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A RANDOM BIOGEOCHEMICAL WALK INTO THREE SODA LAKES OF THE WESTERN USA: WITH AN INTRODUCTION TO A FEW OF THEIR MICROBIAL DENIZENS

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1. Introduction

The Wadi Natrun in Egypt is probably the oldest and best-known soda lake environment for finding polyextremophilic microorganisms adapted to its high salinity, pH, and temperature. It clearly has been a resource and motivational factor for multiple generations of microbiologists interested in exploring the physical/chemical boundaries of microbial life, as is reviewed by Professor Oren in this volume (Oren, 2013). Yet curiosity-driven research is not the only factor egging on a young scientist's aspirations, as was my situation some 35 years ago. I began my professional career at the US Geological Survey (USGS) in 1977 with my dissertation and postdoc background in marine sciences and anaerobic processes. After a very shaky first few years upon coming aboard the USGS, I was told that my research direction should be devoted to inland waters, preferably freshwater streams. About this time three seminal scientific events occurred that got me thinking about extremophiles and their habitats: the publication of a series of papers on Solar Lake (Cohen et al., 1977; Jørgensen and Cohen, 1977), the discovery of chemoautotrophic-based marine life at hydrothermal vent sites (Lonsdale, 1977), and the 1980 eruption of Mount St. Helens followed by the microbial successions observed in nearby Spirit Lake (Baross et al., 1982). I began thinking that there surely must be some interesting environments within a 1-day drive of my laboratory in Menlo Park, California. It was during a chance encounter with my colleagues Yousif Kharaka and Steven Robinson that I was told of such a place: Big Soda Lake, in Fallon, Nevada. Not only was it saline and alkaline, it had been in a condition of meromixis for over 60 years which resulted in hypersaline and extremely reducing anoxic bottom waters. Thus, I could fulfill both my curiosity and obey the letter (but certainly not the spirit) of the "riot act" that was read to me. I would work on a system more saline and far more chemically reducing than what the oceans could provide. I had found my smelly little bit of (obscure) scientific heaven! From there it was only a short conceptual hop to Mono Lake and Searles Lake in California for the scientific purposes of comparisons, as my USGS career blossomed.

2. Big Soda Lake, Nevada

Big Soda Lake (30°31′N; 118°52′W) occupies the remnants of a volcanic crater that last erupted in the Pleistocene (Rush, 1972). In the nineteenth century, the lake level was about 18 m lower than today, and the brine was "mined" by pumping it ashore followed by evaporation for the purpose of obtaining borax. The Newlands Reclamation Project (~1907) diverted water from the Truckee River to the region of Fallon, causing a rise in the local water table with a concomitant influx into the lake of freshwater which eventually resulted in a permanent, density-based stratification (meromixis) of the water column that has persisted since ~1925. A deep chemocline/pycnocline is located at 35 m depth, while the surface water undergoes winter mixing followed by summer stratification and an oxycline/thermocline located at ~20 m depth (Kimmel et al., 1978; Priscu et al., 1982; Cloern et al., 1983a; Oremland and DesMarais, 1983; Kharaka et al., 1984). The lake's dimensions are approximately 1.5 km along its east–west axis by 1.2 km north–south (Fig. 1), with an area of ~1.8 km^2. The lake has a profundal depth of

Figure 1. A satellite image, courtesy of Google Earth, of Big Soda Lake and Little Soda Lake, Nevada. Note the *darker grey* area surrounding the lakes and the discernable line beyond surrounding the local farming community which outlines the volcanic blast zone consisting of extruded lacustrine materials and basaltic debris (Scale, E/W axis of Big Soda Lake boundary = 1.5 km).

Table 1. Some chemical constituents of Big Soda Lake mixolimnion and monimolimnion waters.

Constituent	TDS[a]	pH	Cl^{b}	HCO_3^{-b}	Na^{b}	Ca^{b}	NH_3^{-b}	SO_4^{2-b}	S^{2-b}	Mg^{b}	B^{b}	CH_4^{-c}
Surface	26	9.7	203	67	353	0.13	0	60	0	6.1	4	0.1
Bottom	88	9.7	789	395	1,174	0.02	2.7	73	13.1	0.2	15	55

Modified from Kharaka et al. (1984) and Oremland and DesMarais (1983).
[a]*TDS* (salinity) total dissolved solids (g/L).
[b]mM.
[c]μM.

approximately 62 m. Some chemical characteristics of its surface water (mixolimnion) and its bottom waters (monimolimnion) are given in Table 1.

