A Microbial Arsenic Cycle in Sediments of an Acidic Mine Impoundment: Herman Pit, Clear Lake, California

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ABSTRACT

The involvement of prokaryotes in the redox reactions of arsenic occurring between its +5 [arsenate; As(V)] and +3 [arsenite; As(III)] oxidation states has been well established. Most research to date has focused upon circum-neutral pH environments (e.g., freshwater or estuarine sediments) or arsenic-rich “extreme” environments like hot springs and soda lakes. In contrast, relatively little work has been conducted in acidic environments. With this in mind we conducted experiments with sediments taken from the Herman Pit, an acid mine drainage impoundment of a former mercury (cinnabar) mine. Due to the large adsorptive capacity of the abundant Fe(III)-rich minerals, we were unable to initially detect in solution either As(V) or As(III) added to the aqueous phase of live sediment slurries or autoclaved controls, although the former consumed added electron donors (i.e., lactate, acetate, hydrogen), while the latter did not. This prompted us to conduct further experiments with diluted slurries using the live materials from the first incubation as inoculum. In these experiments we observed reduction of As(V) to As(III) under anoxic conditions and reduction rates were enhanced by addition of electron donors. We also observed oxidation of As(III) to As(V) in oxic slurries as well as in anoxic slurries amended with nitrate. We noted an acid-tolerant trend for sediment slurries in the cases of As(III) oxidation (aerobic and anaerobic) as well as for anaerobic As(V) reduction. These observations indicate the presence of a viable microbial arsenic redox cycle in the sediments of this extreme environment, a result reinforced by the successful amplification of arsenic functional genes (aioA and arrA) from these materials.

Introduction

Over the past two decades considerable scientific attention has been focused on the question of how arsenic is cycled in the environment (e.g., Frankenberger 2002; Nriagu 1994; Santini and Ward 2012). Much of the impetus for this work was prompted by reports of outbreaks of arsenicosis in rural communities of Bangladesh and India resulting from the consumption of As-contaminated groundwater. The hydrological mobility of arsenic is a complex, interdisciplinary question complicated by a number of physical, geochemical, and microbiological factors. The microbiological facet of this question has led to major vertical leaps in understanding the modes of biochemical interaction between microorganisms and this toxic element (Dhuldhaj et al. 2013).

Among these discoveries were the findings that energy conservation in certain prokaryotes could be linked to the reactions associated with the respiratory reduction of arsenate [As(V)] and the oxidation of arsenite [As(III)], the two environmentally relevant redox states of this element. Some of these discoveries were made with new microbial species isolated from “extreme” environments that are unusually rich in arsenic content. These environments typically included hot springs and soda lakes located in geothermal regions, their arsenic abundance owing to the incipient influence of volcanic activity and resultant hydrothermal leaching of the arsenic-containing minerals that occur within the surrounding geologic envelope. However, these “arsenotrophic” processes are also operative in mesophilic organisms, and can be detected in freshwater, marine, soil and subsurface aquifer ecosystems, and are thus widespread in nature (Lloyd and Oremland 2006; Oremland and Stolz 2005). Because of the abiding interest in this phenomenon, a number of comprehensive scholarly reviews have appeared over the past decade that have focused on the microbiology, biogeochemistry, genetics, and biochemistry of these phenomena (e.g., Cavalca et al. 2013; Krüger et al. 2013; Oremland and Stolz 2003; Oremland et al. 2009; Silver and Phung 2005; Slyemi and Bonnefoy 2012; Stolz et al. 2006; van Lis et al. 2013; Yamamura and Amachi 2014; Zhu et al. 2014).

One type of “extreme” As-rich environment that has been somewhat neglected in this context are acid mine drainages (AMDs). Sulfidic ores of geothermal origin that are rich in Au, Ag, Cu, and Zn also have a high associated As content (Cheng et al. 2008; Drewniak and Sklodowska 2013).
Microbial arsenic oxidation occurs in the Giant Mine of Yellowknife, Canada, an arctic environment with As(III) oxidation activity that is more notable for its occurrence at low ambient temperatures rather than at its circumneutral pH (Bromstad and Jamieson 2012; Osborne et al. 2010). The AMD associated with the Carnoulès mine in France has been studied from the perspective of As(III) oxidation occurring along its drainage path (Bruneel et al. 2003), potential bio-remediation via microbial Fe(II)-oxidative interventions (Bruneel et al. 2003; Duquesne et al. 2003), and characterizations of the microbial community made via culture-independent approaches (Bertin et al. 2011; Bruneel et al. 2006; Bruneel et al. 2011).

The biofilms within the gold-rich former Zloty Mine in Poland were characterized using culture-independent molecular approaches, which detected genes for respiratory arsenate reduction (arrA) and arsenite oxidation (aioA). However, it was not evident if this is a low pH or circum-neutral environment, or if there was a concomitant expression of these genes that resulted in a detectable microbial arsenic redox cycle (Tomczyk-Zak et al. 2013). The diversity of arsenic-resistance genes extracted from the headwaters of the acidic (pH ≈ 2.2) and metal contaminated Tinto River in southwestern Spain was studied by Morgante et al. (2014), but investigations related to the presence of arsenotrophy redox genes (e.g., arrA, arrX, aioA) were not pursued.

