Arsenic Release Batch Test for Sulfate-Reducing Bacteria (*Desulfurgbacter postgateii*)

An Undergraduate Honors Thesis

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ABSTRACT

The United States Environmental Protection Agency revised regulations related to the drinking water content of arsenic (As) in 2006 lowering the Maximum Contaminant Level (MCL) from 50 μg/L to10 μg/L. The concentration of arsenic in ground water is associated with iron-reducing, sulfate-reducing and methanogenic conditions. In highly reducing systems, the dominant pathway for arsenic release is poorly understood, but reflects contributions from sulfide minerals (e.g., pyrite) and acid-volatile monosulfides (AVS).

This research will evaluate arsenic release and sequestration processes under highly-reducing conditions, with the goal of identifying mechanisms and pathways responsible for arsenic release from aquifer solids under such conditions using a single culture of sulfate-reducing organisms (insert name). During the 4 week anaerobic incubation, sampling was conducted over 20 days. Samples were analyzed to determine dissolved concentrations of inorganic elements (e.g. As, S,), and concentration of anion such as acetate.

Based on the first 20 days data, the result shows that the iron started being reduced, but sulfate reduction still cannot be clearly observed due to the slow rate of anaerobic bacteria growth. This suggests, more time of sampling and analytical experiments are still needed to be conducted in the following two months.

ACKNOWLEDGEMENTS

I owe many thanks to a great many people who helped and taught me from when I started my research proposal to the end of the research. This research would not have been possible without the help of my advisor, undergraduate trainer and the partners in the lab.

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I express my special thanks to the USGS who gave me generous access to sediment element analysis data.

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CHAPTER 1: INTRODUCTION

Among the elements that comprise the earth's crust, metalloid arsenic (As) ranks 20th in abundance and it is present in more than 245 minerals (Mandal and Suzuki, 2002). Arsenic can be introduced into the environment by natural process, human activity and geochemical process. Also, industrial activities such as mining, smelting of ores, combustion of fossil fuels and use of arsenical pesticides and herbicides can be major sources of arsenic pollution (Wilson and Hawkins, 1978). As a result, there are large amounts of arsenic in seawater, freshwater and sediments. In addition, arsenic becomes a greater concern when it enters the food chain (Azizur et al, 2008).

In general, arsenic compounds in both organic form and inorganic forms have large impacts on the environment. Arsenic compounds can cause very serious and long-term effect on human health and nearby communities at a very low concentration which ranges from a few micrograms to milligrams per liter (Locker, 2012). When the arsenic polluted water or food becomes accessible, it could cause adverse effects on the surrounding environment and organisms. Arsenic is classified as a Class A human carcinogen through inhalation and ingestion (IRIS, 1998), with skin cancer and internal organ cancer such as bladder, kidney, lung and liver cancer being reported to result from arsenic intake.

The U.S. Geological Survey (USGS) has collected and analyzed arsenic data from 18,850 water wells in 595 counties across the United States in the past two decades (Welch et al, 2000). The result shows that the arsenic concentration in groundwater is generally high in the western part of the United States, parts of the

Midwest and the Northwest. Figure 1 shows the occurrence of arsenic within the US according to the US Geological Survey.

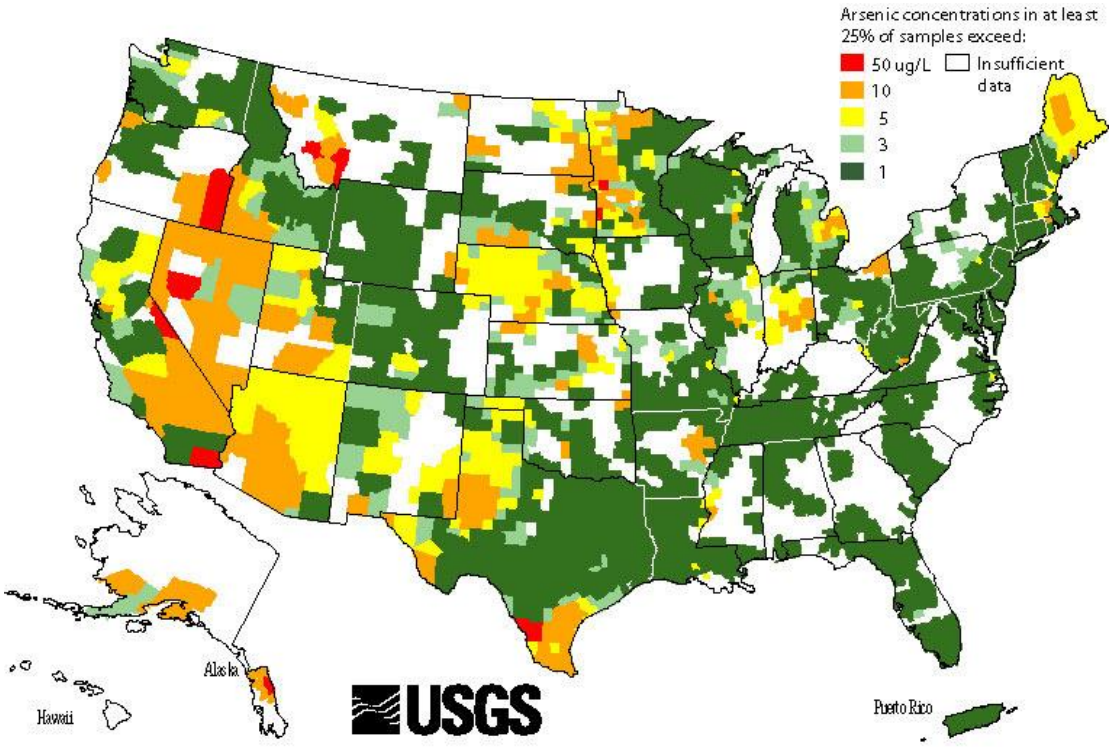

Figure 1: Distribution of arsenic concentration in groundwater (United States Geological Survey, 2011)

