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Facile reduction of arsenate in methanogenic sludge

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Abstract

Due to the recent enactment of a stricter drinking water standard for arsenate, large quantities of arsenate-laden drinking water residuals will be disposed in municipal landfills. The objective of this study was to determine the role of methanogenic consortia on the conversion of arsenate. Methanogenic conditions commonly occur in mature municipal solid waste landfills. The results indicate the rapid and facile reduction of arsenate to arsenite in methanogenic sludge. Endogenous substrates in the sludge were sufficient to support the reductive biotransformation. However the rates of arsenate reduction were stimulated by the addition of exogenous electron donating substrates, such as H_2, lactate or a mixture of volatile fatty acids. A selective methanogenic inhibitor stimulated arsenate reduction in microcosms supplied with H_2, suggesting that methanogens competed with arsenate reducers for the electron donor. Rates of arsenate reduction increased with arsenate concentration up to 2 mM, higher concentrations were inhibitory. The electron shuttle, anthraquinone-2,6-disulfonate, used as a model of humic quinone moieties, was shown to significantly increase rates of arsenate reduction at substoichiometric concentrations. The presence of sulfur compounds, sulfate and sulfide, did not affect the rate of arsenate transformation but lowered the yield of soluble arsenite, due to the precipitation of arsenite with sulfides. The results taken as a whole suggest that arsenate disposed into anaerobic environments may readily be converted to arsenite increasing the mobility of arsenic. The extent of the increased mobility will depend on the concentration of sulfides generated from sulfate reduction.

Abbreviations: AQDS – anthraquinone-2,6-disulfonate; As(V) – arsenate; As(III) – arsenite; BES – 2-bromethanesulfonate; COD – chemical oxygen demand; DMA^V – dimethylarsenic acid; MMA^V – monomethylarsonic acid; TCLP – toxicity characteristic leaching procedure; VFA – volatile fatty acids; VSS – volatile suspended solids

Introduction

The Environmental Protection Agency (EPA) recently lowered the drinking water standard for arsenic (As) from 50 to 10 parts per billion (ppb). The new standard was motivated in part by increasing evidence of cancer risks associated with low levels of As. About 4,000 water systems nationwide are affected by the standard and over 90% are small utilities serving 10,000 or less consumers (US-EPA 2001). The most affected geographic regions are those characterized by As-bearing geological formations together with populations dependent on groundwater for drinking water resources. Nearly all As-treatment technologies will generate solid residues containing As removed from the water supply. Small drinking water utilities will rely mostly on adsorbent based technologies involving the sorption of arsenate after an oxidation pretreatment to convert arsenite to arsenate. The EPA recommends that spent arsenate-laden adsorbent residuals from

small drinking water utilities can be disposed in non-hazardous waste landfills (US-EPA 2001). The recommendation is based on EPA's assessment of the hazard of As-laden adsorbent residuals with the *Toxicity Characteristic Leaching Procedure* (TCLP). The protocol was originally developed as a challenging short-term abiotic extraction of cationic metals from solid waste using an acetic acid buffer under acidic conditions (pH 4.95). In contrast to TCLP, landfills become mildly alkaline upon maturation, have reducing conditions due to elevated anaerobic microbial activities and long hydraulic residence times (Christensen et al. 2001; Kjeldsen et al. 2002). These conditions are expected to be more conducive to the extraction of arsenic compared to the TCLP.

The mobility of arsenic in landfills will depend greatly on arsenic speciation. Pentavalent arsenate (As(V)) is more strongly sorbed by the common adsorbents, activated alumina and granular ferrihydrite, compared to trivalent arsenite (As(III)) (Amy et al. 2000; Lin & Wu 2001; Zobrist et al. 2000). The potential biologically catalyzed reduction of As(V) to As(III) in landfills would therefore impact mobility of arsenic from arsenate-laden drinking water residuals disposed in non-hazardous landfills. A diverse population of anaerobic microorganisms including methanogens, fermentative bacteria, and sulfate- and iron reducers is supported in landfill leachates (Christensen et al. 2001; Ludvigsen et al. 1999; Van Dyke & McCarthy 2002). Additionally the leachates contain organic substrates and thus electron equivalents to sustain the reductive biotransformation of arsenate. High biological oxygen demand (BOD) in leachates values are accounted for largely by volatile fatty acids (VFA). VFA was shown to be responsible for 33 to 89% of the dissolved organic carbon in various leachate samples (Fischer et al. 1997).

A wide variety of organisms are implicated in the reduction of As(V), ranging from fortuitous reduction to purposeful dissimilatory reduction (Oremland & Stolz 2003). Several pure cultures of methanogens were shown to fortuitously reduce As(V) to As(III) and arsine (Michalke et al. 2000; Wickenheiser et al. 1998). A large number of phylogenetically distinct bacteria are known to couple the dissimilatory reduction of As(V) to growth, including sulfate reducing and iron reducing bacteria (Newman et al. 1998; Stolz & Oremland 1999) as well as a thermophilic archaeon (Huber et al. 2000). Additionally, a common strategy in the bacterial resistance to As(V) toxicity ironically involves the reduction of As(V) to the more toxic As(III), since As(III) is the substrate of efflux pumps (Mukhopadhyay et al. 2002; Rosen 2002). Conversion of As(V) to As(IIII) has also been noted in different anaerobic environments, including: salt marsh sediments (Dowdle et al. 1996), lake sediments (Harrington et al. 1998), anaerobic hypersaline lake water (Oremland et al. 2000), and in mixed anaerobic cultures derived from agricultural soil (Jones et al. 2000). Dissimilatory As(V)-reducing bacteria have been observed in wetland, lake and pond sediments at approximately 10^4 cells g^{-1} sediment (Harrington et al. 1998; Kuai et al. 2001).