The well-studied Richmond Mine located within Iron Mountain in northern California has drainages with strongly acidic pH (Drushel et al. 2004; Nordstrom and Alpers 1999). This site has a very high total arsenic content in its waters (0.2–1.8 mM). Yet, other than examining the ability of Acidithiobacillus caldus to leach arsenopyrite materials with regard to release of iron into solution, there has been no published work focused on the arsenic cycle per se (Druschel et al. 2004). Rather the focus of Iron Mountain investigations has been on microbially mediated Fe(II)- and sulfur-oxidation (Edwards et al. 2000), especially as they relate to proton release. Detailed characterizations of the thick biofilms have also been made by culture-independent means (e.g., Bond et al. 2000; Tyson et al. 2004). Nonetheless, it is curious that despite the abundance of arsenic at this site, no arsenic-metabolizing functional genes were annotated in these widely accessible meta-genomic databases (Oremland et al. 2005).

From the examples cited here, it is still not evident if a fully operative arsenic redox cycle can occur under acidic conditions, or even if the potential for such resides in the resident microbial community once restored to circumneutral pH. Furthermore, there are as yet no examples of acidophilic arsenotrophic prokaryotes in culture collections comparable to the halophilic alkaliphiles discovered in soda lakes (Hoeft et al. 2007; Oremland et al. 2004; Switzer Blum et al. 1998). To address these questions we examined sediments collected from the Herman Pit impoundment of the former Sulfur Bank Mine for their ability to carry out two key arsenotrophic microbial reactions, namely anaerobic reduction of arsenate and oxidation of arsenite under aerobic or anaerobic (nitrate-amended) conditions. We now report that these sediments harbor microorganisms capable of dissimilatory reduction of arsenate, and both aerobic and anaerobic oxidation of arsenite. We also carried out successful amplification and sequencing of the functional genes (e.g., arrA and aioA) that drive these processes.

**Materials and methods**

**Site description and sample collection**

The Sulfur Bank Mine (39°00’ N 122°39’ W), currently a US EPA Superfund site, is located on the southeastern bank of Clear Lake, California, a region of geothermal activity. After abandonment this former open-pit mercury mine (cinnabar ore) filled with AMD seepage from the surrounding watershed, which resulted in its present day acidic conditions (Engle et al. 2008; Suchanek et al. 2008; Wells and Ghiorso 1988). The clear, azure-hued waters of the Herman Pit are acidic (pH 2–4), and overlie oxidized sediments that have a distinctive red/orange rustlike coloration indicative of the presence of abundant ferrisiderites and other poorly crystalline Fe(III) minerals (Supplementary Figure S1). There is extensive and continuous ebullitive release of geothermal gases from the lake bottom in the form of numerous constantly flowing seeps which are composed primarily of mixtures of CO₂, CH₄, and H₂S (Nehring 1981).

Bacterial methane oxidation in these sediments has recently been investigated (Baesman et al. 2015). Hydrogen sulfide ebullition is perceptible to the nose, and ambient levels in air at the site can elevate to become an immediate health hazard. Surface waters are oxic (annual range dissolved O₂: 160–218 μM), mesothermal (annual range: 11–22°C), with variable but generally low dissolved arsenic concentrations (annual range: 60–338 nM) (unpublished data, USEPA Sulfur Bank Remedial Investigation and Feasibility Study, 2013; E2 Corporation data report, J. Lucero, personal communication). We collected nearshore surface sediments in March, 2014 using an Eckman grab, and stored the highly porous “soupy” material in completely filled mason jars that were kept refrigerated until experiments commenced (within a few days of sampling).

**Experiments with sediment slurries**

Initial experiments were conducted using 3:1 mixtures of lake water: sediment so as to generate dilute slurries which were subsequently amended with mM levels of electron acceptors (arsenate, nitrate), electron donors (arsenite, acetate, lactate, hydrogen), and incubated under N₂, air, or H₂. Slurries (25 ml) were dispensed into 59-ml serum bottles and crimp-sealed with blue butyl rubber stoppers under air, nitrogen, or hydrogen. Samples were incubated statically, the slurry was periodically sampled (after vigorous hand-shaking) by syringe, and effluent passed through a 0.22-μm filter-fitted centrifuge tube (Corning Costar SpinX, Tewksbury, MA) to remove sediment particles from the liquid analyte before analysis for anions via HPLC or IC (see below). Because adsorption of both As(V) and As(III) proved to be extensive and problematical at this initial water: sediment ratio, in subsequent experiments a further dilution was made whereby slurry aliquots (2 ml) from previously incubated, live samples were transferred into 59-ml serum bottles that contained 30 ml of lake water and treated as described.
here. Killed controls consisted of autoclaved materials (121°C; 30 min).

