eukaryotes (Rosen, 2002). More recently, links between the microbial geochemistry of sulfur and arsenic have been investigated, especially in soda lakes and alkaline thermal springs (Hoeft *et al.*, 2004; Hollibaugh *et al.*, 2005; Fisher *et al.*, 2008b; Planer-Friedrich *et al.*, 2009; Edwardson *et al.*, 2014). One such environment is Mono Lake, a hypersaline soda lake in California, USA. Mono Lake contains elevated levels of arsenic

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(>200 μM) and sulfide concentrations can be high (>1 mM), especially in anoxic bottom waters following prolonged stratification. These conditions lead to the formation of significant concentrations of thioarsenic compounds in these waters (Hollibaugh *et al.*, 2005) where they are stable due to the elevated pH of the lake (Hollibaugh *et al.*, 2005; Planer-Friedrich *et al.*, 2010).

The enzymes involved in dissimilatory transformations of arsenic are well established (Silver and Phung, 2005). Arsenate respiratory reductase (ArrA; Saltikov and Newman, 2003), arsenite oxidase (AioA; Inskeep *et al.*, 2007; Lett *et al.*, 2012) and a more recently identified alternate arsenite oxidase (ArxA; Zargar *et al.*, 2010, 2012) are all members of the complex iron-sulfur molybdoenzyme (CISM) family (van Lis *et al.*, 2013), and assays to examine the genes for these enzymes have been established (Malasarn *et al.*, 2004; Kulp *et al.*, 2006; Zargar *et al.*, 2012). Microbial enzymes used for detoxification purposes include those encoded by the the *ars* operon (Rosen, 2002) and by related proteins such as UspA (Weiss *et al.*, 2009) and Acr3 (Fu *et al.*, 2009).

Molecular markers for both oxidative and reductive pathways of sulfur transformations include the *sox* genes, encoding the thiosulfate/sulfur oxidation enzyme system (Meyer *et al.*, 2007), the reversible dissimilatory sulfite reductase (*dsrAB*; Muller *et al.*, 2015), and adenosine-5′-phosphosulfate (APS) reductase (*aprBA*; Meyer and Kuever, 2007a). APS reductase catalyzes the transformation of APS to sulfite, which is a key step in the pathways of both sulfate reduction and sulfite oxidation to sulfate (Meyer and Kuever, 2007b).

The use of environmental transcripts to identify putatively active microbes and their contribution to biogeochemical cycling in various environments has provided important insights into microbial carbon cycling (Poretsky *et al.*, 2010; Satinsky *et al.*, 2014), the nitrogen cycle (Hollibaugh *et al.*, 2014; Hilton *et al.*, 2015), and the sulfur cycle (Canfield *et al.*, 2010; Stewart *et al.*, 2011). Here, high throughput sequencing of environmental mRNA (metatranscriptomics) was used to gain further insights into the microbial communities responsible for arsenic and sulfur cycling in Mono Lake, CA, USA. We identified transcripts of key enzymes mediating the oxidative and reductive pathways of the sulfur and arsenic cycles and determined their taxonomic affiliations and vertical distributions. This allowed us to examine variation with depth and redox potential of microbially mediated arsenic and sulfur transformations. In addition, our analysis revealed novel lineages of microorganisms potentially involved in arsenic and sulfur cycling in Mono Lake.

Materials and methods

Field site and sampling

We sampled at Station 6 (41 m water column depth) in the southern basin of Mono Lake in July, 2012. We obtained vertical profiles of Conductivity, Temperature, and Depth/pressure (CTD), as well as photosynthetically active radiation (PAR), *in vivo* fluorescence, dissolved oxygen and beam attenuation on July 12 using an SBE19 Seacat CTD equipped with ancillary sensors. We then collected water samples from depths of 10, 15, 18, 25 and 31 m, chosen to sample the major redox zones of the lake, using a Niskin sampler deployed on a rope as described previously (Hollibaugh *et al.*, 2005). Samples for arsenic speciation and sulfide concentration were collected directly from the Niskin sampler and preserved in the field (see Supplementary Methods for details).

Nucleic acid sampling and processing

Samples for RNA analysis were collected by pumping water from 31 m on July 12 and from 10–25 m on July 13. Duplicate samples of total particulate RNA (~0.5–2 l) were collected on 142 mm diameter, 0.2 μm pore-size Supor membrane filters. The filters were placed in 15 ml polypropylene centrifuge tubes and immediately frozen in liquid nitrogen, then transported to the laboratory where they were stored at -80 °C until extracted. The elapsed time from beginning the filtration at a given depth until the filter was placed in liquid nitrogen was <15 min. Total RNA was extracted from particles retained by the filters using bead beating and RNEasy Mini Kits (Gifford *et al.*, 2011 and Supplementary Methods), with internal standards added as described previously (Satinsky *et al.*, 2013).

Water for 16S ribosomal RNA (rRNA) gene analysis and synthesis of subtractive hybridization probes was collected from 5l Niskin bottles into foil-wrapped HDPE bottles with minimal head space and stored in an insulated cooler on ice until processed further. Water was filtered within 8 h of collection through Sterivex-GV 0.22 μm pore-size cartridge filters (EMD Millipore, Billerica, MA, USA) using a peristaltic pump. DNA was extracted from the filters using a lysozyme-proteinase K digestion followed by phenol-chloroform extraction as described previously (Kalanetra *et al.*, 2009). Quantitative PCR (qPCR) of Bacteria 16S rRNA genes in this DNA (Kalanetra *et al.*, 2009) was used to estimate the depth distribution of Bacteria.

Metatranscriptomics

Libraries for metatranscriptomics analysis were prepared from total RNA using a protocol modified from Stewart *et al.*, (2010). Briefly, rRNA was depleted using probes synthesized by PCR from DNA collected at each sampling depth (Supplementary Methods). Messenger RNA was amplified from rRNA-depleted total RNA using random hexamer primers, then double-stranded cDNA was prepared using reverse transcriptase and second strand synthesis kits. Libraries (~225 bp insert) were prepared using Illumina TruSeq technology. Samples were pooled and run on one lane of 150 × 2

Illumina HiSeq 2500 Rapid Run at HudsonAlpha Genomic Services. Reads have been deposited in the NCBI SRA under accession number SRP068308 (Bioproject PRJNA308451).

Bioinformatics

Sequences were processed using a custom pipeline described in more detail in Supplementary Methods. Briefly, paired ends were joined, the ends of combined reads were trimmed to remove low quality base calls, then ribosomal RNA reads and internal standards were counted and removed from the data set. Sequences that remained were annotated by alignment to the NCBI RefSeq database using RapSearch2, retaining only top hits with bit scores >40 and e-values $<10^{-5}$. Taxonomy was assigned and absolute abundances (transcripts L^{-1}) were calculated using counts of internal standards as described previously (Satinsky *et al.*, 2013).

