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Adaptation of a methanogenic consortium to arsenite inhibition

Lucia Rodriguez-Freire^{a,#}, Sarah E. Moore^a, Reyes Sierra-Alvarez^a, and James A. Field^a

^aDepartment of Chemical and Environmental Engineering, The University of Arizona, P.O. Box 210011, Tucson, Arizona, USA

Abstract

Arsenic (As) is a ubiquitous metalloid known for its adverse effects to human health. Microorganisms are also impacted by As toxicity, including methanogenic archaea, which can affect the performance of process in which biological activity is required (*i.e.* stabilization of activated sludge in wastewater treatment plants). The novel ability of a mixed methanogenic granular sludge consortium to adapt to the inhibitory effect of arsenic (As) was investigated by exposing the culture to approximately 0.92 mM of As^{III} for 160 d in an arsenate (As^V) reducing bioreactor using ethanol as the electron donor. The results of shaken batch bioassays indicated that the original, unexposed sludge was severely inhibited by arsenite (As^{III}) as evidenced by the low 50% inhibition concentrations (IC₅₀) determined, *i.e.*, 19 and 90 μ M for acetoclastic- and hydrogenotrophic methanogenesis, respectively. The tolerance of the acetoclastic and hydrogenotrophic methanogens in the sludge to As^{III} increased 47-fold (IC₅₀ = 910 μ M) and 12-fold (IC₅₀ = 1100 μ M), respectively, upon long-term exposure to As. In conclusion, the methanogenic community in the granular sludge demonstrated a considerable ability to adapt to the severe inhibitory effects of As after a prolonged exposure period.

Keywords

Acclimation; adaptation; arsenic; arsenite; inhibition; methanogenic; toxicity

1. Introduction

Arsenic (As) is a metalloid naturally occurring in the crustal Earth; and, it is well known for its human toxicity. Chronic As exposure increases the risk of cancer and cardiovascular diseases, skin lesions, and damage to the hematological, neurological, renal, hepatic, pulmonary, and reproductive systems (ATSDR 2007). Microorganisms are also affected by As toxicity, but they have developed mechanisms to deal with As, and even to use it for their own benefit.

Elevated background As concentrations can be found in hydrothermal veins and sedimentary rocks, where As concentrations can be as high as 40,000 mg/kg in arsenical pyritic copper deposit (Mandal and Suzuki 2002). The release of As into the environment due to anthropogenic activities also contributes to high As concentrations. Mining activity exposes

[#]Corresponding author: Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 210011, Tucson, Arizona, Tel. 520-621-2591, Fax. 520-621-6048, lucia.rodriguez.freire@gmail.com.

reduced As-minerals to the environment that will undergo weathering (with As concentrations in acid rock drainage of up to 11.3 mM (Paikaray 2015)). As-product manufacturing will also contribute to contamination of soil and water bodies, *i.e.* contamination from a pesticide manufacturing facility have led to As aqueous concentrations in groundwater of up to 0.2 mM (O'day et al. 2004); the wastewater effluent from a Gallium Arsenide semiconductor production facility was reported to have an As concentration of 0.8 mM (Li et al. 2015). Exposure to high As levels have obligated many microorganisms to adapt to the inhibitory effects of As, and develop efficient detoxifying mechanisms (*i.e.*, precipitation, chelation, compartmentalization) (Slyemi and Bonnefoy 2012) and As extrusion (Cervantes et al. 1994). Some bacteria will oxidize arsenite, As^{III}, (heterotrophic As^{III}-oxidizing bacteria, HAO) (Ehrlich 2002) or reduce arsenate, As^V, (As^V-resistant bacteria, ARB) (Rosen 2002). Numerous microorganisms can gain energy using As either as electron donor (chemolithotrophic As^{III}-oxidizing bacteria, AOB) (Oremland et al. 2002) or as electron acceptor (dissimilatory As^V-reducing bacteria, DARB) (Ahmann et al. 1994). The microorganisms able to utilize As have demonstrated a great phylogenetic diversity, and they can be found in aquatic and terrestrial environments, as well as in a wide range of environmental conditions (Cavalca et al. 2013, Kruger et al. 2013).