The objective of this study is to characterize the potential of methanogenic consortia to reduce As(V). A stabilized methanogenic granular sludge was used as a simple model to study the conversion of As(V) to As(III) in anaerobic microcosms. Methanogenic conditions occur in mature municipal solid waste landfills (Christensen et al. 2001; Kjeldsen et al. 2002). The impact of exogenous electron donating substrates, As(V) concentration and the presence of sulfur compounds on As(V) biotransformation were evaluated.

Material and methods

Microorganisms

Methanogenic granular sludge was obtained from industrial anaerobic treatment plants treating recycle paper wastewater (Eerbeek, The Netherlands) and distillery wastewaters (Nedalco BV, Bergen op Zoom, The Netherlands). The content of volatile suspended solids (VSS) in the Eerbeek and Nedalco sludge was 12.9 and 10.0%, respectively. The microbial cultures were stored under nitrogen gas at 4 °C.

Batch bioassay

Anaerobic reduction of soluble As(V) was assayed in shaken batch bioassays at 30 °C. Serum flasks (135 ml) were supplied with 50 ml of a basal mineral medium (pH 7.2) containing (in mg l^{-1}): NH_4Cl (280); $NaHCO_3$ (3000); $MgCl_2$ (78), $CaCl_2$ (10), $MgSO_4.7H_2O$ (10); K_2HPO_4 (250); $CaCl_2$ (10); and 1 ml l^{-1} of a trace element solution according to Van Lier et al. (Van Lier et al. 1992). In some experiments, the same basal medium included 100 mg l^{-1} yeast extract. The medium was also supplemented with As(V) (concentrations indicated in Tables and Figures) and an electron donating substrate, typically 10 mM lactate, unless otherwise specified. In selected microcosms,

glucose (10 mM), methanol (10 mM), acetate (10 mM), H_2 (ranging 0.054 to 0.54 atm) or a mixture of volatile fatty acids (VFA) (concentration (as mM): acetate (7.5), propionate (6.1), butyrate (5.1)) equivalent to 2 g chemical oxygen demand (COD) l^{-1}, were provided as the electron donor. In bioassays utilizing H_2 as the sole e-donor, N_2/CO_2 (80:20, v/v) was first used to flush the headspace and medium. Subsequently, H_2 gas was added to the headspace of each flask using a H_2/CO_2 (80:20, v/v) gas mixture. Selected assays also received the methanogenic inhibitor, 2-bromoethane sulfonate (30 mM final concentration) in order to evaluate the involvement of methanogens in As(V) reduction; the redox mediator, anthraquinone-2,6-disulfonate (AQDS) (0 to 1250 μM), in order to evaluate its possible involvement as redox mediator in As(V) reduction; or inorganic sulfur compounds (6.25 mM sulfide or 10 mM sulfate) with the purpose of evaluating the role of sulfide in As(III) deposition. The final pH value of all media was adjusted to 7.2 with NaOH or HCl, as needed. The medium was prepared with minimal sulfur content, unless otherwise specified, to avoid precipitation of As(III) as As_2S_3. Various controls (e.g., abiotic controls and controls with no added e-donor, no added redox mediators, etc.) were included, depending on the experiment. Abiotic controls (lacking microbial inoculum) and killed sludge controls were sterilized by autoclaving, allowed to cool down and then sealed aseptically. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals, and then the headspace was flushed with membrane-filtered, sterile N_2:CO_2 gas (80:20, v/v) to exclude oxygen from the assay. All assays were conducted in triplicate.

Analytical methods

Inorganic and organic arsenic species (arsenite (As(III)), arsenate (As(V)), methylarsonic acid (MMA^V), dimethylarsinic acid (DMA^V) methylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) in liquid samples were analyzed by ion chromatography/inductively coupled plasma/ mass spectrometry (IC/ICP/MS) using a method adapted from Gong et al. (Gong et al. 2001). The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies, Inc.) with a reverse-phase C18 column (Prodigy 3u ODS(3), 150 $\times$ 4.60 mm, Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2mM malonic acid and 4% (v/v) methanol at a flow rate of 1.2 ml min^{-1}. The column temperature was maintained at 50 °C. An Agilent 7500a ICP-MS with a Babington nebulizer was used as the detector. The operating parameters were as follows: Rf power 1500 watts, plasma gas flow 15 l min^{-1}, carrier flow 1.2 l min^{-1}, and arsenic was measured at 75 m/z. The injection volume was 10 μl. The detection limit for the various arsenic species was 0.1 μg l^{-1}. All liquid samples were membrane filtered (0.45 μm) immediately after sampling to minimize exposure to the atmosphere and stored in polypropylene vials (2 ml) to reduce adsorption of arsenic species to the vial. Filtered samples were then stored at -20 °C till analysis was performed in order to reduce changes in arsenic speciation.

Sulfide was analyzed colorimetrically by the methylene blue method (Trüper 1964). Nitrate, nitrite and sulfate were determined by ion chromatography with suppressed conductivity using a DIONEX system equipped with a Dionex AS11-HC4 column (Dionex, Sunnydale, CA) and a conductivity detector. The eluent was 15 mM KOH at a flow rate of 1.2 ml min^{-1}. The injection volume was 25 μl. Before measurement, all samples were membrane-filtered (0.45 μm). Other parameters (e.g., pH, volatile suspended solids) were determined according to Standard Methods (APHA 1998).

Results

The microbial reduction of As(V) was evaluated in methanogenic consortia. This study considered the effect of electron donating substrates, methanogenic inhibitors and As(V) concentration. Due to their presence in landfill leachate, the role of humic substances and sulfur compounds on the conversion was also evaluated. The elimination of As(V) and the recovery of As(III) were measured.