In Ohio, the major source of As comes from groundwater. The concentration of arsenic in the ground water higher than the MCL of 10 µg/L is a general phenomenon in Ohio (OhioEPA 2006). Iron (hydr) oxides exert a domineering control on the dissolved concentration and transport of arsenic within surface and subsurface environments (Herbel and Fendorf, 2005). In Ohio and its surrounding regions, the concentration of arsenic is highly related to reducing conditions in the groundwater aquifer. These are characterized by methanogenic conditions, iron reducing and sulfate reducing conditions. In general, the concentration of arsenic in groundwater is

not determined by the concentration of solid phase arsenic in aquifer solid, instead determined by the redox conditions of ground water and corresponding concentrations of other elements such as iron and sulfur. Aquifers in Ohio that are iron reducing or sulfate reducing result in arsenic concentration elevated in 19 % of the sampled wells (Thomas 2007). Under more highly reducing conditions found in methongenic aquifers, the percent of wells that exceed the MCL approaches 50% (OhioEPA 2006). Microbiological processes are known to be the primary drivers for controlling redox conditions in subsurface environments, but specific details are still lacking of the governing mechanisms.

Acid-volatile monosulfides (AVS) could be seen as an indicator of arsenic mobilization in many sediment studies. AVS exist in several forms which include mackinawite (FeS) and greigite (Fe_3S_4). Both mackinawite and greigite are formed by iron sulfidization under high DOM and Fe-rich environments (Wilkin 2006). AVS phases are described as As release source because they readily oxidize. Arsenic tends to substitute for S in pyrite, and due to this reason, pyrite is a potential stable As sink in reducing environments (Lowers 2007). Also, As(III) is a barrier of AVS transform to pyrite, so the S-Fe ratio is another indicator of higher As mobilization. With the increasing concentration of S(II) and decreasing concentration of reactive iron, As-S phase compounds will form such as orpiment As_2S_3 and realgar As_4S_4 (Bostick 2003). The objective of this research is to evaluate how AVS and pyrite control arsenic release under highly reducing conditions.

Based on previous study of the arsenic release under highly reducing condition, we know that arsenic release is controlled by sulfate reduction in liquid phase. So in this experiment, we used sulfate reducing bacteria and conducted the single-culture experiment to prove our hypothesis. The purpose of this research is to investigate the interaction between single sulfate-reducing bacterial culture and iron minerals, with the goal of evaluating arsenic release and sequestration processes with Fe-S mineral composition. By conducting pure culture experiments, my study will identify whether arsenic release is only related to sediment composition. However, the addition of iron (II) may react with amorphous Fe-S phases and result in further arsenic release.

Chapter 2 ： Methods

2.1 Overview

The research was designed in three types of samples in order to determine the microbiological activities effect that Desulfurbacter postgateii has on the arsenic release under highly reducing conditions. First, Desulfurbacter postgateii was cultured in the medium. When it reached its peak density, it was transferred to prepared media with different types of sediments. Then the sampling of inoculated media containing synthetic groundwater and aquifer sediments at different depths. All the analytical instrument was utilized to characterize liquid-phase sample were provided by Civil and Environmental Engineering Laboratory.

2.2 Preparation of Fe Oxide-Coated Sand

This step can be divided into two parts which were preparation of sand and coating the sand with Fe oxide. The sand used in this research was prepared following the procedures described in Herbel and Fendorf (2006). This entails repeatedly rinsing the sand with deionized water until the rinse water was clear. Once cleaned, the sand was air-dried until further use. Ferrihydrite-coated sand was prepared by follow the procedure described in Iron Oxides in the Laboratory (Schwertmann 1991). Forty grams of $Fe(NO_3)_3*9H_2O$ were dissolved in 500 mL DI water, and added to 330 mL 1M KOH. The pH value was adjusted between 7-8

to induce the formation of ferrihydrite. 100 grams of clean sand was subsequently added to the ferrihydrite solution. The bottle which contained the sand and solution was rotated at 100 rpm for three day for equilibrating. The solution was decanted and the coated sand was rinsed with DI water multiple times until the rinse water was transparent and not any ferrihydrite particles could be observed.

2.2 Loading Fe-coated sand with As

The arsenic content of the Fe-coated sand was prepared to mimic that for sediments from a methanogenic aquifer in northern Preble County, Ohio (Thomas et al., 2007) summarized in Table 1. Values are presented for four different depths (90 ft depth, 91 ft depth, 93 ft depth, and 95 ft depth) to get the average concentration of As, P and S in methanogenic aquifer sediment.

