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Long Term Performance of an Arsenite-Oxidizing-Chlorate-Reducing Microbial Consortium in an Upflow Anaerobic Sludge Bed (UASB) Bioreactor

Wenjie Sun^{*}, Reyes Sierra-Alvarez, and Jim A. Field

Department of Chemical and Environmental Engineering, University of Arizona P.O. Box 210011, Tucson, Arizona

Abstract

A chlorate (ClO_3^-) reducing microbial consortium oxidized arsenite (As(III)) to arsenate (As(V)) in an upflow anaerobic sludge-bed bioreactor over 550 d operation. As(III) was converted with high conversion efficiencies (>98%) at volumetric loadings ranging from 0.45 to 1.92 mmol $\text{As}/(\text{L}_{\text{reactor}} \text{ d})$. The oxidation of As(III) was linked to the complete reduction of ClO_3^- to Cl^- and H_2O , as demonstrated by a molar ratio of approximately 3.0 mol As(III) oxidized per mole of Cl^- formed and by the greatly lowered ClO_3^- -reducing capacity without As(III) feeding. An autotrophic enrichment culture was established from the bioreactor biofilm. A 16S rRNA gene clone library indicated that the culture was dominated by *Dechloromonas*, and *Stenotrophomonas* as well as genera within the family *Comamonadaceae*. The results indicate that the oxidation of As(III) to less mobile As(V) utilizing ClO_3^- as a terminal electron acceptor provides a sustainable bioremediation strategy for arsenic contamination in anaerobic environments.

Keywords

Arsenite oxidation; Chlorate reduction; UASB bioreactor; Clone library; Bioremediation

1. Introduction

Arsenic (As) is a well-known carcinogen and disease-causing contaminant posing a risk to millions of people around the world. The most important source of As to groundwater is from the dissolution of naturally occurring As-bearing geological material (Smedley and Kinniburgh, 2002). In circumneutral aqueous environments, arsenite (As(III) , H_3AsO_3) or arsenate (As(V) , H_2AsO_4^- and HAsO_4^{2-}) are the most common occurring oxidation states of As. The relative binding affinity of As(III) and As(V) depends on the main constituents in the sediment or soil. Typically iron (Fe) (hydr)oxides strongly adsorb both As(III) and As(V) in circumneutral pH environments (Raven et al., 1998). However, As(III) is more rapidly desorbed compared to As(V) (Tufano et al., 2008). Additionally, As(V) is more extensively adsorbed than As(III) on non-iron metal oxides such as aluminum (hydr)oxides (Lin and Wu, 2001). Therefore, the oxidation of As(III) to As(V) can potentially decrease the mobility of As in groundwater (Dixit and Hering, 2003).

^{*}Corresponding author. Tel.: +1-520-626-6781; fax: +1-520-621-6048, alexsun@email.arizona.edu (W. Sun).

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Microbial activities play significant roles in controlling the fate and transformation of As in the environment (Oremland et al., 2005). The key enzymes involved in these transformations are mostly the As(III) oxidase encoded by *aroA* genes (Inskeep et al., 2007) and the dissimilatory As(V) reductase encoded by *arrA* genes (Malasarn et al., 2004). The availability of dissolved oxygen (DO) in the subsurface may be limited due to its consumption, creating anaerobic zones. Recently, several studies have demonstrated that nitrate-dependent As(III) oxidation can be catalyzed by microorganisms under anoxic conditions (Oremland et al., 2002; Rhine et al., 2006; Sun et al., 2009). Although few As(III)-oxidizing nitrate-reducing bacteria have been isolated, the strains reported to date have been isolated from various environments including a salt lake with naturally high As levels (Oremland et al., 2002), arsenic-polluted soils (Rhine et al., 2006), as well as enrichments from pristine sediment and sludge samples (Sun et al., 2009). Some of the As(III) oxidizers have been shown to be autotrophic (Oremland et al., 2002; Rhine et al., 2006; Sun et al., 2010b). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) gene is one of the most common enzymes involved in autotrophy, catalyzing the assimilation of carbon dioxide in the Calvin-Benson cycle (Miziorko and Lorimer, 1983).