Electron-donating substrates

The reduction of As(V) (500 μM) was tested in anaerobic microcosms established with methanogenic granular sludge (2.5 g VSS l^{-1}) in basal inorganic nutrient media containing different electron donating substrates. Figure 1 illustrates the time-course of As(V) reduction to As(III) in the presence of hydrogen, glucose and acetate as electron donating substrates as well as in the absence of exogenous substrate (no added

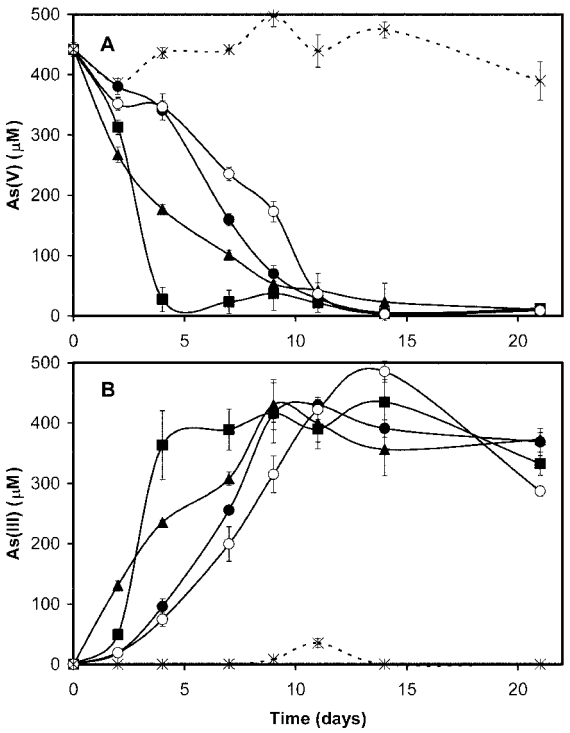

Figure 1. Effect of different electron donating substrates on the time course of arsenate reduction to arsenite in Nedalco anaerobic granular sludge. (A) As(V) concentrations. (B) As(III) concentrations. Legend: ■, hydrogen 0.8 atm; ▲, glucose 10 mM; ●, acetate 10 mM; ○, no added substrate, *, killed sludge (autoclaved).

substrate). In all cases, As(V) was readily reduced to As(III), with greater than 90% removal of As(V) within 12 days. The reduction of As(V) observed in the treatment lacking added exogenous substrate can be attributed to electron donating substrates contributed by the sludge (endogenous substrate), presumably from the slow hydrolysis of microbial biomass. In all treatments, the elimination of As(V) was concomitant with an almost stoichiometric recovery of As(III), indicating that As(III) was the main product of the conversion. MMA^V and DMA^V were also monitored as possible products, but these compounds were not detected in this study.

Glucose and hydrogen decreased the lag period prior to the initiation of As(V) reduction compared to the endogenous substrate control. The rate of reduction was significantly higher with hydrogen as electron donor compared to all other treatments. Additionally, acetate promoted a small enhancement in the rate of As(V) reduction compared to endogenous substrate control. As(V) was not converted in controls with sterile medium or with sterile medium incubated together with H_2 in the headspace (Table 1). Likewise As(V) was not converted by heat-killed sludge (Figure 1, Table 1), confirming that the observed conversions with living sludge were due to biotransformation.

Several other electron-donating substrates were tested, including methanol, lactate and a mixture of volatile fatty acids (VFA) composed of acetate, propionate and butyrate. The results from all substrates are summarized and compared in Table 1 in terms of the maximum rate of reduction observed and the recoveries of As(V) and As(III) on days 4 and 14. Lactate and the VFA mixture permitted high rates of As(V) reduction, which were only slightly less than that obtained with hydrogen. Methanol provided an intermediate rate of reduction. The lag phase prior to rapid As(V) reduction with the VFA and methanol lasted almost four days, accounting for the comparatively low elimination of As(V) on day 4 (Table 1). After 14 days of incubation, approximately 99% or more of the As(V) was converted with the exception of the glucose treatment, which achieved 95.6% As(V) conversion by that time. The As(III) concentrations recovered on day 14 varied from 356 to 484 μM, accounting for approximately 74.5 to 97.5% recovery of the As(V) eliminated. The highest recovery corresponded to endogenous substrate control.

As(V) concentration

The reduction of As(V) at variable concentrations was tested in anaerobic microcosms established with methanogenic granular sludge (2.5 g VSS l^{-1}) in yeast extract – basal inorganic nutrient media with lactate (10 mM) as the electron donor. Figure 2 illustrates the effect of the initial As(V) concentration on the rate of As(V) conversion calculated from two independent sets of measurements. The rates were calculated from the As(V) elimination data or from the As(III) formation data. The graph shows that the As(V) elimination rates corresponded to the As(III) formation rates at all initial As(V) concentrations tested. This finding is in agreement with the nearly stoichiometric conversion of As(V) to As(III) observed in the previous experiment. In the concentration range of 0.2 to 2 mM As(V), increasing As(V) concentrations corresponded to increasing rates of As(V) reduction, reaching a maximum at 2 mM of 20.7 μmol g^{-1} VSS h^{-1}. As the concentration was increased further, the rates declined. At 10 mM As(V), the rates were about half of the maximum values probably as a result of inhibition by either As(V) or As(III). The inhibitory impact

Table 1. The rate of reductive biotransformation of As(V) (500 μM) to As(III) by Nedalco anaerobic granular sludge (2.5 g VSS l^{-1}) and the recovery of arsenicals after 4 and 14 days of incubation with various electron donating substrates in basal inorganic nutrient medium. Also shown is the recovery of As(V) from controls lacking sludge or incubated with killed sludge