Annual primary productivity was estimated at ~500 gC m^{-2}, about 60 % attributable to a winter bloom of pennate diatoms. A significant contribution also came from the region of the summer oxycline, of which 30 % annual carbon fixation stems from bacterial chemoautotrophy with an additional 10 % contributed from anoxygenic photosynthesis associated with a red plate of *Ectothiorhodospira* (Cloern et al., 1983b). While the lake's surface water is only moderately saline, its high pH precludes the presence of fish. Nonetheless, the surface waters host abundant zooplankton (copepods and cladocerans), aquatic insects, and seasonal shoreline rooted macrophytes (*Ruppia* sp.) that become heavily epiphytized by diazotrophic *Anabaena* sp. communities during summer (Oremland, 1983). The water column bacterial communities were described based on microscopic examinations that were done concurrently with measures of heterotrophic activity made with radiotracers (Zehr et al., 1987). The water column was also assayed for sulfate reduction (Smith and Oremland, 1987), methane formation and anaerobic methane oxidation (Iversen et al., 1987), and fluxes of particulate matter across the chemocline (Cloern et al., 1987). The presence of dissolved methane in the monimolimnion, along with a clear ^{12}C-enriched value of $\delta^{13}C$-methane, suggested a biological origin of this gas (Oremland and DesMarais, 1983). Incubated bottom sediment slurries had methanogenic activity, but only amendments with methanol, as opposed to acetate or formate, caused a pronounced stimulation of activity (Oremland et al., 1982a). The high concentrations of sulfate in this system as opposed to seawater (73 vs. 28 mM) gave rise to the concept that certain methylotrophic substrates (e.g., methanol, methylated amines, methylated sulfides) were not vied for with the thermodynamically more efficient sulfate-reducing bacteria and were termed "noncompetitive" methane precursors as opposed to competitive substrates like acetate and H_2 (Oremland and Polcin, 1982; Oremland et al., 1982b; King, 1984; Kiene et al., 1986). The bottom sediments, along with an isolated methylotrophic coccoid methanogen, showed a pronounced pH optimum at 9.7, indicating a clear adaptation to the lake's harsh bottom water conditions (Oremland et al., 1982a). Anoxic sediments from this lake and other sites including Mono Lake and San Francisco

Bay had the capacity to biologically form ethane from ethylated sulfur compounds like ethanethiol and diethyl sulfide (Oremland et al., 1988). Littoral sediments were also scrutinized for denitrification by measuring N_2O reductase activity (Miller et al., 1986). Big Soda Lake remains to be investigated from the perspective of employing culture-dependent and culture-independent methods to better define its resident microbial populations and their adaptations to its ambient conditions. Recent visits to the lake indicate that the hydrological conditions are changing, with a notable sinking of the depth of the deep chemo-pycnocline suggesting a slow breakdown of meromixis.

3. Mono Lake, California

Located in a closed basin formed by geologic subsidence nearly 0.75–1 million years ago (Christensen et al., 1969), Mono Lake (38°N; 119°W) abuts the eastern escarpment of the Sierra Nevada mountain range (Fig. 2). The lake is situated on the western edge of the Great Basin Desert, with its present dimensions running roughly 20.6 km along its east–west axis, and about 13.8 km along its north–south axis (area = ~150 km^2). In the Pleistocene the lake covered five times the area than it does today, contained 18-fold more water, and may have been a branch of the much larger Lake Lahontan that covered a great deal of western Nevada. The change in climate from wet to arid resulted in evaporative contraction of Mono's size and the concentration of its major chemical constituents (Stine, 1990). The currently exposed former lakebeds are clearly visible from the satellite image displayed in Fig. 2. Unlike Big Soda Lake which occupies the remnants of an exploded volcanic caldera, Mono Lake occupies no such caldron. However, volcanism and volcanic features are common in the basin and have contributed to the lake's current morphometry. Volcanism in the region commenced some 750,000 years ago with the mega-scaled eruption of the Long Valley caldera, located ~40 km south of the lake. Volcanic activity migrated northwards over time, and active heat flow persists into the basin (Hill et al., 1985). The Mono Craters (some 40 in all) are prominent features along the lake's south shore, while the its two major islands, Negit and Paoha, were formed as a consequence of volcanic activity, the latter island having hot springs located along its southeastern corner. Mark Twain's classic book "Roughing It" devotes an entire chapter to Mono Lake, mostly dealing with his misadventures on Paoha Island. Early scientific investigations concerning the lake's general limnological properties (Mason, 1967) and sediment organic geochemistry (Reed, 1977) stimulated further research interest. Broader societal interest centered on the consequences of the prolonged diversion of freshwater runoff into the lake to supply drinking water for the city of Los Angeles. These diversions lowered the lake level by some 15 m over four decades, roughly doubling its salinity (from 50 to 90 ppt) and raising concerns that the lake would eventually become too saline to support metazoan invertebrate life (which support large populations of migratory waterfowl) and that its shallow regions

Figure 2. A satellite composite image of Mono Lake, courtesy of Google Earth. Note the striations around the lake's former lakebed, indicative of much higher water stands in the Pleistocene. In contrast, the exposed white region near the lake's current edge is the amount of lake-water volume lost attributable to diversion of freshwater runoff since 1940 (Scale, E/W axis of the lake boundary = 20.6 km).