Although chlorate (ClO_3^-) is not a naturally abundant compound, it has been shown to be an alternative electron acceptor for microbial oxidation of As(III) to As(V) in anaerobic environments as indicated in Eq. (1) (Sun et al., 2010b). Addition of ClO_3^- has been proposed to stimulate the anaerobic biodegradation of aromatic hydrocarbons (Langenhoff et al., 2009; Logan and Wu, 2002). Thus, there is a potential to utilize ClO_3^- to promote the bioremediation of As-contaminated sites through the bio-oxidation of As(III) to As(V). In a previous study, the anoxic oxidation of As(III) linked to chemolithotrophic denitrification was shown to be stable in continuous bioreactors (Sun et al., 2010a). It is well known that (per)chlorate (ClO_4^- and ClO_3^-) reducing bacteria have the ability to oxidize reduced inorganic compounds such as elemental sulfur (S^0), elemental iron (Fe^0), and H_2 (Ju et al., 2008) in batch assays and experimental bioreactors. Microbial reduction of ClO_4^- and ClO_3^- proceeds via a multiple-step process as follows: $\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{O}_2 + \text{Cl}^-$ (Coates and Achenbach, 2004). Disproportionation of chlorite (ClO_2^-) is catalyzed by chlorite dismutase (*clt*) genes to form chloride (Cl^-) and oxygen (O_2), in which intracellular O_2 is immediately metabolized for cell growth (Coates and Achenbach, 2004).

The scope of this research is to explore whether ClO_3^- can support prolonged microbial oxidation of As(III) in a continuous bioreactor. The specific objectives were to demonstrate the interdependence of the anaerobic As(III) oxidation and ClO_3^- reduction, and determine if the stoichiometry of the reaction supports the complete use of electron-accepting equivalents in the reduction of ClO_3^- including both the reduction of ClO_3^- to Cl^- as well as the reduction of intermediate O_2 to H_2O . Also a clone library was used to characterize the As(III)-oxidizing, ClO_3^- -reducing bacteria (AOCRB) in the microbial community from an enrichment culture (EC) derived from the biomass of the bioreactor.

2. Material and methods

2.1. Microorganisms

Anaerobically digested sewage sludge (ADS) was obtained from a local municipal wastewater treatment plant (Ina Road, Tucson, Arizona). The sludge was allowed to settle for 1 h, and the sedimented sludge was collected to be used as inoculum for the bioreactor. The total suspended solids (TSS) and the volatile suspended solids (VSS) content of the

settled sludge was $16.7 \pm 0.2\%$ and $10.0 \pm 0.1\%$ of the wet weight, respectively. The inoculum was stored under nitrogen gas at 4°C .

2.2 Basal medium

The standard basal medium was prepared using ultra pure water (Milli-Q system; Millipore) and contained the following compounds (mg/L): NH_4HCO_3 (3.16); NaHCO_3 (672); CaCl_2 (10), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (40); K_2HPO_4 (300); $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (800); and 0.2 mL/L of a trace element solution containing (in mg/L): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (2,000); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (2,000); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (500); $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (90); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (30); ZnCl_2 (50); H_3BO_3 (50); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (50); $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ (100); $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (50); EDTA (1,000); resazurin (200); and HCl 36% (1 mL).

2.3 Continuous bioreactor

A 2-L bench-scale upflow anaerobic sludge bed (UASB) bioreactor was inoculated with 33 g VSS/L of ADS. The bioreactor was fed with basal mineral medium and As(III) (supplied as NaAsO_2) as the sole energy source (concentrations applied in each operation period are described in Table 1), and ClO_3^- as the sole electron acceptor, which was supplied as NaClO_3 at a concentration of 5.47 ± 0.44 and 3.34 ± 0.29 mM during day 0–105 and day 105–550, respectively. NaHCO_3 (8.0 mM) was supplied as the major carbon source.

The average hydraulic retention time (HRT) of the UASB bioreactor was approximately 1 d. The bioreactor was placed in a climate controlled room at $30 \pm 2^\circ\text{C}$ and covered by aluminum foil to avoid growth of phototrophic bacteria. The initial pH of the influent was adjusted to 7.0–7.2 with NaOH or HCl, as required. The influent was maintained at all times under an N_2 atmosphere to minimize DO from entering the medium which could result in the unwanted aerobic oxidation of As(III). Influent and effluent samples were prepared immediately for analysis to minimize possible changes in As speciation upon exposure to the atmosphere. The pH value was determined immediately after sampling.

