MOLECULAR DETERMINATION OF ARSENATE RESPIRING BACTERIA IN ESTUARINE SEDIMENTS AND GROUNDWATER

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ABSTRACT

Arsenic is a well-known metalloid and carcinogen with both natural and anthropogenic origins. Arsenic occurs naturally in the environment as two primary inorganic forms: arsenate [As(V)] and arsenite [As(III)]. Microbes mediate the presence and quantity of these two arsenic species through oxidation and reduction processes. Dissimilatory As(V) respiring bacteria (DARB) contain As(V) respiratory reductase (*arr*A) genes and reduce As(V) to As(III), therefore enhancing the solubility of arsenic. Thus, the detection of the genes encoding As(V) respiratory reductases could interpret the presence and involvement of DARB in arsenic mobilization. With water samples collected from two NC wells, and sediment collected from Shem Creek, South Carolina the presence of DARB using *arr*A genes as genetic markers were determined and the As(V) reduction activities were monitored via enrichment culture techniques. Based on molecular analysis, the DARB in the NC wells are closely related to *Geobacter uraniumreducens* while those in Shem Creek sediments are related to *Desulfosporosinus* Y5. Anaerobic enrichment cultures were established with NC well water and Shem Creek sediment to determine the rate of As(V) reduction. The rates for all of the sites involved showed that the DARB could have a significant impact on the arsenic levels in the environment. Thus, this study demonstrates the presence and diversity of DARB in these environments and their contributions to the arsenic contamination in NC residential drinking water wells, and in the Shem Creek estuary.

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DEDICATION

I would like to dedicate this thesis to my Granny Shirley who has always taught me that education is something that no one can take away from you. She has supported me throughout everything I have ever done and continues to be my model for the person I want to be when I “grow up.”

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CHAPTER 1: INTRODUCTION TO ARSENIC AND THE PROCESSES INVOLVED IN THE REDUCTION OF As(V) TO As(III)

INTRODUCTION

Relevance and Toxicity of Arsenic

Arsenic is the 20th most abundant element in the earth's crust with an occurrence of about 0.0002%. The main source of natural arsenic in the environment is igneous rock formed from volcanic activity. Other natural sources include weathering, fire (Liu et al., 2004), fossil fuels, marine sedimentary rocks, and various minerals (Stolz and Oremland, 1999). Mean concentrations of arsenic from naturally occurring sources in various systems include approximately 2.6 ppb in seawater and 0.4 ppb in freshwater (Mukhopadhyay et al., 2002). Along with natural sources, arsenic also has various anthropogenic inputs including acid mine drainage, the combustion of coal, pigment production, wood treatments and pesticides. In addition, organic arsenic compounds are used to prevent disease in poultry and swine farming (Oremland and Stolz, 2003).

In the environment inorganic arsenic occurs naturally in four major oxidation states as As^{+5}, As^{+3}, As^{0}, and As^{-3}. The most predominant forms of these species are arsenate [$HAsO_4^{2-}$, As(+5), As(V)] and arsenite [$H_2AsO_3^{-}$, As(+3), As(III)], which are highly soluble in water. As(V) is mostly found in oxic environments and is easily bound to other metals such as iron, manganese, and aluminum, while As(III) is found in anoxic systems in soluble forms (Oremland and Stolz, 2003). Although both are toxic, As(III) is one-hundred times more toxic than As(V) due to the fact that it binds with sulfur atoms which inhibits the functions of various enzymes, including the ones involved in respiration. As(V), as a phosphate analog, is primarily toxic because it affects the main energy producing metabolism for many organisms including humans, by impairing oxidative phosphorylation (National Research Council, 1999).

Arsenic toxicity has been heavily investigated since arsenic trioxide (As_2O_3), an As(III) derivative, was used in industry over the past decade. Arsenic trioxide was primarily used in copper smelting and in treating lumber, as well as a pesticide and an herbicide. It is by far one of the most abundant forms of arsenic on the planet. It was once used as a murder weapon, termed “inheritance powder,” before early forensics developed testing for arsenic levels in tissue (Nriagu, 2001; Bode and Dong, 2002). This deadly compound was also used in small doses, in various areas of medicine, to treat leukemia and protozoan disease. Some arsenic trioxide derivatives such as, salvarsan and its successor neosalvarsan, were two early antimicrobial agents used in the treatment of syphilis until being replaced by penicillin (Lenihan, 1988; Nriagu, 2001). In veterinary medicine, arsenic trioxide is used as a treatment for Chronic Fatigue Syndrome (CFS) in horses, heartworms in dogs, and blackhead disease in turkeys and chickens (Committee on Medical and Biological Effects of Environmental Pollutants, 1977).

Arsenic contamination in drinking water is a serious issue that is getting more and more attention everyday. The most well known place for arsenic contamination in drinking water is Bangladesh, where people are being slowly poisoned by arsenic on a daily basis. This poisoning develops into arsenicosis which is the name for a vast array of symptoms caused by long term arsenic poisoning. These symptoms include changes in the color of a person’s skin, keratosis of the palms and soles of the feet, and many types of cancer (Shrestha et al., 2003). In addition, a number of people in Taiwan developed a condition known as “black foot disease” where the blood vessels in the extremities become diseased and gangrene sets in. The reason for these high arsenic

levels in Bangladesh and in other parts of the world is most likely due to natural geological processes (Nickson et al., 1998) but there is also a theory that the contamination is from fertilizers and chemicals used for agriculture (Acharyya, 1997).

Arsenic contamination is also becoming a major problem in the United States as well as in other parts of the world. The governments of many countries have set limits for the amount of arsenic that can be present in drinking water, and this limit varies from country to country. In Bangladesh, for instance, the upper limit is 50 ppb, while countries such as Canada maintain a legal limit of 25 ppb. Until 2002, the United States held the standard of 50 ppb, but the EPA then revised the limit to the much lower 10 ppb (Silver and Phung, 2005). Although arsenic contamination is present all over the United States, some places such as Michigan and Wisconsin (Mukhopadhyay et al., 2002) and even locally here in North Carolina are having major problems with high arsenic concentrations in residential wells (Pippen, 2005). South Carolina's contamination also occurs in estuarine systems such as Shem Creek, which is a suburban watershed located in Mount Pleasant, South Carolina (Sanger et al., 1999a).

Fate and Transport of Arsenic

The fate and transport of arsenic in sediments and water can be determined by chemical oxidation and reduction. As mentioned earlier, As(V) and As(III) are the predominant forms of arsenic found in the environment. As(V) is the most prevalent of the forms in oxic sediments where the oxidation potential is the greatest, while As(III) tends to be found in anoxic environments where the reducing potential is greater (Mandal and Suzuki, 2002). However, pH also has a major effect on the speciation of these two forms. As(V) exists as H_3AsO^{4-} at pH values of 2-7 but at pHs of 7-12 it exists

primarily as $HAsO_4^{2-}$. As(III), however, is typically found below pH 9.3 in the form of $H_3AsO_3^{\circ}$ (Inskeep et al., 2002). Arsenic complexes also change based on the oxidation/reduction potential of the environment. For example, at the sediment-water interface of an estuary, where the sediments are oxidized, there is a constant supply of Fe(III) and Mn(IV). Due to the fact that As(V) primarily exists in deprotonated forms at neutral pH, similarly to phosphate, it readily binds to the positively charged Fe(III) and Mn(IV) hydroxides. But, deeper in the sediment, under highly reducing conditions, Fe(III) and Mn(IV) become depleted and the As(V) desorbs from these complexes and goes back into the dissolved phase, where it is then reduced to As(III), and binds to the reduced sulfates, as precipitated arsenic-sulfides, also known as arsenopyrite (As_2S_3). These naturally forming arsenic complexes are also affected by pH. At a pH of around 8 As(V) favors sorption to the Fe(III) and Mn(IV) hydroxide complexes, while as the pH decreases the sorption of As(III) to sulfides increases (Inskeep et al., 2002). Another important factor in arsenic speciation is the presence of nitrate. Nitrate promotes the oxidizing of As(III) to As(V) and Fe(II) to Fe(III) (Senn and Hemond, 2002) and denitrification, actually slows down As(V) reduction (Dowdle et al., 1996; Hoeft et al., 2002). Therefore, various metals and redox conditions can change the fate and transport of arsenic in the environment.

The microbial community plays a major role in the release and speciation of arsenic. They do this by either oxidation or reduction processes involving the inorganic forms of arsenic: As(V) and As(III). As(III) oxidation, the conversion of As(III) to As(V), can be either used as a method of detoxification or as a bioenergetic process. As a detoxification mechanism, the oxidation of As(III) to As(V) allows the bacteria to survive

in the presence of arsenic, by making it less toxic. The oxidation is mediated by As(III) oxidase (*aso* or *aox*) enzyme, located on the periplasmic membrane. The *aso*/*aox* enzyme contains two subunits: a larger Mo-pterin one and a smaller Rieske [2Fe-2S] one. Two electrons from the oxidation of As(III) to As(V) transfer to the molybdenum in the large subunit (Mukhopadhyay et al., 2002). The bioenergetic oxidation process uses the As(III) oxidases, coupled to CO_2 fixation, to generate energy (-256kJ/Rx) for chemolithotrophic living for the cell's growth (Santini et al., 2000; Santini and vanden Hoven, 2004).

As stated earlier, there are also processes that involve the reduction of arsenic compounds. Many microbes utilize a detoxification mechanism, while others use a respiratory pathway to reduce As(V) to As(III). The detoxification process occurs in the cytoplasm of the bacteria where As(V) is bound by a cysteine residue of the ArsC protein and reduced to As(III) with electrons donated from the reduced glutathione. The As(III) is then excreted from the cytoplasm by the ATP-dependent *ars* transporter (ArsB) (Oremland and Stolz, 2003). The gene encoding As(V) reduction is the *ars*C gene, which is present in most bacterial genomes, and the other transporter genes are located on the *ars* operon with the *ars*C gene (Silver and Phung, 2005). The other process utilized to reduce As(V) to As(III) is an anaerobic respiratory mechanism. The bacteria that perform this As(V) respiration are classified as dissimilatory As(V) reducing bacteria (DARB). DARB are both taxonomically and metabolically diverse (Figure 1), such as their ability to utilize various carbon substrates including lactate, benzoate, succinate, and formate (Liu et al., 2004), and their capability of reducing iron, selenate, sulfate, and nitrate. The DARB can mobilize arsenic in three different pathways including 1) the

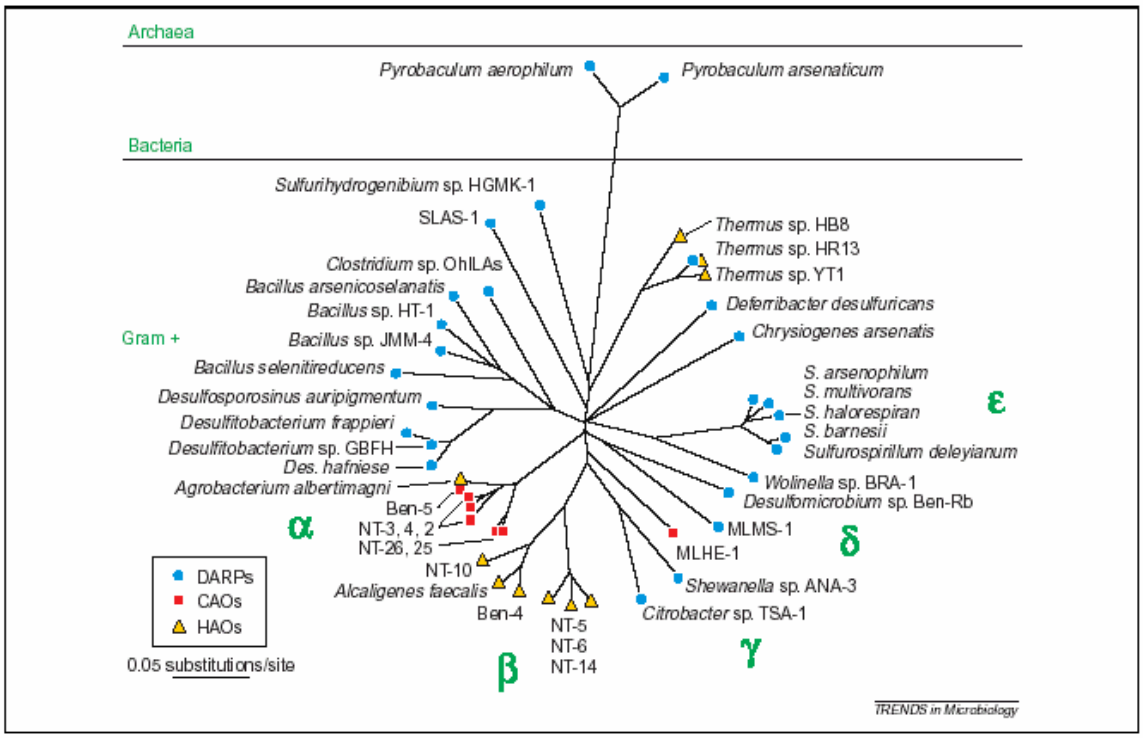

Figure 1. 16S rRNA tree of known As(V) reducing bacteria and archaea (from Oremland and Stolz, 2005)

reduction of Fe(III) to Fe(II) and subsequent reduction of As(V) to As(III), 2) the reduction of bound As(V) to As(III), or 3) the simultaneous reduction of As(V) and Fe(III) (Figure 2). In the first process, iron-reducing bacteria convert Fe(III) to Fe(II). This releases the As(V) from the Fe(III) for further reduction to As(III) by DARB or arsenic resistant bacteria, therefore further enhancing the solubility (Oremland and Stolz, 2005). In the second process, the DARB act alone to reduce bound As(V) to As(III) and due to the high solubility of As(III), it is mobilized in its aqueous phase. In the third mechanism, the DARB can perform both the iron reduction and the reduction of As(V) to As(III) which has the potential for a major increase in the arsenic contamination. All DARB contain an *arr* operon which houses the genes needed for As(V) reduction (Figure 3). The genes encode As(V) respiratory reductase (*Arr*AB) enzymes which allow the bacteria to use both bound and aqueous As(V) as an electron acceptor (Stolz and Oremland, 1999). The *Arr*AB enzyme contains two subunits: a larger mo-pterin subunit and a smaller Rieske subunit with four iron-sulfide cofactors (Figure 4) (Silver and Phung, 2005). The exact biochemistry of the As(V) respiratory reduction process is not fully known, but this process along with the other oxidation and reduction processes contribute to the arsenic contamination in various environments. The *arr*A genes were first detected in *Shewanella* ANA-3 and then the genes were found in various bacteria from gram positive to proteobacteria (Saltikov and Newman, 2003). Degenerate primers were developed based on the gene sequences from the isolated bacteria and since then PCR methods have been employed to detect the *arr*A genes from various environments to show the presence of uncultured DARB (Hoeft et al., 2004; Kulp et al., 2006; Hollibaugh et al., 2006; Lear et al., 2007). The genes have been used to gain an