Identification of complex iron-sulfur molybdoenzyme transcripts

A custom database of 110 amino acid sequences from the catalytic subunit of representative complex iron-sulfur molybdoenzymes (CISM, Supplementary Table S1) was prepared using sequences from various references (Rothery *et al.*, 2008; Schoepp-Cothenet *et al.*, 2012; Denton *et al.*, 2013; Grimaldi *et al.*, 2013). The sequences were downloaded from NCBI and edited in Geneious (Kearse *et al.*, 2012). BLASTx (Altschul *et al.*, 1990) was used to query all reads from each sample against this database. All hits with bit scores >40 were retained (Gifford *et al.*, 2011). Reads that had hits to the custom database were matched with their respective entry in the RefSeq database using RapSearch2. If there was no RapSearch2 hit, BLASTx was used to search against the RefSeq database.

Identification of sulfur cycling transcripts

Amino acid sequences of proteins involved in oxidative and reductive pathways of microbial sulfur metabolism (Supplementary Table S2) were obtained using a text search of the UniRef90 database (Suzek *et al.*, 2015). Additional sequences for some proteins (AprBA, DsrAB, Sat, SorAB and SoxABCDXYZ; Meyer *et al.*, 2007; Meyer and Kuever, 2007a, c; Frigaard and Dahl, 2008; Loy *et al.*, 2009; Mori *et al.*, 2010) were obtained from GenBank and RefSeq. Sequences were clustered at 90% identity with CD-HIT (Li and Godzik, 2006). All reads from each depth were searched against this database using BLASTx, and then processed as described above for the CISM database.

Results

Chemical characteristics of sampling depths

We sampled Mono Lake Station 6, the site of many of the previous microbiological studies of the lake, at five discrete depths (10, 15, 18, 25 and 31 m) selected based on the chemical profile of the lake at the time of sampling (Figure 1). The lake had been stratified for >1 year when sampled. The epilimnion is characterized by the highest temperatures (>15 °C), highest irradiance, highest dissolved oxygen concentrations and is subject to intense grazing by brine shrimp, *Artemia monica* (Jellison and Melack, 1993), which may have contributed to the low abundance of Bacteria there as indicated by quantitative PCR (Figure 1) and direct counts (LeCleir *et al.*, unpublished data). We sampled the base of the epilimnion (10 m), the base of the oxycline (15 m), near the base of the thermocline (18 m) and in the anoxic hypolimnion (25 and 31 m). The dissolved oxygen concentration at 15 m was 0.83 mg l^{-1} and decreased to the instrument's limit of detection (0.68 mg l^{-1}) at 15.8 m, thus the 15 m sample is considered to be suboxic. The concentrations of sulfide, arsenate, arsenite and thioarsenic compounds varied with

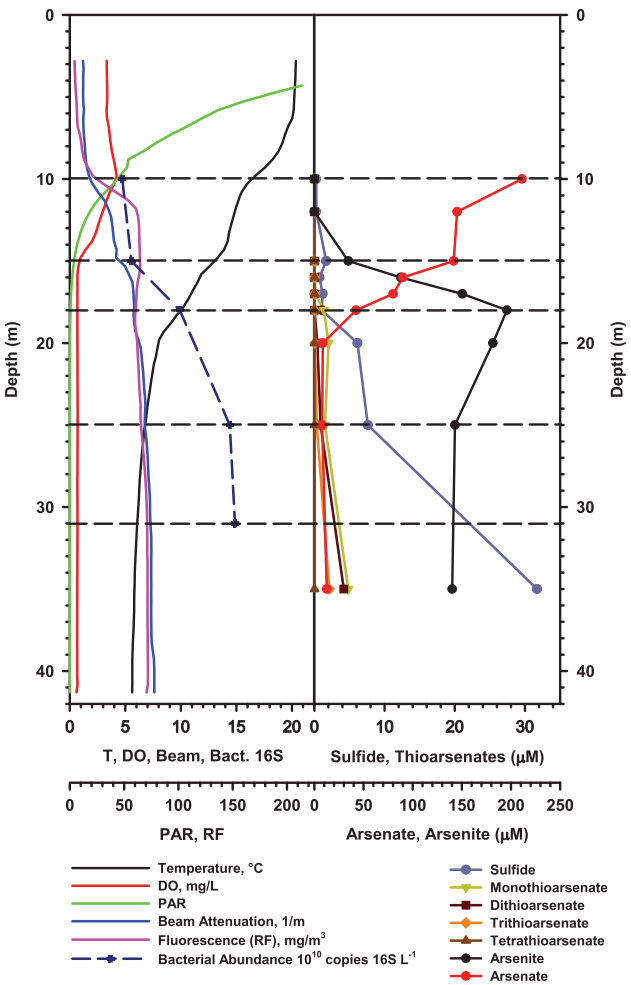

Figure 1 Vertical distribution of biogeochemical variables at Mono Lake Station 6, 12 July 2012. Metatranscriptome sampling depths are indicated by horizontal dashed lines. As, arsenic; Bact., Bacteria; DO, dissolved oxygen; PAR, photosynthetically active radiation; RF, relative fluorescence; S, sulfur; 16S, 16S ribosomal rRNA gene.

depth. The 10 m sample was dominated by the oxyanion arsenate (99.9% of arsenic, 211 μM). The 15 and 18 m samples were from the redox transition zone where arsenic speciation shifts from arsenate (15 m: 80.3%, 142 μM) to arsenite (18 m: 95%, 196 μM). Trace quantities of thioarsenates were present at these depths. The anoxic hypolimnion was sampled at 25 and 31 m. Sulfide appears at these depths (6–30 μM) coincident with a decrease in arsenite concentration (from 196 to 140 μM), and formation of thioarsenic compounds (1–12 μM). Sulfide and thioarsenic concentrations were much lower in July 2012 than recorded in August 2002, which followed 7 years of meromixis and when bottom water sulfide concentrations were >2 mM (Hollibaugh *et al.*, 2005). Although not measured at the time of sampling, sulfate concentration remains generally constant in the lake at ~100 mM throughout the water column (Oremland *et al.*, 2000).

Overview of the metatranscriptome

We obtained 115 million overlapping cDNA reads, with an average read length of 241 nt. Fewer than 1.1% of the transcripts in any sample were from Archaea or viruses, while Eukaryotes contributed 19–41% (Table 1). Twenty-five to forty-seven percent (average 37%) of non-rRNA reads had Rapsearch2 hits to the RefSeq database. Of these potentially protein coding reads, 58–79% were classified as originating from bacteria (see Table 1). The most abundant (>1% relative abundance) bacterial genera in Mono Lake at the time of sampling were affiliated with the Proteobacteria and Firmicutes phyla (Figure 2); especially at 15–31 m. Transcripts assigned to members of the sulfur-oxidizing bacteria genera *Thioalkalivibrio* and *Thioalkalimicrobium* were especially abundant. A literature search to identify bacterial genera with the potential to perform arsenic and sulfur redox transformations (Canfield *et al.*, 2010; Amend *et al.*, 2014; Sorokin *et al.*, 2015) revealed that almost half (27/57) of the most abundant genera we identified contain strains with known capability for dissimilatory sulfur and arsenic metabolism (Figure 2).