Substrate		Sludge	Rate	Arsenicals after 4 days		Arsenicals after 14 days	
type	Conc.		arsenate conversion μmol g $VSS^{-1}.h^{-1}$	arsenate avg* ± std	arsenite avg ± std	arsenate avg ± std	arsenite avg ± std
				—— μM ——		—— μM ——	
H_2	0.8 atm	live	2.38	27.3 ± 20.1	363.3 ± 57.1	3.1 ± 5.0	434.8 ± 37.4
Lactate	10 mM	live	1.81	133.9 ± 12.1	207.6 ± 44.8	3.6 ± 1.8	365.3 ± 11.6
Glucose	10 mM	live	0.54	176.7 ± 7.4	235.1 ± 3.9	22.8 ± 31.6	356.4 ± 43.3
Methanol	10 mM	live	1.33	315.7 ± 26.6	122.3 ± 3.2	2.2 ± 0.9	390.2 ± 9.4
VFA	2 g COD l^{-1}	live	1.77	336.0 ± 20.3	106.7 ± 17.6	1.4 ± 2.4	434.6 ± 54.8
Acetate	10 mM	live	0.91	340.6 ± 15.8	95.9 ± 12.8	5.5 ± 1.9	391.2 ± 14.6
None		live	0.58	346.4 ± 22.1	74.2 ± 10.8	2.8 ± 4.0	485.5 ± 17.7
H_2	0.8 atm	none	0	478.7 ± 52.7	0.7 ± 0.6	531.7 ± 8.8	0.0 ± 0.0
none		none	0	463.4 ± 2.1	0.0 ± 0.0	522.6 ± 17.2	0.0 ± 0.0
none		killed	0	436.0 ± 8.7	0.0 ± 0.0	474.6 ± 13.3	0.0 ± 0.0

* avg = average; std = standard deviation.

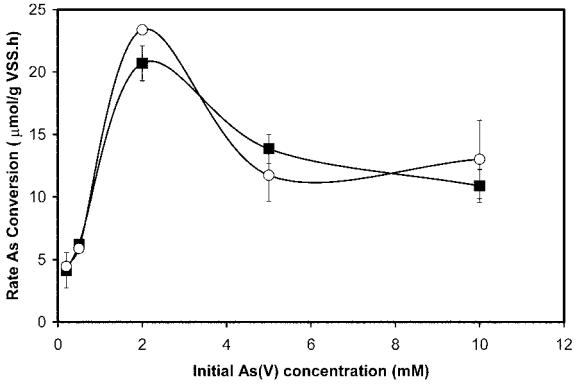

Figure 2. The rate of As(V) reduction to As(III) in Eerbeek anaerobic granular sludge incubated with at variable initial concentrations of As(V) with 10 mM of lactate as electron donor in yeast extract – inorganic basal nutrient medium. Legend: ■, As(V) removal rate; ○, As(III) formation rate.

of high initial concentrations (e.g., 10 mM) was particularly evident after 100 h of incubation when the reaction came to a halt with only one-third of the As(V) converted to As(III).

Methanogenic inhibitor

The compound 2-bromoethane-sulfonate (BES) is a specific inhibitor of methanogenesis (Scholten et al. 2000). In microcosms established with 2 mM As(V), BES was applied to determine the role of methanogens in the reduction of As(V). The experiment was conducted in the presence and absence of added hydrogen as electron donor. Hydrogen was supplied either at 0.054 or 0.54 atm equivalent to 81 or 810 mg L^{-1} chemical oxygen demand (COD), respectively. The results presented in Figure 3 show the effect of BES on the bioconversion of As(V) in the absence and presence of added hydrogen. In microcosms containing exogenous hydrogen, the presence of BES greatly stimulated the reduction of As(V). The result indicates that methanogens are not required for As(V) reduction, rather they interfere with the process. Possibly, the methanogens compete with As(V) reducers for electron donating substrate. The methanogenic activity of the sludge was sufficient to deplete the hydrogen prior to the onset of As(V) reduction. In microcosms lacking exogenous addition of hydrogen, BES has no noteworthy effect on the As(V) conversion. In the latter case, the slow release of electron donor is controlled by the hydrolysis of biomass.

Electron shuttle, anthraquinone-2,6-disulfonate

The effect of the electron shuttle, anthraquinone-2,6-disulfonate (AQDS), on As(V) reduction was tested. AQDS is commonly used as a model of electron transfer by humic substances (Lovley et al. 1996). The effect of variable concentrations of AQDS (5 to 1250 μM) on the rate of As(V) (5 mM) reduction was evaluated with 10mM lactate as electron donor in yeast