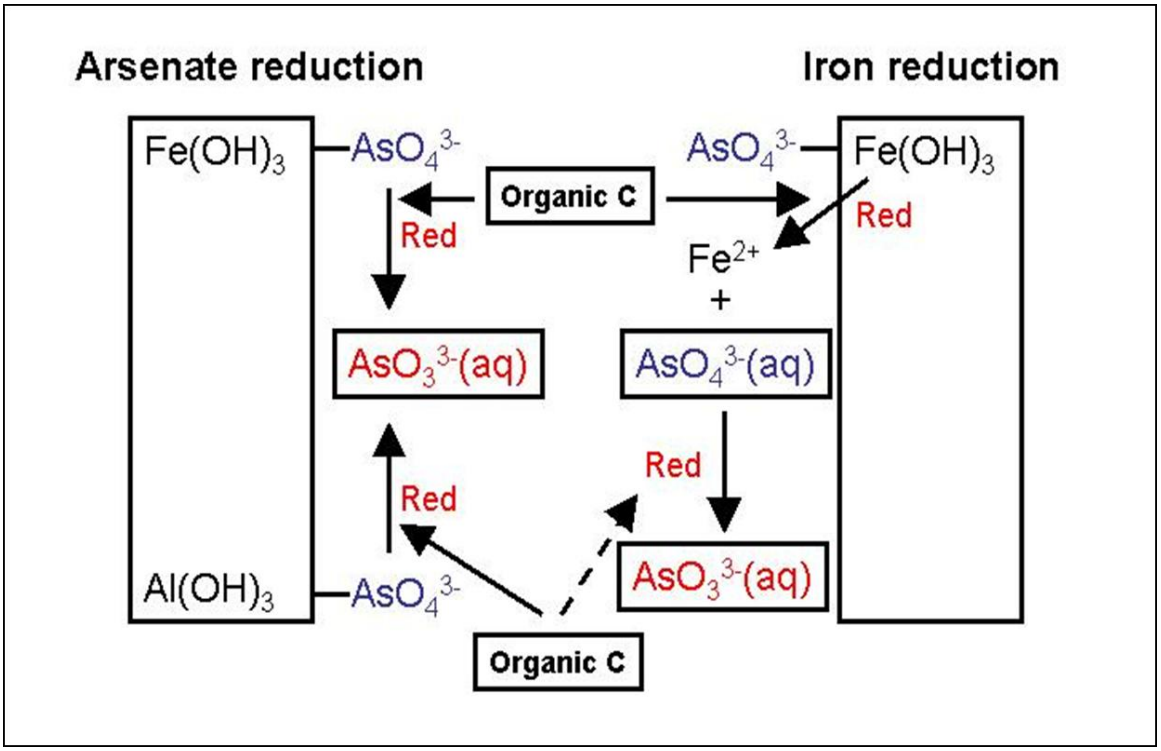

Figure 2. Three major microbial pathways which release arsenic (modified from Inskeep et al., 2002)

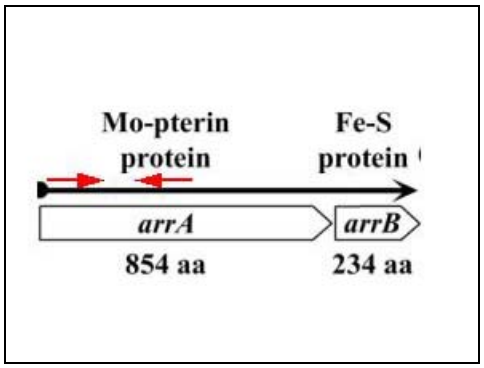

Figure 3. As(V) respiratory reductase (*arr*AB) gene operon (modified from Silver and Phung, 2005)

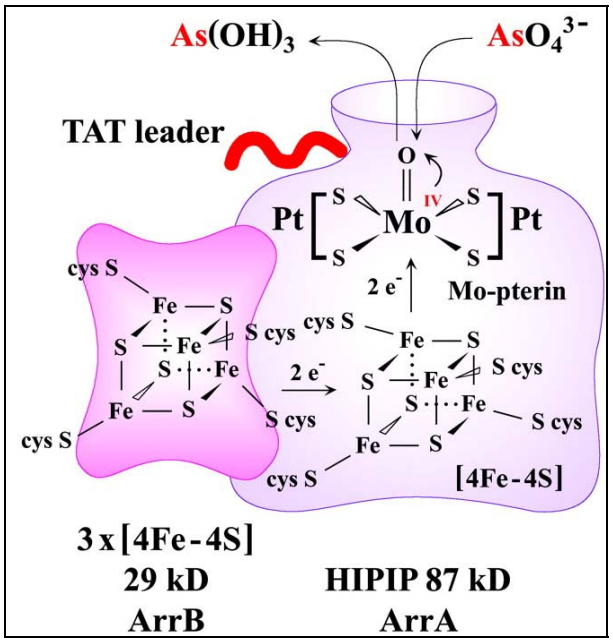

Figure 4. As(V) respiratory reductase and its biochemical pathway (from Silver and Phung, 2005)

understanding of As(V) reducing bacterial distribution as well as the bacterial speciation.

Sampling Site Characterization

The study sites for arsenic contamination were North Carolina's groundwater aquifers and Shem Creek, an estuary in South Carolina. The wells in North Carolina with arsenic contamination are located in the western part of the state, in the piedmont region. Here, the total dissolved arsenic is being detected at levels far surpassing the 10 ppb limit. The reason for this contamination is due to the natural geology of the region, with the contaminated wells falling into a geologic formation known as the Carolina Slate Belt. The geologic units of this area have a high probability of producing water with increased arsenic concentrations. The North Carolina Department of Environment and Natural Resources (NCDENR) has done a series of samplings in this area and found that the exact levels of arsenic in these residential wells ranged from an average of 40 ppb up to a maximum of 110 ppb (Pippen, 2005). This area provides an excellent place to study how the DARB are involved in raising the levels of arsenic in the wells.

For Shem Creek, the problem of arsenic contamination is a serious issue and some residents of South Carolina have even written about the problem such as the poem "Shem Creek: II" by Marjory Heath Wentworth, a South Carolina Poet Laureate. It reads:

> As the creek weaves
> through treeless subdivisions
> strip malls, and concrete,
> it gathers everything
> from oil, soap, and gasoline
> to tires and old refrigerators.

Run-off fills the oyster beds
with unpronounceable toxins.
Arsenic and mercury
drift through the water
in invisible clouds,
as if no one will notice.

This suburban watershed is located in Mount Pleasant, South Carolina and like many coastal regions suffers from a high population density. This is just one of the reasons for the high arsenic levels in this tidal creek's sediments. One natural cause of the arsenic is the fine grain sediments that make up a large portion of this estuary. Most of the cause for the high arsenic concentrations is due to anthropogenic sources such as housing and commercial developments, and a dry stack boat docking facility which houses around 50 commercial fishing vessels. These reasons also cause high PAH contamination in the creek. Shem Creek will make an interesting study site due to the high arsenic levels [3.7-18.2 ppm (µg/g dry weight)] (Sanger et al., 1999a) and due to the high PAH contamination [7.22-7.76 ppm (µg/g dry weight) (Sanger et al., 1999b). It will be interesting to study how PAH affects the mobilization of arsenic in the estuary.

Regardless of the location, and whether of not the arsenic contamination is natural or anthropogenic, As(V) reducing bacteria have the potential to significantly increase the levels of total dissolved arsenic present in both the estuarine and groundwater systems since they have the capability of utilizing both bound and aqueous arsenic to carry out the As(V) respiratory reduction. Thus, this project addresses the initial research to detect the presence of DARB and to examine their activities in both estuarine and groundwater aquifers.

HYPOTHESES

1. *The DARB community significantly contributes to the desorption of arsenic by the reduction of As(V) to As(III) in Shem Creek sediments and NC groundwater aquifers.*

2. *Dissimilatory As(V) reducing bacteria will be detected from Shem Creek sediments and NC groundwater aquifers utilizing molecular techniques.*

3. *Diversity of DARB will have a link to arsenic mobilization in the environment.*

4. *Reduction activities of DARB will have a direct relationship to the amount of arsenic found in the environment.*

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CHAPTER 2: MOLECULAR DETECTION OF ARSENIC REDUCING BACTERIA IN NORTH CAROINA GROUNDWATER AQUIFERS

INTRODUCTION

Arsenic is a naturally occurring metalloid that is widely distributed in the environment. In its inorganic form, it occurs in four major oxidation states: As^{+5}, As^{+3}, As^{0}, and As^{-3}. The most predominant of the inorganic forms are arsenate [As(V)] and arsenite [As(III)], and these two forms also happen to be the most toxic (Oremland and Stolz, 2003). Some natural sources of arsenic are the weathering of rocks, fire (Liu et al., 2004), fossil fuels, marine sedimentary rocks, various minerals (Stolz and Oremland, 1999), and the number one source of natural arsenic is from volcanic activities in igneous rock (Mukhopadhyay et al., 2002). Along with natural sources, anthropogenic point sources also contribute to the amount of arsenic in the environment. Some of these sources include acid mine drainage, coal combustion, pigment production, treated lumber, and farm raised animals that are fed food enriched with arsenic to prevent bacterial diseases (Oremland and Stolz, 2003).

Regardless of the source, arsenic is being detected at high concentrations in many drinking water wells around the world. The most well known place for arsenic contaminated drinking water is Bangladesh, where people are being slowly poisoned by arsenic on a daily basis. This poisoning causes arsenicosis which is the name for a set of symptoms caused by chronic arsenic poisoning. These symptoms include changes in the color of a person's skin, keratosis of the palms and soles of the feet, and many types of cancer (Shrestha et al., 2003). In addition, many people in Taiwan developed a condition known as "black foot disease" where the blood vessels in the extremities become diseased and gangrene sets in. Because arsenic is a carcinogen, chronic poisoning can also cause various types of cancer. The surprising thing is that arsenic is

not just a problem in underdeveloped countries, but also in the United States. The limit for the concentration of arsenic in drinking water for the United States, as set by the Environmental Protection Agency (EPA) in 2002, is 10 parts per billion (ppb) (Silver and Phung, 2005). However, many residential wells are not tested and not maintained to this standard and therefore have arsenic levels much higher than this 10 ppb limit.

North Carolina is one of the places being affected by these high arsenic concentrations. In the western part of the state, in the piedmont region, arsenic is being detected at levels far surpassing the 10 ppb limit, with concentrations ranging from the 1 ppb upwards to 110 ppb. In Stanly, Union, and Chatham counties for example, the probability that a person's arsenic concentration in their well being greater than 1 ppb is 62%, 51%, and 38% respectively (Pippen, 2005). The reason for these high arsenic levels has not been fully understood, but it is certain that one contributing factor is the geology of the region. The place with the highest arsenic concentrations is known as the Carolina Slate Belt, and this region contains various types of geologic units which have a high probability of producing water with increased arsenic levels. Typically, water with arsenic concentrations exceeding 10 ppb is due to one of four geological environments: 1) basins filled with river deposits, 2) volcanic deposits, 3) geothermal systems, and 4) uranium and gold-mining areas (Welch et al., 1988). The high arsenic levels in the NC groundwater are associated with all four of these environmental factors (Goldsmith et al., 1988; Feiss et al., 1993).