Arsenic and sulfur redox activity

The concentration of transcripts, including CISM genes, was low at 10 m (Figure 3), paralleling the abundance of Bacteria (Figure 1), so our detailed analyses focused on of metatranscriptomes from 15 m and below. At 15 m, the alternate arsenite oxidase (*arxA*) was the most abundant CISM transcript. The abundance of *arxA* transcripts was lower in samples from 18, 25 and 31 m, with an increase in abundance of transcripts encoding arsenate reductase, with arsenite oxidation shifting to arsenate reduction as the dominant arsenic transformation. The canonical arsenite oxidase (*aioA*) was detected at very low abundance at all depths (0, 7.1, 3.1, 5.2

Table 1 Summary statistics for metatranscriptome sequencing

Read Statistics	*Library*[a]									
	10a	*10b*	*15a*	*15b*	*18a*	*18b*	*25a*	*25b*	*31a*	*31b*
Raw unpaired[b]	21.36	23.13	25.58	31.67	29.55	20.73	44.06	33.01	32.02	26.91
Paired reads	9029458	10085082	11748031	11524766	10448205	8610511	19077062	13118718	12240380	9116277
Quality trimmed	8382894	9474888	11052641	10396583	9696575	8036024	17974417	12605162	11790853	8727294
Non-rRNA (% of good reads)	4746336 (56.62)	6985620 (73.73)	6122080 (55.39)	6473827 (62.27)	6404351 (66.05)	4987006 (62.06)	9599613 (53.41)	6334622 (50.25)	6453264 (54.73)	4854897 (55.63)
Internal standard recovery %[c]	0.000082	0.000151	0.000074	0.000103	0.000115	0.000087	0.000077	0.000070	0.000054	0.000074
Average read length	251	242	232	247	244	253	246	228	226	236
Number of hits to RefSeq (% of non-rRNA)	1189373 (25)	1881713 (27)	2385704 (39)	2627661 (41)	2556907 (40)	2188271 (44)	4003459 (42)	2228664 (36)	2496333 (39)	1894806 (39)
Domain distribution (B/A/E/V)[d]	58/0.1/40.7/1.1	71.4/0.2/27.4/1	67.8/0.4/30.8/1	68.9/0.4/29.7/1	72.1/0.9/26.1/1	79.1/0.7/19.4/0.8	67/0.7/31.3/0.9	67.5/0.7/31.3/0.4	62/0.7/36.9/0.4	68.4/0.8/30.4/0.4
Number of bacteria hits to RefSeq	689899	1343402	1617784	1809515	1843678	1730129	2682441	1504932	1547612	1295511
Uninformative transcripts (% of RefSeq hits)	361278 (52.4)	696032 (51.8)	832047 (51.4)	806040 (44.5)	957400 (51.9)	987830 (57.1)	1547441 (57.7)	1547441 (56.2)	845854 (58.5)	2682441 (58.2)
Protein encoding transcripts (% of RefSeq Hits)	328621 (47.6)	647370 (48.2)	785737 (48.6)	1003475 (55.5)	886278 (48.1)	742299 (42.9)	1135000 (42.3)	659078 (43.8)	642357 (41.5)	540965 (41.8)

Abbreviation: rRNA, ribosomal RNA.
[a]Number indicates sample depth (m), letter indicates replicate.
[b]Millions.
[c]Number of standards counted in 'good' non-rRNA reads/number of standards added (average of two standards).
[d]A = archaea, B = bacteria, E = eukaryota and V = virus.

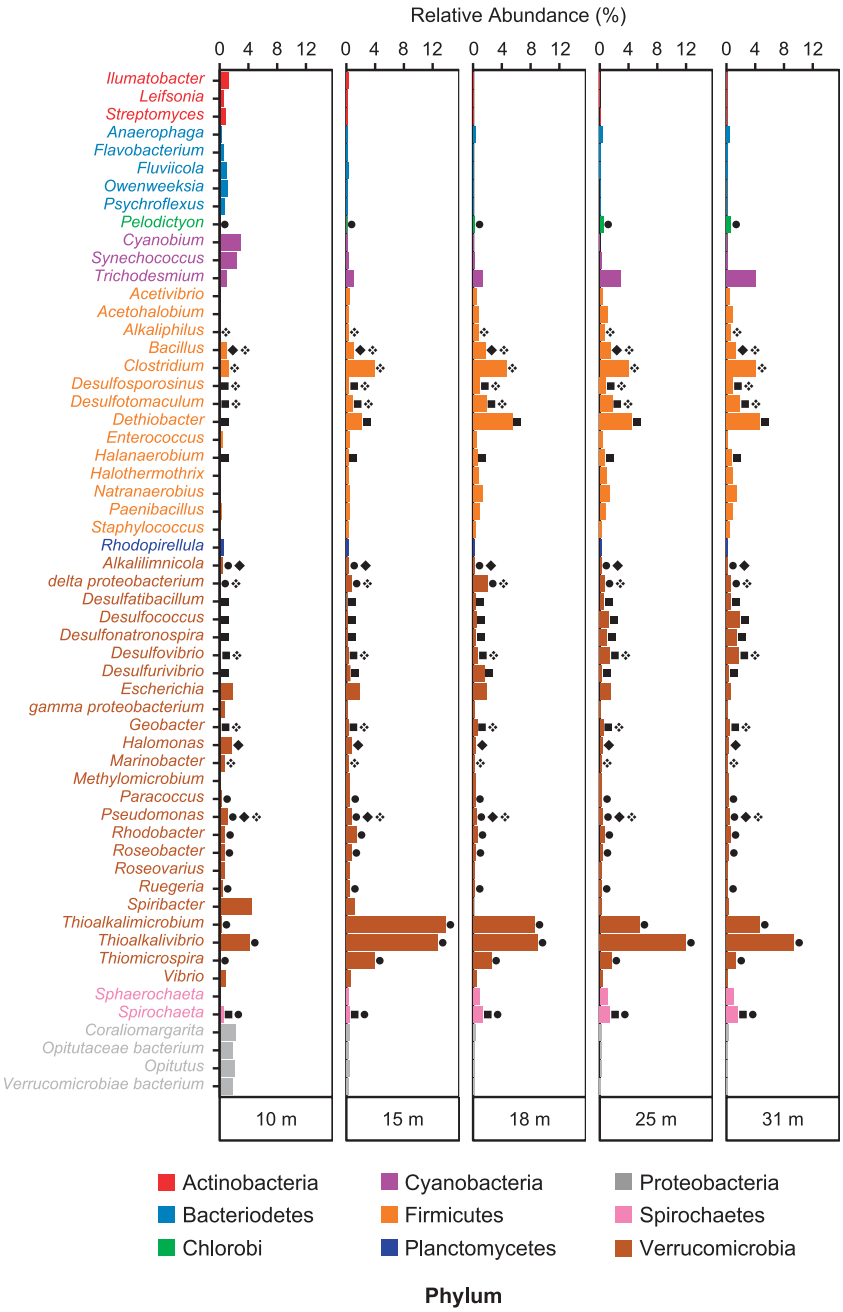

Figure 2 Taxonomic affiliations of transcripts recovered from 5 depths in the water column of Mono Lake, CA, USA. Relative abundance of transcripts (mean of 2 metatranscriptomes) assigned to individual genera is shown by the length of the bar opposite the taxonomic designation. Symbols next to bars indicate genera containing isolates known to perform the following arsenic and sulfur transformations: sulfur oxidation, circle; sulfate reduction, square; arsenite oxidation, hatched diamond; arsenate reduction, solid diamond.

and 2.3×10^6 copies per litre at 10, 15, 18, 25 and 31 m; respectively). In addition to arsenic redox enzymes, the most abundant CISM transcripts recruited to open reading frames annotated as different types of formate dehydrogenases (*fdhA, fdhN* and *fdnG*).