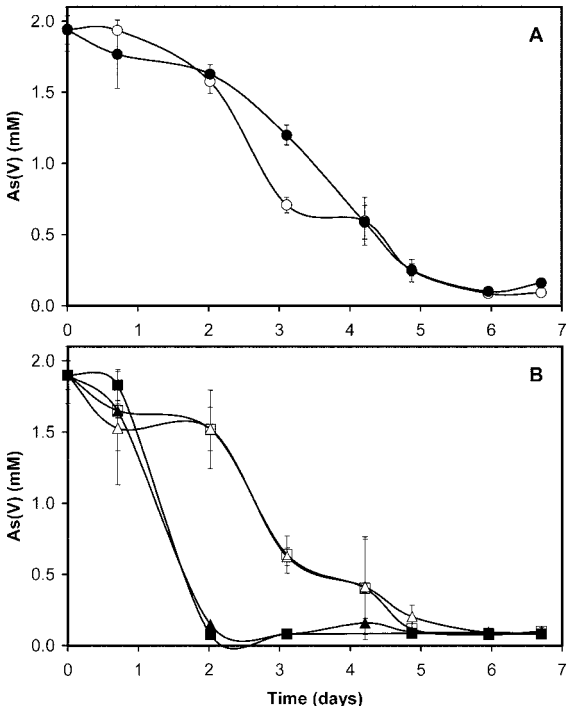

Figure 3. Effect of 30 mM of 2-bromoethane sulfonate (BES) on the reduction of 2 mM arsenate in the absence and presence of hydrogen gas as electron donor by Eerbeek methanogenic sludge (1 g VSS L^{-1}). (A) As(V) reduction in the absence of electron donating substrate: ●, with BES; ○, without BES. (B) As(V) reduction in the presence of hydrogen at two concentrations: Legend: ▲, 0.054 atm H_2 with BES; ■, 0.54 atm H_2 with BES; △ 0.054 atm H_2 without BES; □, 0.54 atm H_2 without BES.

extract – inorganic nutrient medium. The rates of As(V) elimination and As(III) formation are plotted as a function of AQDS concentration in Figure 4. The figure illustrates that AQDS at 500 and 1250 μM significantly increased the reductive biotransformation rate. The rate was maximally increased by approximately 2-fold with the treatment containing 1250 μM AQDS compared with the treatment lacking AQDS.

Sulfur compounds

Sulfur compounds such as sulfate are common in anaerobic environments, including landfills (Christensen et al. 2001). Sulfate is an electron acceptor of anoxic respiration for sulfate-reducing bacteria and thus could possibly compete with As(V) as an electron acceptor. Biogenic sulfides produced from sulfate reduction could potentially impact the rate of As(V) reduction and the yield of soluble As(III). Sulfide is known to chemically reduce As(V) and precipitate As(III), forming orpiment (Rochette et al. 2000).

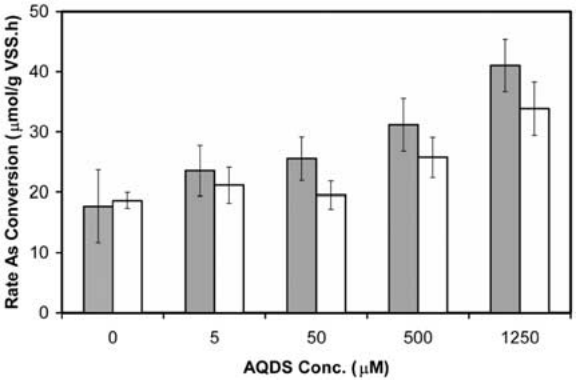

Figure 4. Effect of variable anthraquinone-2,6-disulfonate (AQDS) concentrations on the rate of As(V) (5 mM) reduction to As(III) in Eerbeek anaerobic granular sludge with 10 mM lactate as electron donor in yeast extract – inorganic basal nutrient medium. Legend: shaded bars, As(V) elimination rate; unshaded bars, As(III) formation rate.

Therefore, the effect of different sulfur compounds on As(V) reduction and As(III) product yield were evaluated. The basal inorganic nutrient medium was utilized with an initial As(V) concentration of 500 μM and a mixture of volatile fatty acids (2 g COD l^{-1}) was utilized as the electron donating substrate. In Figure 5, the results of the sulfide-amended treatments are compared with the treatments lacking any addition of sulfur compounds. In the absence of sulfide, As(V) was readily converted with the maximum rates of conversion starting after 4 days. By day 7, the formation of soluble As(III) was stoichiometric and thereafter there were marginal losses of As(III). In the presence of sulfide, As(V) was also readily converted and the maximum rates were achieved immediately without any lag phase, probably due to the sudden decrease of the redox potential of the culture medium with sulfide. Comparatively little soluble As(III) accumulated in the media. The maximum accumulation of As(III) was 60 to 67 μM occurring on days 4 through 7. The lack of any significant accumulation of As(III) indicates that the As(III) formed from As(V) reduction was precipitated. In the killed-sludge control, As(V) was relatively stable in the presence of sulfide, confirming the absence of any significant abiotic reduction or precipitation of As(V).

The results of the sulfate-amended treatment are shown in Figure 6. As(V) was readily and preferentially converted in the presence of sulfate with approximately the same time-course as in the treatment lacking added sulfur compounds (Figure 5). Thereafter, sulfate elimination occurred due to sulfate

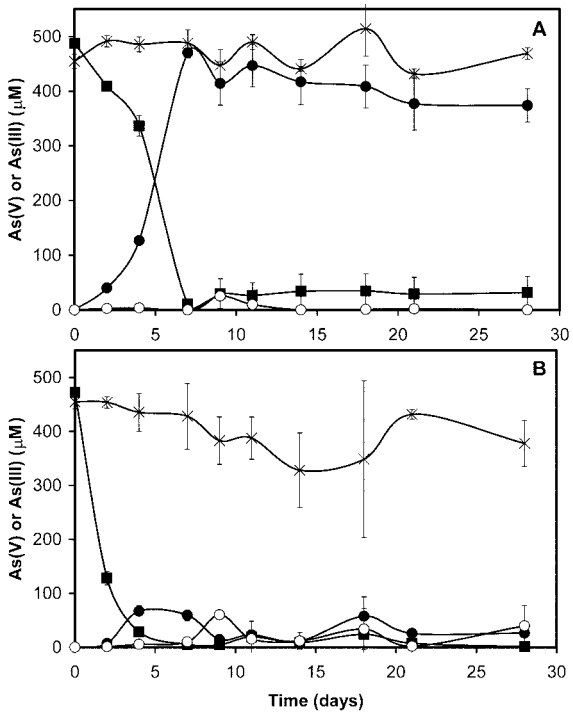

Figure 5. The time course of As(V) reduction to As(III) in Nedalco anaerobic granular sludge incubated with a volatile fatty acid mixture (2 g COD l^{-1}) in the absence and presence of sulfide (A) No sulfide added. (B) Sulfide (6.25 mM) included in medium. Legend: ■, As(V) with living sludge; *, As(V) with killed sludge (autoclaved); ●, As(III) with living sludge; ○, As(III) with killed sludge.