	90 ft	**91ft**	**93 ft**	**95 ft**	**Average**
As	7 ppm	8 ppm	10 ppm	9 ppm	9 ppm
P	345 ppm	380 ppm	418 ppm	381 ppm	380 ppm
S	0.12%				0.12g/100g

Table 1: Solid element analysis under methanogenic aquifer at northern Preble County, Ohio (Thomas et al., 2007)

In order to facilitate the experimental approach, however, it was decided to prepare the sand to contain 900 ppm of arsenic which is 100 times higher than the solid under natural environment. We assume that arsenic in solution is 100% adsorbed. I added 1.872 g of Na_3AsO_4, 0.870 g of Na_2HPO_4, 2.663 g of Na_2SO_4 and 1.260g of

$NaHCO_3$ to 1 L of DI water and mixed with 100 g of sand. The solution was allowed to equilibrate with the sand at 23 ℃ for 3 days on an orbital shaker at 250 rpm. Analyses of the solution after equilibration with the solids showed that the about 60% of As remaining in the solution and only 40% adsorbed by sand. So the final concentration of arsenic on iron coated sand was 360 ppm.

2.3 Synthetic Groundwater

The sampling was conducted with the assistance of the USGS in mid-August 2012, and the groundwater came from 90 ft depth from a well in northern Preble County, Ohio with known arsenic contamination. Solid analysis was conducted and reported by Mary Ann Tomas scientist from USGS in 2008. After the first day of sampling, the groundwater sample's geochemistry was characterized, with wlement concentrations which affect growth of bacteria listed in the table 2.

Ba	Ca	Fe	K	Mg	Mn	Na	P	S	Si	Ba
0.01	67.07	**2.68**	1.38	31.17	0.01	15.68	0.16	0.22	12.55	0.01

Table 2: Composition of the natural groundwater in 90 depth in northern Preble County, Ohio (All unit is in mg/L)

When the stock solution was prepared, each single element stock solution was made with 100 times concentration higher than the natural environmental groundwater. One liter of solution was made by combining the K, P, S, Si, NH_4, yeast and acetate stock solution together, deoxygenized and autoclaved. The pH was adjusted to 7 and add 876 mL of DI water. Before autoclaving, the solution was

purged for 15 minutes. Ca, Mg, Vitamin and Mineral solutions were filtered by 0.25μm filter prior to being added to the autoclaved solution.

	K	P	S	Si	NH4	yeast	acetate	HCO3	H2O	pH Adjust	Ca	Mg	vitamin	mineral
mg/L	1.38	0.16	0.22	12.55		1%				1N HCl	67.07	31.17		
mmol/L	0.04	0.01	0.01	0.45	0.10			3.37			1.68	1.30		
Stock (mg/L)	35.44	5.07	6.81	44.84	95.55	10		337			167.67	129.86		
Synthetic (mL/L)	1	1	1	10	1	10	50	10	876		10	10	10	10

Table 3: Composition of the stock solution and stock solution/ synthetic groundwater

2.4 Pure Culture Cultivation- *Desulfobacter postgatei*

The methods to culture those organisms followed Bryant 1972. This method utilizes test tubes sealed with rubber stoppers. Rubber is suitable as culture tube enclosures. Gasses that enter the cannula are scrubbed free of oxygen by passage through heated copper fillings. A gas mixture of 80% H_2 and 20% CO_2 was used for culture (Bryant, M. P., 1972)

The *Desulfobacter postgatei* media was prepared by following recipe in Appendix: Table A1. Once media was prepared, 1 mL of D. postgatei in media were added to 100 mL of FW media.

Cultured cell population was measured at a wavelength of 600 nm.

2.5 Sampling and analytical procedure

The microcosm studies were conducted using nine 125 bottles with 100 mL of synthetic groundwater and 5 grams of sediment. Sampling was scheduled on day 1, 3, 7, 10, 14 and 21. Each time sampling, 8.5 mL of samples were taken from each bottle.

Iron concentration, was determined by ratio of iron II and iron total. The concentration of both iron II and iron total was determined by using the Ferrozine Method (Ferrozine in HEPES) which comes from the method in Stookey (1970). Sulfide concentration was determined by using the Hach kit, the procedure followed the Hach kit manual. Both sulfate and acetate concentration were determined using a sample in 1:20 dilution (sample: DI water) which was measured on ICS-2100 ion

chromatography. The concentration of As were determined by analyzing the filtered samples which were filtered by a disposable cartridge. Disposable cartridges were packed with 2.5 g of selective aluminosilicate adsorbent for the separation of arsenate and arsenite in water sample. Arsenic speciation was performed by passing approximately 50 mL of water through the cartridges at a flow rate of 60±30 mL per min using a 50-mL syringe. As(V) in the water samples was removed by the cartridges and As(III) remained in the filtrates.(Meng and Wang, 1997). Then the arsenic concentration was determined using Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES) and Varian Atomic Absorption Spectrometer after acidification with 5% concentrated nitrate acid. Analyses of specific elements were also conducted using ICP-AES.

Chapter 3: Result and Discussion

3.1 Culturing Cells in medium

As the Figure 1 shows, the relationship between OD 600 and carbon source was reversed. During the four-day culturing, the concentration of acetate, which was the carbon source for the *Desulfobacter postgatei* decreased from 5500 mg/ L to 3000 mg/L. On the other hand, the *Desulfobacter postgatei'*s growth rate was comparatively fast. OD 600 value start from 0.006 at day 0 and it reached its peak density at day 4.