Microorganisms able to utilize As and methanogens are both widespread in anaerobic environments (Li et al. 2013). Furthermore, methanogenic activity has been correlated with high As concentrations in groundwater systems (Wang et al. 2015). In wastewater treatment plants, methanogenic activity is critical for the stabilization of excess activated sludge generated by anaerobic digestion, since methanogens are the organisms responsible for the conversion of organic intermediates (H₂ and acetate) from the anaerobic conversion of organic compounds to methane (CH₄) (Tchobanoglous et al. 2003). Different contaminants can inhibit methanogenesis, including inorganic species such as ammonium, sulfide, metal ions and heavy metals, and some organic compounds (Chen et al. 2008). Few studies have addressed the inhibitory impact of As on methanogenic microorganisms, and prior to this study no research has investigated the ability of methanogens to adapt to elevated As concentrations. Sierra-Alvarez et al. (2004) investigated the toxicity of inorganic As species to different mixed methanogenic cultures using short-term bioassays and showed that As^{III} concentrations as low as 15.5 µM caused 50% inhibition of the methanogenic activity. In contrast, As^V concentrations greater than 500 µM were required to cause similar levels of inhibition. The ability of methanogenic microorganisms to adapt to arsenic toxicity has received little attention. However, it is known that a greater tolerance to toxic metals can be achieved after a period of exposure due to changes in the microbial community structure (*i.e.* enrichment of a new dominant species or death of more sensitive species) (Giller et al. 1998). The main objective of this study was to evaluate the adaptation capacity of a methanogenic granular sludge consortium to As^{III} toxicity, after being exposed to 1 mM As^{III} during 160 d in a bioreactor (Rodriguez-Freire et al. 2015).

2. Materials and methods

2.1. Source of microorganisms

The microorganisms unexposed to arsenic were obtained from an anaerobic granular sludge from the up-flow anaerobic sludge bed (UASB) reactor of a beer brewery wastewater treatment plant (Mahou, Guadalajara, Spain). A further description of the reactor is described in the previous study by Rodriguez-Freire et al. (2015). The arsenic exposed microorganisms were obtained from a continuous-flow laboratory scale UASB bioreactor inoculated with the granular sludge (13.3 g volatile suspended solids (VSS)/l) and fed with 1.02 mM arsenate (As^{V}) and 4.40 mM ethanol as electron donor for 160 d. The endogenous activity of the inocula was measured to be $8 e^- \text{ meq/g VSS}$. The methanogenic sludge was continuously exposed to arsenite (As^{III}) (ca. 0.92 mM) which was formed by microbial reduction of the supplied As^{V} . A summary of the operational conditions in the bioreactor is presented in Table 1.

2.2. Batch methanogenic inhibition bioassays

The inhibitory effect of arsenite (As^{III}) on a methanogenic granular sludge was evaluated before and after extended exposure (160 days) to high levels of As^{III} (0.92 mM). The toxicity of As^{III} to methanogens was studied either using acetate (acetoclastic methanogenesis) or hydrogen (H_2) (hydrogenotrophic methanogenesis) as substrates.

Methanogenic inhibition by As^{III} was evaluated in shaken batch bioassays utilizing acetate (CH_3COO^-) or hydrogen (H_2) as the substrate, using the method modified from Sierra-Alvarez et al. (2004). The methanogenic transformation of these substrates can be summarized as:

The batch experiments were carried out in serum bottles (50 ml) containing basal medium (10 ml) and inoculum (1 g VSS/l). The mineral medium (pH 6.5) contained (mg/l): NH_4Cl (280), NaHCO_3 (4,000), K_2HPO_4 (250), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (10), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (100), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (100); yeast extract (100), and 1 ml/l of trace element solution (Rodriguez-Freire et al. 2014). Acetoclastic methanogenic bioassays were also spiked with 25 mM of acetate (as CH_3COONa). Subsequently, the liquid medium in the serum bottles was flushed with N_2/CO_2 (80:20, v/v) for 5 min. Next, the bottles were closed using butyl rubber septa and aluminum seals, and the headspace was flushed for an additional 5 min with N_2/CO_2 to ensure anaerobic conditions. In the hydrogenotrophic methanogenic bioassays, H_2 was supplied as overpressure using H_2/CO_2 (80:20, v/v) to a final partial pressure of 1.4 atm of H_2 (56 mmol $\text{H}_2/\text{l}_{\text{liq}}$). All the bottles were pre-incubated overnight at 30°C in an orbital shaker at 120 rpm. Following the pre-incubation, a known volume of As^{III} was added from a stock solution prepared using NaAsO_2 to each flask to obtain at least five different concentrations of As^{III} in each experiment. The volume of liquid added was completed (if

needed) to 2.5 mL with ultra-pure water, for a total volume of liquid in the treatments of 12.5 ml. The bottles were flushed again with N₂/CO₂ (80:20), and H₂/CO₂ (80:20) was added to a final partial pressure of 1.4 atm in the hydrogenotrophic methanogenic activity assays. Controls with no As^{III} were run in quadruplicate, and the rest of the treatments were run in duplicate.