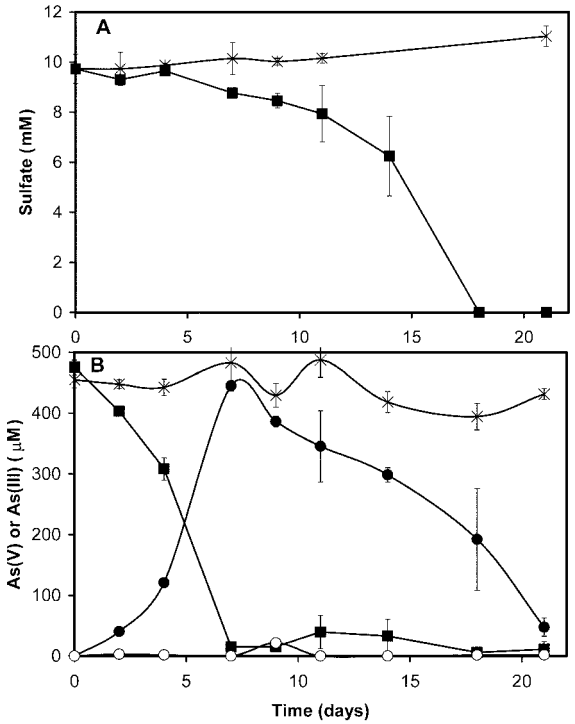

Figure 6. The time course of As(V) reduction to As(III) in Nedalco anaerobic granular sludge incubated with a volatile fatty acid mixture (2 g COD l^{-1}) and sulfate (10 mM). (A) Sulfate concentrations. Legend: ■, living sludge; *, killed sludge (autoclaved). (B) Arsenic concentrations. Legend: ■, As(V) with living sludge; *, As(V) with killed sludge; ●, As(III) with living sludge; ○, As(III) with killed sludge.

reduction (Figure 6a) as was evidenced by the formation of sulfides (result not shown). The onset of rapid sulfate reduction was clearly associated with a decrease in the accumulated As(III) concentration (Figure 6b), indicating As(III) precipitation with the biogenic sulfides.

Discussion

The present study demonstrates that As(V) is readily converted to As(III) in methanogenic consortia. The inocula utilized were obtained from full-scale upward-flow anaerobic sludge bed reactors treating either recycle paper or distillery wastewaters. These effluents presumably were not contaminated with significant concentrations of As(V). Therefore the consortia were most likely exposed for the first time to high As(V) concentrations during the bioassays conducted in this study. Since the consortia immediately reduced the As(V), the results suggest that no major enrichment of specialized As(V) reducing microorganisms was required. In sterile medium or in medium containing heat-killed sludge, no conversion of As(V) was observed, clearly eliminating the possibility for abiotic mediated transformation under the conditions tested. The results therefore indicate that either a fortuitous reduction process was occurring or that constitutive enzymes were present that are involved in As(V) respiration or detoxification. The findings are in agreement with the fact that bovine rumen fluid and hamster feces harbored microorganisms capable of reducing As(V) without any or very little lag phase (Forsberg 1978; Herbel et al. 2002).

Microbiological and biochemical basis of As(V) reduction

Many anaerobic microorganisms have been identified that are capable of linking As(V) reduction to growth (Newman et al. 1998; Oremland & Stolz 2003; Stolz & Oremland 1999), referred to as dissimilat-

ory arsenate-respiring prokaryotes (DARPs) . To date, DARP strains have been isolated from eleven eubacterial genera and one archaeon genus (Oremland & Stolz 2003). Microorganisms closely related to the currently known DARPs have been detected in methanogenic bioreactor communities (including granular sludge), such as *Desulfotomaculum* spp. (Hristova et al. 2000; Imachi et al. 2000), *Desulfitobacterium* spp. (Raskin et al. 1996), *Clostridium* spp. (Fernandez et al. 2000; Frigon et al. 1997; Godon et al. 1997), *Citrobacter* spp. (Frigon et al. 1997; Vieira et al. 2001), *Wolinella* sp. (Wallace et al. 1996); *Bacillus* spp. (Godon et al. 1997; Sekiguchi & Nakamura 1998), and *Thermus* spp. (Godon et al. 1997; Sekiguchi & Nakamura 1998).

Little is known about the reductases implicated in dissimilatory As(V) respiration. The As(V) reductase purified from an acetate utilizing As(V) respiring bacterium, *Chrysiogenes arsenatis* (Krafft & Macy 1998), was specific for As(V). Likewise, As(V) was required to induce enzyme expression. Membrane-bound As(V) reductases were also described from the iron-reducing *Sulfurospirillum barnesii* (Newman et al. 1998) and *Shewanella* sp. strain ANA-3 (Saltikov & Newman 2003) as well as the sulfate reducing, *Desulfomicrobium* sp. (Macy et al. 2000). The membranes of both *Sulfurospirillum barnesii* and *Desulfomicrobium* sp. contain cytochrome type proteins, suggesting their possible involvement in As(V) respiration (Macy et al. 2000; Newman et al. 1998). In support of this possibility, the reduced cytochromes of *Desulfomicrobium* sp. were shown to be reoxidized by As(V) (Macy et al. 2000).

