## Chapter 6

# Effects of adsorbed arsenic on HFO aggregation and bacterial adhesion to surfaces

## **6.1 Introduction**

Trace elements can be released to sediment porewaters upon reductive dissolution of iron (Fe) oxides. The rate and extent of Fe(III) oxide reductive dissolution is dependent on crystallinity, mineral solubility, surface area, and the presence of adsorbed ions (Bondietti et al. 1993; Roden and Zachara 1996; Larsen and Postma 2001; Bonneville et al. 2004; Pedersen et al. 2006). Adsorbed anions such as arsenate (As(V)) and arsenite (As(III)) can retard reductive dissolution by chemical reductants, but the effect of adsorbed As on microbial Fe(III) reduction has not been studied in detail.

In incubations with *Shewanella* species strain ANA-3, microbial Fe(III) reduction was enhanced by the presence of As(III) adsorbed onto the surface of hydrous ferric oxide (HFO), an amorphous Fe oxyhydroxide (see Chapter 5). *Shewanella* sp. strain ANA-3 wild-type (WT) was incubated with hydrous ferric oxide (HFO) pre-equilibrated with As(III) (HFO/As(III)) or As(V) (HFO/As(V)) or without As (HFO only). ANA-3  $\Delta arrA$ , a mutant unable to respire As(V), was incubated with HFO/As(V) as a control for As(V) reduction. Interestingly, the rates of Fe(III) reduction for the  $\Delta arrA$  mutant on HFO/As(V) and ANA-3 WT on HFO were similar, while the rates of ANA-3 WT grown on HFO/As(III) and HFO/As(V) were significantly higher. ANA-3 WT reduced the majority of adsorbed As(V) to As(III) within 40 hours of incubation. Once As(III) was the dominant species on the surface, the rates of Fe(III) reduction was comparable to the rates of ANA-3 WT incubated with HFO/As(III). The presence of adsorbed As(III) on HFO increased the rate of Fe(III) reduction. An explanation for this result was not immediately obvious, and could be due to HFO surface properties, aggregation, bacterial adhesion and/or genetic factors. The purpose of this study is to investigate the properties of HFO with adsorbed As(III) compared to HFO with adsorbed As(V) and HFO without adsorbed As in an attempt to explain increased rates of microbial Fe(III) reduction in the presence of adsorbed As(III). Surface properties were investigated by light microscopy and environmental scanning electron microscopy (ESEM). The rates of bacterial and chemical reduction was measured and the extent of bacterial adhesion onto the surface in the presence and absence of As was compared.

#### 6.2 Materials and Methods

All chemicals used were reagent grade and used without further purification. Solutions were prepared with 18 M $\Omega$ -cm deionized water (Milli-Q, Millipore) and stored in plastic containers that had been washed in 2-5% nitric acid. For bacterial incubations, all solutions were autoclaved before use with the exception of the bicarbonate buffer, which was filter-sterilized (0.2 µm pore size) and added to the autoclaved medium. The bacterial minimal growth medium (Appendix C) was buffered with 50 mM bicarbonate and had a total phosphate concentration of 50 µM and an ionic strength of 0.06 M.

#### 6.2.1 Arsenic-equilibrated HFO preparation

HFO was prepared by the drop-wise addition of 0.5 M NaOH to 0.067 M  $Fe(NO_3)_3$  until the solution stabilized at pH 8 (Schwertmann and Cornell 1991). The suspension was equilibrated for >4 h under constant stirring, adjusting any pH drift as necessary with 0.5 M NaOH. The HFO was then washed three times with sterile water and centrifuged. The HFO was not autoclaved after synthesis to avoid changes in mineralogy.

After the final wash, the HFO was resuspended in an As solution, and equilibrated overnight with constant stirring. For the ESEM experiments, 1 g of HFO was equilibrated in 200 mL of 0.01 M of Na<sub>2</sub>HAsO<sub>4</sub> (Sigma) or NaAsO<sub>2</sub> (Sigma) at pH 8.0. For all other experiments, 0.25 g of HFO was equilibrated in 40 mL of 0.015 M As(III) or As(V) solution. These conditions ensured that all available surface sites for As adsorption were saturated with As at pH 8. The HFO was washed once with sterile water to remove excess As and resuspended in bacterial minimal medium (pH 8) to a final slurry concentration of 3  $g_{HFO}/L$ . In the case of HFO without any adsorbed As, the solid was resuspended directly in bacterial medium after the initial washing and adjusted to pH 8. A subset of HFO, HFO/As(III) and HFO/As(V) solids were resuspended in water rather than bacterial medium as a control for the ESEM experiments.