A closer examination of the taxonomic affiliation of *arxA* and *arrA* transcripts (Supplementary Figure S1) showed that the *arxA* transcripts were dominated by *Thioalkalivibrio* (47–51% relative abundance) and *Halomonas* (25–39%), with transcripts most similar to genes from *Thioalkalivibrio nitratireducens*, *Halomonas boliviensis* and *Halomonas* sp. A3H3. The taxonomic affiliations of *arrA* transcripts were more diverse, with representatives from at least 7 phyla. Dominant bacterial genera include *Desulfurispirillum* (Phylum Chrysiogenetes), *Desulfitobacterium* and *Desulfosporosinus* (Phylum Firmicutes), *Aeromonas*, *Ferrimonas* and *Thioalkalivibrio* (Class Gammaproteobacteria).

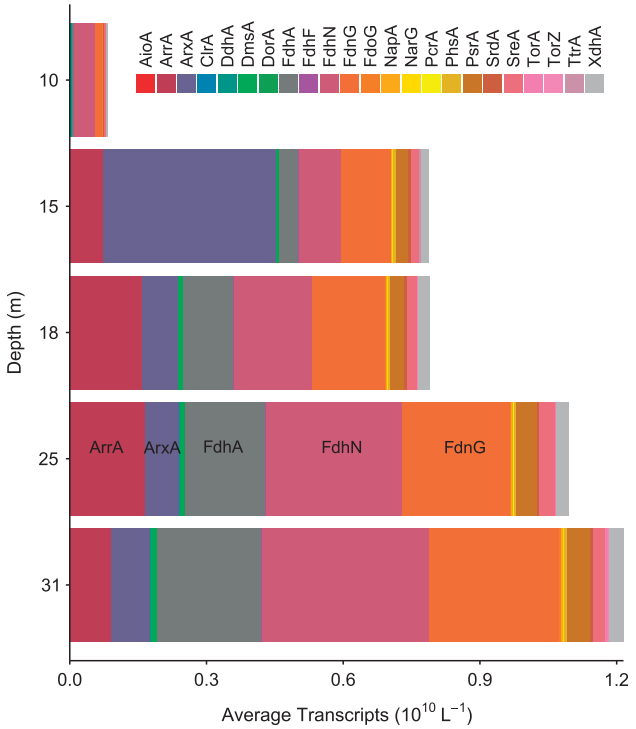

Figure 3 Average abundance (transcripts per litre) of hits to the complex iron-sulfur molybdoenzyme (CISM) custom database by depth in the water column of Mono Lake, CA, USA. Abbreviations of CISM protein names are: AioA, arsenite oxidase; ArrA, arsenate reductase; ArxA, anaerobic arsenite oxidase; ClrA, chlorate reductase; DdhA, dimethylsulfide dehydrogenase; DmsA, membranous DMSO reductase; DorA, DMSO reductase; FdhA, formate dehydrogenase subunit alpha; FdhF, formate dehydrogenase-H; FdhN, formate dehydrogenase; FdnG, NAD-dependent formate dehydrogenase; FdoG, formate dehydrogenase-O; NapA, periplasmic nitrate reductase; NarG, membrane-bound nitrate reductase; PcrA, perchlorate reductase; PhsA, polysulfide/thiosulfate reductase; PsrA, polysulfide/thiosulfate reductase; SerA, selenate reductase; SrdA, selenate reductase; SreA, sulfur reductase; TorA, TMAO reductase; TorZ, TMAO reductase (System III); TtrA, tetrathionate reductase; XdhA, xanthine dehydrogenase.

A BLASTp search against the NCBI database of the amino acid consensus sequences of assemblies yielding full-length *arrA* and *arxA* sequences (Figure 4) showed that the ArrA sequence (PROKKA_00186) was most closely related to *Halarsenibacter silvermanii* (59% AA identity), a strain isolated from nearby Searles Lake (Blum *et al.*, 2009), and *Natranaerobius thermophilus* (55% AA identity), a haloalkalithermophile isolated from Wadi An Natrun, a soda lake in Egypt (Mesbah *et al.*, 2007). The ArxA sequence (PROKKA_00030) was most closely related to Oceanospirillales bacteria including *Nitrincola lacisaponensis* and *Halomonas* strains (71–73% AA identity). In addition, we detected transcripts related to arsenic resistance enzymes (Supplementary Figure S2) with increasing abundance to 25 m, corresponding to the increase in arsenite concentration. The abundance of these transcripts was an order of magnitude lower than *arxA* and *arrA*.

Sulfur redox cycling

The vertical distribution by taxon of transcripts that were assigned to genes in sulfur metabolism pathways (*aprA*, *dsrA* and *soxB*; Supplementary Figure S3) was similar to that of genes from the arsenic metabolism pathways. Transcripts in samples from 15 m were mostly affiliated with *Gamma-* and *Deltaproteobacteria* (38–95% and 12–15% respectively), with taxonomic assignments for *aprA* and *dsrA* transcripts dominated by *Thioalkalivibrio* (Supplementary Figure S4, 65 and 35% respectively). *sox* transcripts were most abundant at 15 m (Supplementary Figure S3) and Supplementary Figure S4 shows that 66% of *soxB* transcripts were most similar to genes from *Thioalkalimicrobium*, although transcripts most similar to genes from *Thioalkalivibrio* were also abundant (27%). The abundance of *soxB* transcripts decreased with depth, whereas transcripts from *dsrA* and *aprA* increased with depth. *aprA* transcripts from Deltaproteobacteria were present at 15 m, but they increased in relative abundance at 18 m (12–65%) and the population was dominated by sequences most similar to genes in Deltaproteobacteria strain MLMS-1 (Supplementary Figure S4). *aprA* transcripts from 25 and 31 m were most similar to proteins from the Deltaproteobacteria *Desulfatibacillum*, *Desulfococcus*, *Desulfonatronospira*, and *Desulfovibrio* (Supplementary Figure S4, total 63% and 78% relative abundance, respectively). Sequences most similar to genes from these organisms also contributed to the *dsrA* transcript pool (17 and 23%); however, the *dsrA* transcript pool contained more reads affiliated with the sulfate-reducing Clostridia, including *Desulfotomaculum*, *Natranaerobius*, *Desulfurispora* and *Dethiobacter* (21% at 25 m and 18% at 31 m). The proportion of *dsrA* transcripts affiliated with unclassified Thermoplasmatales Archaea increased with depth (15 m: 4%, 18 m: 9%, 25 m: 16% and 31 m: 14%). d*srA* transcripts from 15 m contained sequences most similar to genes from Clostridia (23%) and Deltaproteobacteria (15%) as well as from Gammaproteobacteria (38%), but as depth increased, the contribution of sequences most similar to Gammaproteobacteria genes decreased (10 at 18 m to 2% at 31 m).