Experiments were also conducted using this dilution scheme to ascertain the pH range for viable As(V) reduction and As (III) oxidation. For As (V) reduction 30-ml aliquots of the artificial medium of Silverman and Lundgren (1959) containing (g/L): NaCl (3.0), K2HPO4 (0.5), KCl (0.1), MgSO4•7H2O (0.5), Ca(NO3)2 (0.1), plus (ml/L): NMS trace elements (Dalton and Whittenbury 1976) (1.0), 3.8% FeEDTA (0.1), 0.1% NaMoO4•2H2O (0.5) were dispensed into 59-ml serum vials. The pH was adjusted by addition of varying combinations of NaOH was also added. The incubation temperature was 23°C. For As(III) oxidation, 25 ml Herman Pit sediment slurried 3:1 with lake water (pH = 4.5) as above was dispensed into 59-ml serum bottles and crimped sealed either under air (aerobic incubation) or N2 (anaerobic incubation with 5 mM NaNO3), after which 5 mM As(III) was injected by syringe resulting in pH increases to 6.7 and 7.0, respectively. The pH was poised after which 5 mM As(III) was injected by syringe resulting in 57°C (aioA) or 60°C (arxA) for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR detection of the arsenate respiratory reductase, arrA, was done using a semi-nested PCR procedure and primers reported elsewhere (Ranganathan 2005; Stolz et al. 2011).

The first round PCR was conducted using primers arrAUF1 (5'-TGGAAGGTTAATCDBDTCGG-3') and arrAUR5 (5'-GGTTTCRCTTRAAACTCTAAAHT-3'). The second PCR was done with the same forward primer (arrAUF1) and an internal reverse primer, arrAUR3 (5'-GCWGGCCAYTCVGGN-3'). For the first round PCR (arrAUF1 and arrAUR5), a 25-μl reaction was prepared consisting of ~45 ng of DNA, 2X Taq mix, and 0.6 μM of each primer. The following thermocycler profile was used: initial denaturing at 95°C for 5 min followed by 30 cycles of denaturation of 95°C for 30 s, annealing at 57°C (aioA) or 60°C (arxA) for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 5 min. This yielded a 1825 bp PCR amplicon using DNA from an arr-containing strain Shewanella sp. str. ANA-3. The second round semi-nested PCR (using arrAUF1 and arrAUR3) consisted of 3 μl of the first PCR as a DNA template, 2X Taq mix, and 0.6 μM each primer adjust to 25 μl total volume with nuclease free water.

The thermocycler profile consisted of: initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation of 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min with a finishing extension at 72°C for 5 min, yielding a 850 bp PCR amplicon. The optimal annealing temperatures were identified by gradient PCR on an Opticon2 DNA Engine (MJ Research Inc., Watertown, MA) using Shewanella sp. str. ANA-3 genomic DNA. PCR products were examined by UV fluorescence after electrophoresis in a 1.0% agarose gel containing a 1 μg ml⁻¹ ethidium bromide, in TAE buffer at 108 V for 45 min.

**Detection of 16S rRNA gene**

The 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GCGGCTGTGTACCTT-3'). PCRs consisted of: 20 ng of template DNA, 12.5 μl of 2X Taq mix (Promega), 0.2 μM of each primer, and nuclease-free water to a final volume of 25 μl. PCR was performed with the following thermocycler profile: an initial denaturing step at 95°C for 5 min, 30 cycles of denaturation (95°C for 30 s), annealing (52°C for 30 s) and extension (72°C for 1.5 min) followed by a final extension at 72°C for 5 min. PCR products (5 μl) were analyzed by electrophoresis in a 1% agarose gel prepared in 1X TAE with 1 μg ml⁻¹ ethidium bromide. After electrophoresis at 108 V for 45 min, gels were visualized by UV fluorescence.

**PCR amplification of aioA, arxA, and arrA**

PCRs for the arsenite oxidase aioA were prepared using the following primers: AOX-F-A2 (5'-TGCATCGTCGGCTGYGGNTAY-3') and AOX-R-E2 (5'-TTCGGGATATAAGCCGNNCRKTRTT). For the arxA anaerobic arsenite oxidase, the following primers were used: arxA_Deg_F_B (5'-CCATCWSCTGRACGGACCCYTSG-3') and arxA_Deg_R_B (5'-GTWGTGTAGGGCCGGAAS-3'). The composition of PCRs for aioA and arxA were similar to those for 16S rRNA gene. The thermocycler profiles for both aioA and arxA consisted of: initial denaturing step at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C (aioA) or 60°C (arxA) for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR detection of the arsenate respiratory reductase, arrA, was done using a semi-nested PCR procedure and primers reported elsewhere (Hofert et al. 2004; Sulfate and arsenite were measured by ion chromatography (Herbel et al. 2003).
products, plasmids (24 clones per microcosm sample) were screened for the correct insert size using M13F and M13R vector primers. The diversity of the clones were screened for RFLPs by digesting plasmids with HaeIII (New England Biolabs) followed by agarose gel electrophoresis analysis using 1.5% agarose gels prepared in 1X TEA. The RFLPs were chosen for Sanger sequencing at Sequentech (Mountain View, CA).

Clone libraries for functional genes were constructed and analyzed similarly to the 16S rRNA gene PCR amplicons. For aioA, 28 clones were screened for the proper plasmid insert size. Of the 28 purified plasmids, 6 clones representing different RFLPs were chosen for sequencing. For the cloned arrA PCR amplicons, 24 clones per sample were screened for the proper plasmid insert size. Sequencing was done by the Sangar dideoxynucleotide termination method at either UC Davis DNA Sequencing Facility (CA) or Sequetech DNA Sequencing Service (Mountain View, CA).