#### 6.2.2 Incubations with ANA-3

40mL of each type of HFO slurry were transferred into a sterile tube with a screw-cap lid and placed in an anaerobic chamber (80%  $N_{2}$ , 15%  $CO_{2}$ , 5%  $H_{2}$ ).

Shewanella sp. strain ANA-3 and Shewanella sp. strain ANA-3 $\Delta arrA$ , a mutant with a deletion in *arrA*, were used as inocula. See Chapter 5 for more information on the bacterial strains. A summary of the experimental conditions is presented in Table 6.1. Each tube was inoculated with  $2 \times 10^4$  cells of ANA-3 WT or the  $\Delta arrA$  mutant, or left uninoculated. Each condition was measured in duplicate. All tubes were incubated at  $30^{\circ}$ C in the dark in the anaerobic chamber.

At each time point, dissolved Fe(II) and total Fe(II) concentrations were determined by the ferrozine method (Stookey 1970). A 500  $\mu$ L aliquot was filtered through a microcentrifuge filter with a 0.2  $\mu$ m pore size (Costar, nylon Spin-x) in the anaerobic chamber for dissolved Fe(II) analysis. Ten  $\mu$ L of slurry was added directly to 90  $\mu$ L of 1 M HCl in a 96-well plate for total Fe(II) determination. Time points were collected after 55 hours and 91 hours of incubation.

**Table 6.1.** Experimental conditions. Samples incubated in the time course are denoted "TC" and s samples run on ESEM are denoted "ESEM". Each condition was performed in duplicate.

	ANA-3 WT, minimal medium	<i>∆arrA</i> mutant, minimal medium	no bacteria, minimal medium	no bacteria, water only
HFO	TC, ESEM	TC, ESEM	TC	TC, ESEM
As(III)	TC, ESEM	TC, ESEM	TC	TC, ESEM
As(V)	TC, ESEM	TC, ESEM	TC	TC, ESEM

#### 6.2.3 Environmental Scanning Electron Microscopy (ESEM)

ESEM is a technique particularly suited for imaging surface structures of hydrated solids because the beam can be operated at relatively high pressures. The structure is preserved when the solid is frozen and the water slowly evaporated from the surface.

After 91 hours of incubation, all tubes were centrifuged and washed once with sterile deoxygenated water to remove the bacterial minimal medium. The solids were then resuspended in 20 mL of water, and transferred into a sterile Balch tubes. The tubes were capped before removal from the anaerobic chamber.

ESEM images were taken at the Jet Propulsion Laboratory (JPL) on a Phillips XD30 scanning electron microscope, operating in environmental mode. The samples were extracted from the Balch tubes with a syringe and placed on a sample holder. Excess water was quickly wicked away from the sample before placing the sample holder on a Peltier stage cooled to  $3.2-3.3^{\circ}$ C. Conditions inside the chamber were maintained at 4.5 torr (~75% relative humidity), and the vacuum was applied such that the water was slowly removed from the sample surface (~15 min). The beam was operated at 20 kV and 332 µA.

#### **6.2.4 Chemical Rates of Reduction**

The chemical rates of reduction of HFO, HFO/As(V), and HFO/As(III) were compared by measuring the Fe(II) produced by reaction with l(+)-ascorbic acid (EM Science) at pH 8. All solutions were deoxygenated and the experiment was carried out in an anaerobic chamber. 18 mL of 100 mM of ascorbic acid in HEPES buffer (pH 8) was added to a concentrated stock solution of HFO, HFO/As(V) or HFO/As(III) to a final slurry concentration of 0.3 g/L. Each condition was performed in duplicate. At each time point, 10  $\mu$ L of sample was added to 90  $\mu$ L of 1 M HCl in a 96-well plate for ferrozine analysis of total Fe(II).

#### 6.2.5 Bacterial adhesion assay

A Shewanella sp. strain ANA- $3\Delta arrA$  derivative expressing unstable green fluorescent protein (GFP) was created by transferring pTK4 (Teal et al. 2006) into ANA- $3\Delta arrA$  through conjugation and selection on 15 µg/mL tetracycline. pTK4 expresses a variant of GFP, GFP(AAV), containing a C-terminal oligopeptide extension that makes it susceptible to fast degradation by native intracellular proteases. The *gfp*(AAV) gene is driven by the *Escherichia coli rrnB* P1 promoter, which is growth regulated such that cells fluoresce when they are actively growing.