2.4 Batch bioassay for As(III) inhibition

Batch bioassays were performed in shaken flasks, which were incubated in a dark climate-controlled room at $30 \pm 2^\circ\text{C}$. Serum flasks (160 mL) were supplied with 120 mL of a basal mineral medium containing bicarbonate as the only carbon source, as described above. The medium was also supplemented with As(III) as electron donor and ClO_3^- as the electron acceptor. Biomass taken from bioreactor was added to the assays at 10 g wet weight per liter of medium. The As(III) concentrations ranged from 0.005 to 10 mM, and the ClO_3^- was supplied as 10 fold excess for each initial As(III) concentration tested based on the electron equivalent. Sufficient sampling times were used to evaluate the activity of As(III) oxidation so as to obtain kinetic data normalized based on the lowest As(III) concentration tested of 0.005 mM. Flasks for these anaerobic assays were sealed with butyl rubber stoppers, and then the medium and headspace were purged with N_2/CO_2 (80/20, v/v) for 20 min to exclude oxygen from the assay. All assays were conducted in triplicate. Liquid samples were analyzed for the concentration of electron acceptor and biotransformation products (ClO_3^- , ClO_2^- , and Cl^-) and As species (As(III) and As(V)).

2.5 Enrichment culture

Under strict anaerobic conditions, a chemolithoautotrophic enrichment culture was established by adding biomass from a UASB bioreactor (sampled on day 530) to the assays at 10% (v/v) of culture medium. The basal medium was amended with 0.5 mM As(III) as electron donor, 3.0 mM ClO_3^- as electron acceptor, and 8.0 mM NaHCO_3 as the major carbon source. The procedure for enrichment process was described elsewhere (Sun et al.,

2009), in which a 5% (v/v) dilution of the active culture was serially transferred to fresh medium once complete oxidation had occurred as evidenced by measuring the As(V) formation. The enrichment process was maintained for 16 transfers.

2.6 16S rRNA gene clone library

Community genomic DNA was extracted from the enrichment culture, and then 16S rRNA gene was amplified with polymerase chain reaction (PCR) using universal bacterial primers 27F and 1492R. A clone library was established to characterize the microbial community composition of enrichment. Details of the clone library procedure used in this work are described by Sun (Sun et al., 2009). Selected clones representing each phylotype obtained in each culture have been deposited in the GenBank database with accession numbers GU557149-GU557154 corresponding to AOCRB-EC-2, AOCRB-EC-1, AOCRB-EC-3, AOCRB-EC-7 AOCRB-EC-6 and AOCRB-EC-13, respectively. The accession numbers for reference sequences used to prepare Fig. 5 are as follows: *Dechloromonas* sp. JM, AF323489; *Dechloromonas* sp. HZ, AF479766; *Dechloromonas* sp. CL, AF170354; *Dechlorimonas agitated* strain CKB, AF047462; *Dechloromonas* sp. strain ECC1-pb1, GU202936; an uncultured bacterium clone EC1-17, EU708503; *Stenotrophomonas* sp. T23, EU073089; *Stenotrophomonas* sp. SY2, EU073113; *Stenotrophomonas* sp. G10, DQ342275; *Acidovorax* sp. TS7, EU073073; uncultured bacterium clone EC1-1, EU708507; *Diaphorobacter* sp. MC-pb1, FJ514095; *Gemmatimonas aurantiaca* T-27, AP009153, *Dechloromonas aromatica* strain RCB, CP000089. Sequence data were aligned with ClustalX, and a tree was constructed using PAUP* version 4.0b10 employed with the neighbor-joining algorithm. Tree confidence was tested using the Hasegawa method (Hasegawa et al., 1985).