Methane (CH₄) production was monitored three or four times a day until the maximum methane production was reached. Liquid samples were collected at the beginning and at the end of the experiment to detect any change in As speciation. Hydrogen gas was measured at the beginning of the experiment to determine the exact amount of H₂ added in the treatments. The actual VSS concentration in each flask was analyzed at the end of the experiments.

The maximum specific methanogenic activity (mg CH₄-COD per mg VSS per day) was calculated for each assay. The normalized methanogenic activity (NMA) was calculated as the ratio of maximum methane production rates in each specific treatment (for each As^{III} concentration) and the control (no As^{III}), as shown in equation 3:

$$\text{NMA}(\%) = \left(\frac{\text{Maximum CH}_4 \text{ production rate at tested As}^{\text{III}} \text{ concentration}}{\text{Maximum CH}_4 \text{ production rate of the control}} \right) \times 100 \quad \text{Eq. 3}$$

The As^{III} that caused 50% of inhibition of the normalized methanogenic activity compared with the control treatment (IC₅₀) was determined for each assay, by plotting the NMA against the actual As^{III} concentration, and graphically interpolating the As^{III} concentration that caused the 50% inhibition.

2.3. Analytical techniques

Total As concentration was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES, Optima 2100 DV, Perkin-Elmer TM, Shelton, CT, USA) at wavelength 193.7 nm. As^{III} was measured using a high pressure liquid chromatography system (HPLC, Agilent 1100 HPLC, Agilent Technologies, Inc., Santa Clara, CA, USA) linked to an ICP-mass spectrometer (ICP-MS, Agilent 7500ce). The HPLC (was fitted with a reverse-phase C18 column (Gemini 5 μ C18 110A, 150 x4 .60mm, Phenomenex, Torrance, CA, USA) and a guard cartridge. The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2 mM malonic acid and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50°C and samples were kept at 4°C in a thermally controlled autosampler. The ICP-MS was operated as follows: Rf power 1500 watts, plasma gas flow 15 l/min, carrier flow ~ 0.9 l/min, 0.15 l/min makeup. Arsenic was detected at 75 m/z. The low detection limits for the measured species are 0.05 mg/l total As and 0.03 mg/l As^{III}.

Acetate, H₂ and CH₄ were analyzed by gas chromatography (Agilent Technologies 7890A GC) by thermal conductivity detection (TCD) (H₂) and flame ionization detection (FID) (acetate and CH₄). Head space samples were taken with a pressure lock gas tight syringe (1710RN, 100 μ l (22s/2"/2), Hamilton Company, Reno, NV, USA). H₂ was detected using a

Carboxen[™] 1010 Plot fused capillary column (30 m × 0.53 mm) and N₂ as the carrier gas. The temperature of the injection port, column and detector were 220, 100 and 230 °C, respectively. Acetate and CH₄ were monitored using a Restek Stabilwax[®]-DA column (30 m × 0.35 mm, ID 0.25 µm), and He as the carrier gas (5.2 ml/min). For acetate determination, the temperature of the injector port and the detector was 280 °C. The initial temperature of the column was 100 °C and it was increased up to 150 °C at a rate of 8 °C/min. During CH₄ measurements, the temperature of the column, injector port, and detector was 140, 200, and 275 °C, respectively.

Volatile suspended solids were determined as described by the Standard Methods for the Examination of Water and Wastewater (APHA 1999).

3. Results and discussion

3.1. Arsenite toxicity to the unexposed methanogenic granular sludge

The presence of As^{III} impacted methane (CH₄) production by the methanogenic granular sludge. Fig. 1 shows the time course of the CH₄ production by the unexposed granular sludge as a function of time for the different As^{III} doses. Exposure to increasing As^{III} concentrations led to a decrease in the rate of CH₄ production by both the acetoclastic and hydrogenotrophic methanogenic communities, however the former was more sensitive to As^{III} concentration. In the acetogenic methanogenesis (Fig. 1A), the CH₄ concentration in the treatments with no As^{III} increased at a rate of 0.47±0.03 mmol CH₄/(g VSS • h), reaching the maximum CH₄ concentration of 21.69±0.45 mmol CH₄/g VSS in less than 48 h. The CH₄ production was partially inhibited at an As^{III} concentration as low as 14.4±0.9 µM, and the CH₄ production rate decreased by 57.4±0.8% when the As^{III} concentration was 19.7±0.2 µM. The methanogenic community was completely inhibited (more than 95% inhibition) at As^{III} concentrations greater than 82.5±1.3 µM. Hydrogenotrophic methanogens were more resistant to As^{III} toxicity than acetoclastic methanogens. The maximum specific hydrogenotrophic methanogenic activity in the absence of As^{III} was 0.53±0.05 mmol CH₄/(g VSS • h). In this case, 30.0±7.1% inhibition was observed when the As^{III} concentration was 39.1±2.4 µM, and the CH₄ production was fully inhibited when the As^{III} concentration exceeded 2000 µM (Fig. 1B).