As(V) reduction has another important physiological function aside from its role as a terminal electron acceptor. Microorganisms that tolerate high concentrations of As(V) rely on non-respiratory As(V) reductases. As(V), analogous in chemistry with phosphate, enters the cell via active uptake systems for phosphate. Resistance to As(V) is usually afforded by a microorganism's ability to pump arsenic out of the cell and As(III) is the substrate of all the known efflux pumps (Mukhopadhyay et al. 2002). Therefore, As(V) reductases are integral components of resistance mechanisms, converting intracellular As(V) to As(III) prior to becoming pumped out. An important example of an As(V)-resistance reductase is ArsC from the *Escherichia coli* resistance plasmid (R773) (Gladysheva et al. 1994). Recently, genes homologous to *arsC* of *E. coli* have been shown to be responsible for the As(V) reducing activity of various anaerobes such as the sulfate reducing bacterium, *Desulfovibrio* (Macy et al. 2000), and the iron reducing bacterium, *Shewanella* sp. strain ANA-3 (Saltikov et al. 2003).

Reduction of As(V) by pure cultures of methanogens has also been observed. Cultures of *Methanobacterium formicicum* converted As(V) to As(III) but this product was not quantified (Wickenheiser et al. 1998). The biochemical basis for the reduction of As(V) in methanogens is not yet known.

In this study, it was observed that arsenate reduction occurred immediately with methanogenic consortia not previously acclimated to As(V). This observation coincides with the fact that many DARPs have the ability to use alternative electron acceptors which can support the growth of DARPs in environments lacking As(V). Two sulfate reducing bacteria, *Desulfotomaculum auripigmentum* (Newman et al. 1997b) and *Desulfomicrobium* sp. (Macy et al. 2000), can utilize As(V) as an alternative electron acceptor with lactate as electron donor. Interestingly, sulfate grown cells of *Desulfomicrobium* sp. had constitutive As(V) reductase activity, emphasizing the possibility of As(V) reducing activity by bacteria previously not exposed to As(V). The dissimilatory iron reducing bacteria, *Sulfurospirillum barnesii* (Laverman et al. 1995), *Shewanella* sp. stain ANA-3 (Saltikov et al. 2003) and *Desulfitobacterium* sp. (Niggemyer et al. 2001), were shown to utilize As(V), manganese(IV), and nitrate for respiration as well as several sulfur compounds, such as sulfur, thiosulfate and sulfite. *Shewanella* sp. strain ANA-3 could also utilize the humic substance analogue, AQDS, as an electron acceptor (Saltikov et al. 2003).

As(V) reduction in anaerobic microbial communities

The observation in this study that As(V) is readily converted in methanogenic consortia adds to the list of anaerobic microbial communities in which As(V) reduction has been observed. Previously, conversion of As(V) to As(III) was observed in anoxic sediments obtained from a salt marsh (Dowdle et al. 1996), mining-impacted lake sediments (Harrington et al. 1998), anoxic hypersaline lake water (Hoeft et al. 2002; Oremland et al. 2000), and anaerobic enrichment cultures from agricultural soils (Jones et al. 2000). Microbial communities from various gastrointestinal (GI) tracts, such as bovine rumen fluid, hamster feces and termite hindgut were all able to readily reduce As(V) to As(III) (Herbel et al. 2002). As was observed in the

present study, the GI organisms readily reduced As(V) without any previous exposure to As(V).

Various inhibitors have been applied to characterize the populations and redox conditions required for As(V) reduction in anaerobic microbial communities. Nitrate and elemental oxygen inhibited As(V) reduction, at least as long as these alternative electron acceptors were present (Dowdle et al. 1996; Hoeft et al. 2002). Specific inhibitors of certain ecological significant populations, such as molybdate for sulfate reducing bacteria, have not caused substantial inhibition of As(V) reduction (Dowdle et al. 1996; Harrington et al. 1998; Hoeft et al. 2002). Also in the methanogenic consortia evaluated in the present study, a specific inhibitor of methanogenesis, BES, did not cause any inhibition of As(V) reduction. The data taken as a whole suggest that neither methanogens nor sulfate reducing bacteria are major contributors of the As(V) reducing activity. Attempts have been made to demonstrate the importance of respiratory As(V) reductases in anaerobic microbial communities using tungstate as a selective inhibitor of molybdenum-containing enzymes (Dowdle et al. 1996; Herbel et al. 2002). Molybdenum is an important transition-metal in respiratory As(V) reductases (Krafft & Macy 1998; Saltikov & Newman 2003; Stolz & Oremland 1999) and it is not present in ArsC (Mukhopadhyay et al. 2002). Tungstate does cause inhibition of As(V) reduction in anaerobic microbial communities, suggesting the possible involvement of respiratory As(V) reductase. However, the inhibition observed is generally not severe and the tungstate levels applied are quite high (10 mM) (Dowdle et al. 1996; Herbel et al. 2002; Hoeft et al. 2002).

Role of electron donors

The results of the present study indicate that addition of electron donors can improve the rate of As(V) reduction in a methanogenic consortium. Many of the electron-donating substrates were found to greatly improve the reduction rates, including hydrogen, lactate, methanol, and a VFA mixture. Of all the electron donors tested, hydrogen followed by lactate clearly had the greatest stimulatory effects. These findings were in agreement with those observed previously with bovine rumen fluid (Herbel et al. 2002) or anoxic sediments (Dowdle et al. 1996) in which hydrogen and lactate were shown to increase the As(V) reduction rate. Also a mixture of organic acids (VFA, benzoate and lactate) stimulated As(V) reduction in lake sediments (Harrington et al. 1998). Glucose on the other hand did not increase As(V) reduction rates in the methanogenic consortium, in contrast to findings in lake sediments (Dowdle et al. 1996). However, glucose did shorten the lag phase prior to the commencement of As(V) reduction. In this study acetate stimulated the rate of As(V) reduction, although previously acetate had no effect on reduction rates in lake sediments (Dowdle et al. 1996).