Functional genes including *cld*, RuBisCO, *aroA* and *arrA* genes were PCR amplified with specifically designed primer sets. Two sets of PCR primers were used to target the *cld* gene, including primer set DCD-F and DCD-R and primer set UCD-238F and UCD-646R (Bender et al., 2004). Four primer sets were used to target the As(III) oxidase (*aroA*) gene (Table S1 in the Supplementary Information) in the ECs and pure cultures. The primer sets included *aroA*1-85F and *aroA*1-593R (set #1) (Inskeep et al., 2007), *aroA*2-85F and *aroA*2-602R (set #2) (Inskeep et al., 2007), *aroA*3-69F and *aroA*3-1374R (set #3) (Rhine et al., 2007) and *aroA*4-85F and *aroA*4-622R (set #4) (Hoeft et al., 2007). Two primer sets (Table S1) were used to amplify the conserved regions of As(V) reductase genes (*arrA*), which included CHarrAfwd and CHarrArev (set #1) (Malasarn et al., 2004) and HAarrA-D1F and HAarrA-G2R (set #2) (Kulp et al., 2006). Two PCR primer sets were used to identify RuBisCO genes, both *cbbL* and *cbbM* type genes (Elsaied and Naganuma, 2001). Details of the PCR methods are reported in the Supplementary Information. To verify the integrity of the amplification, both positive, negative (no template DNA) and original inoculum samples were included. The PCR products were purified using a Quick PCR purification kit (Qiagen, Valencia, CA), and then run using gel electrophoresis on a 1.5% agarose gel containing 1×Tris-borate-EDTA buffer (Intermountain Scientific Corp., Kaysville, UT). The gels were stained with ethidium bromide (0.5 µg/mL) and stained DNA was visualized under ultraviolet illumination in a MultiDoc-it imaging system (UVP, Upland, CA). The identities of amplicons were confirmed by verifying their molecular weight on the gel with electrophoresis.

2.7 Analytical methods

Arsenic speciation (As(III) and As(V)) was analyzed by high performance liquid chromatography–inductively coupled plasma–mass spectroscopy (HPLC-ICP-MS). The system consists of an HPLC (Agilent 1100) and an ICP-MS (Agilent 7500a) with a Babington nebulizer as the detector. The operating parameters were as follows: Rf power

1,500 watts, plasma gas flow 15 L/min, carrier flow 1.2 L/min, As was measured at 75 m/z and terbium (IS) measured at m/z 159, three points per peak; 1.5 s dwell time for As, 1.5 s dwell time for Tb; number of repetitions = 7. The injection volume was 10 μ L. The detection limit for the various As species was 0.1 μ g/L.

ClO_3^- , ClO_2^- , Cl^- and As(V) were analyzed by suppressed conductivity ion chromatography (IC). The Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 high capacity analytical column (4 $\times$ 250 mm) and an AG11 high capacity guard column (4 $\times$ 50 mm). During each injection the eluent (20 mM KOH) was used for 20 min. Other analytical determinations (*e.g.*, pH, TSS, VSS, *etc.*) were conducted according to Standard Methods (APHA, 1999).

3. Results and discussion

3.1 Microbial oxidation of As(III) to As(V) linked to ClO_3^- reduction in the UASB bioreactor

To determine if the process of As(III) oxidation linked to ClO_3^- reduction could be sustained in a continuous bioreactor, a 2-L UASB bioreactor was initiated. The bioreactor was operated for a period of 550 d and the operation was divided into seven periods, which were distinguished based on the feeding concentration of As(III) and ClO_3^- as shown in Table 1. During the whole operation of the UASB bioreactor, the pH of effluent was constant in the range of 6.8–7.0, which is close to the initial influent pH range of 7.0–7.2. The microbial oxidation of As(III) and the concomitant formation of As(V) in the bioreactor as a function of operation time are illustrated in Fig. 1. The removal efficiency of As(III) in the bioreactor averaged $95.9 \pm 4.3\%$ considering the data of all the operation periods when As(III) was supplied in the feed. Fig. 2 also illustrates the average influent and effluent concentrations of As(III) and As(V) in the bioreactor for each operation period. The conversion efficiency of influent As(III) to effluent As(V) averaged $98.3 \pm 4.0\%$ during the same periods, which indicates that As(V) was the main product of the conversion.