Fig. 2 shows the normalized methanogenic activity (NMA) as a function of the As^{III} concentration measured in the different bioassays. The IC₅₀ of As^{III} toward the acetoclastic community in the non-exposed granular sludge was 19 µM (Fig. 2A); whereas, the IC₅₀ for the hydrogenotrophic methanogens in the same sludge was 4.7-fold greater, 90 µM (Fig. 2B). The IC₅₀ obtained for acetoclastic methanogenesis in the unexposed granular sludge is in close agreement with the value (15.5 µM) reported in a previous study that evaluated the toxicity of As on two granular sludges from anaerobic industrial wastewater treatment plants (Sierra-Alvarez et al. 2004). On the other hand, the hydrogenotrophic methanogens in the original granular sludge in this study were 3.3-fold more resistant to As^{III} toxicity than that reported by Sierra-Alvarez et al. (2004).

3.2. Increased tolerance to As^{III} inhibition following extended exposure of the methanogenic granular sludge to arsenic

The inhibitory impact of As^{III} on the arsenic-exposed granular sludge was evaluated by performing the same bioassays and data analysis as that used for the unexposed sludge. CH₄ concentration was measured as a function of time for the As^{III}-free treatments and for different As^{III} concentrations, and the NMA was calculated. Fig. 3 represents the NMA of the pre-exposed sludge as a function of the As^{III} concentration in the treatment. Exposure to As increased the tolerance of the methanogenic microorganisms to the inhibition by As^{III}. The IC₅₀ calculated for As^{III} in the acetoclastic bioassays was 910 µM in the pre-exposed granular sludge (Fig. 3A), a value which is almost 47-fold greater than for the unexposed sludge. Similarly, the IC₅₀ determined for the hydrogenotrophic methanogens after extended exposure to arsenic was 1100 µM, which is 12-fold greater compared to the value determined for the original, unexposed granular sludge (Fig. 3B). Consequently, while very low As^{III} concentrations will initially inhibit the CH₄ production by a methanogenic granular sludge, the methanogenic activity can be restored after an initial period of acclimation, as evidenced by the increment in the As^{III} IC₅₀ in the pre-exposed granular sludge.

Arsenite toxicity has been attributed to its affinity for sulfide groups. Arsenite association with thiol groups in enzymes can interfere with their normal function and expression, affecting the intracellular homeostasis, deoxyribonucleotide synthesis and repair, protein folding, and xenobiotic detoxification (Slyemi and Bonnefoy 2012). There are two possible mechanisms by which the microorganism communities could have gained resistance to As^{III}: (1) enrichment of As^{III}-tolerant methanogens; or, (2) induction of enzymes responsible for As^{III} resistance (Chen et al. 2008) in the existing methanogen population. Organisms have evolved to deal with As since the primordial life (Oremland et al. 2009). The main As detoxification mechanism is the efflux of As^{III} out of the cell, expressed by the *Ars* operon, which expels As^{III} from the cell through an ArsB periplasmic protein pump (Slyemi and Bonnefoy 2012).

The natural widespread occurrence of As and As-utilizers have facilitated that a large diversity of organisms have the ability to detoxify and respire As (Yang et al. 2012), including methanogenic archaea as proven by the presence of the *Ars* operon genes in the genomes of *Methanosarcina mazei*, and *Methanothermobacter thermautotrophicus*, among others (Bini 2010) and the ability of methanogenic consortia to reduce As^V (Field et al. 2004, Rodriguez-Freire et al. 2015). Recently, Wang et al. (2014) isolated the gene *MaarsM* in the archaeon *Methanosarcina acetivorans*. Expression of the *MaarsM* gene is known to increase the tolerance of *Escherichia coli* to As^{III} concentrations up to 100 µM, through As^{III} methylation. Fernandez et al. (2008) investigated the microbial community of the methanogenic granular sludge from the same wastewater treatment plant using fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and cloning. Their study shows that *Methanospirillum*-like methanogen, *Methanosarcina*, and *Methanosaeta concilii* were present in the sludge. Thus, the ability of methanogenic granular sludge to gain tolerance to As^{III} could potentially be explained by the intrinsic capability of

the methanogens to express the required genes, such as *Ars* operon or *MaarsM* (found in *Methanosarcina*), in response to exposure to high As^{III} concentrations.