Cultures of ANA-3 $\Delta$ *arrA* carrying pTK4 were grown to midexponential phase on LB supplemented with tetracycline at 15 µg/mL. These cells were washed twice in LB with no antibiotic and resuspended in anaerobic minimal medium to remove tetracycline, which can form complexes with HFO and increase solubility (Gu and Karthikeyan 2005). Under anaerobic conditions, 100 µL of washed cells at ~5 x 10<sup>8</sup> cells/mL were mixed with 100µL of HFO, HFO/As(III), or HFO/As(V) slurry and stored at room temperature anaerobically in a glove box for 1 hr. Samples were resuspended by gentle pipetting and 5µL were placed on a slide with no cover slip and brought out of the glove box for microscopy. Images were obtained using a Zeiss Axioplan microscope with a 20x NEOFLUAR plan objective lens using light (exposure time 0.05 seconds) and fluorescent excitation (exposure time 10 seconds).

## **6.3 Results and Discussion**

#### 6.3.1 Biological and chemical rates of reduction

The rate and extent of Fe(II) production for ANA-3 WT (HFO, HFO/As(III), HFO/As(V)) and ANA-3  $\Delta arrA$  mutant (HFO/As(V)) is consistent with the more detailed time course in Chapter 5. The highest concentrations of Fe(II) are found in incubations with ANA-3 WT with HFO/As(V) and HFO/As(III). ANA-3 WT incubated with HFO only produces significantly less Fe(II) than ANA-3 WT incubated with HFO/As(III) and HFO/As(V). Iron(II) production in ANA-3  $\Delta arrA$  mutant incubations with HFO only and HFO/As(III) are similar. However, increased Fe(II) production is evident in the ANA-3  $\Delta arrA$  mutant HFO/As(III) incubations. Both ANA-3 WT and ANA-3  $\Delta arrA$  mutant incubations confirm the that the presence of adsorbed As(III) increases the rate of Fe(II) production (Table 6.2). Only a background amount of Fe(II) was measured in the control tubes over the course of the experiment, indicating that bacterial contamination was negligible.

**Table 6.2.** Total Fe(II) concentrations (mM) from the incubations used in the ESEM experiment after 55 and 91 hours of incubation. Tubes were incubated at  $30^{\circ}$ C in an anaerobic chamber. The slurry concentration in all tubes is 3 g/L and was buffered at pH 8. The data reported is an average of duplicate tubes.

Bacterial incubations							
	ANA-3 WT	ANA-3 ⊿arrA mutant					
Incubation							
time	55 h	91 h	55 h	91 h			
HFO	0.23	0.65	0.20	0.38			
As(III)/HFO	0.65	1.04	0.67	1.01			
As(V)/HFO	3.00	4.24	0.25	0.47			
Control tubes (no bacteria)							
	Bacterial medium		Water				
Incubation							
time	55 h	91 h	55 h	91 h			
HFO	0.15	0.15	0.14	0.15			
As(III)/HFO	0.14	0.14	0.13	0.14			
As(V)/HFO	0.12	0.16	0.11	0.16			

The amount and rate of Fe(II) production by ascorbic acid is similar for HFO, HFO/As(V), and HFO/As(III) (Figure 6.1). Therefore, the chemical rates of reduction are independent of adsorbed As.



**Figure 6.1.** Total Fe(II) production during the chemical reduction of HFO, As(V)/HFO and As(III)/HFO by 100 mM ascorbic acid at pH 8. The error bars indicate the standard deviation of duplicate samples.

#### 6.3.2 Effect of adsorbed As on HFO aggregation

Adsorbed As(III) made HFO more bioavailable for reduction, presumably by effecting HFO aggregation or surface properties. Slower settling was observed from HFO with adsorbed As(III) (Figure 6.2), suggesting smaller particle size or less efficient aggregation. When imaged by light microscopy after vigorously shaking the tubes, the particle sizes in all three cases appear to be the same size (~50  $\mu$ m) (Figure 6.5). However, imaging the supernatant after allowing the solid to settle for >24 hours, only the HFO/As(III) tube had traces of particles that would not settle (Figure 6.3). Although they may comprise only a small fraction of the total mass of HFO/As(III) in the tube, the presence of the small particles may explain the increased rates of Fe(III) reduction.



**Figure 6.2.** Tubes of HFO only (left tube), HFO/As(III) (middle tube), and HFO/As(V) (right tube) immediately after shaking (A), after 2 hours (B), and after >24 hours of settling (C). The HFO in all tubes is suspended in bacterial medium (no inoculation, pH 8, ionic strength = 0.01 M).