**Phylogenetic analysis**

The phylum and genus level affiliations for the partial 16S rRNA gene sequences were determined using the RDP Classifier tool and Seqmatch tools (http://rdp.cme.msu.edu). Phylogenetic inferences were made based on top hits to selected type strains in the RDP following the Seqmatch tool. Various known bacterial type strains were included that showed the highest similarity scores within the RDP. The retrieved RDP alignment file was used as an input to PhyML (Guindon et al. 2010). The HKY85 nucleotide substitution model was used to find the tree with the optimal maximum likelihood. Alignments and phylogenetic analyses of the predicted partial AioA and ArrA sequences were conducted as previously described (Zargar et al. 2010).

**Results**

**Anaerobic arsenate reduction**

Initial experiments with sediment slurries made note of their strong adsorptive capacity for added arsenic anions (Table 1).

In the case of arsenate, a cumulative total of 14 mM As(V) was added incrementally to slurries over the first three days of the incubation, but no As(V) was ever evident in solution in either the live (Table 1) or heat-killed controls (not shown) over the entire course of the 3 month incubation. With time, however, live slurries demonstrated the appearance of a small amount of As(III) in the aqueous phase which represented a recovery of only ~ 2% of the added As(V). Complete consumption of lactate was complimented by the recovery of acetate, which also was entirely consumed by the end of the incubation. In contrast, killed controls neither consumed lactate nor produced acetate (data not shown).

In a parallel experiment with acetate as the electron donor, essentially the same results were obtained, namely no detection of any of the added 14 mM As(V) in solution, and the transient appearance of As(III) accounting for at most only 7% of the As(V) added (Table 1). When incubated with 100% H2 as the electron donor, a negative pressure developed over time which indicated its consumption, but again there was no evidence of the 14 mM added As(V) ever appearing in solution. As in the preceding experiments with organic acids, by day 18 variable and small amounts of As(III) were evident in the duplicate live slurries over the incubation. The results with H2 were, therefore, consistent with the two other electron donors employed.

In an attempt to overcome the As(V) adsorption to better discern the influence of microorganisms on As(V) reduction, sub-samples of live slurries from the experiment described above were transferred into lake water and incubated as before (Figure 1). Here As(V) was detected in solution and its consumption over a prolonged incubation was notable along with the emergence of As(III), although there was not a 1:1 correspondence between them. Also evident was the oxidation of a comparatively small amount of lactate to acetate, yet there was no notable consumption of sulfate (Figure 1A). In the absence of added arsenate, there was obvious sulfate consumption and lactate oxidation to acetate was more extensive (Figure 1B). Without As(V) incubated sediments turned black, indicating the formation of an FeS precipitate, which contrasted with the orange color that persisted in the As(V)-amended slurries (Supplementary Figure S2). No notable reduction of As(V) to As(III) or removal of sulfate occurred in a live control without added lactate (Figure 1C). A third dilution of live slurry into lake water and incubated over a 2-month period resulted in good agreements between As(V) lost and As(III) recovered, as well as acetate recovered from lactate oxidation (Figure 1D).

The oxyanions arsenate and arsenite, each being differentially negatively charged over broad pH ranges have an inherent, strong buffering capacity (Smedley and Kinne-burgh 2002). Moreover, any biochemically mediated oxidative shift from As(III) to As(V) or reductive shift from As(V) to As(III) entails a significant drop in pH in the first instance, and a rise in pH associated with the latter instance. For example, a rise in pH to ~ 7.0 was observed upon addition of 5 mM As(III) to Herman Pit lake water (initial pH = 4.5). The initial pH measurements for the first As(V) reduction experiment shown in Table 1 were 6.5, 7.4, and 7.3 for the lactate, acetate and hydrogen amended conditions, which by the end of the incubation rose to 7.9, 7.8,

| Table 1. Time course of Herman Pit sediment slurries incubated in lake water with amendment of 14 mM arsenate as electron acceptor, and lactate, acetate, or hydrogen as electron donor. |
|------------------|------|------|------|------|
| **Solute (mM)** | T (0 days) | T (18 days) | T (27 days) | T (94 days) |
| **Lactate addition** | 14* | 0 | 0 | 0 |
| Arsenate | 0 | 0.567 ± 0.026 | 0.756 ± 0.026 | 0 |
| Arsenite | 4.0 ± 0.2 | 0 | 0 | 0 |
| Acetate | 4.9 ± 0.4 | 3.4 ± 1.3 | 0 |
| **Acetate addition** | 14* | 0 | 0 | 0 |
| Arsenate | 0 | 0.33 ± 0.02 | 1.0 ± 0.02 | 0.8 ± 0.01 |
| Arsenite | 4.4 ± 0.3 | 3.0 ± 0.4 | 0.5 ± 0.5 | 0 |
| **H2 addition** | 14* | 0 | 0 | 0 |
| Arsenate | 0 | 0.27 ± 0.004 | 1.02 ± 0.13 | 0.24 ± 0.05 |

*Initial amount of As(V) added. The results represent the average of duplicate slurries with error expressed as the range of observed values.
and 7.9, respectively. Hence, to determine if a truly acidophilic or even an acid-tolerant capacity exists in the arsenotrophic community of the Herman Pit samples, it was necessary to add a range of buffers to the slurries to counteract the pH effects diverging from ambient values caused by the addition of arsenic oxyanions or by their redox changes during incubation.