Table 2. Some chemical constituents of Mono Lake.

Constituent[a]	TDS	pH	Cl	HCO_3^-	Na	Ca	Mg	SO_4^{2-}	As	B	CH_4[b]
–	90	9.8	500	400	1,300	0.05	1.3	130	0.2	46	40

Compiled from Dana et al. (1977), Oremland et al. (1993), and Johannesson and Lyons (1994).
[a]Given as mM (or g/L for TDS) unless noted otherwise.
[b]μM as dissolved in the bottom water.

would become exposed dry playas that would generate toxic dust storms. A political settlement was eventually reached with the goal of stabilizing the lake level at its 1941 level by allowing more freshwater runoff into the lake. Some of the major chemical constituents of Mono Lake are given in Table 2.

Our interest in Mono Lake was piqued in the early 1980s by the observation that the high precipitation and snow melt runoff of freshwater into the lake (1982–1984) caused by a strong El Nino event resulted in a dilution of surface water salinity by ~10 %. This set up a meromictic condition that was to persist for 8 years (Jellison and Melack, 1993; Jellison et al., 1993; Miller et al., 1993), a situation that was to recur during the El Nino winter of 1994–1995 and persist for another 5 years (Humayoun et al., 2003). We made two serendipitous observations during our early visits to Mono Lake that led to a couple of unexpected discoveries with regard to (1) multiple sources of methane outgassing from the lake (Oremland et al., 1987) and (2) a small-sized (1–3 μm) eukaryotic phytoplankter in the water column that was responsible for most of the lake's primary production (Roesler et al., 2002).

Despite the great abundance of sulfate ions in its waters, Mono Lake has considerable dissolved methane present within its bottom waters during stratification (Table 2) along with an abundance of sulfide (~2 mM). Methanogenic activity was detectable both in the lake's pelagic and littoral sediments (Kiene et al., 1986; Oremland and King, 1989; Oremland et al., 1993; Oremland and Miller, 1993) arising primarily from "noncompetitive" precursor substrates (e.g., methylated amines, methylated sulfides, and methanol), ultimately derived from breakdown of osmoregulatory compatible solutes like dimethylsulfoniopropionate and glycine betaine. A small amount of biogenic ethane could also be formed from precursors like ethanethiol and diethyl sulfide (Oremland et al., 1988). Sediment core profiles taken from the pelagic region indicated methane saturation was reached in the upper 60 cm, where there was still abundant sulfate present (25–70 mM) (Oremland et al., 1987). This pore water methane was isotopically "light" ($\delta^{13}CH_4 = -75‰$) and contained detectable amounts of ethane and propane ($C_1/C_2 + C_3 = 600–750$), indicating biogenic origins. Sulfate reduction, as determined with ^{35}S-radiotracer, also was detectable in these cores, although rates measured in the top 3 cm (250 μmol L^{-1} day^{-1}) declined exponentially over the upper 10 cm and were about 100-fold higher than most elsewhere in the core (Oremland et al., 1993). Methane-oxidation activity, both aerobic and anaerobic, was measured in the stratified water column of the lake (Oremland et al., 1993; Joye et al., 1999), as was oxidation of methyl bromide (Connell et al., 1997). Culture-independent methods detected the presence of methanogens (Scholten et al., 2005), methanotrophs (Carini et al., 2005; Lin et al., 2005; Nercessian et al., 2006), and nitrifiers (Ward et al., 2000; Carini and Joye, 2008) in the water column.

The first surprise in this context was our observation of gas seeps around the lake. Intuition argued against them being composed of methane because of the lake's high sulfate content. But paradoxically, they turned out to be methane rich. Mono Lake therefore leaks methane to the atmosphere in the form of continuous bubble ebullition from thousands of seeps located around the lake (Oremland et al., 1987). What differentiates these seeps from the methane cycle described above is that they are radiocarbon dead (age >> 20,000 years), while methane in the bottom water, which has its origin in the anoxic sediments, is not.

Hence, the seeps are associated with a natural gas deposit underlying the lakebed that escapes its confinement along geologic fracture lines. Ebullition rates for individual seeps ran from ~50 mL $CH_4\,min^{-1}$ to as high as 4 L $CH_4\,min^{-1}$. Seeps located in the vicinity of the hot springs and active heat flow on the southeastern corner of Paoha Island had a strong thermogenic character (methane depleted in ^{12}C and contained abundant ethane, propane, and butanes), while seeps located elsewhere had a strong biogenic character (methane enriched in ^{12}C; lacking ethane and propane).