During periods I, II, III and IV, the influent concentration of As(III) was gradually increased (0.5, 1.0, 1.75 to 2.0 mM, respectively). After day 20, the dominant As species in the effluent of bioreactor was As(V), indicating the occurrence of microbial As(III) oxidation linked to ClO_3^- reduction under anaerobic conditions. In period V, no As(III) was included in the influent of the bioreactor in order to measure the endogenous consumption of ClO_3^- (and production of Cl^-) with organic matter naturally present in the biofilm of the bioreactor serving as the electron donor. In periods VI, VII and VIII, As(III) was re-introduced in the reactor feed and its concentration was increased step-wise (0.5, 1.0 and 2.0 mM, respectively). After 112 days of operation without As(III), microorganisms in the anaerobic bioreactor readily converted As(III) to As(V) indicating full recovery of the population after the As(III) feeding interruption. Upon resumption of the As(III) feeding at the start of period VI, stable removal of As(III) was achieved after only 7 d. The average removal efficiency for the remainder of period VI was 95.3%, which was comparable to that of periods I to IV. Residual As(III) in effluent increased noticeably from period VI to VIII due to large increases in the influent As(III) concentrations. Table 1 presents a summary of the performance data for the total duration of this study.

3.2 ClO_3^- reduction coupled to anaerobic oxidation of As(III) in the UASB bioreactor

The feed of anaerobic bioreactor was supplemented with ClO_3^- (5 mM) during the initial 150 days of operation. The concentration of ClO_3^- was decreased to 3 mM after day 150 to facilitate the measurement of ClO_3^- consumption (Fig. 3). The ClO_3^- concentration was supplied in excess of the concentration required for the stoichiometric conversion of As(III) to As(V) (the theoretical requirements for 0.5, 1.0, 1.75 and 2.0 mM As(III) would be 0.17,

0.33, 0.58 and 0.67 mM ClO_3^- , respectively). In period V (the period of the As(III) feed interruption), the ClO_3^- consumption (and Cl^- production) significantly decreased indicating that ClO_3^- reduction was dependent on the presence of As(III) in the feed. The endogenous consumption of ClO_3^- was measured as 0.26 ± 0.28 mM, which corresponded to the formation of nearly stoichiometric concentrations of Cl^- (0.23 ± 0.15 mM). After day 423, the consumption of ClO_3^- increased again due to the reintroduction of As(III) to the feed. The endogenous ClO_3^- consumption (and Cl^- production) was used to correct the ClO_3^- consumption and Cl^- production during periods in which As(III) was fed. The corrected ClO_3^- consumption was attributed to As(III) oxidation, and used to calculate the ratios of As(III) removal and As(V) formation to ClO_3^- consumption or similar ratios made with Cl^- production. During the whole operation, the molar ratios of As(III) removal or As(V) formation to corrected ClO_3^- consumption or Cl^- formation (Fig. 4) were close to the theoretical ratio of 3.0 indicated in Eq. (1) for As(III) oxidation linked to complete ClO_3^- reduction, including the reduction of intermediate O_2 to H_2O . ClO_2^- , a possible intermediate product from the microbial degradation of ClO_3^- , was not detected in the effluent of the reactor throughout the experiment.

3.3 Microbial community composition of AOCRB enrichment culture

The microbial community composition in the established AOCRB EC was analyzed by preparing a 16S rRNA gene clone library. Rarefaction analysis for the clone library (Fig. S1, Supplementary Information) indicated that a total of six unique phylotypes were identified from this culture with 36 clones sufficiently capturing the community composition. The 36 clones analyzed fell into four phylogenetic categories, either one of three classes in the phylum *Proteobacteria* (α -*Proteobacteria*, β -*Proteobacteria*, and γ -*Proteobacteria*) or the phylum *Gemmatimonadetes*, accounting for 8.3%, 75.0%, 11.1% and 5.6% of all the clones from this culture, respectively. The phylogenetic relationships among the phylotypes recovered are shown in Fig. 5.