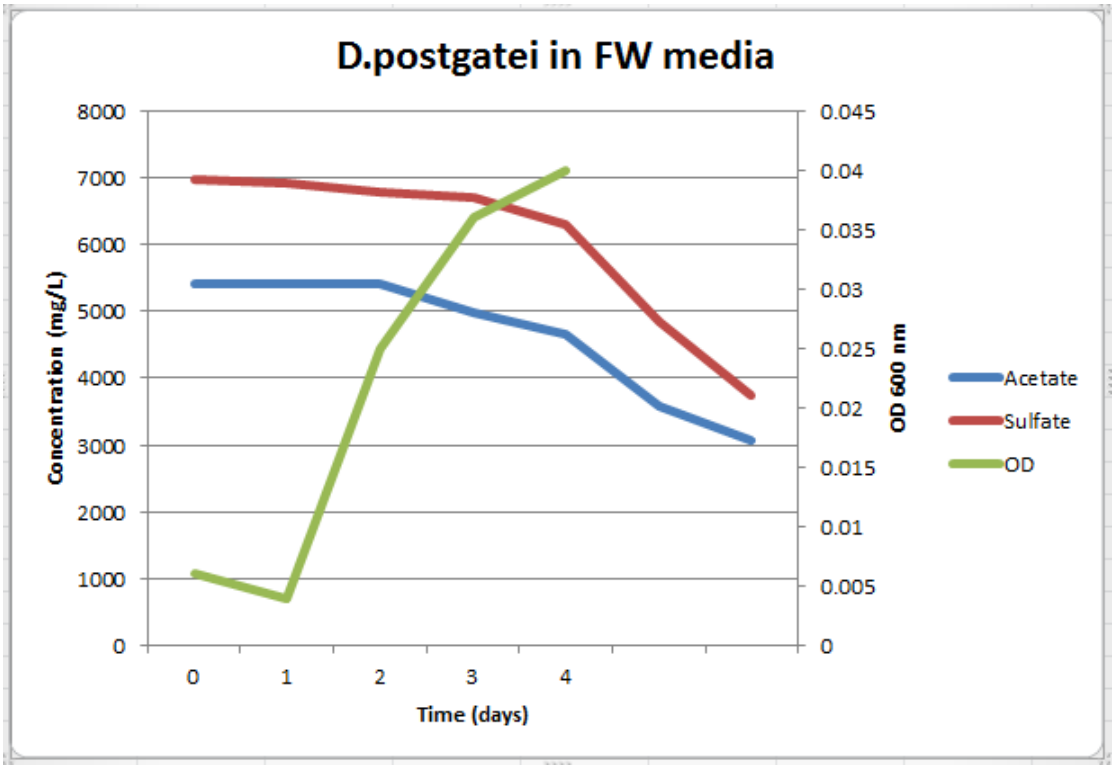

Figure 1: Growth curve of *Desulfobacter postgatei* and reduction of acetate & sulfate (Evert 2012)

3.2 Sampling of Sediments, Sand with synthetic groundwater

The following three figures show the change of total iron and iron II during first 20 days incubation in three types of sediments and sand. The sediments come from 80 - 90ft depth at northern Preble County, Ohio. Sand was coated with iron which is mentioned in previous.