ESEM was used to observe the surface morphology of HFO in the presence and absence of adsorbed As(III) and As(V). All three solids had similar morphologies, but there were some subtle differences. HFO and HFO/As(V) had clusters of ball-shaped mounds (Figure 6.4A,B). These mounds were actually collections of smaller, nanoparticles of HFO, consistent with previous studies of HFO aggregates (Cornell and

Schwertmann 1996). HFO/As(III) had a slightly different surface structure, exhibiting smoother surfaces and fewer ball-shaped mounds (Figure 6.4C).



**Figure 6.3**. Light microscope image of the particles in the supernatant of HFO/As(III) that had been left undisturbed for >24 hours.

ESEM images were also taken after 91 hours of incubation with ANA-3 WT and  $\Delta arrA$  mutant. The morphology of all three solids (HFO, HFO/As(III), HFO/As(V)) after incubation with either ANA-3 WT or ANA-3 mutant was similar to the starting material (Figure 6.5 A,B,C). In the case of HFO/As(V) incubated with ANA-3 WT, most of the As(V) had been reduced to As(III) after 91 hours of incubation. Even though the majority of the As on the surface was As(III), the morphology was more similar to HFO/As(V). Therefore, the change in oxidation state from As(V) to As(III) did not significantly affect the morphology of the surface.



**Figure 6.4.** ESEM images of HFO only (A), HFO with As(V) (B) and HFO with As(III) (C) without bacteria. The solids were suspended in water. Samples for imaging were taken immediately after shaking the tubes.



**Figure 6.5.** ESEM images of HFO only (A), HFO with As(V) (B), and HFO with As(III) (C) after 91 hours of incubation with *Shewanella* sp. strain ANA-3 WT. Samples for imaging were taken immediately after shaking the tubes.



**Figure 6.6.** Adhesion of active *Shewanella* sp. strain ANA-3 $\Delta arrA$  onto HFO. Light microscope images of solid HFO (A), As(III)-sorbed HFO (B), and As(V)-sorbed HFO (C). Fluorescent images of GFP(AAV)-expressing ANA-3 $\Delta arrA$  in the same field of view corresponding to light images in the adjacent panel (D to F).

#### **6.3.3 Bacterial adhesion**

To understand the contribution of cell adhesion to the differences in iron reduction rates, adhesion assays were carried out using ANA-3  $\Delta arrA$  expressing a growth-regulated unstable GFP variant. Performance of this assay used a high cell number for a short amount of time to allow visualization to be completed in the absence of growth, which would have resulted in differences in total cell number. Use of the growth-regulated, unstable GFP allowed direct observation of cells that were metabolically active.

After incubating the cells with the HFO samples for 1 hour, actively growing fluorescent cells were found attached to particles under all three conditions (Figure 6.6). This suggests that the differences in surface properties due to adsorbed As(III) did not significantly affect cell adhesion.

#### 6.3.4 Conclusion

The presence of adsorbed As(III) on the surface of HFO significantly increased the rate of Fe(III) reduction by ANA-3 WT and ANA-3  $\Delta arrA$  mutant, but did not affect the rates of chemical reduction by ascorbic acid. The reduction of As(V) to As(III) by ANA-3 WT did not change the surface morphology, and there was no evidence for preferential bacterial adhesion to HFO/As(III). While most of the particles in all three cases are approximately the same size, the presence of colloidal HFO/As(III) particles may explain the increased rates of Fe(III) reduction. The colloidal particles may be about the same size or smaller than the bacterial cells, which may make the Fe(III) particles more bioavailable for reduction.

Colloidal stabilization of Fe(III) oxides by As(III) adsorption has not been reported in the literature and it would be valuable to investigate this property further (e.g., effects of pH on colloidal stability and the effect of varying As(III) concentration) to determine whether this observation may be relevant to natural sediments. Although the As concentrations used in this study are higher than observed in most natural environments, the presence of As(III)-stabilized Fe oxide colloids may have environmental implications, even at lower concentrations. In Haiwee Reservoir, As(III) is the dominant species in the Fe reduction zone. Even if only a small fraction of the solid phase is affected by As(III)-stabilized colloids, it may impact the overall rate of Fe reduction. The fraction of Fe and As in the sediments that would have to be mobilized in order to support the observed porewater concentrations is <2.5% (Hering and Kneebone 2001). Thus, even as a small fraction of the total solid phase, the presence of As(III)-stabilized colloids may affect the bioavailability of the Fe phase, and ultimately the mobilization of As.

## **6.4** Acknowledgements

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