The β -*Proteobacteria* accounted for the majority of all the clones and three of a total of six phylotypes identified. These β -*Proteobacteria* phylotypes were most closely related to members of the genus *Dechloromonas*, *Acidovorax* and *Alicyciphilus* and they accounted for 58.3, 11.1 and 5.6% of all the clones in the clone library, respectively. The *Dechloromonas* phylotype showed 99% 16S rRNA gene sequence similarity to an As(III)-oxidizing strain *Dechloromonas* sp. strain ECC1-pb1, which is a chemolithotrophic bacteria gaining growth energy from the reaction of As(III) oxidation coupled to ClO_3^- reduction (Sun et al., 2010b). This phylotype also had a high 16S rRNA gene sequence similarity to several well-studied *Dechloromonas* species of (per)chlorate reducing bacteria such as *Dechloromonas* sp. JM (99%) (Miller and Logan, 2000) and *Dechloromonas* sp. HZ (99%) (Zhang et al., 2002). The *Dechloromonas* clones have 98% similarity to *Dechloromonas agitata* strain CKB, which is able to oxidize Fe(II) with (per)chlorate under strictly anaerobic conditions (Lack et al., 2002). Regarding *Acidovorax* and *Alicyciphilus* species, these members of *Comamonadaceae* family are reported to play a role in oxidizing As(III) linked under both aerobic and anaerobic conditions. One *Comamonadaceae* cluster phylotype of this study (clone AOCRB-EC-13) has 97% 16S rRNA gene sequence similarity to an As(III)-resistant strain *Acidovorax* sp. TS7, which was isolated from soils highly contaminated with arsenic (Cai et al., 2009). The other *Comamonadaceae* cluster phylotype detected in this study (clone AOCRB-EC-7) was related to the genus *Alicyciphilus* and has 96% of 16S rRNA gene sequence similarity to a strain *Diaphorobacter* sp. MC-pb1, with a demonstrated ability to oxidize As(III) in the presence of NO_3^- (Sun et al., 2009).

One phylotype within the class α -*Proteobacteria* was most closely related to *Rhodobacter*. Another phylotype in the phylum *Gemmatimonadetes* was most closely related to

Gemmatimonas. The last phylotype within the class γ -*Proteobacteria* was most closely related to *Stenotrophomonas*. Recently the *Stenotrophomonas* phylotype was illustrated to have the ability to oxidize As(III) in various environments. The phylotype observed in this study shows 99% 16S rRNA gene sequence similarity to an uncultured bacterium clone EC1-17, which was identified from an EC oxidizing As(III) under denitrifying condition (Sun et al., 2009). This phylotype was also closely related to two aerobic As(III) oxidizers, *Stenotrophomonas* sp. TS23 and SY2 (99% and 98% 16S rRNA gene sequence similarity, respectively), which were As(III)-resistant bacteria identified from As contaminated soil samples (Cai et al., 2009). In addition, the clone had 99% 16S rRNA gene sequence similarity with *Stenotrophomonas* sp. G10, which utilizes acetate to reduce ClO_3^- as the electron acceptor (Weelink et al., 2007).

3.4 Functional gene PCR

Functional gene PCR targeting *cld*, RuBisCO, *aroA* and *arrA* genes were performed on the established AOCRB EC originated from the bioreactor biomass. The electrophoresis gels (data not shown) showed that *cld* and RuBisCO genes were present in this EC. On the other hand, various primer sets were used to detect the existence of arsenic metabolizing genes, *aroA* or *arrA*. The presence of *cld* genes proved additional evidence that the enriched AOCRB culture has the capacity to reduce the ClO_2^- to Cl^- , a key step in the ClO_3^- reduction pathway (Coates and Achenbach, 2004). The existence of RuBisCO genes provided DNA-based evidences that this culture is autotrophic. The results from this study confirmed that chemolithotrophic ClO_3^- reduction to the benign products, Cl^- and H_2O , was responsible for the oxidation of As(III) to As(V) in the UASB bioreactor.

The *arrA* genes were successfully detected with the primer set #1 (Table S1, Supplementary Information). The identities of amplicons were confirmed by the amplicons length of approximate 160–200 base pair (bp) on the gel as expected for the primer set. However, no evidence could be found for the presence *aroA* genes with any of the primer sets tested. Although there was no *aroA* genes detected in the enriched AOCRB culture developed from the bioreactor, the presence of *arrA* genes provides the molecular evidences for the biological nature of As(III) oxidation (Richey et al., 2009). Previously, *arrA* genes in the As(III)-oxidizing nitrate reducer *Alkalilimnicola ehrlichii* strain MLHE-1 have been shown to function not only as dissimilatory As(V) reductase but also as an As(III) oxidase (Richey et al., 2009). As(III) oxidase (Aro) and As(V) respiratory reductase (Arr) are enzymes within the same family (dimethyl sulfoxide reductase family). The gene *arxA* has been identified as a unique clade encoding for the oxidase activity in strain MLHE-1 that is closer to *arrA* than to *aroA* (Zargar et al., 2010).