The second surprise was our observation of an optically turbid layer located in the vicinity of the oxycline/pycnocline/redoxcline of the lake's water column. We assumed this to be caused by a dense "plate" of photosynthetic bacteria, like the *Ectothiorhodospira*-dense layer that occurs in Big Soda Lake. But rather than being pink, the water recovered from this depth (~16 m) was green-tinged. Microscopic examination revealed the abundant presence of unicellular eukaryotic algae in the 1–3-μm size range. A study was launched by C.W. Culbertson to investigate the ecophysiology of this microbe. It was dubbed "Mickey" because its spherical cytoplasmic main body had two superimposed smaller "ears" that contained its chlorophyll, giving it a resemblance to Mickey Mouse when viewed head on, thereby facilitating direct counting via epifluorescence microscopy because of its unique morphology. "Mickey" could be cultivated in the lab and was designated as *Picocystis* sp. strain ML (Roesler et al., 2002). Strain ML exhibited growth over a wide range of pH (5–11; optimum 6–8) and salinity (0–250 ‰; optimum = 50‰). Cells contained both dimethylsulfoniopropionate (DMSP) and glycine betaine (GBT) as internal osmolytes. Internal DMSP levels showed no trend when cells were grown over a salinity range of 19–123‰ (0.08–0.14 pg $cell^{-1}$), but GBT increased markedly at salinities above 40‰ and became the dominant osmoregulant at the highest salinity (~0.8 pg $cell^{-1}$ at 123‰). Strain ML only demonstrated light-dependent growth, but was able to grow well under initially anoxic (N_2 headspace) and reducing conditions when supplemented with 100 μM sulfide plus 100 μM ammonia, thereby mimicking the sub-oxic environment of the 16 m chemocline. Cells contained primarily chlorophyll *a*, but also substantial amounts (~30 %) of chlorophyll *b*, as well as several ancillary pigments (several xanthins and carotenes). When captured brine shrimp (*Artemia monica*) from the lake were placed in a suspension of "Mickey," their numbers decreased rapidly over a 15-h feeding incubation, thereby indicating the importance of strain ML in trophic energy transfer to the lake's dominant zooplankter. Highest cell densities of "Mickey" (2×10^5 cells mL^{-1}) occurred in the chemocline during water column stratification. Annual primary production during meromixis was estimated at 22.4–38.5 mol C m^{-2}, but runs roughly 50 % higher during the lake's "normal" monomictic (one turnover per year) cycle (Jellison and Melack, 1993). Estimates of the contribution that "Mickey" makes to the lake's annual photoautotrophic carbon fixation were determined by conducting seasonal water sample incubations made with ^{14}C-bicarbonate incorporation followed by size fractionation. Results indicated that this microorganism holds importance beyond its small

size and contributes between 25 and 50 % of annual primary productivity. Mono Lake surface water contains abundant phosphate (~600 μM; J. T. Hollibaugh, 2003, personal communication) but is severely nitrogen-limited. A diazotrophic community was detected that inhabited the internal matrices of a small, tufted free-floating algal assemblage (*Ctenocladus circinnatus*) found in the lake (Oremland, 1990). Nitrogen fixation was attributed to both a light-driven, aerobic component, presumably cyanobacterial, and a dark/anaerobic component presumably bacterial. However, the estimated activity did not appear to make a quantitatively relevant contribution to sustaining water column primary productivity.

The issue of the contamination of the western portion of the San Joaquin Valley's soils with naturally occurring selenium salts prompted the next phase of this work. We were seeking extremophiles better suited to grow in saline/alkaline agricultural wastewaters so as to efficiently remove selenium from the aqueous phase via its precipitation as Se(0). Since we had first isolated a selenate-respiring bacterium, *Sulfurospirillum barnesii*, from a freshwater slough (Oremland et al., 1994; Laverman et al., 1995; Stolz et al., 1999), we turned to Mono Lake as a source of anaerobes better suited to more extreme conditions. Two new species of low G + C haloalkaliphilic anaerobes were isolated from the sediments of Mono Lake, *Bacillus arsenicoselenatis* strain E1H (renamed *B. arseniciselenatis*) and *B. selenitireducens* strain MLS10 (Switzer Blum et al., 1998). Both grew by oxidizing lactate to acetate + CO_2, with strain E1H growing via reduction of Se(VI) to Se(IV), and strain MLS10 via reduction of Se(IV) to Se(0). Both were adapted to high pH (optima between 8.5 and 10.0) and salinity, with strain E1H having growth between 20 and 120‰, while strain E1H had a much broader range (20–220‰). Both species also proved capable of growing via dissimilatory reduction of As(V) to As(III), and the respiratory arsenate reductase (*arrA*) of *B. selenitireducens* was shown to align with the family of Mo-containing arsenate reductases of other mesophilic arsenate respirers (Afkar et al., 2003). Both species also proved capable of growth via dissimilatory reduction of oxyanions of tellurium, but they were sensitive to the presence of concentrations >1 mM Te-oxyanions added to the medium (Baesman et al., 2007). Therefore, an isolation protocol was imposed by using 10 mM tellurite [Te(IV)] as the enrichment selection factor to find a more robust organism in this regard. The result was isolation of *B. beveridgei* strain MLTeJB to honor of the late Professor Terry Beveridge. This microorganism could use Te(IV), Te(VI), Se(IV), Se(VI), and As(V) (amongst others) as respiratory electron acceptors to sustain growth with the concomitant oxidation of lactate (Baesman et al., 2009).