More careful phylogenetic analysis revealed that the custom database search recruited paralogous sequences of a 4Fe-4S ferredoxin of unknown function, but possibly an assimilatory sulfite reductase, most similar to genes from the Firmicutes and Thermoplasmatales Archaea. Although these prokaryotes likely contribute to sulfur cycling in the lake, it is unclear if they can gain energy from the reduction of sulfur compounds or if they contain the dissimilatory sulfite reductase enzyme complex. All sequences that contributed >1% of the *dsrA* transcripts at every depth are members of either the oxidative ('reverse') group, and most similar to genes from *Thioalkalivibrio* (35% at 15 m) or the Deltaproteobacteria orders *Desulfovibrionales*

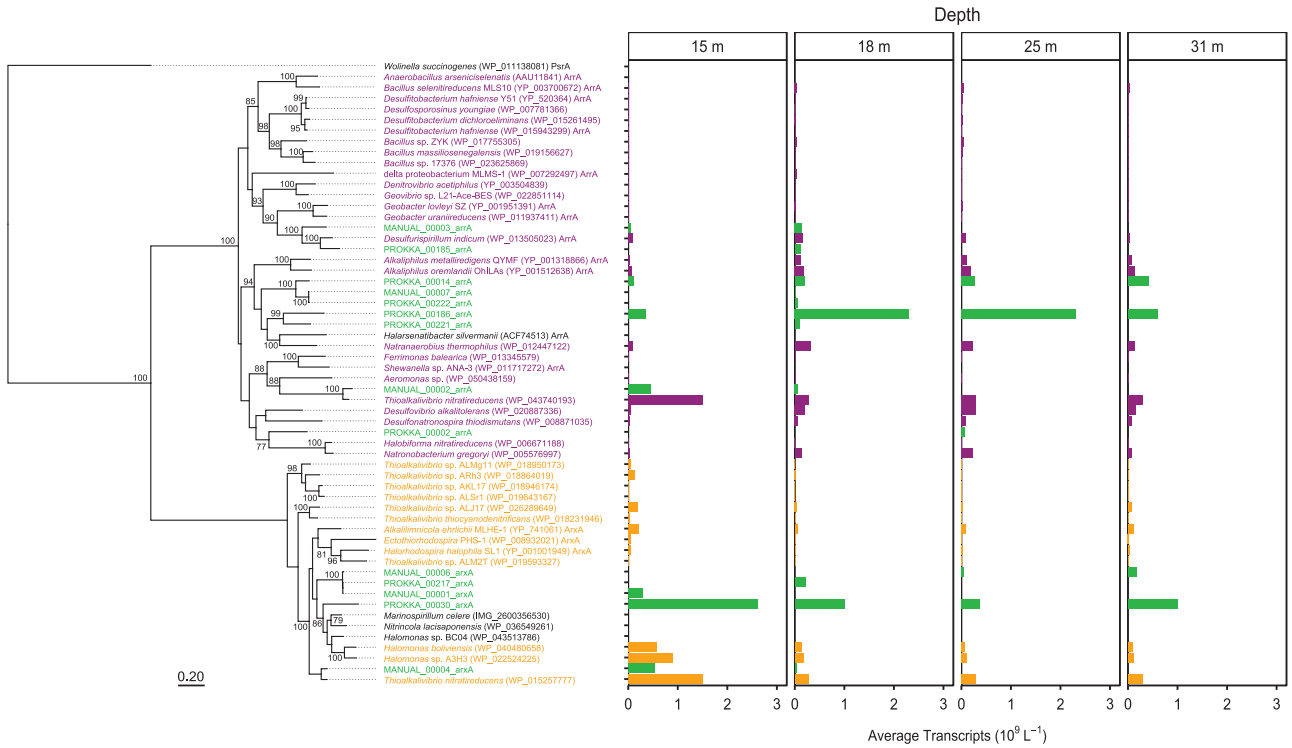

Figure 4 Maximum likelihood tree of known arsenate reductase (ArrA) and arsenite oxidase (ArxA) proteins (indicated as such in the tree) along with proteins found in reference genomes and representative (90% identity cluster) inferred amino acid sequences of open reading frames found in contigs assembled from Mono Lake transcripts (green). Hits to reference proteins (ArrA, purple; ArxA, orange) are indicated by organism and RefSeq accession number. Reference sequences from organisms known to reduce arsenate or oxidize arsenite are indicated with ArrA and ArxA. *Wolinella succinogenes* polysulfide reductase (PsrA) was used as the outgroup. The LG+I+G model was used and bootstrap support (100 replicates) >75% is shown.

(25 m: 14%, 31 m: 20%) and *Desulfobacterales* (25 m: 13%, 31 m: 15%). Two *aprA* consensus sequences: PROKKA_00232, most closely related to genes from Deltaproteobacteria strain MLMS-1 (95% AA identity); and PROKKA_00196, most closely related to genes from *Thioalkalivibrio nitratireducens* (94% AA identity), were abundant in assemblies from 15 and 18 m. These transcripts were also abundant at 25 m, as were transcripts related to five genes from sulfate-reducing Deltaproteobacteria (Supplementary Figure S5), highlighting the transition between sulfide oxidation and sulfate reduction between 18 and 25 m. This parallels the transition from transcripts related to arsenite oxidation to transcripts involved in arsenate reduction at these depths. Transcripts of *dsrA* displayed an almost identical trend (Figure 5). A general trend in the distribution of transcripts from sulfur-cycling genes is a shift from transcripts of sulfur oxidation genes most closely related to *Desulfurivibrio alkaliphilus* and strain MLMS-1 at 15 and 18 m to transcripts most similar to genes from the sulfate-reducing Desulfovibrionales group (*Desulfovibrio* and *Desulfonatronospira thiodismutans*) at greater depths. The Desulfobulbaceae (including MLMS-1 and *Desulfurivibrio*) are known sulfide oxidizers (Hoeft *et al.*, 2004; Hollibaugh *et al.*, 2006; Pfeffer *et al.*, 2012), thus, sulfide oxidation occurs at 15–18 m, with a switch to sulfate reduction at 25 and 31 m. This is consistent with previous rate measurements (Oremland *et al.*, 2000).