3.5 As(III) inhibition to As(III) oxidation linked to ClO_3^- reduction

A batch experiment inoculated with AOCRB biofilm sampled from the bioreactor (on day 530) was monitored at various initial As(III) concentrations to study the substrate inhibition. The oxidation of As(III) was dependent on the presence of ClO_3^- and inoculum as evidenced by the absence of any significant conversion in incubations lacking ClO_3^- or sludge inoculum (data not shown). The specific activities for all the concentrations applied were measured to evaluate the inhibitory effects. The anaerobic oxidation of As(III) coupled to ClO_3^- reduction was inhibited by increasing As(III) concentrations as illustrated by the decreasing in specific activity. Compared to the activity observed at 0.005 mM As(III), the maximum specific activities were inhibited by 50% (IC50) at 0.29 mM As(III). The activity inhibition by As(III) at 5 and 10 mM was 97 and 98%, respectively. Although the inhibition was severe, As(III) as high as 10 mM could be completely oxidized by anaerobic microorganisms after a prolonged lag phase of 25 d.

The microbial toxicity of As is well established. Compared with As(V), As(III) shows much higher toxicity (Sierra-Alvarez et al., 2004). As(III) binds to sulfhydryl groups, inactivates/denatures the normal functions of many proteins and causes cell damage (Mukhopadhyay et al., 2002). As(III) is known to interfere essential enzymatic functions and transcriptional events, and it also binds to the vicinal thiols in pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase thereby inhibiting respiration (Mukhopadhyay et al., 2002). The IC₅₀ of As(III) to methanogenic activity in sludge has been reported to be as low as 15 μ M (Sierra-Alvarez et al., 2004). In this study, As(III) was found to be an inhibitory substrate of AOCRB as was demonstrated previously with As(III)-oxidizing, denitrifying bacteria (Sun et al., 2010a).

Despite the high level of substrate inhibition, AOCRB in this study were nonetheless able to oxidize As(III) up to the highest tested of 10 mM. This might be due to the presence of As(III)-resistant bacteria, such as *Stenotrophomonas*, *Dechloromonas* and *Gemmatimonas*. The *Stenotrophomonas* phylotype identified in this study has a close sequence similarity to two *Stenotrophomonas* sp. TS23 and SY2, which have high minimal inhibitory concentrations (MICs) ranging from 10 to 20 mM As(III). Both of these *Stenotrophomonas* strains contain As(III) transporter genes (*ACR3*), which have been shown to confer the As resistance (Cai et al., 2009). Moreover, the *Dechloromonas* clones and *Gemmatimonas* phylotypes analyzed in this study have 96% and 90% similarity to *Dechloromonas aromatica* strain RCB and *Gemmatimonas aurantiaca* T-27, respectively, which contain the *arsC* gene (Complete genome, Genbank database with accession number CP000089 and NC_012489). The *arsC* gene, an As(V) reductase, mediates the reduction of As(V) to As(III) in the cytoplasm, which is an important component of the *ars* operon involved in As tolerance (Mukhopadhyay et al., 2002). In the UASB bioreactor, the As(III) was oxidized to As(V) at influent concentrations of As(III) up to 2.0 mM without obvious inhibition observed. The apparent difference in As(III) inhibition observed in the batch experiments compared to the bioreactor may be due to the prolonged exposure of low amount of As(III)-oxidizing bacteria to untransformed As(III) in the batch assays; whereas, As(III) was quickly converted to As(V) by higher concentration of As(III)-oxidizing biomass in the bioreactor. The incoming As(III) would be mixed with the bioreactor contents, which would contribute to lowering the effective concentration of As(III) to which the bacteria would be exposed. In addition, bacteria inside biofilms typically tolerate higher concentrations of toxic substances than freely suspended cells. The dense extracellular matrix and the outer layer of biofilms protect the interior microbial community (Hall-Stoodley et al., 2004). A slow or incomplete penetration/diffusion of the As(III) into the biofilm may have also contributed to a lower As(III) exposure.