The high arsenic concentrations in the groundwater are also directly related to microbial activities. Microbes are capable of performing various oxidation and reduction processes involving arsenic, as well as other metals, but the most important microbes

affecting the arsenic speciation and concentrations in well water are As(V) reducing bacteria. Since As(V) is usually found bound to ferric [Fe(III)] (hydr)oxides at the pH of most natural groundwater, the reduction of As(V) to the more mobile As(III), leads to an increase in the arsenic concentration of the groundwater. There are two different metabolic processes the bacteria can utilize to reduce As(V) to As(III), such as the detoxification or anaerobic respiratory pathways. The detoxification process occurs in the cytoplasm of the bacteria where As(V) is reduced to As(III). The As(III) is then excreted from the cytoplasm by the *ars* transporter (ArsB) (Oremland and Stolz, 2003). The gene encoding As(V) reduction is the *ars*C gene, which is present in most bacterial genomes (Silver and Phung, 2005). The bacteria that reduce As(V) to As(III) as part of their respiration processes are known as dissimilatory arsenate reducing bacteria (DARB). Arsenate reducing bacteria can be significantly involved in arsenic mobilization through three different pathways 1) the reduction of Fe(III) to Fe(II) and subsequent reduction of As(V) to As(III), 2) the reduction of bound As(V) to As(III), or 3) the simultaneous reduction of As(V) and Fe(III) (Figure 2). In the first process, iron-reducing bacteria convert Fe(III) to Fe(II). This releases the As(V) from the Fe(III) for further reduction to As(III) by DARB or arsenic resistant bacteria, therefore further enhancing its solubility (Oremland and Stolz, 2005). In the second process, the DARB act alone to reduce bound As(V) to As(III), which is mobilized in aqueous phase due to the higher solubility of As(III). In the third mechanism, the DARB can perform both the iron reduction and the reduction of As(V) to As(III) which has the potential for a major increase in the arsenic contamination. Thus, DARB could significantly impact arsenic

mobilization mediated by As(V) respiratory reductase (*arr*AB) enzymes capable of reducing both bound and aqueous As(V) (Silver and Phung, 2005).

Although arsenate reduction is considered to be an important process in the arsenic contamination in groundwater aquifers, the involvements of DARB were not quantitatively measured related to the arsenic levels in groundwater. In order to understand the significance of DARB in the arsenic contamination, we conducted this study with two different water samples collected from drinking water wells located at the western part of North Carolina where high levels of arsenic were detected due to the desorption of arsenic from natural sources. We determined the presence and abundance of DARB with molecular methods, monitored As(V) reducing activities in well water using enrichment culture techniques, and isolated As(V) reducing bacteria to gain a better understanding of DARB in North Carolina groundwater aquifers.

MATERIALS AND METHODS

Sample Collection

Water samples were collected from two contaminated wells in the western part of North Carolina. The first well (Rushing) was in Wingate, NC which had arsenic levels of 150 ppb and the second well (Deese) was in Monroe, NC which had levels of 73 ppb (Figure 5) (Table 1). Water was pumped directly out of the well into 5 liter jugs at each site and placed in a cooler until arriving back to the lab.

DNA Extraction from Filters and Quantification

The water samples (1 L) from both the Rushing and Deese wells were filtered onto 0.22 µm Millipore Sterivex™ fliter units (Millipore Corporation; Billerica, MA) and the DNA was extracted using the Gentra's Puregene® Genomic DNA Purification Kit (Gentra Systems, Inc.; Minneapolis, MN). All of the extracted DNA was ran on a 1% agarose gel with 0.15 mg/mL ethidium bromide to confirm that the extraction worked. Also, the DNA was quantified using Quant-iT™ dsDNA HS Assay Kit, according to the manufacturer's protocol (Invitrogen; Eugene, OR).

PCR Detection of *arr*A Genes

After verifying that the DNA was present, a nested Polymerase Chain Reaction (PCR) was set up to amplify the As(V) respiratory reductase (*arr*A) gene from the environmental DNA. The DNA from *Desulfitobacterium hafniense* DCB-2 was used as the positive control. The PCR reaction was setup in 0.6 mL PCR tubes, with 2.5 µL of 10x Advantage 2 PCR Buffer, 0.4 µM of primers As1F and ArrA1R (Table 2), 20 µM of dNTPs, 0.5 µL of 50x Advantage® 2 polymerase, and 1 µL of DNA template. The samples were vortexed and given a quick spin, then placed in an MJ Thermocycler (MJ

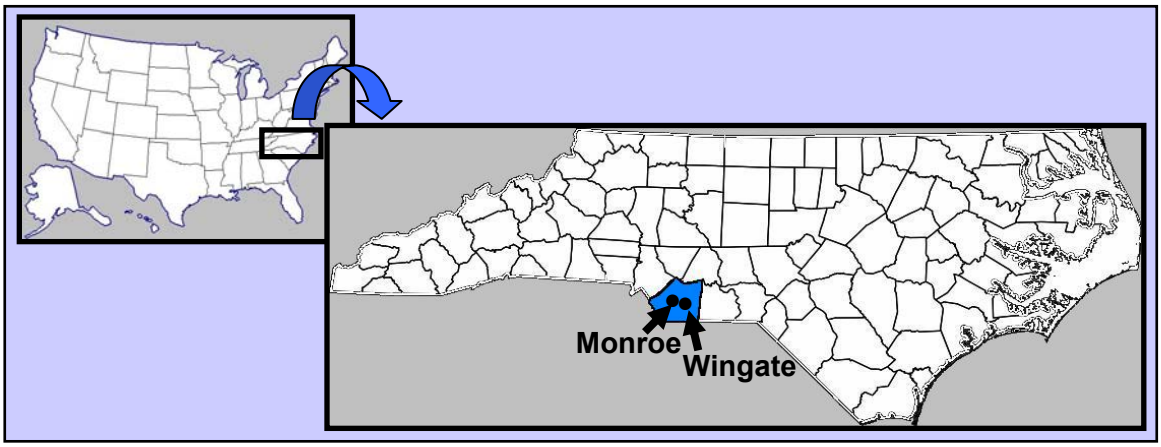

Figure 5. Map of well water sampling sites in western North Carolina

Table 1. Levels of arsenic and other metals in NC wells

Metals	Rushing (µg/L)	Deese (µg/L)
Aluminum	50U	50U
Arsenic	150	73
Cadmium	2U	2U
Chromium	25U	25U
Copper	17	3.8
Iron	50U	50U
Magnesium	6,100	8,800
Manganese	150	10U
Nickel	10U	10U
Lead	10U	10U
Selenium	19	14
Zinc	46	10U

U=levels below detection limit

Table 2. Primer sequences used in this study

Primer Name	Primer Sequences
ArrA1R	5’ ATANGCCCARTGNCCYTGNG 3’
As2F	5’ CTCCCNATBASNTGGGANRARGCNMT 3’
As1F	5’ GAAGTTCGTCCCGATHACNHGG 3’
As1R	5’ GGGGTGCGGTCYTTNARYTC 3’
NCAs1F	5’ CACAGCGCNATYTGCGCCGARGC 3’
NCAs1R	5’ CCRTCCTTGAARTCRCCGACRAAYTC 3’

Research; Reno, NV). The PCR started with an initial denaturation step of 95 °C for 5 minutes and 35 cycles of a 95 °C denaturation for 1 minute, a 55 °C annealing step for 30 seconds, and a 72 °C elongation for 2 minutes. Once this reaction has finished, the tubes were removed and the nested PCR was set up using 2.5 µL of 10x PCR Buffer (500 mM KCl, 200 mM Tris-HCl [ph 8.4]), 1.5 mM of a 25 mM $MgCl_2$, 0.4 µM of both As2F and As1R primers (Table 2), 20 µM dNTP, and 1U Taq polymerase. The first reaction (1 µL) was used as template for each reaction. The samples were vortexed and spun down briefly, then put back in the thermocylcer using the same cycle except the 2 minutes of 72 °C elongation was changed to 1 minute.

After the PCR was complete, 5 µL of each PCR product was ran on an 1% agarose gel. All of the samples were positive (approximately 627 bp) for the As(V) respiratory reductase (*arr*A) gene, except for the negative control. These samples were gene cleaned using the protocol from the Perfectprep® Gel Cleanup Kit (Eppendorf AG; Hamburg, Germany). After doing this, the gene cleaned samples were ran on a 1% agarose gel with 0.15 mg/mL of ethidium bromide to verify that the samples were not lost in the process of the gene clean.

Cloning and Sequencing

The gene cleaned samples were cloned according to the protocol outlined in the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation; Carlsbad, CA). The ligated plasmids were transformed in high-transforming-efficiency *Escherichia coli* TOP10® cells (Invitrogen Corporation; Carlsbad, CA) according to the manufacturer's protocol and then plated on Lauria Broth agar plates containing 10 µg/mL of kanamycin and 100 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). The

clone colonies grew overnight and the next day the white colonies were used for a colony check reaction. The colony check was set up as follows: 2.5 µL of 10x PCR Buffer, 1.5 mM of 25 mM $MgCl_2$, 0.4 µM of each M13R and T7 primers, and 22 µM dNTP. At least 10 colonies were picked from each plate and used as the template in the colony check reactions. One of the reactions contained no template and was used as a negative control. The tubes were placed in the thermocycler on the colony check program with an initial denaturation of 95 °C for 10 minutes and 30 cycles of a 95 °C denaturation for 1 minute, a 55 °C annealing step for 30 seconds, and a 72 °C elongation for 1 minute. When finished, the samples were run on a 1% agarose gel with 0.15 mg/mL of ethidium bromide to check for approximately 750 bp size products.

The positive clones were used to set up a sequencing reaction by adding 2 µL 10x Sequencing Buffer, 0.5 µL of primer (either M13R or T7), 1 µL Big Dye Terminator, 5.5 µL PCR water into 0.6 mL tubes, and using 0.5 µL of the colony check reaction as the template. The samples were placed in the thermocycler on the sequencing program which heats to 96 °C at 1 °C/sec and denatures the samples for 1 minute, then cools to 50 °C at a rate of 1 °C/sec and anneals for 5 seconds, then heats to 60 °C at a rate of 1 °C/sec and elongates for 4 minutes. The annealing step and elongation are repeated 25 times followed by a final annealing at 50 °C for 5 seconds and an elongation at 60 °C for 4 minutes. The reactions were then precipitated for sequencing by adding 40 µL of 75% isopropanol to each sample, vortexing briefly, and let stand at room temperature for 15 minutes. Then the samples are spun down at 14,000 rpms for 15 minutes in a microcenterfuge. The samples were then aspirated and 125 µL more of 75% isopropanol was added to wash the DNA. The reactions were spun down again at

14,000 rpms for 5 minutes. The isopropanol was then aspirated and the tubes were placed in a 95 °C heating block with the lids open and left to dry for 1 minute. Formamide was added to resuspend the pellet and the samples are denatured in the heating block for 1 minute. Immediately after the denaturation, the samples are cooled on ice for 1 minute, plated, and sequenced using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems; Foster City, CA).

Phylogenetic and DOTUR Analysis of *arr*A Genes

The sequences obtained were blast searched using NCBI's BLAST-X (http://www.ncbi.nih.gov) search engine. The positive sequences were assembled using DNASTAR Lasergene SeqMan Program (DNASTAR, Inc., Madison, Wisconsin) and were aligned using ClustalW (www.ebi.ac.uk/clustalw/) for phylogenetic analysis. The alignment file was put into MEGA 3.1 (Molecular Evolutionary Genetics Analysis) to make a neighbor-joining phylogenetic tree to examine diversity within the sequences.

The DOTUR (Distance-Based OTU and Richness) program was utilized to compare the diversity of the *arr*A gene sequences from each sampling site (Schloss and Handelsman, 2005). A 2% difference in nucleotide sequences was used to define the OTU's (Operational Taxonomic Units), which was determined in DOTUR by the furthest neighbor algorithm comparison. Diversity analyses including Chao[1] and Shannon index numbers were determined for each sampling site using the DOTUR program.

Development of Specific Primers for Q-PCR

Specific primers were designed by aligning the Rushing and Deese sequences to target the conserved regions of *arr*A gene for a Q-PCR analysis. The primers, NCAs1F and NCAs1R (Table 2), were used with the environmental DNA from the Rushing and

Deese sites. The reaction was set up as follows: 10 µL SYBR® GreenER™ qPCR SuperMix Universal, 1 µL of both NCAs1F and NCAs1R, 0.3 µLROX Reference Dye, 0.3 µL Taq Polymerase, 5.4 µL water, and 2 µL of template totaling a 20 µL reaction. Standards for the quantification reaction were plasmids extracted from one positive Rushing and one positive Deese clone. The standards were serially diluted from 1 ng/µL down to .0001 ng/µL and 2 µL of the diluted standards were also set up with the same reaction. The 7500 Real Time PCR System (Applied Biosystems; Foster City, CA) was used and the program began with an initial denaturing step of 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute. Then, there was one final cycle of 95 °C for 15 seconds, 55 °C for 1 minute, and 95 °C for 15 seconds. The Q-PCR data was analyzed with 7500 Analysis Program (Applied Biosystems; Foster City, CA).

Enrichment Cultures of Arsenate Reducing Bacteria in Groundwater

The well water from both the Rushing and Deese wells was used to make a set of enrichment cultures from each site. In place of media, 150 mL of the site water was put into 150 mL serum bottles. Fifteen bottles from each site were filled, bubbled with argon gas, and sealed the same way as the Shem Creek cultures. Four different conditions of substrates were added to each set in triplicate: 1) 1 mM Lactate and 1 mM As(V); 2) 1 mM Lactate and 1 mM NO_3^-; 3) 0.1x y.s.t. (50 µg/mL of yeast extract, 30 µg/mL of succinic acid, and 50 µg/mL of tryptone peptone) and 1 mM As(V); and 4) 1 mM As(V). A fifth set was used as a sterile control and contained 1 mM Lactate, 1 mM As(V), 1 mM NO_3^-, and 0.1x y.s.t. and was autoclaved twice to kill the bacteria.