As(V) was also readily reduced when no exogenous electron donating substrate was added. This observation suggests that endogenous substrates in sludge supplied to the bioassay supplied electrons to support microbial As(V) reduction. The endogenous substrate level in the sludge was estimated from the methane production after incubating the sludge with inorganic basal medium for 30 days. The methane yield corresponds in value to 150 mg l^{-1} chemical oxygen demand (COD) of endogenous substrate. This concentration of endogenous substrate was in large excess of the 1 mmol l^{-1} electron equivalents (= 8 mg l^{-1} COD) required to reduce 500 μM of As(V) to As(III). Endogenous substrates in rumen fluid and anoxic sediments were also shown to support As(V) reduction albeit at slower rates than treatments with exogenous electron donor (Dowdle et al. 1996; Harrington et al. 1998; Herbel et al. 2002).

Niggemyer et al. (2001) summarized the available literature data with respect to electron donors utilized by various isolates of known As(V) respiring microorganisms. Lactate and pyruvate are utilized by almost all known isolates. Hydrogen (most commonly with acetate as carbon source) is used by many of the isolates, except those from the genus *Bacillus*. Butyrate, representative of a typical VFA, was tested with two of the isolates and was found to support As(V) respiration. Acetate, on the other hand, has generally not supported As(V) respiration with the sole exception of *Chrysiogenes arsenatis*, the only known acetate-oxidizing As(V) respiring organism (Macy et al. 1996). Glucose has also only been found to be used by one isolate, *Bacillus selenitireducens* (Blum et al. 1998).

Role of electron shuttles

Quinone substructures of humic substances are implicated in aiding microorganisms with the transfer of electrons for metal reduction (Cervantes et al. 2002; Lovley et al. 1998; Scott et al. 1998). AQDS has commonly been used as a model of the redox-active

quinone substructures (Lovley et al. 1996). In this study, AQDS at a concentration as low as 500 μM significantly stimulated the rate of As(V) reduction (5 mM) in a methanogenic consortium supplied with lactate. A similar methanogenic consortium was previously shown to reduce AQDS to its corresponding hydroquinone (AH_2QDS) at the expense of lactate oxidation (Cervantes et al. 2000). AH_2QDS may have transferred electrons to As(V) either abiotically or via a biologically catalyzed reaction. The abiotic reaction is not known. However, AH_2QDS is known to serve as electron donor supporting the microbial reduction of As(V) by the bacterium *Wolinella succinogenes* (Lovley et al. 1999).

Role of sulfur compounds

Sulfate is a common anion in landfills (Christensen et al. 2001), thus the impact of sulfate reduction on the reduction of arsenate was assessed. The addition of sulfate to the anaerobic microcosms did not alter the rate at which As(V) was reduced. The fact that sulfate did not effect As(V) reduction is in keeping with the favorable thermodynamics of As(V) reduction compared to sulfate reduction (Newman et al. 1997b; Oremland & Stolz 2003). However, inclusion of sulfate in the medium decreased the recovery of As(III). The initial temporal stoichiometric yield of soluble As(III) gradually disappeared in parallel with sulfate reduction. When the experiment was conducted in the presence sulfide instead of sulfate, only very low yields of soluble As(III) were measurable, accounting for only 10% of the As(III) recovery in the absence of added sulfur compounds. The results are in agreement with the precipitation of As(III) with sulfide. Previously, the bacterium *Desulfotomaculum auripigmentum* was shown to form orpiment (As_2S_3) precipitates due to its ability to consecutively reduce As(V) followed by sulfate reduction (Newman et al. 1997a). Sulfate reduction in anaerobic microcosms prepared from metal contaminated sediments also confirmed precipitation of As(III) associated with sulfate reduction (Rittle et al. 1995). Biological reduction of As(V) on agar plates can be made visual by applying sulfide which causes As(III) formed near dissimilatory As(V) reducing colonies to precipitate (Kuai et al. 2001). Immobilization of As(III) by sulfide is a complex matter, as orpiment is in equilibrium with soluble forms of arsenic sulfides and depends greatly on pH and sulfide concentrations (Helz et al. 1995; Inskeep et al. 2002; Newman et al. 1997a; Webster 1990).

In redox gradients, the mobility of As(V) in sediments and sludges is the greatest under mild reducing conditions that favor dissimilatory iron and As(V) reduction without favoring sulfate reduction (Carbonell-Barrachina et al. 2000; McCreadie & Blowes 2000; Meng et al. 2001). Under highly oxidizing conditions, As(V) is strongly adsorbed by iron oxides. Under highly reducing conditions, the sulfides formed from sulfate reduction cause the formation of solid arsenic sulfides species with As(III) (McCreadie & Blowes 2000; Meng et al. 2001). Sulfide is also known to cause the direct abiotic reduction of As(V) to As(III) and subsequent precipitation of As_2S_3 (Rochette et al. 2000). However, the kinetics of abiotic reduction are only rapid at low pH. The slow kinetics at circumneutral pH were confirmed in this study by the lack of any significant conversion of 500 μM As(V) in the presence of 6.25 mM sulfide during a month-long incubation with heat-killed sludge.

Conclusions

The results of the present study indicate the rapid and facile reduction of As(V) to As(III) in methanogenic sludge. The results taken as a whole suggest that As(V) disposed in anaerobic environments may readily be converted to As(III), increasing the mobility of arsenic. The extent of the increased mobility will depend on the concentration of sulfides generated from sulfate reduction.

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