4. Conclusions

The potential of methanogenic granular sludge to increase their tolerance to As toxicity after an acclimation period is a novel finding, not previously reported in the literature. It demonstrates that well-adapted methanogenic sludge can be used for anaerobic digestion or even anaerobic wastewater treatment plants exposed to high As concentrations, such as effluents from mine drainage and semiconductor industries. The methanogenic activity of a granular sludge which was not previously exposed to As^{III} was greatly inhibited by the addition of As^{III}, as indicated by the very low IC₅₀ values determined for acetoclastic and hydrogenotrophic methanogenesis, of 19 and 90 µM, respectively. However, the methanogenic community showed a remarkable capacity to adapt to As^{III} inhibition after an exposure period in which the granular sludge was in contact with approximately 0.92 mM As^{III} for 160 days. After this exposure, the IC₅₀ of As^{III} toward the acetoclastic and hydrogenotrophic methanogens increased to 910 and 1100 µM, respectively. These results can be explained by either the ability of existing methanogens to develop resistance to As toxicity or the enrichment of methanogens with greater As tolerance. In practice, a prior acclimation period for methanogens to As may be required in treatment plants treating wastewater with relatively high As levels.

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Fig. 1. Methane (CH₄) production in the experiments assessing the toxicity of varying concentrations of arsenite (As^{III}) (expressed as μM) on: **(A)** acetoclastic methanogens from the unexposed granular sludge: (□) 0; (x) 14; (◇) 20; (■) 39; (△) 82; (+) 111; (—) 152; and (○) 213; **(B)** hydrogenotrophic methanogens from the unexposed granular sludge: (□) 0, (x) 39, (◇) 207, (■) 777, (△) 1343, and (+) 1861. The horizontal dotted black lines represent the maximum theoretical concentration of CH₄ that can be produced. The error bars represents the standard deviation of duplicates assays, or quadruplicates in the case of the controls.

Fig. 2. Arsenite (As^{III}) toxicity to methanogens. Normalized maximum specific acetoclastic (**A**) and hydrogenotrophic (**B**) methanogenic activity of the unexposed, original granular sludge, as a function of the initial concentration of soluble As^{III} . The 50% inhibition concentration (IC_{50}) values are shown in the corresponding graph. The error bars represent the standard deviation of duplicate assays, or quadruplicate in the case of the controls.

Fig. 3.

Adaptation of methanogens to arsenic toxicity. Normalized maximum specific acetoclastic- (A) and hydrogenotrophic (B) methanogenic activity of the granular sludge exposed to 0.92 mM arsenite (As^{III}) (due to arsenate (As^V)-reduction) for 160 d in a continuous-flow bioreactor fed with 4.4 mM ethanol and 1.02 mM As^V as a function of the initial concentration of soluble As^{III} . The 50% inhibition concentration (IC_{50}) values are shown in the corresponding graph. The error bars represents the standard deviation of duplicates assays, or quadruplicates in the case of the controls.

Table 1

Bioreactor measured operational parameters: Arsenate (As^{V}), arsenite (As^{III}), ethanol, acetate, methane (CH_4), volatile suspended solids (VSS), pH, and hydraulic retention time (HRT)

	Influent	Effluent
As^{V}	$1.02 \pm 0.06 \text{ mM}$	$0.09 \pm 0.06 \text{ mM}$
As^{III}	$0.03 \pm 0.07 \text{ mM}$	$0.92 \pm 0.08 \text{ mM}$
Ethanol	$4.40 \pm 0.30 \text{ mM}$	$0.02 \pm 0.06 \text{ mM}$
Acetate	$0.50 \pm 0.38 \text{ mM}$	$1.32 \pm 1.46 \text{ mM}^{\#}$
CH_4	---	$4.68 \pm 1.80 \text{ mmol CH}_4/\text{L}_{\text{liq}}$
VSS	13.3 g VSS/L	10.5 g VSS/L
pH	6.36 ± 0.10	6.47 ± 0.08
HRT	$24.23 \pm 0.49 \text{ h}$	

[#] Acetate concentration high standard deviation was due to a change in acetate utilization throughout the operation in the reactor. At the beginning of the operation, acetate accumulated at 3.31 mM and CH_4 production was negligible. After an initial period of adaptation that lasted 22 d, acetate utilization and CH_4 production increased progressively with the operational time (Rodriguez-Freire et al. 2015).