A time course of As(V)-reduction over a pH range was attempted using dilute sediment slurries incubated in artificial medium (Figure 2). After 2 weeks incubation (the time of the first sampling) activity with regard to As(V) consumption was evident in the samples held at pH 4.8 and 6.8, while samples incubated at pH 4.0 lagged and activity only initiated between days 35 and 50 (Figure 2A). No activity occurred at pH 3.3 over the course of the incubation, while activity did occur for samples incubated under slightly alkaline conditions (pH 8.9) although it lagged slightly when compared to the conditions at 4.8 and 6.8. By the end of the experiment, there was a clear upward shift of \([H^+]|\) by an order-of-magnitude in most samples with the exception of minor changes at the lowest and highest values. Thus samples initially poised at 3.3, 4.0, 4.8, 6.8, and 8.9 had final measured pH values of 3.7, 5.3, 5.8, 7.8, and 8.8, respectively.

In all cases of active As(V)-reduction there was a quantitative equivalency between As(V) removed and recovery of As(III), and there was also a general balance for lactate consumption with recovery of acetate (Figure 2). There was also a good balance achieved between electrons produced from lactate oxidation to acetate with the electron sink of As(V) being reduced to As(III). For example, in the case of pH 4.0 samples, 12 mM equivalent electrons were formed from oxidation of 3 mM lactate to 3 mM acetate plus 3 mM CO₂, which was balanced by the reduction of 6 mM As(V) to its recovery as 6 mM As(III), thereby consuming all of the 12 mM equivalent electrons. These results underscore that dissimilatory arsenate reduction, rather than a reductive detoxification reaction (i.e., \(arsC\)), is the biochemical process responsible for the observed arsenic redox reactions.

**Arsenite oxidation**

In an experiment that ran concurrently with As(V)-reduction (Table 1), the oxidation of added As(III) was examined in sediment slurries incubated under both aerobic and anaerobic (nitrate amended) conditions. A total of 13 mM As(III) was added incrementally to both the aerobic and anaerobic slurries over a 26-day time course. Immediately after each pulsed addition of As(III) (~2–5 mM) to aerobic or anaerobic slurries, approximately 20% of the As(III) remained in solution. Over time each pulse of As(III) disappeared but an appearance of As (V) in solution was never observed over the entire time course of the incubation. In addition, for the anaerobic slurry nitrate...
disappeared by the end of the 26-day incubation, while nitrate concentrations remained unchanged in the autoclaved control (data not shown).

In an aerobic experiment made with diluted live slurries from the preceding experiment, the consumption of several injected pulses of As(III) resulted in the accumulation of As(V) although only about half of the total added former (12 mM) accumulated as the latter (7 mM), indicating there still was persistent As(V) adsorption (Figure 3A). A subsequent incubation experiment with a further dilution of these live materials into lake water resulted in a 1:1 correspondence of arsenite consumed (~9 mM) with that recovered in solution as arsenate (~9 mM) (Figure 3B). Arsenite oxidation was also detected in anaerobic slurries with a concurrent removal of nitrate (Figure 3C).

For the pH range experiments, with the exception of the two alkaline pH values (8.9 and 10.5) As(III) consumption in aerobic sediment slurries generally proceeded linearly over the time course of the incubation (Figure 4A), and was associated with the formation of arsenate in all cases (not shown). The optimal pH for arsenite oxidation was 6.7, although there was slower but still significant activity at pH 2.7 and at 7.2 (Figure 4B). Little activity was noted at or above pH 8.9. Under anaerobic conditions, qualitatively similar results were obtained, although the rates themselves were only about half those of the aerobic conditions (Figure 4D). Curiously, despite As(III) removal being evident along with accumulation of 1.3, 1.5 and 2.0 mM As (V) in the pH 7.0, 7.3, and 4.6 incubations, respectively (not shown), no consumption of the 5 mM nitrate present in the slurries occurred, an observation that was different from the previous experiments (e.g., Figure 3C).

**Microbial diversity of incubated sediments**

Microbial diversity was assessed in the Herman Pit sediment enrichment experiments by sequencing representative restriction fragment length polymorphisms (RFLP) of 25 cloned 16S rRNA gene fragments (~1,500 base pair length) from PCR amplified DNA extracted from the pH ~5 and ~6.5 enrichments. These sediments were anaerobically incubated with lactate and arsenate (Figure 5). The majority of the Gram-positive OTUs were most similar to *Bacillus*, *Desulfosporosinus*, and *Clostridium* genera. The OTUs related to *Clostridium* and *Desulfosporosinus* were mostly associated with the near pH 5 enrichments, whereas the *Bacillus*-like OTUs were found in the higher pH ~6.5 incubations. Members of each of these genera are known to respire arsenate (Liu et al. 2004; Switzer-Blum et al. 1998). The *Bacillus* species that respire arsenate are haloalkaliphilic isolates from Mono Lake, CA.