3.2.1 Iron Reduction

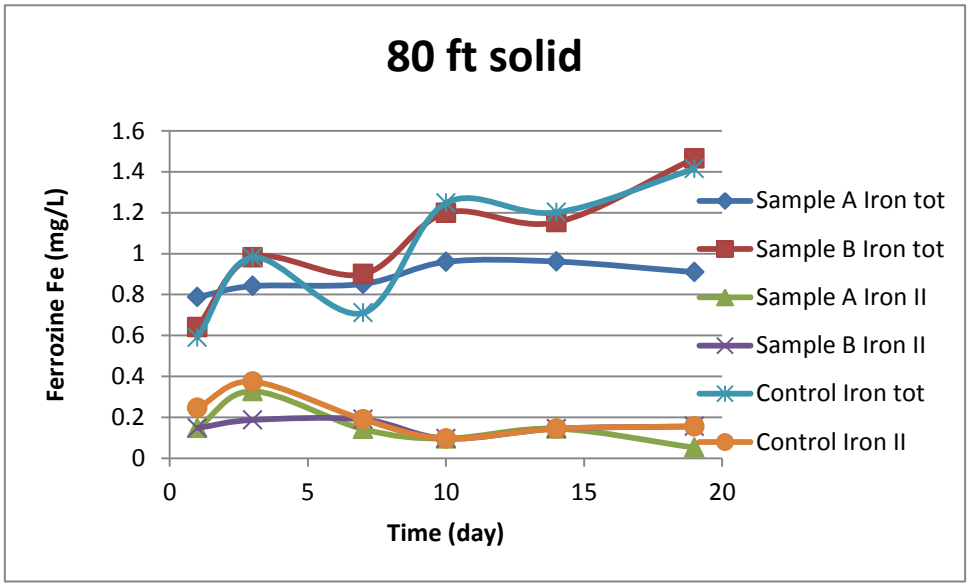

Figure 1: Iron concentration for 80 ft depth sediment

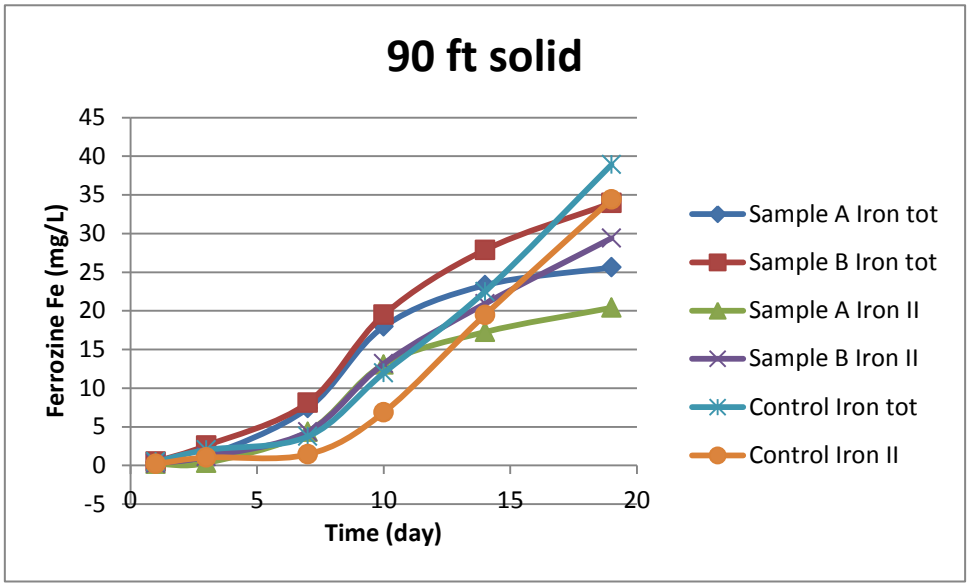

Figure 2: Iron concentration for 90 ft depth sediment

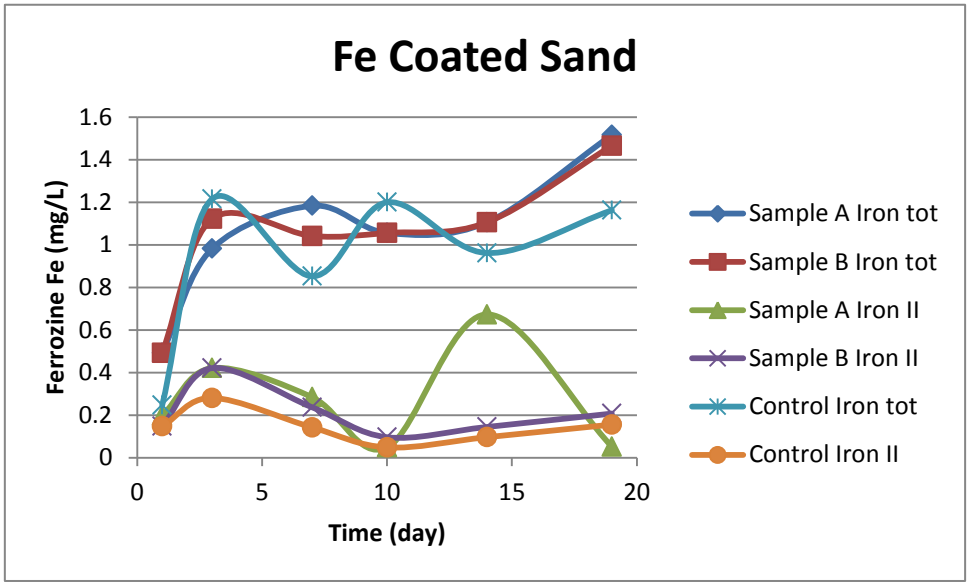

Figure 3: Iron concentration for iron coated sand

During the 20 days incubation, iron release was observed from all three types of sediments. All samples showed an increasing trend of iron. The release rate happened in 90ft Depth Sediment much faster compare to other two types of sediment. The concentration of iron total in 90ft depth reached 33 mg/L on average at day 20. The iron total concentration in 80ft depth and iron coated sand remain under 2 mg/ L during the entire study, but still increased, albeit gradually.

The results show that the iron released from the 90ft depth sediment sample was almost completely reduced to iron II. On the other hand, there was less iron reduction happened in 80ft depth sediment and iron coated sand.

Since the same pure culture was added to all of the samples, much more iron was released in the 90 ft depth sediment samples. The iron concentration in control sample even increase at higher rate than biotic samples. This might imply that 90 ft depth sediment samples were contaminated by a kind of iron reducing bacteria which exist in the sediment. In Appendix 1A, we can see the acetate, carbon source of the bacteria, did not decrease meaning that bacteria did not consume the acetate. While unlikely, it is possible that the growth of iron reducing bacteria consumed the organic matter in the sediment as carbon source instead of acetate. There was less iron release in the 80 ft depth sediment samples and iron coated sand might due to chemical reaction, because the concentration of iron in those samples did not increase after three days.