The observation that our isolates from Mono Lake all had a clear affinity to employ arsenate as an electron acceptor coupled with the unusual abundance of this oxyanion in the lake water (Table 2) led us to conduct experiments designed to measure biological As(V) reduction in the lake's stratified water column by employing the radiotracer ^{73}As(V) (Oremland et al., 2000). Field incubations demonstrated that anoxic samples recovered from below the chemocline were able to reduce ^{73}As(V) to ^{73}As(III), yielding rate estimates ranging between

0.5 and 5.9 μmol L^{-1} day^{-1} with the highest rates evident just below the chemocline. It was estimated that this vertically integrated activity could mineralize as much as ~14 % of annual primary productivity occurring during meromixis. In a subsequent study that used a consideration of seasonal mass balances of arsenic oxyanions, a similar estimate was arrived at (~10 %) (Hollibaugh et al., 2005). Both studies found cell densities of arsenate-respiring bacteria in the monimolimnion, as determined by MPN techniques, to be in the 10^2–10^3 cells mL^{-1} range. The next question was what were the types of bacteria and/or archaea that were responsible for the observed activity? A water column culture-independent microbial diversity study was made using 16S-based rRNA genes cloned from DNA collected at various depths (Humayoun et al., 2003). There was a fundamental difference observed between the populations in the surface waters as opposed to the more richly diverse population in the monimolimnion, but this approach did not tackle the question of assigning species-specific responsibility for arsenic metabolism. Aspects of the earlier work conducted on arsenic biogeochemistry of Mono Lake were reviewed by Oremland et al. (2004).

While conducting experiments with manipulated anoxic bottom water aimed at assigning the responsibility mentioned above two serendipitous discoveries of were made: (1) the nitrate-linked biological oxidation of arsenite and (2) the sulfide-linked biological reduction of arsenate. The first case led to the isolation and characterization of *Alkalilimnicola ehrlichii* strain MLHE-1, a haloalkaliphilic gammaproteobacterium capable of chemoautotrophic growth via nitrate respiration using arsenite, hydrogen, or sulfide as electron donors, and of aerobic, heterotrophic growth upon acetate (Oremland et al., 2002; Hoeft et al., 2002, 2007). This microbe was to prove seminal in further discoveries because its genome lacked homologs of arsenite oxidase (*aoiB*) but had two evident homologs of respiratory arsenate reductase (*arrA*) even though the strain showed no capacity for As(V) reduction. The arsenate reductase was found to be operating in a reverse functionality in vivo (Richey et al., 2009), and knockout mutants of the strain demonstrated that one of the annotated arsenate reductases was essential for cell growth on arsenite plus nitrate (Zargar et al., 2010). A new clade of anaerobic arsenite oxidase genes were assigned and designated *arxA* because of their proximity to arsenate reductases in lieu of aerobic arsenite oxidases (*aoiB*). The second discovery led to the isolation of strain MLMS-1, an anaerobic deltaproteobacterium that was also an obligate chemoautotroph and the first example of an obligate arsenate respirer (Hoeft et al., 2004). Further work with culture-independent and culture-dependent molecular characterization approaches indicated a greater diversity amongst the deltaproteobacteria of Mono Lake that had this capacity to oxidize sulfide with arsenate (Hollibaugh et al., 2006).