In contrast, the arsenate-respiring *Desulfosporosinus* species was isolated from Onondaga Lake sediment (Syracuse, NY), a lake with a long history of environmental pollution problems (Liu et al. 2004). *Alkaliphilus oremlandii* str. OhILAs (formerly *Clostridium* sp. str. OhILAs) was isolated from Ohio River (PA) sediments. Also detected in the Herman Pit sediment experiments were OTUs associated with Gram-negative genera such as *Desulfurella*, *Geobacter*, and *Paludibacter*. Although most similar to the
latter genus, the sequences associated with the "C" RFLP were only similar by 88.2% to *Paludibacter propionicigenes* type strain WB4^T^, which is an obligate anaerobe that ferments sugars into acetate and propionate (Ueki et al. 2006). The other genera, *Desulfurella* and *Geobacter*, are strict anaerobes and known to respire inorganic substrates. Moreover, certain *Geobacter* species may also be able to respire arsenate (Héry et al. 2015).

**Functional genes associated with arsnotrophy**

In addition to the 16S rRNA gene, all samples were analyzed for the presence of functional genes relevant to arsenic biogeochemical cycling: *arrA*, arsenate respiratory reductase, *aioA*, aerobic arsenite oxidase, and *arxA*, anaerobic arsenite oxidase. For *arrA*, known primer pairs developed by Song et al. (2009), Malasarn et al. (2004), and Kulp et al. (2007) were tested on various sediment incubations. The Song primers yielded non-specific PCR amplicons even after considerable optimization of the PCR conditions. Cloning and sequencing confirmed that these were indeed non-specific PCR amplification products because none showed any resemblance to *arrA* sequences. No PCR amplicons were detected using Malasarn and Kulp *arrA* primer sets. Additional *arrA* primer sets from Stolz et al. (2011) and Ranganathan (2005) were tested.

Various primer combinations tested using *Shewanella* sp. ANA-3 as a control strain, and a successful semi-nested PCR amplification protocol was eventually developed that yielded a positive PCR amplicon of the expected 850 bp size using *Shewanella* sp. ANA-3 (Supplementary Figure S3). Using these new primer sets and a semi-nested PCR protocol, *arrA* was detected only in the pH > 6.5 lactate *C*arsenate experiment and not in the pH < 5 counterpart. After cloning and sequencing, the predicted partial ArrA sequence was shown to cluster closest to Gram-positive genera *Bacillus*, *Desulfosporosinus* and *Desulfbacterium*. All three contain members known to respire arsenate (Figure 6).

In contrast to arsenate-respiratory reduction, evidence for arsenite oxidation has been detected across a wide pH spectrum. In the environment arsenite oxidation occurs through either an *aioA*- or *arxA*-dependent pathway. For the latter, we were unable to detect *arxA* in any of our samples. Because of the recent identification of *arxA*, primer development for the anaerobic arsenite oxidase is in its infancy and will likely progress as more reference organisms are identified. Conversely, *aioA* was detected in all the primary enrichment cultures (containing either arsenate and arsenite) but not in experiments with sequential transfers into arsenate-amended media. The *aioA* PCR amplicons from the nitrate + arsenite enrichments and those from the aerobic + arsenite incubation conditions were cloned and sequenced. Analysis of the sequence data revealed that the translated partial *aioA* sequences were most similar to the AioA of *Thiomonas* species (Figure 7). *Thiomonas arsenitoxydans* str. 3As is an *aioA*-containing arsenite-oxidizing acidophile isolated from an acid-mine drainage stream in France (Slyemi et al. 2011; Slyemi et al. 2013).

**Discussion**

The geochemistry of acid mine drainages is a complex subject entailing interrelated aspects of sulfur and iron redox cycles in proton generation, the formation, precipitation and dissolution of secondary minerals, and the sorption/desorption of toxic metals and metalloids (Nordstrom et al. 2015). A key class of
Figure 4. Arsenite oxidation in sediment slurries at adjusted/buffered pH values. Aerobic incubations: Left two panels, mean of 3 samples ± 1 std. dev. presented. A) Time course. B) Calculated rates over pH range from triplicate slurries, with bars indicating ± 1 std. dev. Anaerobic arsenite oxidation in slurries incubated with nitrate: Right two panels. C) Time courses of mean rates of 3 samples plotted for each pH condition ± 1 std. dev. D) Rates extrapolated from time courses over the pH range tested, with bars indicating ± 1 std. dev. Note Y-axis scale differences between panels B and D.

Figure 5. Phylogenetic analysis of partial 16S rRNA gene sequences retrieved from Herman Pit sediment incubations, pH 5 and 6.5. The OTUs in bold-faced font represent sequences determined for a particular RFLP. Numbers in parenthesis indicate the number of clones sequenced within that corresponding RFLP and pH treatment. The Genbank accession numbers are shown trailing the species name. Bootstrap percentages for 1000 replicate sub-samples of the alignments are indicated on the nodes only if the value exceeds 50%.
secondary minerals specifically relevant to arsenic is the ferric hydroxide phase which, depending upon pH and involvement (or not) of Fe(II)-oxidizing microbes, can give rise to such minerals as ferrihydrite, goethite, jarosite, and lepidocrocite. These types of Fe(III)-minerals are well-known for their ability to strongly and differentially adsorb arsenic oxyanions, which is especially relevant in acid-mine drainages as well in understanding the hydrogeochemistry of more conventional sediment/aquifer systems (Burton et al. 2014; Cheng et al. 2009).