A second sampling of the Rushing well site was performed a year after the first sampling and from that trip another set of enrichments were made. These sets were made the same way as the first set except only a 50 mL volume of water was used. Also, only four different conditions were used: 1) 0.1x y.s.t. + 1 mM As(V); 2) 1 mM SO_4^{2-} + 1 mM As(V); 3) 1 mM As(V); and 4) 0.1x y.s.t. + 1 mM SO_4^{2-} + 1 mM As(V) that was used as a sterile control and autoclaved twice to kill the bacteria. These cultures were sampled daily for one week and the As(V) reduction measured by HPLC.

Isolation of As(V) Reducing Bacteria

DARB were isolated from the Deese and Rushing enrichment cultures using anaerobic shake tubes made with ATCC *1957 Geobacter* media (Table 3) with 0.5% agar. A total of 5 mM concentration of sodium arsenate was added to the media and it was dispensed under argon gas into each Hungate tube. The tubes were autoclaved and cooled upside down in a 55 °C water bath. While the media was still in liquid form, 1 mL of the inocula was serially diluted from 1:10 up to 1:100,000 ratios. The tubes were inverted a few times and then cooled seal side down in ice so that the agar would solidify quickly, trapping the bacteria throughout the media. The shake tubes were removed from the ice and placed in a 28 °C incubator for a period of time.

The colonies grew separately in the agar and 4 colonies for each site were taken out with a syringe and resuspended into a liquid media of ATCC *1957 Geobacter* (Table 3) with a 1 mM concentration of arsenate in a new Hungate tube. The optical density of these tubes were measured daily by a spectrophotometer to monitor the growth of the isolates. After about one month, samples were taken from the Hungate tubes and spun down to pellet the cells. The supernatant was transferred to a clean tube and the As(III)

Table 3. ATCC *1957 Geobacter* media components

Basal media:

Component	Amount
NH_4Cl	1.5 g/L
NaH_2PO_4	0.6 g/L
KCl	0.1 g/L
$NaHCO_3$	2.5 g/L
Wolfe's mineral solution (see below)	10.0 mL/L
V-7 vitamin solution (see below)	2 mL/L (added after media cooled)
Agar (optional)	15 g/L

Wolfe's mineral solution (100x):

Component	Amount
Nitrilotriacetic acid	1.5 g/L
$MgSO_4 \cdot 7H_2O$	3.0 g/L
$MnSO_4 \cdot H_2O$	0.5 g/L
NaCl	1.0 g/L
$FeSO_4 \cdot 7H_2O$	0.1 g/L
$CoCl_2 \cdot 6H_2O$	0.1 g/L
$CaCl_2$	0.1 g/L
$ZnSO_4 \cdot 7H_2O$	0.1 g/L
$CuSO_4 \cdot 5H_2O$	0.01 g/L
$Al(SO_4)_2 \cdot 12H_2O$	0.01 g/L
H_3BO_3	0.01 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.01 g/L

V-7 Vitamin Solution (500x):

Component	Amount
Cyanocobalamin (B12)	100 mg/mL
Pyridoxin-HCl	300 mg/mL
Thiamin-dichloride	200 mg/mL
Ca-D-Pantothenate	100 mg/mL
p-Aminobenzoic acid	80 mg/mL
D-Biotin	20 mg/mL
Nicotinic acid	200 mg/mL

concentrations were measured via HPLC to test whether or not the isolates were capable of reducing As(V).

HPLC Analysis of As(III)

The HPLC with UV detector was used to determine the As(III) concentrations that are produced by the bacterial reduction of As(V) to As(III) in the enrichment produced by the bacterial reduction of As(V) to As(III) in the enrichment cultures (Liu et al., 2004). A Hamilton PRP-X100 anion exchange column was used with an isocratic, 30 mM monobasic sodium phosphate, adjusted to pH 6 with NaOH, as the mobile phase. A 10 µL volume of the As(III) standards and the enrichment culture samples were injected. The As(III) was detected at 1.5 min using a flow rate of 1.5 mL/min and a wavelength of 200 nm on a Waters UV-vis detector. The detection limit of As(III) was 0.1 mM.

Statistical Analysis

For the Rushing and Deese As(III) concentration data that was obtained from the HPLC-UV analysis, the standard deviation was taken of the As(III) concentrations for both the y.s.t. + As(V) cultures and the lactate + As(V) cultures for each day of sampling. This standard deviation was then used to calculate the standard error.

RESULTS

PCR Detection of *arr*A Genes from Groundwater Samples

The DNA extraction from the filters containing biomass from the Deese and the Rushing wells yielded 1.46 ng/μL and 0.166 ng/μL of DNA, respectively. The initial PCR with the primers As1F and ArrA1R did not amplify any products. A nested PCR with the primers As2F and As1R yielded the amplicons with 627 bp size, which was the same size as the positive control of *Desulfitobacterium hafniense* DCB-2.

Phylogenetic and DOTUR Analyses of *arr*A Genes

Clonal libraries of the amplified *arr*A genes were constructed in 96 wells. A total of 20 clones from Rushing and 19 clones from Deese libraries were fully sequenced and used for phylogenetic analysis. The DNA sequences were translated to amino acid sequences for further analysis. A phylogenetic tree of *arr*A genes showed that the Rushing clones formed two groups, a large one (Rushing A) and a small one (Rushing B), while the Deese clones grouped in one large group (Deese A) and two smaller groups (Deese B and C). The clones in Rushing A shared ~95% amino acid sequence identity to each other and ~74% to *Geobacter uraniumreducens* Rf4. This same group had ~64% amino acid sequence identity to *Desulfosporosinus* Y5, while the Rushing B sequences shared 76% to *Geobacter uraniumreducens* Rf4, and ~63% to *Desulfosporosinus* Y5. The Deese A group was ~100% identitical to itself based on amino acid sequences, ~78% identical to *Geobacter uraniumreducens* Rf4, and ~65% identical to *Desulfosporosinus* Y5. The sequences in Deese B shared ~99% identity to themselves, ~60% to Delta proteobacterium MLMS-1, and were ~63% similar to *Clostridium* sp. OhILAs. The clones in the Deese C group had ~96% identity to each

other and were only ~61% similar to the outgroup *Magnetospirillum magnetotacticum* (Figure 6).

DOTUR (Distance-Based OTU and Richness) program was employed to examine the diversity as a measurement of species richness based on the nucleotide sequences from the Rushing and Deese sites. At a 2% difference comparison, the Rushing site had more diverse DARB than the Deese site based on the *arr*A gene sequences. The Rushing sequences had 8 OTU's, while the Deese site had 4 OTU's which are both shown in the rarefaction analysis of phylotypes (Figure 7). The Chao[1] estimate showed the predicted level of phylotype richness. The Chao[1] estimate for Rushing was 13 and for Deese was 4 (Table 4). The Shannon index numbers indicated the diversity of *arr*A genes in both wells. The Shannon index number for Rushing was 1.69 and for Deese was 1.06 (Table 4).

Q-PCR of *arr*A Genes

Q-PCR with the primers NCAs1F and NCAs1R was set up to quantify the *arr*A gene numbers present in both the Rushing and Deese well sites. The PCR was not successful in detecting the *arr*A genes in the Rushing well, but the Deese well had 6.9 x 10^3 ± 2 x 10^3 copies of *arr*A genes per ng of DNA (Table 4). It should also be noted that three other NC well sites were tested and no quantity was determined due to no amplification. The reason for this is that the primers were probably designed to be too specific.

Arsenate Reducing Activities in Groundwater

The first set of enrichment cultures were monitored once a week for a time period that lasted four weeks. The HPLC-UV analysis was performed and both Rushing and

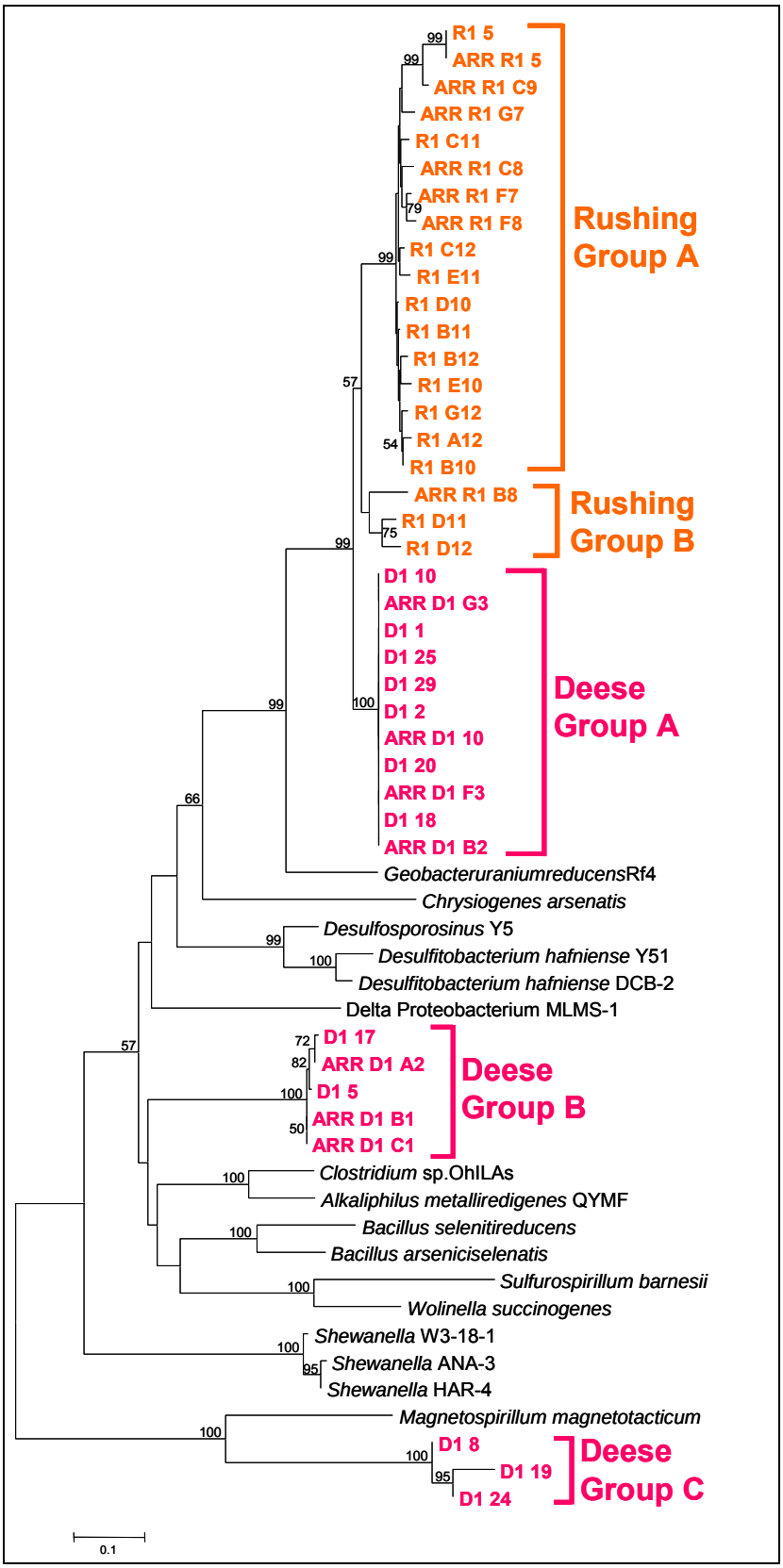

Figure 6. Phylogenetic tree of *arr*A genes from the Rushing and Deese wells

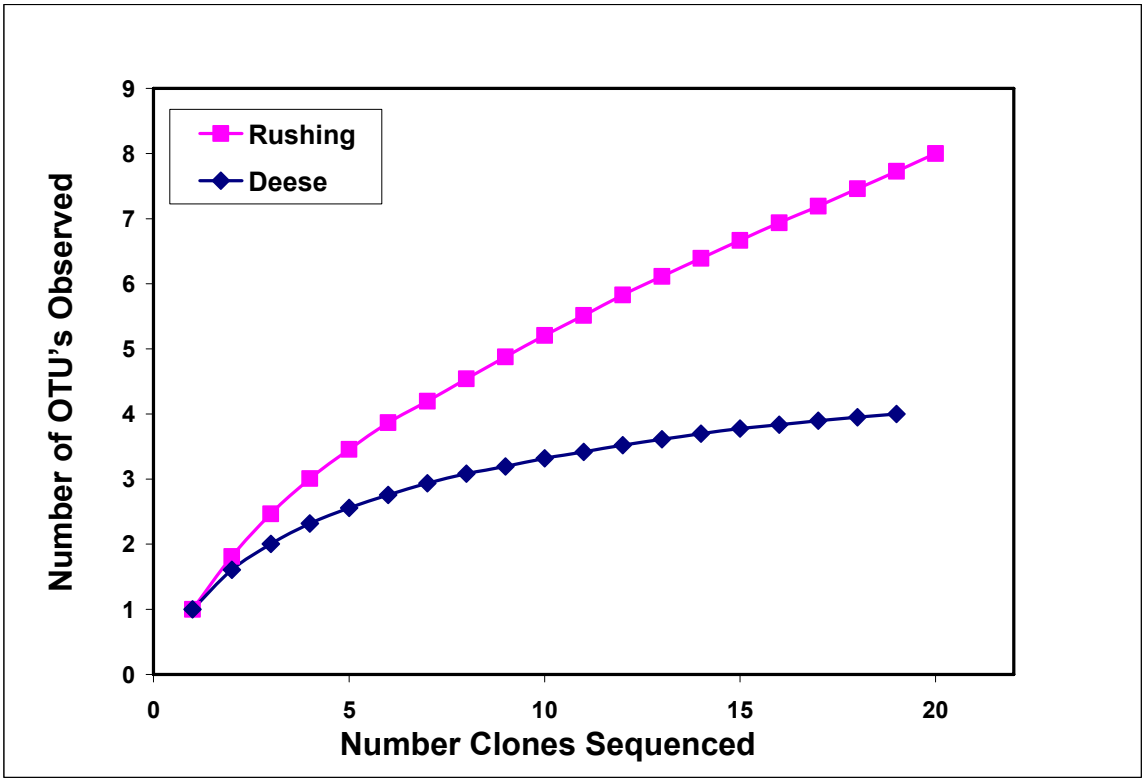

Figure 7. Rarefaction analysis of *arr*A genes detected from Rushing and Deese wells