In our early sojourns to the shores of Paoha Island, a mass of volcanically uplifted bottom sediments located in the middle of Mono Lake, we noted the presence of and smelled the sulfide emanating from a series of small hot springs located on the shoreline of the island's southeast corner. When examined more closely, these springs, most of which had temperatures of ~45 °C, were adorned

with red biofilms. We thought it likely that they were composed of anaerobic bacteria carrying out anoxygenic photosynthesis by using the aqueous sulfide present as their primary electron donor. Yet these anoxic waters also contained an abundance of other chemical reductants in addition to sulfide, which included dissolved methane and notably ~100 μM arsenic, mostly in the form of As(III). We then initiated investigations using the live biofilm materials with the goal in mind of determining if As(III) could also serve an electron donor to support anoxygenic photosynthesis (Kulp et al., 2008). These early results were successful, in that we observed light-driven anaerobic As(III) oxidation, which did not occur in dark-incubated controls. We hypothesized that As(III) could also support growth, and Shelley Hoeft eventually isolated a pure culture of a red-pigmented gammaproteobacterium from the biofilm, preliminarily named *Ectothiorhodospira* strain PHS-1, which demonstrated As(III)-dependent growth, forming As(V) as a product in proportion to the amount of As(III) consumed. When PCR tested for the presence of arsenite oxidase using primers appropriate for aerobic As(III)-oxidizing bacteria (*aoiB*), no amplicons were obtained, yet strain PHS-1 successfully produced PCR amplicons when primers for respiratory arsenate reductase (*arrA*) were employed. The sequenced product aligned closely with that of the *arxA* of *A. ehrlichii*. A further characterization of the biofilm material revealed that it is mostly, but not entirely, composed of *Ectothiorhodospira*-like organisms and harbors functional genes of both the classic *arrA* type and the *arxA* type (Hoeft et al., 2010). The former gene is involved in employing As(V) as a respiratory oxidant formed by the latter's light-driven activities. These are carried out by different bacterial populations, with As(V) respiration being driven by chemoautotrophy using sulfide or H_2 as electron donors. The *arxA* was also upregulated in strain PHS-1, and the *arxA* gene itself was identified as a new clade of anaerobic Mo-containing arsenite oxidases phylogenetically more closely related to the *arrA* arsenate reductases than to the *aoiB* aerobic arsenite oxidases (Zargar et al., 2012).

One more finding concerning unusual microbes discovered in Mono Lake certainly bears mentioning, namely, the claim that a halomonad, strain GFAJ-1, could get by using arsenate instead of phosphate to sustain growth (Wolfe-Simon et al., 2011). This aerobic organism was isolated from shoreline mud by using arsenate in lieu of phosphate in the medium, and growth experiments indicated it could grow reasonably well with 40 mM As(V) substituting for the normally employed 1.5 mM phosphate. It certainly grew better and faster with this amount of phosphate than the As(V), but the implications of the growth experiments were profound, namely, that arsenate may successfully substitute for phosphate in nucleic acids. It implied that life may not be constrained to only the six major known elements (C, N, S, O, P, and H) that make up the bulk of living biomass. A variety of follow-up experiments and analyses in the original paper supported this claim, which included distribution of ^{73}As(V) radiolabel in the cells, EXAFS/XANES spectra, and nano-SIMS analysis of extracted DNA. The prepublication (on-line version) of the paper in the journal Science in December 2010 coincided

with a widely disseminated NASA-sponsored press conference and media follow-up. The mood of the scientific and lay community quickly changed in a matter of a couple of days from astonishment to disbelief and anger, especially in the on-line "blogosphere" but also from reputable scientific news sources and commentaries in professional journals (e.g., Rosen et al., 2011; Silver and Phung, 2011). I will not recount my perspective on these details in this chapter, other than to say it was an extremely harrowing experience. Nonetheless, such extraordinary claims must be tested experimentally, especially by disinterested parties, to either support or refute the original contentions. The genome annotation of strain GFAJ-1 was recently published (Phung et al., 2012) along with commentary on its possible significance, or lack thereof, regarding the question of arsenic in DNA (Kim and Rensing, 2012). I will say at this juncture that the key item to be concerned with is the background level of phosphorus contained in the impurities of the reagents employed for culturing this microorganism. Discounting all the personalities involved, the media circus and the many strong (and at times quite nasty) opinions expressed on-line, the scientific process will resolve the ultimate controversy. In this regard, it is incumbent on me to briefly discuss the most recent experimental publications that have appeared after the first draft of this chapter was submitted.

The results of Reaves et al. (2012) and Erb et al. (2012) clearly do not support the original observations made in my laboratory with the newly isolated strain GFAJ-1 which gave evidence for arsenate-dependent growth at low background levels of P (Wolfe-Simon et al., 2011). Because we originally observed no growth at these background phosphorus concentrations (~3 μM), we concluded that added arsenate insinuated itself into the bioenergetic and genetic operation of this microorganism and was carrying out P-related functions under conditions of severe P-limitation. We provided the strain to two established culture collections (ATCC and DSMZ), as well as to a number of labs that requested it for further testing either in confirmation or refutation of the original hypothesis posed. In recent weeks my own lab has reexamined this question after eliminating the problem of background P, and we too have failed to see any evidence for arsenate-dependent growth in the absence of phosphorus, although we have observed a modest arsenate-growth enhancement at 1–3 μM phosphate. This evidence in itself is clearly not sufficient to support our original hypothesis, but it does bear closer scrutiny in the months to come.