Trying to dissect the specific contribution(s) of different physiological groups of microorganisms in this process is a daunting task. It can be attempted by tracking temporal or spatial changes in chemical species (both in the dissolved and solid phases), in experiments with manipulated mesocosms, or within actual impacted field sites and along stream flow-paths (e.g., Diez-Ercilla et al. 2014; Kirk et al. 2010; O’Day et al. 2004; Saalfield and Bostick 2010; Vaxevanidou et al. 2015). To discern the possible involvement of specific microbial guilds, culture-independent methods can also be employed to identify active components of the putative participatory microbial flora (Bruineel et al. 2006; Majzlan et al. 2014). When we undertook our investigations of the Herman Pit materials at the outset we simplified our approach by attempting to answer a basic question: Are any microorganisms present in this complex milieu that can either oxidize As(III) or reduce As(V)?

Hence the approach we employed was one we had used many times before in other environments, namely add high concentrations (millimolar) of the oxyanion and monitor its redox changes over time using simple, manipulated mesocosm incubations (e.g., Switzer Blum et al. 1998). Although it is now clear from our results that such arsenotrophic microorganisms do exist within the Herman Pit milieu, we had to adapt our methods to overcome the unanticipated strong adsorptive...
capacity of the assayed materials. As such, our results cannot be extrapolated for the purpose of deconvolution of chemical reactions ongoing or the participatory arsenotrophs under in situ conditions, but merely as a first step to see if such things are at all possible.

When we first observed the orange-hued color of the collected sediments (Supplementary Figure S1), we inferred a pronounced abundance of poorly crystalline Fe(III)-type minerals present therein. Because such oxidized Fe-, Mn-, and Al-mineral phases are strongly adsorptive of As(V) [and to a lesser extent As(III)] from the aqueous phase (e.g., Fuller et al. 1993; Moore et al. 1990; Smedley and Kinniburgh 2002; Ying et al. 2013; Zobrist et al. 2000) we designed experiments to attempt to counter this effect so as to allow us to be able to discern the occurrence of arsenic redox changes in the aqueous phase that could be attributable to microbial respiratory (“dissimilatory”) reduction of As(V) to As(III) [“DAsR”]. Therefore, for our initial anoxic incubations we diluted these highly porous sediments with lake-water and amended the slurries with a high amount of As(V).

However, even with these steps As(V) could not be detected in the aqueous phase over the course of the initial incubations implying it was all adsorbed to the sediment phase (Table 1). We did, nonetheless, observe a transient appearance of As(V) that was bioavailable to DAsR processes that could release the more mobile As(III) into solution. However, the small quantities of As(III) observed released coupled with its disappearance over time suggested that this chemical species was also subject to adsorption and/or precipitation under these experimental conditions. The complete consumption of the added electron donors lactate and acetate (Table 1) and the consumption of H₂ from our observation of negative pressures in the serum bottles all indicated behavior that was qualitatively consistent with the presence of an active community of As(V)-respiring prokaryotes (e.g., Oremland and Stolz 2003). Yet, in order to make a better case for their active presence, we needed to be able to account for a balance in solution between these oxyanions and the provided electron donors. Therefore we undertook further incubations at higher dilutions using small amounts of anoxic sediment as inoculum from these early incubations to initiate the reactions.

The subsequent experiment did achieve a clear presence of all the added As(V) in the aqueous phase, and although there was consumption of about 5 mM noted with time, only about half was balanced by recovery as As(III) (Figure 1A) suggesting the possibility of a continued solid phase sink for As(III). In the absence of added As(V) sulfate-reduction occurred, supporting evidence that sulfate was consumed and lactate was quantitatively oxidized to acetate (Figure 1B). Acidophilic sulfate-reducers, such as Desulfovosporus acididurans, occur in AMD environments, such as the Tinto River (Sánchez-Andrea et al. 2015).

It was clear that the presence of abundant As(V) inhibited sulfate reduction, presumably as a preferred electron acceptor (Kulp et al. 2007) and that without added electron donor at this sediment dilution, neither As(V) reduction nor sulfate-reduction could proceed (Figure 1C). The 4 mM sulfate consumed (~ 32 mM equivalent e⁻) was exceeded by the electrons generated from oxidation of 12 mM lactate (~ 48 mM equivalent e⁻) suggesting the presence of an additional electron sink, most likely as Fe(III), a supposition reinforced by the blackening of these slurries, indicating formation of abundant FeS (Figure 1B; Supplementary Figure S2). Yet, only upon a further incubation of live sediment material in a subsequent (third) incubation was there a rough 1:1 equivalence between As(V) and lactate consumed and As(III) plus acetate produced that was balanced, and a 2:1 ratio evident between lactate oxidized versus As(V) reduced giving much clearer evidence for the presence of a DAsR-active flora.