Analysis of transcription by Thioalkalivibrio

We performed additional analyses of transcripts in the top genome bin, *Thioalkalivibrio nitratireducens*, due to their significance in both arsenic and sulfur cycling pathways. All hits from each depth that were assigned to the *Thioalkalivibrio* taxonomic bin were aligned to the *T. nitratireducens* (*Tnat*) genome by BLASTx against all *Tnat* protein sequences. Our assumption was that transcripts binning to genes from *Thioalkalivibrio* would have homologs in the *Tnat* genome. The goal of this exercise was to capture the functional diversity of the *Thioalkalivibrio* genus, since it is unlikely that the *Thioalkalivibrio* found in Mono Lake are identical to *T. nitratireducens*. We found that 86% of all transcripts assigned to *Thioalkalivibrio* had hits in the *T. nitratireducens* genome. The average inferred amino acid identity of the transcripts to proteins from *T. nitratireducens* was 78%, which corresponds approximately to the genus level (Luo *et al.*, 2014).

We used differential transcription analysis to look for genes that might be transcribed more at one depth than another. Transcription of genes most similar to those in the *T. nitratireducens genome* at 10, 18, 25 and 31 m was compared to transcription at 15 m,

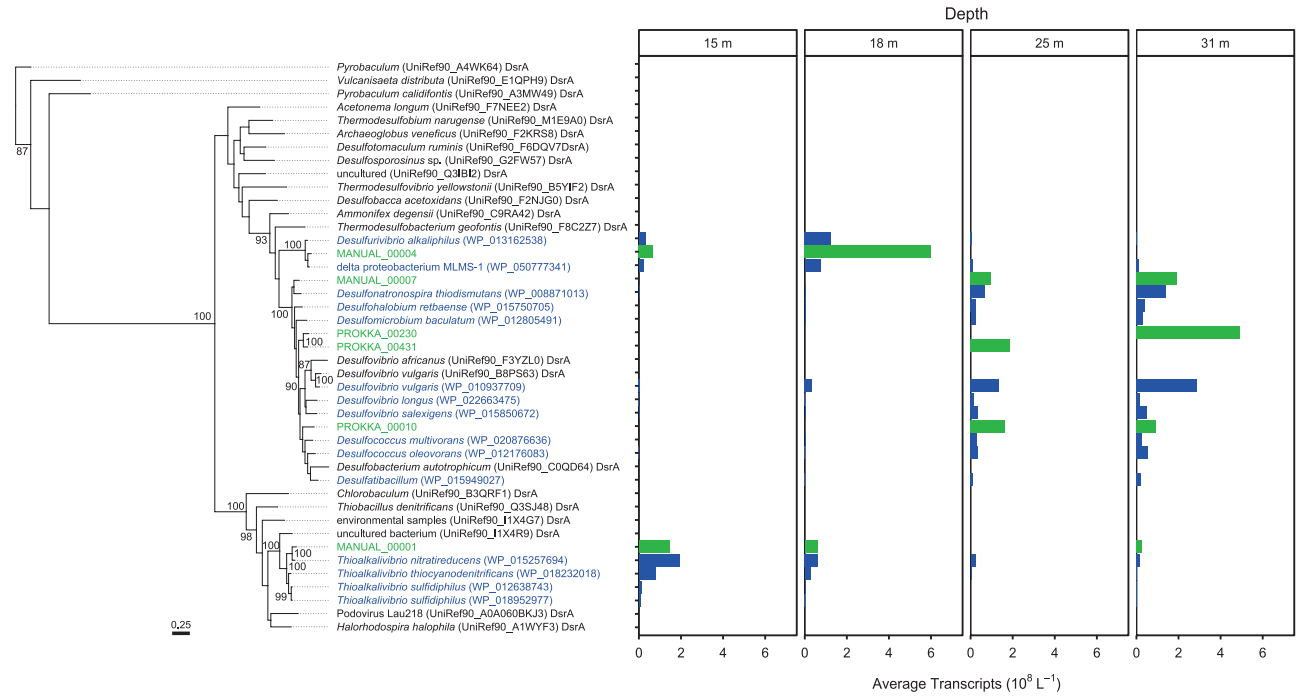

Figure 5 Maximum likelihood tree of reference dissimilatory sulfite reductase (DsrA) proteins along with proteins found in reference genomes and representative (90% identity cluster) inferred amino acid sequences of open reading frames found in contigs assembled from Mono Lake transcripts (green). Hits to reference proteins (blue) are indicated by organism and RefSeq accession number. Nodes labeled with 'DsrA' are sequences from the reference database. *Pyrobaculum* DsrA was used as the outgroup. The LG+I+G model was used and bootstrap support (100 replicates) >75% is shown.

arbitrarily chosen as a reference depth for comparison purposes. The top 20 statistically significant (adjusted *P*-value <0.05) differentially transcribed genes for each depth relative to 15 m are listed in Supplementary Table S3.

Transcription of genes known to be involved in sulfur and arsenic metabolism were analyzed separately (Supplementary Table S4). Transcripts of sulfite and arsenite oxidation genes (*dsr* and *arx* operons) were among those with the greatest change in transcription between 10 vs 15 m. The genes that were the most highly transcribed at 15 m (relative to the other depths) include members of the dissimilatory sulfite reductase operon and proteins involved in electron transport and respiration, indicating respiration of sulfite at 15 m. In addition, differential transcription analysis of inferred arsenate reductase and arsenite oxidase genes show that arsenite oxidase and arsenate reductase operons are both more highly transcribed at 15 m relative to other depths.

Discussion

Overview

Mono Lake is a complex geochemical system with 3 major electron acceptors (oxygen, arsenate and sulfate) and electron donors ranging from organic carbon and methane to reduced As and S compounds and ammonium (Oremland *et al.*, 2000; Hollibaugh *et al.*, 2005). Our results indicate that organisms involved in the metabolism of inorganic sulfur and arsenic compounds are abundant in the water column of Mono Lake, especially at 15 m and below. Fermentation and disproportionation reactions are also likely important, especially in anoxic bottom waters. Some of these interactions are shown in the conceptual model presented in Supplementary Figure S7, which relates the vertical distribution of potential electron donors and acceptors to microbial processes and the genes that are the focus of this study.

Some of the results were unexpected, for example, the abundance of *arrA* transcripts in samples from depths in the lake where no arsenate is detected, and the failure to detect *aioA* or *ars* genes in the surface layer of the lake. These highlights suggest that a similar analysis of transcripts characteristic of other geochemical processes, such as aspects of the N cycle, may reveal additional unexpected processes and relationships.

CISM genes

The most frequent hits of transcripts to reference sequences in the CISM database were to formate dehydrogenases (Figure 2). These enzymes catalyze the conversion of formate to CO_2 and occur in a wide variety of organisms (Grimaldi *et al.*, 2013). Comparison of the formate dehydrogenase sequences used in the CISM database to RefSeq returned top hits to two general types of formate dehydrogenase (data not shown). One is membrane bound and known to be

involved in generating a proton motive force tied to electron transport driven by nitrate reductase (Jormakka *et al.*, 2002), and the other is a formate dehydrogenase known to be associated with the formate hydrogen lyase complex (McDowall *et al.*, 2014).