3.2.2 Sulfate Reduction

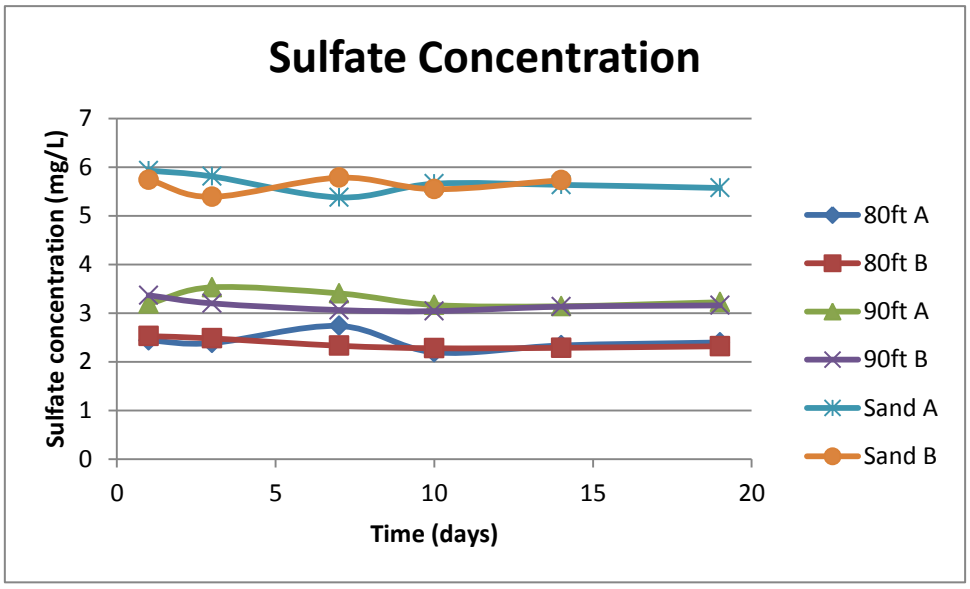

Figure 4: Sulfate concentration for all of biotic samples

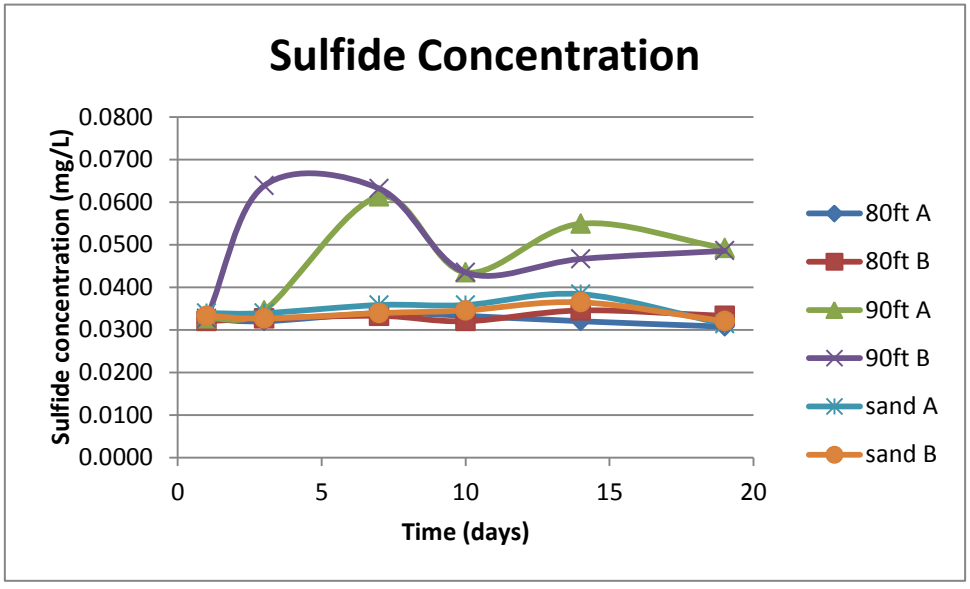

Figure 5: Sulfide concentration for all of biotic samples

As the figures above show the concentrations of both sulfate and sulfide did not have obvious changes during first 20 days' incubation. This fits results described in M. Barlett and K. Zhuang (2012), which mentioned that sulfate reducing bacteria grow slowly and don't reach dominance until 30 and 40 days. The previous studies show

that growth of Sulfate Reducing Bacteria (SRB) was slow but steady. So there is no significant change in sulfate and sulfide concentration could be observed in the first 20 days. According to Barlett and Zhuang, reduction of Fe (III) does not affect the growth of SRB. So that is also the reason why the sulfate was not been reduced in 90ft depth sediment a lot, even a large amount of iron was reduced in 90ft depth sediment.

For the remainder of the incubation, I anticipate the concentration of sulfate to be reduced to half of the current amount between day 40- day 60.