Table 4. Summary of abundance and diversity of DARB in NC wells

Measurement	Deese	Rushing
Relative Abundance	$6.9 \times 10^3 \pm 2 \times 10^3$ (copies per ng DNA)	Not Measurable
Shannon Index	1.06	1.69
Chao 1	4	13

Deese had As(V) reduction activity present in the cultures that contained the lactate + As(V) and the ones that had the y.s.t. + As(V). No measurable reduction took place in the As(V) only, or in the sterile control enrichments. The As(III) concentrations for both the Deese and Rushing cultures that had reduction were plotted and the standard error was calculated for each sample set (Figures 8 and 9). For both the Rushing and Deese enrichments, the cultures that contained the y.s.t. were able to reduce the As(V) to As(III) earlier than the lactate cultures, which took anywhere from day 8 to day 17. In the case of the Deese enrichments, the lactate cultures ended up reducing just as much As(V) as the y.s.t. cultures and both had a final average As(III) concentration of around 0.34 mM. The Rushing y.s.t. cultures' final As(III) concentration almost doubled that of the Deese with an average final As(III) concentration of 0.62 mM and the Rushing lactate culture had an average concentration of 0.57 mM As(III).

The rate of As(V) reduction was calculated for each site and for each condition. The final average rate of As(V) reduction for the Rushing lactate cultures was 16.3 μM/day and the Deese cultures had a faster rate of As(V) reduction with the lactate cultures having an average rate of 28.9 μM/day. The y.s.t cultures for Deese had a rate of 38.9 μM/day, while the y.s.t. cultures for Rushing had a rate of 62.2 μM/day. All of the rates were measured over an 8 day time period except for the Rushing lactate + As(V) cultures which were measured over a 25 day time period because in one of the replicates, no reduction took place until day 25. The reason the rest of the cultures were measured at day 8, even though the experiment lasted for 35 days, is because most of the As(V) reduction occurred over the first 8 days.

A second set of enrichment cultures were made from a second sampling at the

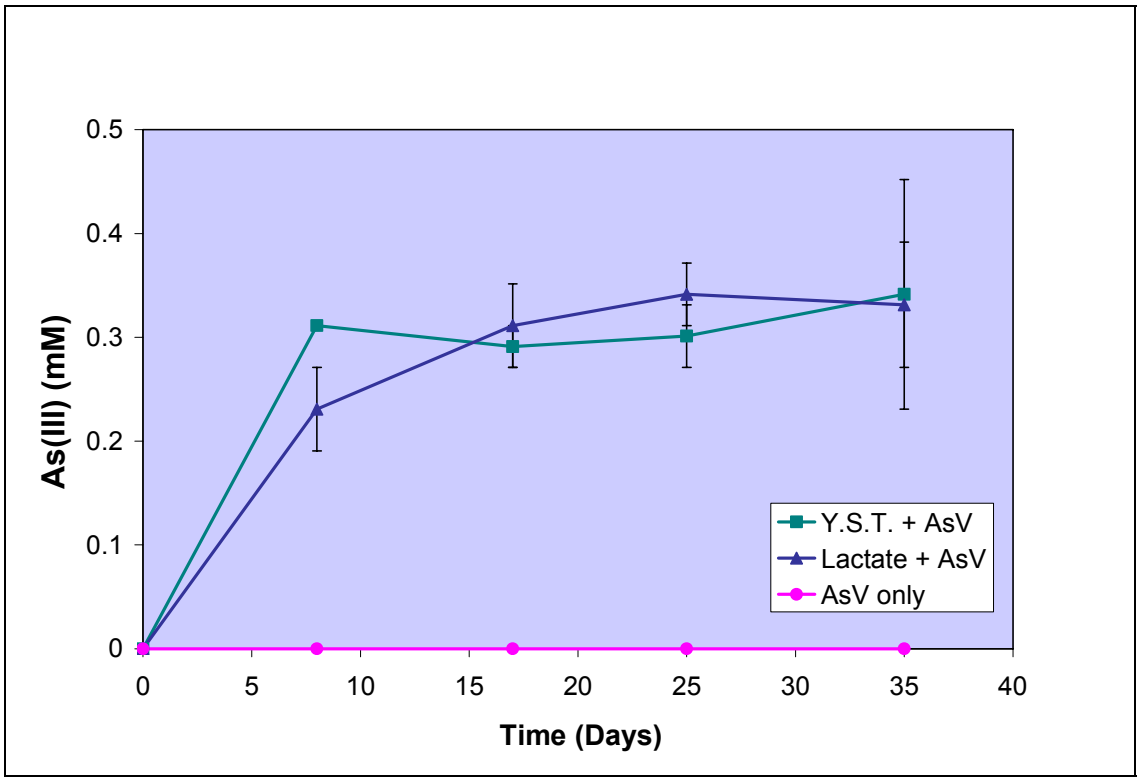

Figure 8. As(V) reduction in anaerobic enrichment cultures established with Deese well water

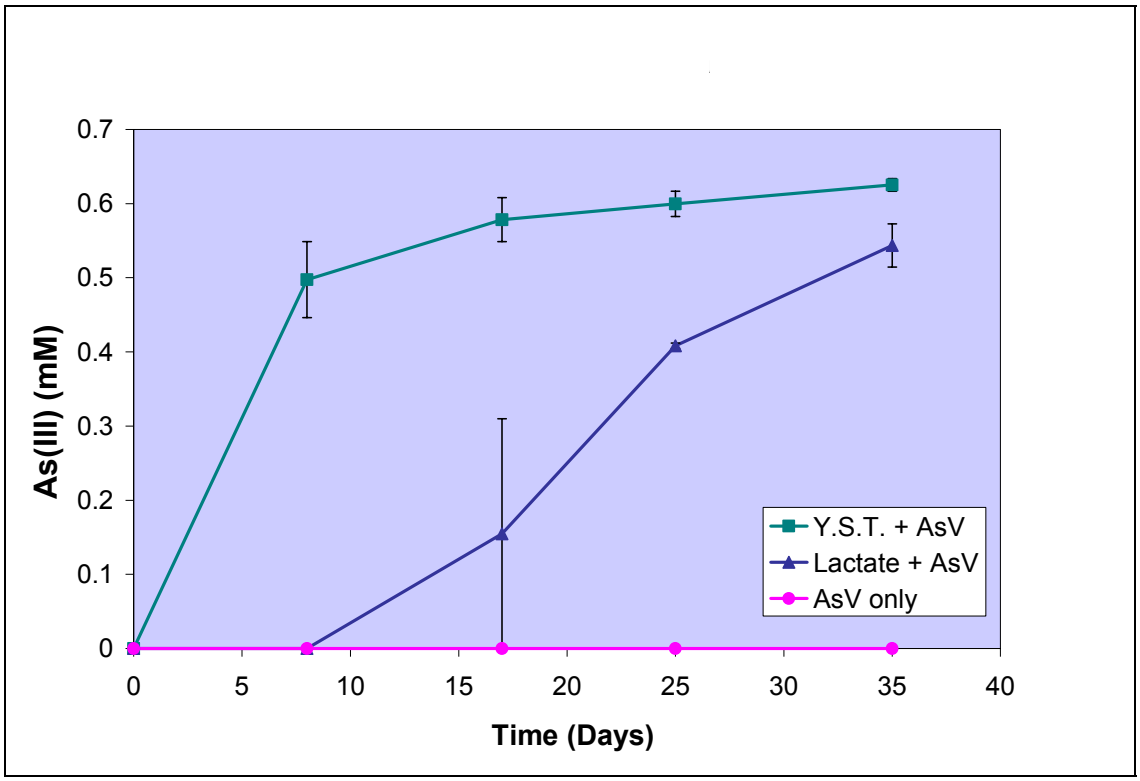

Figure 9. As(V) reduction in anaerobic enrichment cultures established with Rushing well water

Rushing well site and based on the findings from the first set, the cultures were sampled daily for one week so that a better understanding of the As(V) reduction rate could be determined. The cultures with As(V) only and the sterile control had no measurable As(V) reduction take place. The cultures that contained y.s.t + As(V) and the ones that had y.s.t. + As(V) + SO_4^{2-} were the only ones capable of reducing As(V). The As(III) data for these two conditions were plotted with error bars to show the variation in the triplicate cultures (Figure 10). Both conditions ended up with similar As(III) concentrations of 0.71 mM for the y.s.t. + As(V) culture and 0.73 mM for the culture containing SO_4^2. The big difference in these two conditions, was that the SO_4^{2-} culture did not start reducing As(V) until after day 1, where as the culture with just y.s.t. + As(V) started reducing As(V) the first day. The average rate of As(V) reduction for y.s.t. + As(V) culture was 126.8 μM/day and the rate for the SO_4^{2-} culture was a little higher at 134.8 μM/day based on the day 6 concentrations of As(III). The rates from this set of enrichment cultures more than doubled the highest reduction rates from the first incubation experiment.

Isolation of Arsenate Reducing Bacteria

The enrichment cultures from Deese and the first incubation of Rushing used as inocula for the anaerobic shake tubes. The 1:10,000 dilution had large colonies with enough separation to be able to take them out of the tube without disturbing the other colonies. Four colonies were taken from both the Rushing and Deese shake tubes and injected into liquid media. The optical density measurements that were taken from these isolates growth were very low, with the highest one being 0.07 absorbance at 600 nm wavelength from the Rushing isolate, #2. Due to the fact that the growth could not

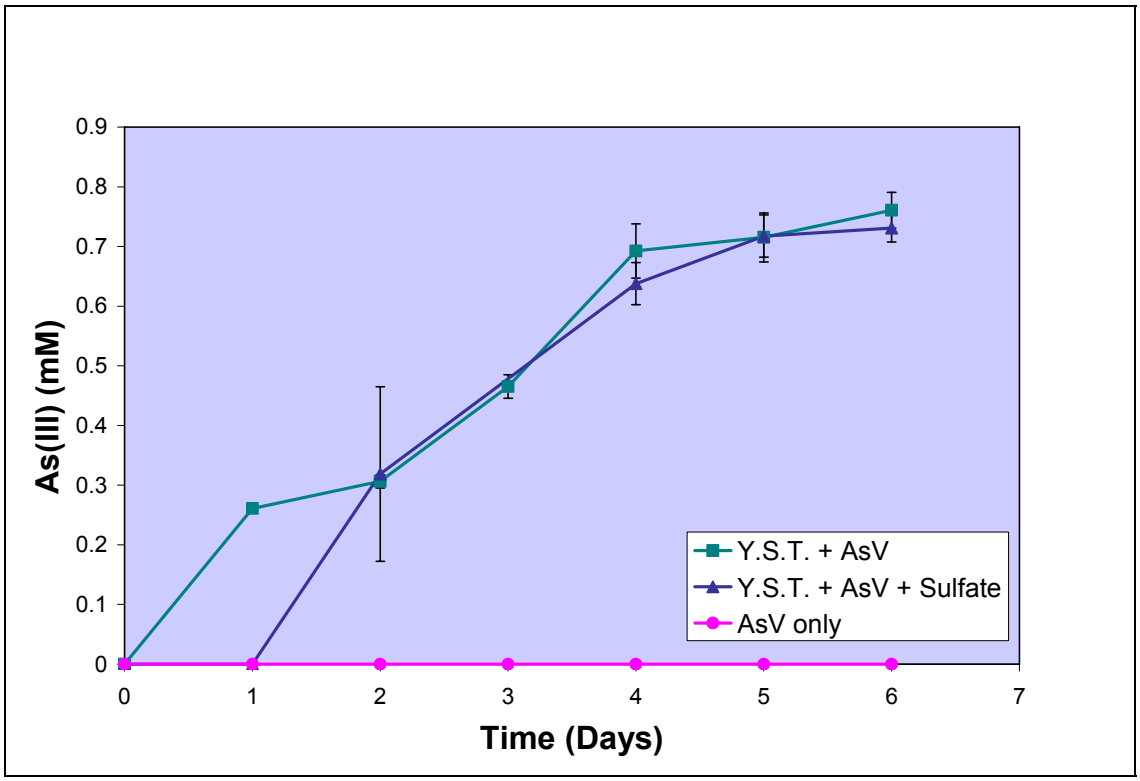

Figure 10. As(V) reduction in the second anaerobic enrichment cultures established with Rushing well water

be determined based on the optical density and to prove that the isolates could reduced As(V), samples of the isolate culture were taken on day 28. The samples were ran on HPLC and were not found to reduce As(V).