So what can account for this key disparity between the original growth data published in Wolfe-Simon et al. (2011) and that of Reaves et al. (2012) and Erb et al. (2012) as well of those of my own lab's recent efforts? One possibility is that this trait has been lost over time (~18 months) with the continued transfers of the cell line made into media either replete with arsenate or with phosphate. Another opinion, as suggested by Basturea et al. (2012), is that a scavenging of ribosomal phosphate occurs in strain GFAJ-1. These workers demonstrated with *E. coli* that added arsenate at low background P resulted in similar growth to that originally observed with strain GFAJ-1 held under similar conditions. Finally, Kang et al.

(2012) noted that the induction of arsenite oxidation genes in *Agrobacterium tumefaciens* occurs only at low P concentrations (<5 μM), a phenomenon which presumably somehow aids in the scavenging of phosphate from the surrounding environment and might be applicable to the case of GFAJ-1.

So where do things stand now? While the progress of science is usually thought to move in a straightforward, logical fashion, in reality it often progresses more like that of a pinball in an arcade game. While the results of Reaves et al. (2012) and Erb et al. (2012) may eventually firmly close the door on the validity of the original arsenic-life hypothesis, at this point I would say it is still just a tad ajar, with points worthy of further study concerning the metabolic effects of arsenate upon cells encountering severe P-limitation.

4. Searles Lake, California

Located in the Mojave Desert of southeastern California (35°45′ N, 117°22′ W) by the small town of Trona, Searles Lake occupies the basin floor of Searles Valley (dimensions ~16 km N/S and ~10 km E/W). The basin consists of broad alkaline playas, evaporation ponds, salt flats, and an ephemeral shallow lake that owes its variable surface water content to the extent of any runoff from local winter precipitation coupled with industrial discharge water stemming from Searles Valley Minerals, Inc. (http://www.svminerals.com/default.aspx). These operations extract a number of valuable chemical salts (e.g., borax, soda ash, sodium sulfate) from heated brine solutions. The brines are pumped up from the subsurface underlying the lakebed. Hence, the extent of the lake's area is seasonally variable and as pictured below (Fig. 3) runs roughly 3.5 km along the W/SE axis and 2.1 km N/S axis, with a depth of ~1.0 m. In our first excursion to the lake in 2004 led by Larry Miller, the region sampled had no surface water visible and consisted only of a thick (~12 cm) salt crust beneath which lay a salt-saturated, dense brine perhaps 30 cm deep, underlain further by anoxic sediments. When we returned early the following spring after a rainy winter, there was approximately 50 cm of water overlying the salt crust which was, despite this water's presence, still quite firmly intact. Table 3 gives the major constituents of the Searles Lake brine.

The impetus for examining Searles Lake came out of our interest in arsenate-respiring prokaryotes. As part of our review paper on arsenic metabolism (Oremland and Stolz, 2003), John Stolz produced a 16S rRNA gene sequence phylogenetic tree which provided a map of "DARPs" or dissimilatory arsenate-respiring prokaryotes. While there were over 20 species of diverse DARPs in the Domain Bacteria, the Domain Archaea had but two closely related species of hyperthermophiles, *Pyrobaculum aerophilum* and *P. arsenaticum*. Reasoning that there must be other examples of DARPs from the Archaea out there, we began a search for other "extremophile" conditions, assuming (incorrectly) that anaerobic, halophilic Archaea would thrive in such locales. We settled on the arsenic-rich environment of Searles Lake. After contacting some of the folks at Searles Valley

Figure 3. A satellite image of Searles Lake and the surrounding Searles Valley courtesy of Google Maps. A *push pin* notes the lake's general location, which is surrounded by dry playa, salt flats, and evaporation ponds (Scale, W/SE axis of the lake's boundary = ~3.5 km).

Table 3. Major constituents of the Searles Lake brine underlying its salt crust.

Constituent[a]	TDS	pH	Cl^-	HCO_3^{-b}	Na^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	As[c]	B	CH_4[d]
–	~350	9.8	5,250	640	7,430	ND	ND	730	4	460	<1

Compiled from Smith (1979), Felmy and Weare (1986), and Oremland et al. (2005).
ND not determined.
[a]Units are as milli-molal (millimoles per kilogram of solvent).
[b]Carbonate plus bicarbonate.
[c]Millimolar.
[d]Micromolar.