3.2.3 Arsenic Release

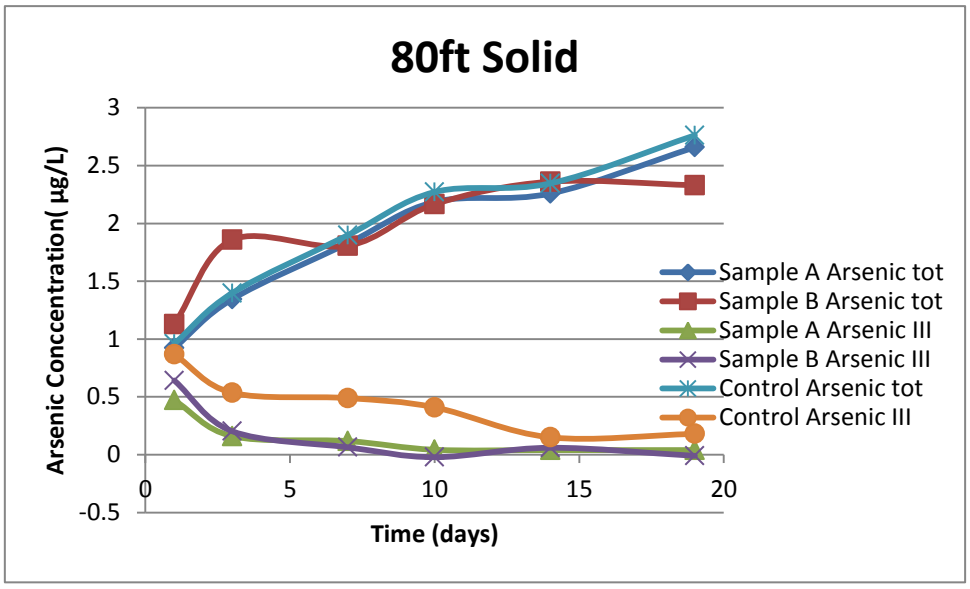

Figure 6: Arsenic concentration for 80 ft depth sediment

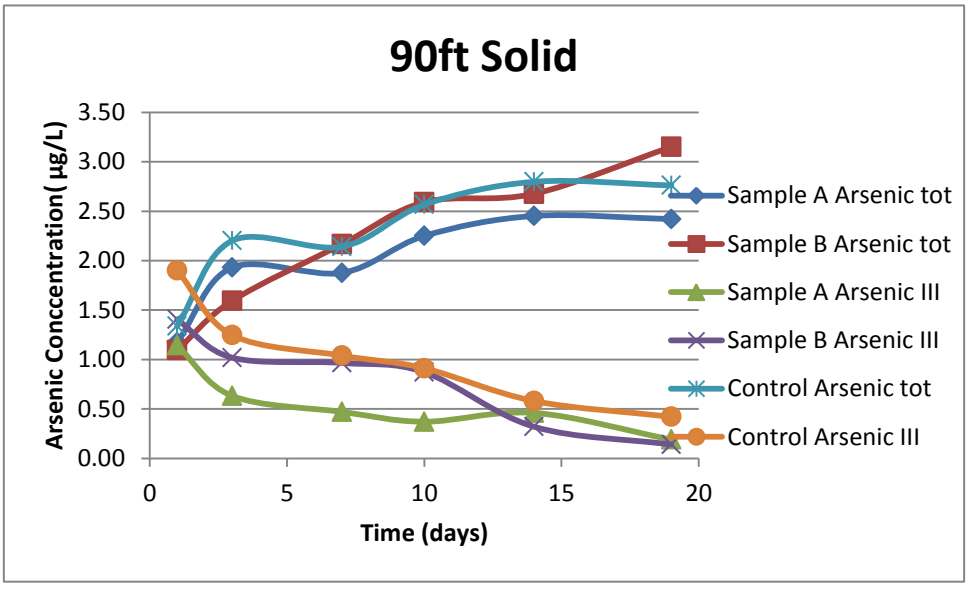

Figure 7: Arsenic concentration for 90 ft depth sediment

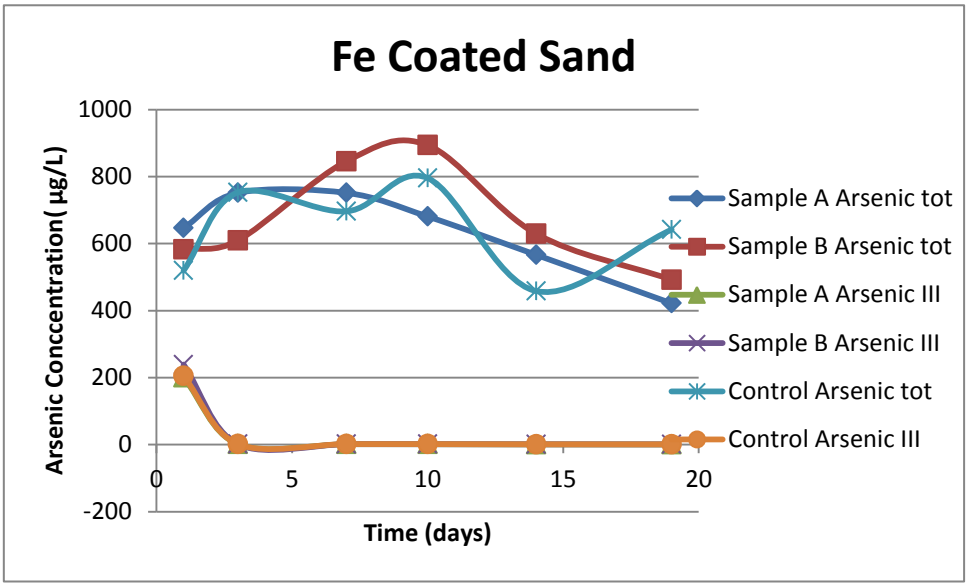

Figure 8: Arsenic concentration for iron coated sand

In all three types of sediment, iron coated sand released the highest concentration of arsenic (total) compared to other types of sediments which come from natural environment. Likely, this reflects the amount of arsenic equilibrated with iron coated sand was 100 times higher than the sediment under natural environmental which is 900 ppm. It also reflects that preparation of the iron oxide coated sand entailed drying the sample without rinsing thereby leading to the formation of arsenic salts on the media which were readily dissolved upon equilibrating the solids with a solution. Although the concentration of arsenic in iron coated sand was the highest, the trend was still unclear during the first 20 days incubation.