Arsenic cycle transcripts

Aerobic arsenite oxidation is mediated by AioA (Lett *et al.*, 2012). We detected very few *aioA* transcripts in our samples, even in oxic surface waters: 0, 0.09, 0.04, 0.05 and 0.02% of all transcripts sampled at 10, 15, 18, 25 and 31 m, respectively; and some of the apparent *aioA* hits may be bioinformatic artefacts. As Mono Lake surface water communities are dominated by poorly characterized Bacteriodetes and Actinobacteria (Humayoun *et al.*, 2003), one possibility is that *aioA* genes (and likely others) in Mono Lake are divergent and thus not retrieved by BLAST against RefSeq.

In contrast, *arxA* accounted for to 0, 48, 10, 7 and 7% of the transcripts recovered for the same samples. Although transcript abundance does not necessarily correlate directly with enzyme activity, and AioA and ArxA may turnover at different rates, this distribution suggests that arsenite diffusing upward from anoxic waters is rapidly oxidized in the oxycline by an electron acceptor other than oxygen. Indeed, arsenite was not detected in the 10 m sample (Figure 1). These observations also suggest that arsenite production in the surface layer by the *ars* pathway is not significant, despite arsenate concentrations $>200\,\mu M$. This is borne out by the near absence of *ars* transcripts in the 10 m sample (Supplementary Figure S2). Although Mono Lake surface waters contain high concentrations of arsenate, they also contain high concentrations of phosphorus (~570 μM, Hollibaugh, unpublished data). Assuming the P is primarily in the form of inorganic phosphate, the high concentration (P:As ratio of 2.85) may competitively inhibit uptake of arsenate by the lake's biota, and thus reduce their dependence on the *ars* detoxification pathway on the one hand, and production of arsenite on the other.

The *arxA*-type arsenite oxidase was found throughout the water column below 10 m, raising the question of the identity of the electron acceptor for arsenite oxidation at depth in the lake. The water below the pycnocline is sulfidic and devoid of oxygen (Figure 1) and there is no evidence of springs near the station that might supply electron acceptors (for example, oxygen or nitrate) via groundwater. The sampling station is kilometers from the edges of the lake where edge-mixing might enhanced the flux of electron acceptors across the pycnocline (MacIntyre *et al.*, 1999; Vidal *et al.*, 2013). The water column of the lake was stably stratified at depth so downward transport of active cells from the pycnocline by mixing seems unlikely. Similarly, it seems unlikely that a flux of sinking cells could support this distribution of transcripts.

So far, *arxA* has only been found in anaerobes (Zargar *et al.*, 2012; van Lis *et al.*, 2013) growing chemoautotrophically with electron acceptors such as nitrate and Fe(III), yet *arxA* was the most abundant arsenic cycle transcript encountered under microaerophilic conditions at 15 m (Figure 2). The taxonomic affiliations of most of the *arxA* hits were to *Thioalkalivibrio* and *Halomonas* species. We have no direct evidence from this study that *Thioalkalivibrio* is able to oxidize arsenite, but two other relatives in the *Ectothiorhodospiraceae* family, *Ectothiorhodospira* sp. PHS-1 (Kulp *et al.*, 2008) and *Alkalilimnicola ehrlichii* MLHE-1 (Hoeft *et al.*, 2007) both oxidize arsenite and transcribe the *arxA* gene (Zargar *et al.*, 2010, 2012). Arsenite was oxidized photoautotrophically by an Ectothiorhodospira-dominated enrichment culture raised from a Mono Lake inoculum (Budinoff and Hollibaugh, 2008). Photoautotrophic arsenite oxidation by *Ectothiorhodospira* sp. PHS-1 has been shown to depend on *arxA* (Hernandez-Maldonado *et al.*, 2017), and three other *Ectothiorhodospira* strains containing the *arxA* gene have been shown to oxidize arsenite photoautotrophically (Hoeft McCann *et al.*, 2017). It is likely that there was sufficient irradiance at 15 m when we sampled to have supported anoxygenic photosynthesis. *Halomonas* species from Big Soda Lake, NV (a lake with chemistry similar to Mono Lake) have been shown to oxidize arsenite and to contain *arxA* (A. Conrad, unpublished thesis) and an arsenite-oxidizing, *arxA*-containing strain related to *Halomonas* (designated ANAO-440) has been isolated from a Mongolian soda lake (Hamamura *et al.*, 2014). Thus, *Halomonas* and *Thioalkalivibrio* strains appear to be important members of the arsenite-oxidizing community in Mono Lake.

Transcripts of arsenate reductase (*arrA*) were found throughout the water column at all depths below the oxic surface layer sampled at 10 m. We assume that the presence of transcripts from this gene in suboxic and microaerophilic samples at 15 and 18 m either reflects organisms associated with particles with anoxic interiors, or possibly temporal shifts in the vertical distribution of oxygen produced by photosynthesis. The microalga *Picocystis* forms a dense 'plate' in the oxycline (Figure 1; Roesler *et al.*, 2002; Humayoun *et al.*, 2003), where hypoxia provides a refuge from grazing by Artemia. Alternatively, *arrA* transcription may not be inhibited under microaerophilic conditions.

In contrast to the limited taxonomic range of *arxA* transcripts (Gammaproteobacteria), transcripts related to *arrA* were more diverse (Supplementary Figure S2). We retrieved *arrA* transcripts from a number of bacterial and archaeal genera including *Desulfurispirillum*, *Desulfitobacterium*, *Desulfosporosinus*, *Aeromonas*, *Ferrimonas* and *Thioalkalivibrio.* Strains of those genera have been shown directly (Niggemyer *et al.*, 2001; Pérez-Jiménez *et al.*, 2005; Nakagawa *et al.*, 2006; Rauschenbach

et al., 2012) or indirectly (Pepi *et al.*, 2007) to be able to reduce arsenate. Six different *arrA* clades each had >10% relative abundance at 15–31 m: *Thioalkalivibrio nitratireducens*, *Desulfurispirillum indicum*, *Desulfovibrio alkalitolerans*, *Natronobacterium gregoryi*, *Alkaliphilus oremlandii* and *Natranaerobius thermophilus* (Figure 4). Of these, only *Alkaliphilus oremlandii* and *Desulfurispirillum indicum* have been shown to reduce arsenate (Fisher *et al.*, 2008a; Rauschenbach *et al.*, 2012). An arsenate-reducing haloarchaeal biofilm community containing an ArrA operon most similar to *Natronobacterium gregoryi* has been described (Rascovan *et al.*, 2016) and we detected an haloarchaeal arsenate reductase most similar to a gene from *Halobiforma* sp. in our samples. To our knowledge none of the other organisms that our analysis suggests may contain *arrA* have been tested for their ability to use arsenate as an electron acceptor.