The balance between electrons generated from the oxidation of electron donor (lactate) and reduction of As(V) to As(III) thereby giving further evidence of DAsR was again extended in an experiment conducted in artificial medium in lieu of lake-water (Figure 2). Moreover, it was apparent that As(V) respiration could occur over a broad range of pH, from 4.0 to 8.9. Only at pH 3.3 was there no detectable activity, while lags were noted at pH 4.0 and 8.9 which would suggest an optimum lying somewhere between pH 4.8 and 6.8. These results imply that the (V)-respiring microbial population was slightly acidophilic or possibly acidotolerant, but was readily adaptable to and eventually tolerant of substantial (2–3 units) pH excursions on either side of neutrality. As was discussed in the previous section, strong evidence for the presence of DAsR bacteria in these sediment enrichments was underscored by the amplification of 16S rRNA gene sequences, many of which were closely aligned with clades having As(V)-respirers (Figure 5), a result further supported by the arrA sequences obtained (Figure 6). Isolation of novel acidophilic or acid-tolerant DAsR bacteria from this milieu remains an ongoing goal of this work.

The sediment adsorptive problems observed with the DAsR work also extended to testing for the presence of active microbially mediated As(III) oxidation. In the initial aerobic experiment, only ~2.6 mM As(III) of the total 13 mM added could be detected in the aqueous phase and although this was consumed over time in the live samples, none of the As(V) presumed to have been formed ever appeared in solution, implying extensive adsorption onto the solid phase and consistent with our observations made in the DAsR experiments (e.g., Table 1). As in the DAsR experiments, only the second experiment with a further diluted sample was the consumption of added ~12 mM As(III) roughly balanced by the accumulation of ~9 mM As(V) (Figure 3A). This situation further improved with yet another (third) dilution (Figure 3B), where there was a good agreement between the cumulative amount of As(III) consumed (~9.5 mM) and As(V) accumulated (~9.2 mM).

The occurrence of this process in the absence of added organic electron donors strongly indicated that a chemautotrophic population was being carried. The anaerobic (nitrate) incubation also demonstrated a sustained, cumulative As(III) removal (~7 mM) but with accumulation of only ~2 mM or 29% as As(V) (Figure 3C). There was also a complete consumption of the added 3.5 mM nitrate, an electron sink for ~15.5 mM equivalent e⁻ if linked to its reduction to N₂ and therefore in approximate balance with the 14 mM equivalent e⁻ generated from As(III) oxidation. Nitrate reduction has been shown to couple to As(III) oxidation in Mono Lake water.
samples (Oremland et al. 2002), soil isolates (Rhine et al. 2006), and for the growth of the chemooautotroph Alkalilimnicola ehrlichii (Hoeft et al. 2007).

Both the aerobic and the anaerobic As(III) oxidation experiments exhibited activity over a broad range of pH (Figure 4), but highest rates were obtained at neutral pH, although there was significant activity at the acidic pH values tested (2.7 and 4.6, respectively). These results would suggest an acid-tolerant population, but amenable to any upward excursions towards neutrality. There was, however, a paradoxical result in that there was no loss of nitrate in these anaerobic experiments, suggesting abandonment of the nitrate and employment of another oxidant like the abundant Fe(III) or possibly Mn(VI). Experiments with Fe(III)- and Mn(VI)-supplemented anoxic Mono Lake water indicated that such a possibility exists (Oremland et al. 2002).

Re-oxidation of Fe(II) linked to nitrate respiration is also a potential, complicating phenomenon (Benz et al. 1998; Carlson et al. 2013; Staub et al. 1996; Weber et al. 2001). In agreement with these observations, we did not obtain amplicons using primers for the arxA gene, implying there was a dearth of organisms having this mode of As(III) oxidation, although nitrate-linked As(III) oxidation can also be mediated via aiaA homologs in Azotobacter strain DAO1 and Sinorhizobium strain DAO10 (Rhine et al. 2006; Rhine et al. 2007). However, our aiaA amplicons aligned with various species of thiomonads (Figure 7), microorganisms that have been previously identified as mediators of As(III) oxidation in the AMD of the Carnoules mine in France (Bruneel et al. 2003, Bruneel et al. 2006, Bruneel et al. 2011).

The primary goal of this work was to overcome the adsorption problems posed by the Fe(III)-rich sediment matrix so as to obtain clear evidence for the presence of arsenotrophs, especially those that could respire As(V) as well as those that oxidize As(III). In this regard the work was successful, although whether or not these biogeochemical processes and implicated microorganisms are active under in situ conditions would require a much different, more analytically detailed approach than what we have attempted herein. Clearly efforts employing X-ray spectroscopy (e.g., EXAFS/XANES) and extraction of DNA and RNA from freshly recovered and incubated (manipulated) solid phase materials would be needed to pursue this question (Burton et al. 2014; Majzalan et al. 2014; Morgante et al. 2014). Nonetheless, based on our observations, such an effort would be justified to determine if a true biochemical arsenic cycle occurs in the Herman Pit, and to delineate its interactions with parallel cycles of iron and sulfur.

Acknowledgments

We thank Prof. Lily Young for helpful comments on this manuscript.

Funding

This work was supported in part by the USGS, and to RSO grants from the U.S. EPA, and NASA Exobiology, and to CWS from NSF (EAR-1349366).

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