Minerals, they agreed to ship us anoxic sediment from which Jodi Switzer Blum isolated strain SLAS-1, a haloalkaliphilic anaerobe capable of growth at salt saturation (~350 g/L) and respiring arsenate. But alas, the isolate was not a member of the Archaea but belonged to the *Haloanaerobiales* of the Domain Bacteria. Nonetheless, it was clearly a polyextremophile, demonstrating growth only at alkaline pH (optimum ~9.4) and only at high salinities (>200 g/L) including at salt

saturation (~350 g/L) (Oremland et al., 2005; Switzer Blum et al., 2009). Strain SLAS-1 had a mixed metabolism, able to grow either as a chemoorganotroph (e.g., lactate/arsenate) or as a chemoautotroph (sulfide/arsenate) using inorganic carbon as its cell C source, although cell extracts lacked RuBisCo activity.

Tom Kulp found that sediments from Searles Lake also demonstrated clear biological activity with regard to their ability to reduce As(V) to As(III) under anoxic conditions, as well as their capability to oxidize As(III) back to As(V) in aerobic incubations. Larry Miller and Tom Kulp, along with the rest of my project (Shelley Hoeft and Shaun Baesman), made a field excursion to Searles Lake in order to obtain sediment cores, which, when processed, gave clear evidence for a reduction of As(V) to As(III) with vertical transit from surface oxic conditions into the anoxia prevalent in the sediments. This was written up in our paper in Science, which for the purpose of logical "storytelling" described the reverse order of the actual chronology of events (i.e., sediment profiles sediment slurry experiments isolation and characterization of strain SLAS-1). It still remains to be determined if an arsenate-respiring extreme halophile from the Domain Archaea exists. Nonetheless, *Halarsenatibacter silvermanii* strain SLAS-1 is a most unusual organism, especially with regard to its sinuous motility which is more akin to that of an eel than the "twiddle and run" of a "typical" bacterium like *E. coli*.

The enrichment culture from which we isolated *H. silvermanii* also had the clear ability to form sulfide, and ^{35}S-sulfate radiotracer demonstrated it was produced via sulfate reduction. Subsequent work resulted in the isolation of strain SLSR-1 from the enrichment, which had the capacity to grow via sulfate reduction, as well as via arsenate respiration (Switzer Blum et al., 2012). The organism is also an extreme halophile, capable of growth (and sulfate reduction) at salt saturation, which has been a topic of scientific curiosity for those interested in hypersaline ecosystems (Oren, 1999, 2011).

The question of respiratory arsenate reduction and sulfate reduction in Searles Lake sediments was investigated further and compared with similar experiments conducted in Mono Lake (Kulp et al., 2006, 2007). Although both sulfate reduction and arsenate reduction were robust in Mono Lake, only arsenate reduction could be detected in Searles Lake. Despite a number of attempts and manipulations to enhance sulfate reduction in the latter environment (e.g., substrate additions, lowering background sulfate, lowering the salinity), no activity could be solicited. Addition of 200 mM borate ions to Mono Lake sediments resulted in ~80 % diminishment in sulfate reduction as compared with controls, while increasing the salinity from 25 to 325 g/L completely eliminated detectable sulfate reduction. By extrapolation to Searles Lake, these results indicated that the combined effects of high salinity and high borate concentrations in the brine conspired against expression of sulfate reduction in situ. Nonetheless, free sulfide was detected in Searles Lake sediments, albeit at levels between 10- and 100-fold lower than in comparable Mono Lake sediments (e.g., 0.1–0.3 vs. 5–10 mM). The origin of this sulfide is not clear, but it could stem from a partially constrained

sulfur cycle associated with the redox reaction of lower oxidation states of sulfur (e.g., thiosulfate, sulfite, elemental sulfur) that give rise to sulfide upon reduction (or disproportionation) and do not require expenditure of energy to activate, as does sulfate. These processes have been studied closely in the soda lakes of Russia and have been recently reviewed in detail (Sorokin et al., 2011).

One final point worth bearing in mind as a reason to study the microbial ecology and biogeochemistry of hypersaline lakes is that they can be considered potential terrestrial analogs of possible biomes for life elsewhere in our Solar System. Hence, Mars is currently a cold and exceptionally dry environment. Any liquid water encountered upon or well beneath its surface is likely to be highly saline. Similar arguments can be made for the dense brines existing beneath the ice sheets of Europa (satellite of Jupiter) or of Enceladus (satellite of Saturn). Detection of in situ life, if these brines could be remotely sampled, would pose a challenge akin to that encountered for the Viking Mission to Mars in the 1970s, namely, what would constitute strong proof (or disproof) for an active microbial population? To this end we tested the ability of isolated anaerobes (*B. selenitireducens* and *H. silvermanii*) and anoxic sediments from Mono and Searles Lakes to generate electricity by using an anode of a microbial fuel cell as an electron acceptor. Power generation occurred in live samples and was greater in the lower salinity samples and cultures from Mono Lake than from Searles Lake. Nonetheless, biological electricity generation was notably occurring in the latter, suggesting that this approach to life detection has merit (Miller and Oremland, 2008).

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