DISCUSSION

The molecular portion of this research provided promising data which can be used to better understand the bacteria present in these underground aquifers. The DNA extraction, although it yielded small amounts of DNA, worked well considering how little biomass is in drinking water. The nested PCR of this environmental DNA worked very well to amplify the *arr*A genes present in these well environments. The cloning and sequencing of these *arr*A genes showed the similarity of the DARB present in both well systems, which were closely related to the *arr*A gene found in *Geobacter uraniumreducens* Rf4. The *Geobacter* bacteria have been shown to reduce iron and they may also have the capacity to reduce arsenate (Lear et al., 2007). This would be favorable in well environments as As(V) is usually bound to Fe(III). In addition, based on the sequencing data, the Deese well contained two different groups of As(V) reducers that were not found in the Rushing well. This shows that the two individual aquifers have unique bacterial community structures.

According to the rarefaction analysis, the Rushing well has a two fold higher number of OTU's than the Deese well, 8 and 4 respectively. This number is based on a 2% difference in phylotype, which means that if the sequences are more than 2% different based on nucleotides, they are assigned as one OTU. Although the Deese well has three distinct groups (A, B, and C) present based on the phylogenetic analysis and that those amino acid sequences are closely related to five species of bacteria, the sequences within groups A, B, and C are very closely related, with 100%, 99%, and 96% similarity, respectively. The Rushing sequences, however, form just two groups based on the phylogenetic analysis that are closely related to only two bacterial species,

but the 95% similarity of the Rushing sequences, is lower than that of the Deese sequences, making the Rushing well more diverse. Therefore, the molecular data shows that the Rushing well has a more diverse As(V) reducing bacterial communities present than does the Deese well.

Enrichment culture studies showed that the As(V) reduction rates were very different depending on the growth condition and the sampling site. The rate variations under different growth conditions are obvious because some bacteria are capable and more favorable of utilizing different carbon compounds as growth substrates. However, the remaining question is what causes the variation of the As(V) reduction rate in different samples, collected from two different wells, even though the enrichment cultures are under the same condition. The Rushing enrichment cultures amended with y.s.t. + As(V) yielded a final As(III) concentration of 0.62 mM in the first enrichment and 0.73 mM in the second enrichment with similar rates of As(V) reduction. But, when compared to the Deese enrichment cultures, the As(III) concentration is nearly half of the Rushing enrichments' with 0.34 mM of As(III). This discrepancy might be directly related to the diversity of DARB present in both wells. The Rushing well has a more diverse group of DARB, which leads to the higher activity in the enrichments, while the DARB in the Deese well are less diverse and have a lower activity in the enrichments. This finding might explain different levels of arsenic found in both wells. The Rushing well has 150 ppb of arsenic, while the Deese well has 73 ppb. The higher diversity and activity of DARB in the Rushing well could lead to the presence of higher arsenic concentrations in contrast to the Deese well.

The abundance of DARB measured by Q-PCR showed that the Deese well has $6.9 \times 10^3 \pm 2 \times 10^3$ copies of the *arr*A gene per ng of DNA present. Although the Rushing well's DARB abundance could not be measured, based on the lower As(V) reduction activity in the Deese well, the Rushing well must have a higher abundance of As(V) reducers. Even though the Deese well was detected on Q-PCR, this could be because of bias due to the primers being too specific. The lower activity in the Deese well might also be related to competition from other bacteria competing for substrate. If both wells have the same amount of carbon substrate and the same amount of As(V), they theoretically should have the same final As(III) concentration. But, since the Rushing well's concentration is approximately twice as high as the Deese well, some other activity, such as fermentation, must be going on in the Deese microcosms, which takes away valuable carbon from the As(V) reducing bacteria.

When attempting to isolate the As(V) reducing bacteria for the first time, it was clear that we had obtained some fermenters in the Deese culture because there were gas bubbles present throughout the anaerobic agar shake tubes. This shows that fermentation is a potential opponent to As(V) reduction because it uses up the carbon in the enrichments and potentially in the environmental wells themselves, based on the higher activity of the Rushing well in all facets. Eventually, when using a more specific media targeting the *Geobacter* community, this problem was alleviated in the isolation efforts. But with just using site water in the enrichment cultures to grow the bacteria, the fermenters could be a potential for competition regarding the carbon substrate as is seen in the activity data.

In conclusion, as the sequencing data reveals, there are clearly As(V) reducing bacterial communities present in these wells containing high arsenic levels. The DARB at both wells actively reduce As(V) to As(III) with the presence of carbon. Based on the reduction rates seen in the enrichment cultures and the amount of As(III) that can be produced from this reduction, these bacteria have the potential to significantly impact the levels of arsenic in groundwater systems.

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CHAPTER 3: MOLECULAR DETECTION OF ARSENIC REDUCING BACTERIA IN SHEM CREEK ESTUARINE SEDIMENTS

INTRODUCTION

An estuary is a semi-enclosed body of water where the freshwater of the streams and rivers meets the saltwater of the ocean. Estuarine environments are areas of tidal fluctuation that are the home to many species of fish, birds, mammals, plants, and other life (EPA, 2007). The estuary buffers the elements from these organisms and allows them to have a place to live, feed, and reproduce (Hackney et al., 1976). Approximately 65% of commercial fish, shellfish, and marine sportfish spend part or all of their lives in estuaries (Smith, 1966). Estuaries are aesthetically pleasing, drawing millions of tourists (Miller, 1993; Miller and Auyong, 1991) and growing population levels (Culliton et al., 1990) to their banks each year. These growing population levels have contributed to a decline in habitat through an increase in fisheries, boating, and pollution (Vitousek et al., 1997). Pollution, brought on by industrial and commercial development near estuaries, is by far the most significant of these three impacts. Many estuaries all over the world are becoming polluted by industrial waste, sewage, dredged material, and oil spills (Capuzzo et al., 1978). These pollutants lead to organic loading, heavy metal contamination, and PAH contamination to name a few (Kennish, 1992).

Shem Creek, located in Mount Pleasant, South Carolina, is one of many estuaries suffering from a high population density (15.7 people per hectare) and it is also impacted by commercial development, a major highway, and a boat docking facility at the lower reaches of this creek. This suburban tidal creek has high levels of arsenic present in its sediments (Sanger et al., 1999a) and high PAH contamination (Sanger et al., 1999b). The PAH contamination is most likely associated with the watershed, including residential and commercial land uses, and from the anthropogenic sources

downstream, including commercial fishing and recreational boating (Sanger et al., 1999b). The high arsenic levels, 3.7 ppm (µg/g dry weight) in the upper reaches of the creek and 18.2 ppm (µg/g dry weight) in the lower reaches of the creek, are most likely associated with the fine grain sediments and housing developments (Sanger et al., 1999a) (Table 5).

Arsenic, a carcinogenic metalloid, occurs naturally in the environment in organic, inorganic, and methylated forms. The inorganic forms are the most toxic and are found in four major oxidation states: As^{+5}, As^{+3}, As^{0}, and As^{-3}. The most predominant of the inorganic forms are As(V) [As(V)] and As(III) [As(III)], and these two forms also happen to be the most toxic (Oremland and Stolz, 2003). Arsenic cycling in estuaries is a combination of many biological, chemical, and geological processes. For example, in oxic sediments As(V) is the most prevalent due to the high oxidation potential, while As(III) is usually found in anoxic environments where the reducing potential is greater (Mandal and Suzuki, 2002). At the sediment-water interface of an estuary, where the sediments are oxidized, there is a constant supply of Fe(III) and Mn(IV). Since As(V) primarily exists in deprotonated forms at neutral pH, similarly to phosphate, it readily binds to the positively charged Fe(III) and Mn(IV) hydroxides. But, deeper in the sediment, under highly reducing conditions, Fe(III) and Mn(IV) concentrations diminish and the As(V) desorbs from these complexes and goes back into the dissolved phase, where it is then reduced to As(III), and binds to the reduced sulfates, as precipitated arsenic-sulfides, known as arsenopyrite (As_2S_3) (Inskeep et al., 2002).

As stated earlier, the microbial community plays a major role in the release and speciation of arsenic through various oxidation and reduction processes. Although the

Table 5. Levels of metals and PAHs in Shem Creek

Contaminants	Upper Creek ppm (µg/g)	Lower Creek ppm (µg/g)
Aluminum	2.26	5.29
Arsenic	3.7	18.2
Cadmium	0.11	0.28
Chromium	36.9	84.9
Copper	16.4	47.6
Iron	1.45	3.22
Lead	17.3	36.0
Manganese	96.0	212.8
Nickel	6.7	23.3
Zinc	58.6	140.2
Naphthalene	0.01	0.01
Phenanthrene	0.31	0.25
Fluoranthene	1.37	1.13
Pyrene	1.09	0.93
Total PAHs	7.76	7.22

oxidation processes are important, the focus of this research is on the reduction mechanisms. There are two processes that reduce As(V) to As(III), a detoxification mechanism and an anaerobic respiratory pathway. The bacteria that perform this As(V) respiration are classified as dissimilatory As(V) reducing bacteria (DARB). The DARB can mobilize arsenic in three different pathways including 1) the reduction of Fe(III) to Fe(II), 2) the reduction of bound As(V) to As(III), or 3) a combination of both processes. In the first process, iron-reducing bacteria convert Fe(III) to Fe(II). This releases the As(V) that was bound to Fe(III) for further reduction to As(III) by DARBs or arsenic resistant bacteria (Oremland and Stolz, 2005). In the second process, the DARB act alone to reduce bound As(V) to As(III) and due to the high solubility of As(III), it is mobilized in its aqueous phase. In the third mechanism, the DARB can perform both the iron-reduction and the reduction of As(V) to As(III) which has the potential for a major increase in the arsenic contamination. All DARB contain As(V) respiratory reductase (*arr*AB) enzymes which allow them to use both bound and aqueous As(V) as an electron acceptor (Stolz and Oremland, 1999). DARB are both taxonomically and metabolically diverse, such as their ability to utilize various carbon substrates including lactate, benzoate, succinate, and formate (Liu et al., 2004), and their capability of reducing iron, selenate, sulfate, and nitrate (Oremland and Stolz, 2005).

Due to the ability of these DARB to significantly increase the levels of arsenic in estuaries, there is a need to study these organisms so that the limitations of these pathways can be determined. Since Shem Creek has high arsenic levels in its sediments, it is a great place to study As(V) reducing bacteria and due to the high PAH concentrations, it would be worthwhile to see if the DARB can use PAHs as carbon and

energy sources for the As(V) respiration. The objectives of this project are to determine the presence of DARB using molecular methods, to examine the community structure of DARB in Shem Creek using Terminal Restriction Fragment Length Polymorphism (T-RFLP), to monitor As(V) reducing activities in Shem Creek sediments using anaerobic sediment enrichment culture techniques, and to isolate As(V) reducing bacteria.

MATERIALS AND METHODS

Sample Collection

Samples were collected from Shem Creek located in Mount Pleasant, South Carolina. Sediment was obtained from three sites within the creek: Shem Creek 1, Shem Creek 2, and Shem Creek 3. Site 1 is located in the upper reaches of the creek, site 2 in the middle, and site 3 is located at the lower reaches (Figure 11). Duplicate sediment samples were collected from each site from the top 5 cm. The samples were placed in coolers and transported back to lab for immediate refrigeration and freezing depending on the analysis.

DNA Extraction from Soil

Environmental DNA was extracted from the Shem Creek sediments using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.; Carlsbad, CA). All of the extracted DNA was ran on a 1% agarose gel with 0.15 mg/mL ethidium bromide to confirm that the extraction worked.

PCR Detection of *arr*A Genes

After verifying that the DNA was present, a nested Polymerase Chain Reaction (PCR) was set up to amplify the As(V) respiratory reductase (*arr*A) gene from the environmental DNA. The DNA from *Desulfitobacterium hafniense* DCB-2 was used as the positive control. The PCR reaction was setup in 0.6mL PCR tubes, with 2.5 µL of 10x Advantage 2 PCR Buffer, 0.4 µM of primers As1F and ArrA1R (Table 2), 20 µM of dNTPs, 0.5 µL of 50x Advantage 2 polymerase, and 1 µL of DNA template. The samples were vortexed and given a quick spin, then placed in an MJ Thermocycler (MJ Research; Reno, NV). The PCR started with an initial denaturation step of 95 °C for 5

Figure 11. Map of sampling sites located at Shem Creek, South Carolina

minutes and 35 cycles of a 95 °C denaturation for 1 minute, a 55 °C annealing step for 30 seconds, and a 72 °C elongation for 2 minutes. Once this reaction has finished, the tubes were removed and the nested PCR was set up using 2.5 µL of 10x PCR Buffer (500 mM KCl, 200 mM Tris-HCl [ph 8.4]), 1.5 mM of a 25 mM $MgCl_2$, 0.4 µM of both As2F and As1R primers (Table 2), 20 µM dNTP, and 1U Taq polymerase. The first reaction (1 µL) was used as template for each reaction. The samples were vortexed and spun down briefly, then put back in the thermocylcer using the same cycle except the 2 minutes of 72 °C elongation was changed to 1 minute.