Sulfur cycle transcripts

Microbial oxidation of sulfur compounds (sulfide, sulfite, thiosulfate and elemental sulfur) is performed by both phototrophic and lithotrophic bacteria (Friedrich *et al.*, 2005; Frigaard and Dahl, 2008). Oxidative and reductive transformations of sulfur compounds are mediated by a large number of enzymes, but recent molecular surveys have focused on *soxB* (thiosulfate oxidation), *aprBA* (conversion of sulfite to sulfate or the reverse through APS) and *dsrAB* (conversion of sulfite to sulfide or the reverse; Meyer *et al.*, 2007; Meyer and Kuever, 2007b; Muller *et al.*, 2015). This history led us to focus on these genes in our analysis. We performed a search of metatranscriptome reads against a custom database of common sulfur oxidation and sulfate reduction pathway proteins (Apr, Dsr, Sox, Sat, Sor; Supplementary Table S2), then hits to AprA, DsrA and SoxB were examined more closely (Supplementary Figures S3 and S4).

Oxidation of reduced sulfur compounds (HS^-, S (0), thiosulfate) was represented by transcripts of key genes encoding proteins of sulfur oxidation pathways (reverse DsrA and AprA, SoxB) in organisms known to oxidize sulfur compounds. More transcripts from the 15 m samples were assigned to SoxB, indicating thiosulfate oxidation (Meyer *et al.*, 2007), than to AprA or DsrA. *soxB* transcripts from genes most closely related to *Thioalkalivibrio nitratireducens*, to a member of the Gammaproteobacteria family Piscirickettsiaceae, and contig PROKKA_00002 were most abundant at 15 and 18 m, which is similar to the distribution of *arxA* transcripts from genes most similar to *Thioalkalivibrio* and *Halomonas*. In contrast, transcripts most similar to *soxB* genes from *Thioalkalimicrobium cyclicum* and contig PROKKA_00004 were abundant throughout the anoxic water column, suggesting different functions for this protein in these two clades. Although the genes in both organisms are annotated as *soxB*, they only shared 46% amino acid identity. The *soxB* in *Thioalkalimicrobium* and other related non-Chromatiales Gammaproteobacteria is found in a conserved gene cluster, while the *soxB* gene in *Thioalkalivibrio* is not in this cluster.

Thioalkalivibrio and *Thioalkalimicrobium* were two of the most transcriptionally active prokaryotes in Mono Lake (Figure 2). Representative species *Thioalkalivibrio jannaschii* and *Thioalkalimicrobium cyclicum* have been isolated from Mono Lake (Sorokin *et al.*, 2002). Their abundance in Mono Lake is consistent with findings from other soda lake environments (Sorokin *et al.*, 2007, 2011). Growth studies indicate that these species are aerobic or perhaps prefer microaerophilic conditions (Sorokin *et al.*, 2002), thus the abundance and diversity of transcripts from the sample at the bottom of the oxycline (15 m) is to be expected. The presence of a large number of *soxB* transcripts affiliated with *Thioalkalimicrobium* and contig PROKKA_00004 in samples from anoxic depths (18–25 m) suggests thiosulfate oxidation coupled to an alternative electron acceptor, or possibly disproportionation of thiosulfate, although no Gammaproteobacteria have been shown to perform this process (Finster, 2008; Poser *et al.*, 2013). An alternative is oxidation and/or disproportionation of thioarsenic compounds mediated by SoxB (Edwardson *et al.*, 2014); however, oxidation would still require an electron acceptor (Tourova *et al.*, 2013).

An additional indication of the significance of sulfide oxidation at 18 m is the abundance of transcripts for DsrA and AprA associated with Desulfobulbaceae (Strain MLMS-1 and *Desulfurivibrio alkaliphilus*). Although these organisms fall into 'sulfate reducing' groups based on their DsrA phylogeny (Muller *et al.*, 2015), MLMS-1 is known to oxidize sulfide with arsenate as the electron acceptor (Hoeft *et al.*, 2004; Hollibaugh *et al.*, 2006) as well as disproportionate thioarsenate compounds (Planer-Friedrich *et al.*, 2015). Sulfide oxidation coupled to arsenate reduction has been shown to be mediated by *Desulfobulbaceae* in enrichment cultures from Mono Lake (Hollibaugh *et al.*, 2006). *Desulfurivibrio alkaliphilus* is unable to reduce sulfate (Sorokin *et al.*, 2008) but reduces (or disproportionates) thiosulfate and performs disproportionation of elemental sulfur and polysulfides (Poser *et al.*, 2013).

Sulfate reduction in Mono Lake appears to be dominated by Deltaproteobacteria that are members of the order Desulfovibrionales (Figure 4,Supplementary Figures S4–S9). This conclusion is consistent with previous studies of the distribution of sulfur-reducing bacteria in Mono Lake by Scholten *et al.*, (2005). This study of the distribution of diagnostic 16S rRNA genes found that sulfate-reducing bacteria were abundant in the anoxic depths of the lake; although they were not able to corroborate this finding with markers based on functional genes (*dsrAB* and *aprA*). A previous study of sulfate and arsenate reduction rates in the Mono Lake

water column hypothesized that arsenate and sulfate reduction were occurring simultaneously or that there was a potential shift in SRB activity between sulfate and arsenate reduction (Oremland *et al.*, 2000). We found transcripts most similar to genes from *Desulfovibrio* and *Desulfonatronospira* indicating arsenate (*arrA*) and sulfate reduction (*dsrB* and *aprA*) in the same sample. Therefore, it is plausible that these organisms are capable of performing sulfate and arsenate reduction simultaneously.

Depth distribution of transcripts related to Thioalkalivibrio

Our analysis of differential transcription of genes most similar to those in *Thioalkalivibrio* genomes showed that sulfur and arsenic cycle genes are transcribed significantly more than expected at 15 m relative to other depths. This reinforces conclusions from transcript counts and indicates that arsenite and sulfite oxidation are both important to *Thioalkalivibrio* at 15 m. Additional *Thioalkalivibrio* genes that were more highly transcribed at 15 m relative to other depths were related to growth (ribosomal proteins and ATP production), respiration and electron transport (ubiquinone and cytochrome), and carbon assimilation (RuBisCO). Based on this evidence, we hypothesize that *Thioalkalivibrio*-like organisms are growing and/or respiring more rapidly at 15 m than at other depths. Twelve of the 28 genes that were transcribed more strongly at 18–31 m than at 15 m were annotated as hypothetical proteins, although predictions of their functions can be made by conserved domain analysis (see Supplementary Table S4). Although *Thioalkalivibrio*-like genes are transcribed at 18–31 m, a suitable electron acceptor (oxygen, nitrate or potentially arsenate) may not be available and thus the cells may not be able to respire or grow.

The use of a custom database to identify ambiguously annotated CISM family genes revealed transcripts for putative ArxA and ArrA proteins in organisms for which pathways for arsenic transformations have not been identified. However, phylogenetic analysis of these protein sequences clearly place them in groups of prokaryotes known to contain arsenate reductases and arsenite oxidases. Our analysis has thus revealed greater diversity of organisms involved in arsenic cycling in Mono Lake, and presumably in other similar habitats, than has been realized previously. In addition, transformations of both sulfur and arsenic compounds appear in some cases to be mediated by the same organisms. These results highlight the complex interplay between the arsenic and sulfur geochemical cycles in Mono Lake.

Conflict of Interest

The authors declare no conflict of interest.

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