4. Conclusions

The results of this study demonstrate that ClO_3^- can be used as an electron acceptor in a sustained fashion by anaerobic As(III) oxidizers immobilized in the biofilm of a UASB bioreactor. Thus, ClO_3^- injection into groundwater could potentially be considered as a means of remediating As contamination, since the arsenite-oxidizing-chlorate-reducing microbial community would be expected to readily develop and be stable over long periods of time. The results taken as a whole indicate that the utilization of ClO_3^- as alternative electron acceptor to O_2 provide a strategy for bioremediation of As contamination in anaerobic environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi XXX

Fig. 1. The removal of As(III) (panel A) and the formation of As(V) (panel B) in the continuous bioreactor linking the anoxic oxidation of As(III) to ClO_3^- respiration as a function of time: (●) Influent, (○) Effluent.

Fig. 2. The influent and effluent concentrations of As(III) and As(V) for each period of the total operation in the continuous bioreactor linking the anoxic oxidation of As(III) to ClO_3^- respiration.

Fig. 3. The removal of ClO_3^- (panel A) and the formation of Cl^- (panel B) in the continuous bioreactor linking the anoxic oxidation of As(III) to ClO_3^- respiration as a function of time: (●) Influent, (○) Effluent.

Fig. 4.

Summary of molar ratios of As(III) removed and As(V) formed compared to ClO_3^- consumed and Cl^- formed as determined in the continuous bioreactor linking the anoxic oxidation of As(III) to ClO_3^- respiration for all operational periods. Legends: As(III)/ ClO_3^- (○), As(III)/ Cl^- (□), As(V)/ ClO_3^- (△) and As(V)/ Cl^- (◇). Theoretical ratio if ClO_3^- is reduced to Cl^- , and intermediate O_2 is reduced to H_2O (—); Theoretical ratio if intermediate O_2 is reduced to H_2O (----). ClO_3^- consumed and Cl^- formed were corrected for endogenous ClO_3^- consumption and Cl^- formation measured in period V fed with ClO_3^- in the absence of As(III), respectively.

Fig. 5. Phylogenetic tree for the clones identified in this As(III)-oxidizing, ClO_3^- -reducing EC established from bioreactor. Reference sequences were obtained from GenBank and the accession numbers were included in the Supplementary Information. The dendrogram was constructed using PAUP* version 4.0b10 employed with the neighbor-joining algorithm. The scale bar represents the evolutionary divergence unit, which is equal to 10% difference.

Table 1

Results summary of operation periods of the UASB reactor

Period	Days	As(III) loading rate (mg/(L _{reactor} d))	As(III) removed (mM)	As(V) formed (mM)	As(III) removal efficiency (%)	ClO ₃ ⁻ consumed (mM)	Cl ⁻ formed (mM)	Cl ⁻ formed /ClO ₃ ⁻ consumed (mol/mol)
I	0-88	33.8±6.0	0.45±0.08	0.40±0.08	92.69±6.94	0.40±0.09	0.38±0.05	1.00±0.26
II	88-211	81.8±15.0	1.09±0.20	1.04±0.19	97.31±2.91	0.66±0.11	0.56±0.06	0.87±0.18
III	211-278	126.0±10.5	1.68±0.14	1.64±0.11	97.97±1.24	0.83±0.17	0.72±0.07	0.91±0.21
IV	278-301	139.5±3.0	1.86±0.04	1.84±0.03	96.75±0.94	0.76±0.20	0.84±0.06	1.18±0.39
V	301-423	N/A [‡]	N/A	N/A	N/A	0.25±0.18 [‡]	0.26±0.10 [‡]	1.12±0.26
VI	423-453	35.2±1.5	0.47±0.02	0.47±0.02	95.28±1.31	0.42±0.03	0.38±0.02	0.90±0.09
VII	453-500	67.5±11.3	0.90±0.15	0.87±0.13	95.05±0.83	0.58±0.10	0.52±0.04	0.91±0.06
VIII	500-550	144.0±11.3	1.92±0.15	1.95±0.05	92.84±3.59	0.90±0.11	0.85±0.07	0.95±0.07

[‡] The endogenous consumption of ClO₃⁻.

[‡] N/A means not applicable since there was no As(III) fed in period V.