After the PCR was complete, 5 µL of each PCR product was ran on an 1% agarose gel. All of the samples were positive (approximately 627 bp) for the As(V) respiratory reductase (*arr*A) gene, except for the negative control. These samples were gene cleaned using the protocol from the Perfectprep® Gel Cleanup Kit (Eppendorf AG; Hamburg, Germany). After doing this, the gene cleaned samples were ran on a 1% agarose gel with 0.15 mg/mL of ethidium bromide to verify that the samples were not lost in the process of the gene clean.

Cloning and Sequencing

The gene cleaned samples were cloned according to the protocol outlined in the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation; Carlsbad, CA). The ligated plasmids were transformed in high-transforming-efficiency *Escherichia coli* TOP10® cells (Invitrogen Corporation; Carlsbad, CA) according to the manufacturer's protocol and then plated on Lauria Broth agar plates containing 10 µg/mL of kanamycin and 100 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). The clone colonies grew overnight and the next day the white colonies were used for a

colony check reaction. The colony check was set up as follows: 2.5 µL of 10x PCR Buffer, 1.5 mM of 25 mM $MgCl_2$, 0.4 µM of each M13R and T7 primers, and 22 µM dNTP. At least 10 colonies were picked from each plate and used as the template in the colony check reactions. One of the reactions contained no template and was used as a negative control. The tubes were placed in the thermocycler on the colony check program with an initial denaturation of 95 °C for 10 minutes and 30 cycles of a 95 °C denaturation for 1 minute, a 55 °C annealing step for 30 seconds, and a 72 °C elongation for 1 minute. When finished, the samples were run on a 1% agarose gel with 0.15 mg/mL of ethidium bromide to check for approximately 750 bp size products.

The positive clones were used to set up a sequencing reaction by adding 2 µL 10x Sequencing Buffer, 0.5 µL of primer (either M13R or T7), 1 µL Big Dye Terminator, 5.5 µL PCR water into 0.6 mL tubes, and using 0.5 µL of the colony check reaction as the template. The samples were placed in the thermocycler on the Sequencing Program which heats to 96 °C at 1 °C/sec and denatures the samples for 1 minute, then cools to 50 °C at a rate of 1 °C/sec and anneals for 5 seconds, then heats to 60 °C at a rate of 1 °C/sec and elongates for 4 minutes. The annealing step and elongation are repeated 25 times followed by a final annealing at 50 °C for 5 seconds and an elongation at 60 °C for 4 minutes. The reactions were then precipitated for sequencing by adding 40 µL of 75% isopropanol to each sample, vortexing briefly, and let stand at room temperature for 15 minutes. Then the samples are spun down at 14,000 rpms for 15 minutes in a microcenterfuge. The samples were then aspirated and 125 µL more of 75% isopropanol was added to wash the DNA. The reactions were spun down again at 14,000 rpms for 5 minutes. The isopropanol was then aspirated and the tubes were

placed in a 95 °C heating block with the lids open and left to dry for 1 minute. Formamide was added to resuspend the pellet and the samples are denatured in the heating block for 1 minute. Immediately after the denaturation, the samples are cooled on ice for 1 minute, plated, and sequenced using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems; Foster City, CA).

Phylogenetic and DOTUR Analysis of *arr*A Genes

The sequences obtained were blast searched using the BLAST-X search engine (http://www.ncbi.nih.gov). The positive sequences were assembled using DNASTAR Lasergene SeqMan Program (DNASTAR, Inc.; Madison, Wisconsin) and were aligned using (www.ebi.ac.uk/clustalw/) for phylogenetic analysis. The alignment file was put into MEGA 3.1 (Molecular Evolutionary Genetics Analysis) to make a neighbor-joining phylogenetic tree to examine diversity within the sequences.

The DOTUR (Distance-Based OTU and Richness) program was utilized to compare the diversity of the *arrA* gene sequences from each sampling site (Schloss and Handelsman, 2005). A comparison of a 2%, 3%, and 5% difference in the nucleotide sequences was used to define the OTU's (Operational Taxonomic Units), which was determined in DOTUR by the furthest neighbor algorithm comparison. Diversity analyses including Chao[1] and Shannon index numbers were determined for each sampling site using the DOTUR program.

T-RFLP Analysis (Terminal Restriction Fragment Length Polymorphism)

Nested PCR was set up with a fluorescence (6'-FAM) labeled forward primer (As2F) and an unlabeled reverse primer (As1R) as described above. After the PCR was

finished the samples were again run on an 1% agarose gel with ethidium bromide and gene cleaned.

The gene cleaned products from the Shem Creek samples were digested with 5U of restriction enzyme *HAEIII* overnight at 37 ºC. The following day, the samples were precipitated just as they were for sequencing, except 10 µL of formamide with GS500 ROX size standard (Applied Biosystems; Foster City, CA) was added. They were plated and a gene scan for Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was then performed using the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems; Foster City, CA) to look at the community structure.

Enrichment Culture of Arsenate Reducing Bacteria

Enrichment cultures were set up for each sampling site from Shem Creek using a 10% inoculum of sediment. A total of 1 L of 1x *Thauera aromatica* (TA) (Table 6) media (Tschech and Fuchs, 1987) was poured into a 2 L Erlenmeyer flask. Ten percent (100 mL) of the media was removed and a line was drawn on the flask to mark the 900 mL mark, then the media was added back to the flask. The media was boiled on a hot plate until it had evaporated down to the 900 mL mark. The media was then cooled on ice under argon gas and once cool, sediment from one Shem Creek sampling site was added to the media up to the 1000 mL level. Aliquots of 50 mL of the slurry was transferred into serum bottles under argon gas, which was plugged with a butyl stopper, and then fastened with an aluminum seal. This process was repeated with the sediment from the other two sampling sites, with a final number of 15 enrichments being made for each sampling site.

All of the Shem Creek enrichment cultures were spiked with a 5 mM

Table 6. *Thauera aromatica* (TA) media components

Basal media (1x):

Component	Amount
$Na_2HPO_4 \cdot 2H_2O$	2.0 g/L
KH_2PO_4	1.0 g/L
NH_4Cl	0.5 g/L
K_2SO_4	0.06 g/L
SL-10 trace element solution (see below)	1.0 mL/L
V-7 vitamin solution (see below)	1.0 mL/L (added after media cooled)
Mg/Ca solution (see below)	0.5 mL/L

SL-10 trace element solution:

Component	Amount
$FeCl_2$	7.5 g/10 mL of 25% HCl
$ZnCl_2$	350 mg/L
$MnCl_2 \cdot 4H_2O$	500 mg/L
H_3BO_3	30 mg/L
$CoCl_2 \cdot 6H_2O$	950 mg/L
$CuCl_2 \cdot 2H_2O$	10 mg/L
$NiCl_2 \cdot 2H_2O$	120 mg/L
$Na_2MoO_4 \cdot 2H_2O$	180 mg/L

V-7 vitamin solution:

Component	Amount
Cyanocobalamin (B12)	100 mg/mL
Pyridoxin-HCl	300 mg/mL
Thiamin-dichloride	200 mg/mL
Ca-D-Pantothenate	100 mg/mL
p-Aminobenzoic acid	80 mg/mL
D-Biotin	20 mg/mL
Nicotinic acid	200 mg/mL

Mg/Ca solution:

Component	Amount
$MgCl_2 \cdot 6H_2O$	325.3 g/L
$CaCl_2 \cdot 2H_2O$	29.4 g/L

concentration of sodium As(V). Carbon substrates were added separately to each enrichment culture set in triplicate as 100 µM of benzoate, 100 µM of naphthalene, and 100 µM of succinate. This yielded 3 benzoate, 3 naphthalene, and 3 succinate cultures per set of enrichment cultures. Three serum bottles from each set were designated as the sterile controls and they contained all three carbon substrates along with the 5 mM of sodium As(V). They were autoclaved in triplicate on three consecutive days. The cultures were incubated at room temperature in the dark and 1 mL of the slurry was taken out weekly, spun down, and the supernatant was placed in 1 mL glass vials the -20 °C freezer and saved for future analysis.

At the end of the enrichment, two cultures from the Shem Creek 3 set were used to extract DNA. The naphthalene replicate 1 and the background replicate 1 were both opened and their contents were poured into two, 50 mL, Falcon tubes. These tubes were centrifuged for 15 minutes and the supernatant was poured off. 1 g of the remaining sediment slurry was taken and used to extract the DNA as described earlier. The *arr*A genes were amplified from the DNA and the products cloned and sequenced as described above. These sequences were included in all the molecular analysis performed during this project.

After the first enrichment experiment was complete, the inital enrichment cultures from the Shem Creek 2 sediment were used as inocula to establish 1:10 diluted enrichment cultures. The initial enrichment culture used as the inocula was monitored for naphthalene degradation coupled to As(V) reduction. ATCC *Geobacter* 1957 liquid media (Table 3) was made and pipetted into twelve, 50 mL, serum bottles under argon gas. The bottles were plugged with a butyl stopper and secured with an aluminum seal.

Once cool, 5 mM of As(V) was added to all 12 cultures and 500 µM of benzoate, 200 µM naphthalene, and 500 µM succinate were added to two cultures each. In four of the cultures all three carbon substrates were added, and in two of the cultures, no carbon substrate was added at all. A total of 5 mL of the initial enrichment culture slurry was added into every culture except two of the cultures that contained all three carbon substrates, which was left as a no cell control. The two cultures that were spiked with all three carbon substrates and As(V) were autoclaved in triplicate and used as a sterile control. All of the cultures were sampled weekly according to the protocol described for the first enrichment

Isolation of As(V) Reducing Bacteria

Cultures were isolated from the Shem Creek enrichment cultures using anaerobic shake tubes made with 1957 *Geobacter* agar media (Table 3). A 5 mM concentration of sodium arsenate and 0.1x y.s.t. was added to the media and it was dispensed under argon gas into each Hungate tube. The tubes were autoclaved and cooled upside down in a 55 °C water bath. While the media was still in liquid form, 1 mL of the inocula was serially diluted from 1:10 up to 1:100,000 ratios. The tubes were inverted a few times and then cooled seal side down in ice so that the agar would solidify quickly, trapping the bacteria throughout the media. The shake tubes were removed from the ice and placed in a 28 °C incubator for a period of time.

The colonies grew separately in the agar and 4 colonies for each site were sucked out with a syringe and resuspended into a liquid ATCC 1957 *Geobacter* media (Table 3) with a 5 mM concentration of arsenate in a new Hungate tube. The optical density of these tubes were measured daily by a spectrophotometer to look at the

growth of the isolates in the arsenic enriched media. After about one month, samples were taken from the Hungate tubes and spun down to pellet the cells. The supernatant was transferred to a clean tube and the As(III) concentrations were measured via HPLC to test whether or not the isolates were capable of reducing As(V).

HPLC Analysis of As(III)

An HPLC with UV detector was used to determine the As(III) concentrations that are produced by the bacterial reduction of As(V) to As(III) in the enrichment cultures (Liu et al., 2004). A Hamilton PRP-X100 Column was used with an isocratic, 30 mM Monobasic Sodium Phosphate Buffer, adjusted to pH 6 with NaOH, as the mobile phase. A 10 µL volume of the As(III) standards and the enrichment culture samples were injected. The As(III) was detected at 1.5 min using a flow rate of 1.5 mL/min and a wavelength of 200 nm on a Waters UV-vis detector.

HPLC Analysis of Benzoate and Naphthalene

An HPLC with UV was used to monitor the degradation of benzoate and naphthalene coupled to the reduction of As(V). A Supelcosil™ LC-18 Column with Supelguard™ LC-18 guard column was used with an isocratic, 70% acetonitrile/30% water, mobile phase. A 10 µL volume of benzoate and naphthalene standards, and enrichment culture samples were injected. Benzoate was detected at 5 min and the naphthalene was detected at 7 min using a flow rate of 1.0 mL/min and a wavelength of 280 nm on a Waters UV-vis detector.

Statistical Analysis

The standard deviation was taken of the As(III) concentrations obtained from the Shem Creek HPLC data for both sets of enrichments for the benzoate, naphthalene,

and succinate cultures containing As(V) for each sampling day. This standard deviation was then used to calculate the standard error.

RESULTS

PCR Detection of *arr*A Genes from Estuarine Sediments

The DNA was extracted from Shem Creek sediments for site 1, 2, and 3. The initial PCR targeting the *arr*A genes, with the primers As1F and ArrA1R, did not amplify any products. A nested PCR with the primers As2F and As1R yielded the 627 bp amplicons, which were the same size as the positive control of *Desulfitobacterium hafniense* DCB-2. In addition, the *arr*A genes were successfully amplified from the sediment slurries which were collected from two enrichment cultures established with the Shem Creek 3 sediments.