The concentrations of arsenic (total) in the 80ft and 90ft depth sediments were much lower than the concentration arsenic (total) in iron coated sand. But the figures above indicate that the trend of the arsenic keeps increasing.

The arsenic III concentrations in all of samples were under 1 µg/L and during the most time of the incubation, the concentrations of arsenic III was under 0.5µg/L which is under the detection limit. According to arsenic release mechanism, arsenic V

should be reduced to arsenic III under highly reducing condition. Comparing to all types of samples and figures above, the arsenic III concentration did not grow with the increasing concentration of total arsenic. It seems possible that Desulfobacter postgatei still have not grown in all nine samples based on the amount of arsenic release we observed. Twenty days sampling need to be conducted to see if there will have an increasing arsenic III in samples.

4 Conclusion

Arsenic as a carcinogenic element, still exist concern when its concentration over the MCL in groundwater which has potential to be used by resident. Arsenic biogeochemistry, especially in chemically natural groundwater systems are still not greatly understand.

Through this research, insight into arsenic release due to sulfate reducing microbes was gained. This research shows that Desulfobacter postgatei did cause arsenic release under sulfate reducing aquifers in northern Preble County, Ohio.

In all of the samples, the arsenic started to release accompany with iron release. But the sulfate reducing phase still cannot be observed in the first 20 days because of the microbial activity reaction rate.

Further research and sampling will need to be conducted to see if the sulfate will start being reduced after 30-40 days incubation. Also, the concentration of arsenic V and arsenic III will be measured to see if it increases to a higher level because of sulfate reduction.

APPENDIX: Figures and Tables

Table A1: Comparison of acetate concentrations change in all samples

Acetate	BA	BB	BC	CA	CB	CC	SA	SB	SC
day 1	0.431	0.4365	0.4211	0.4393	0.4492	0.451	0.4601	0.4474	0.4546
day 3	0.4575	0.4566	0.4609	0.4551	0.4818	0.4511	0.4599	n.a.	0.4692
day 7	0.4614	0.4594	0.4695	0.4634	0.4747	0.4554	0.4427	0.4538	0.4644
day 10	0.4244	0.4388	0.4606	0.4589	0.4541	0.4654	0.4574	0.4595	0.4528
day 14	0.4542	0.4571	0.4455	0.4602	0.4574	0.4641	0.4647	0.4432	0.4614
day 19	0.4739	0.457	0.4576	0.4687	0.4726	0.4535	0.462	0.4551	0.4527

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Solution A is boiled for a few minutes, cooled to room temperature, gassed with 80% N_2 + 20% CO_2 gas mixture to reach a pH below 6, then autoclaved anaerobically under the same gas mixture. Solutions B, D, E and F are autoclaved separately under nitrogen. Solution C is filter-sterilized and flushed with 80% N_2 + 20% CO_2 to remove dissolved oxygen. Solution B to F are added to the sterile, cooled solution A in the sequence as indicated. The complete medium is distributed anaerobically under 80% N_2 + 20% CO_2 into appropriate vessels. Final pH of the medium is 7.1 - 7.4. Addition of 10 - 20 mg sodium dithionite per liter (e.g. from 5% (w/v) solution, freshly prepared under N_2 and filter-sterilized) may stimulate growth at the beginning. For transfers use 5 - 10% inoculum.

Solution A		
Distilled water	870.0	ml
Resazurin	1.0	mg
$CaCl_2 \times 2\ H_2O$	0.15	g
KCl	0.5	g
$MgCl_2 \times 6\ H_2O$	1.3	g
NaCl	7.0	g
NH_4Cl	0.3	g
KH_2PO_4	0.2	g
Na_2SO_4	3.0	g

Solution B		
Trace element solution SL-10	1.0	ml
Solution C		
Distilled water	100.0	ml
$NaHCO_3$	5.0	g
Solution D		
Distilled water	10.0	ml
Na-acetate x 3 H_2O	2.5	g
Solution E		
Vitamin solution	10.0	ml
Solution F		
Distilled water	10.0	ml
Na_2S x 9 H_2O	0.4	g

Trace element solution SL-10		
Distilled water	990.0	ml
Na_2MoO_4 x 2 H_2O	36.0	mg
$NiCl_2$ x 6 H_2O	24.0	mg
$CuCl_2$ x 2 H_2O	2.0	mg
$CoCl_2$ x 6 H_2O	190.0	mg
H_3BO_3	6.0	mg
$MnCl_2$ x 4 H_2O	100.0	mg
$ZnCl_2$	70.0	mg
$FeCl_2$ x 4 H_2O	1.5	g
HCl (25%; 7.7 M)	10.0	ml
First dissolve $FeCl_2$ in the HCl, then dilute in water, add and dissolve the other salts. Finally make up to 1000.0 ml.		
Vitamin solution		
Distilled water	1.0	l
Lipoic acid	5.0	mg
p-Aminobenzoic acid	5.0	mg

Vitamin B12	0.1	mg
D-Ca-pantothenate	5.0	mg
Nicotinic acid	5.0	mg
Riboflavin	5.0	mg
Thiamine-HCl x 2 H_2O	5.0	mg
Pyridoxine-HCl	10.0	mg
Folic acid	2.0	mg
Biotin	2.0	mg

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