Phylogenetic and DOTUR Analyses of *arr*A Genes

Clone libraries were constructed in 96-well plates from the amplified *arr*A genes from all three Shem Creek sites. A total of 38 clones (8 from Shem Creek 1, 4 from Shem Creek 2, 7 from Shem Creek 3, 14 from the Shem Creek 3-1 naphthalene enrichment culture, and 5 from the Shem Creek 3-1 background enrichment culture) were fully sequenced, and the DNA sequences were translated into amino acid sequences for further analysis. A neighbor-joining phylogenetic tree was constructed based on the amino acid sequences using MEGA 3.1 (Molecular Evolutionary Genetics Analysis). The phylogenetic tree (Figure 12) shows three distinct groups present in the Shem Creek samples. These groups were labeled A, B, and C. The 34 sequences belonging in group A shared 96% amino acid sequence identities and also had ~74% amino acid sequence identity to *Desulfosporosinus* Y5. The sequences in group B shared an ~88% identity to each other and ~73% to *Desulfosporosinus* Y5. The sequences in group C had a ~98% identity to each other, ~94% identity to

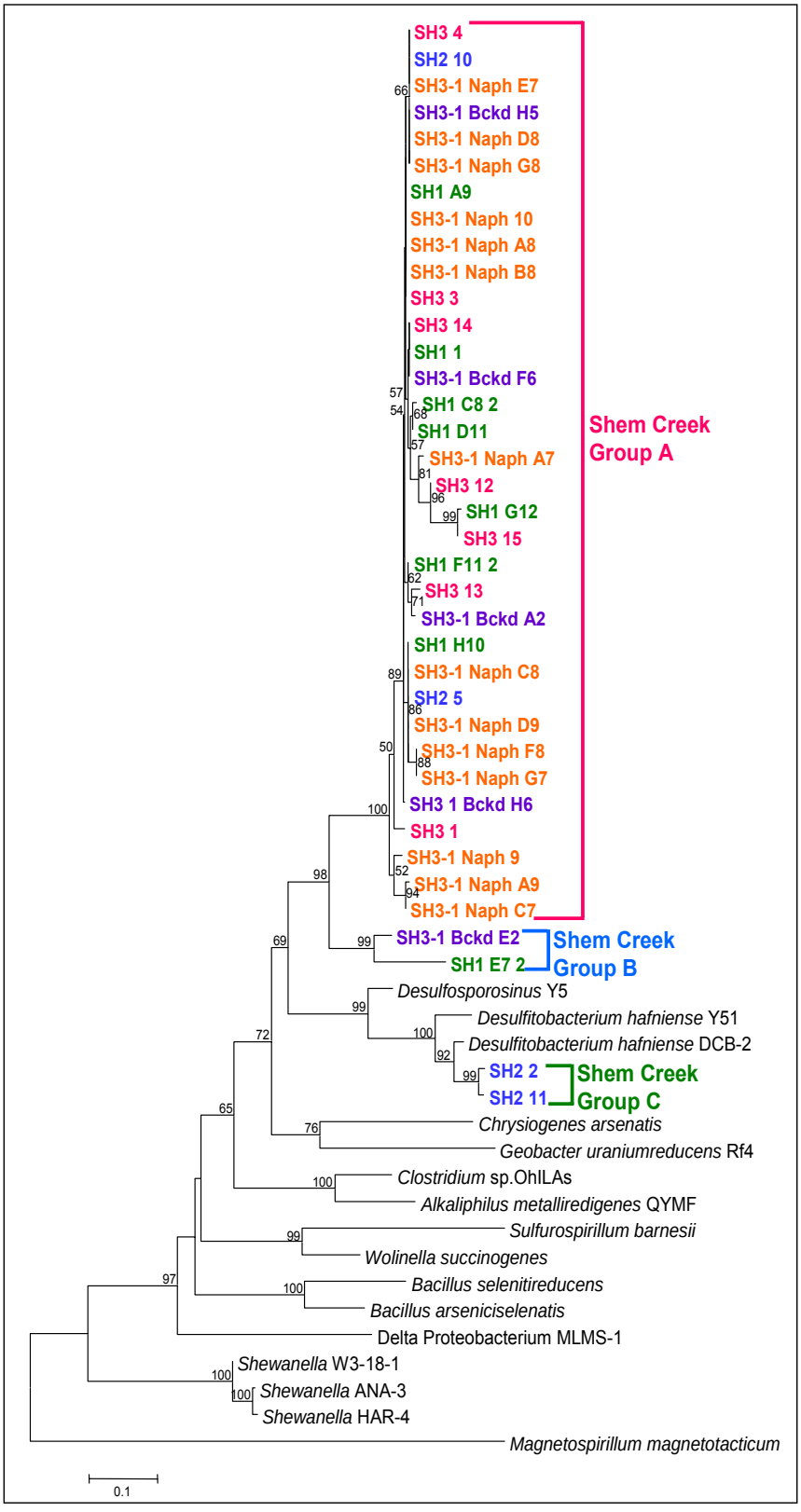

Figure 12. Phylogenetic tree of *arr*A genes detected from Shem Creek sediment

Desulfitobacterium hafniense DCB-2, ~87% to *Desulfitobacterium hafniense* Y51, and ~84% to *Desulfosporosinus* Y5.

DOTUR (Distance-Based OTU and Richness) was used to examine the diversity as a measure of species richness based on the nucleotide sequences from the Shem Creek sites. At a 2% cutoff for OTU determination, there were 9 OTU's, for the 3% comparison there were 8 OTU's, and for the 5% comparison there were 7 OTU's. The OTU's for the 2%, 3%, and 5% difference are all shown in the rarefaction analysis (Figure 13) and by the Chao[1] estimator and Shannon index numbers which measure species richness and diversity (Table 7). As the % of OTU determination increases the diversity of arsenate reducers decreases.

T-RFLP Analysis (Terminal Restriction Fragment Length Polymorphism)

Community structures of arsenate reducing bacteria in Shem Creek sediments were examined using T-RFLP analysis. Each peak in the T-RFLP data represents a different arsenate reducing population present in the three sampling sites (Figure 14). It is clear that similar As(V) reducing communities are present in all three sites from Shem Creek, as the same sizes of T-RF peaks (21 bp, 95 bp, 180 bp, 394 bp and 534 bp) were present. It can also be seen that some peaks in Shem Creek 1 and 3 are not present in Shem Creek 2. Only the T-RF peak with a 95 bp size matched the *arr*A gene sequences detected in Shem Creek sediments. Based on the T-RFLP analysis and number of peaks present, it appears that Shem Creek 2 is the least diverse out of all of the sites, followed by Shem Creek 1. Shem Creek 3 appears to have the most diverse As(V) reducing communities present in the estuary.

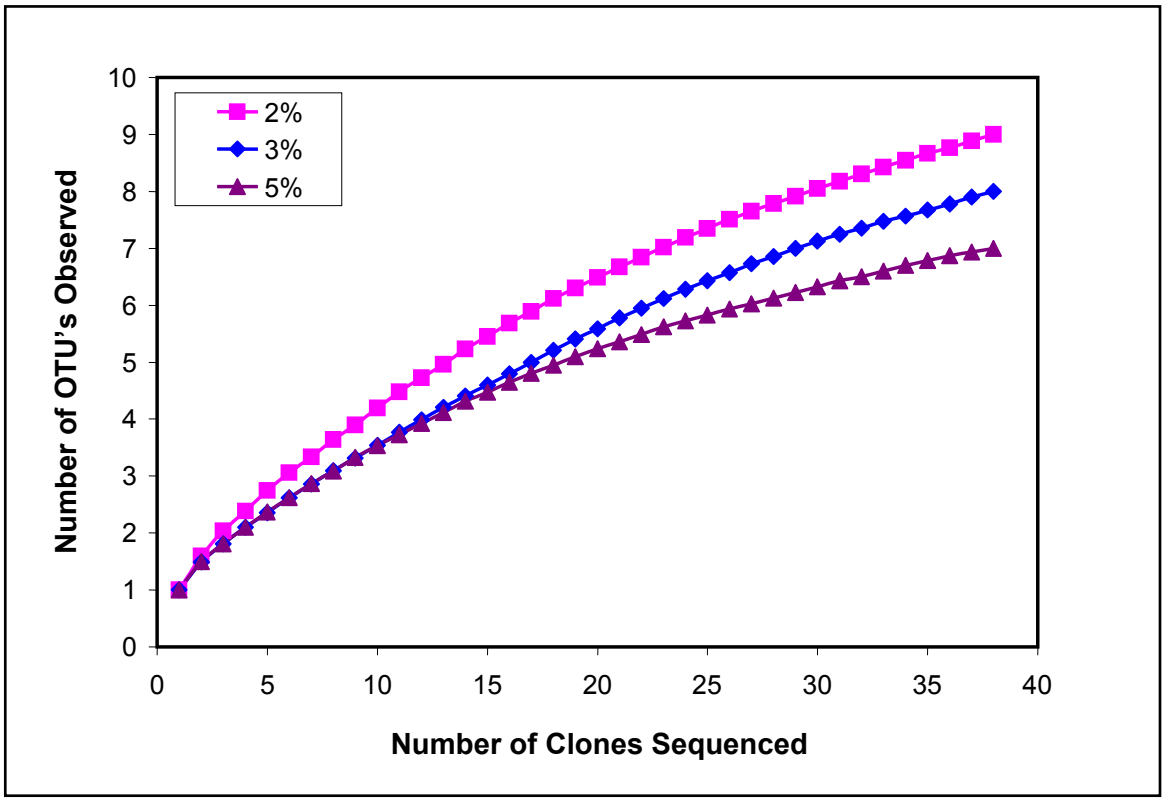

Figure 13. Comparison of the rarefaction analysis of *arr*A genes at a 2%, 3%, and 5% similarity detected from Shem Creek sampling sites

Table 7. Summary of diversity of DARB for Shem Creek based on *arr*A gene detection

Percent Difference	Shannon Index	Chao[1]
2%	1.38397	11
3%	1.13611	10
5%	1.08586	8.5

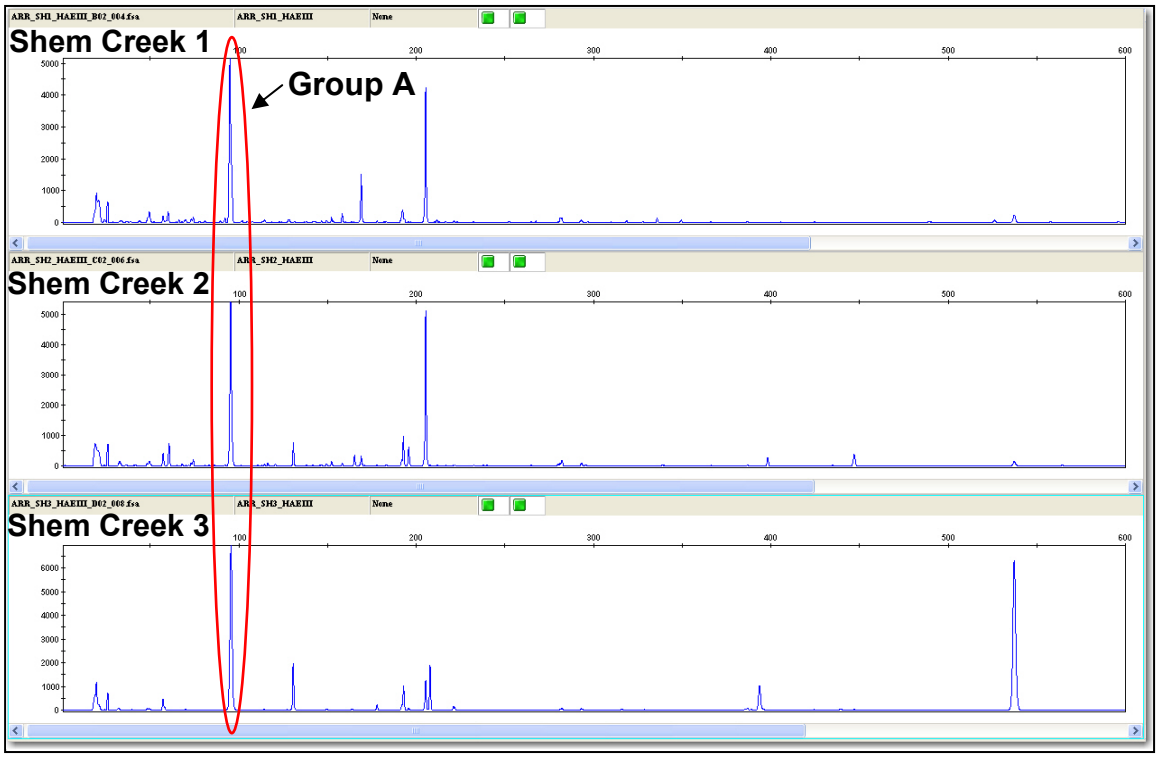

Figure 14. T-RFLP analysis of *arr*A genes detected from Shem Creek sites 1, 2, and 3

Arsenate Reducing Activities in Shem Creek Sediment

Arsenate reduction activities in Shem Creek sediment communities were measured by establishing anaerobic enrichment cultures, which were sampled once a week for four weeks. The elevation of As(III) concentrations were monitored using HPLC with UV detection for the enrichment cultures spiked with benzoate, naphthalene, and succinate (Figure 15 and Figure 16). For the Shem Creek 1 enrichment cultures, the succinate cultures had the most reduction take place within the first week with an average As(III) concentration of 1 mM, but by the end of the enrichment, the benzoate culture had the most reduction take place with an average As(III) concentration of 2.86 mM. The same pattern occurred in the Shem Creek 2 enrichments with a 0.75 mM average concentration of As(III) in the succinate culture at the end of the first week, and a 2.26 mM average of As(III) in the benzoate culture for the final week. The final As(III) concentration for succinate in Shem Creek 1 was 2.14 mM and in Shem Creek 2 was 1.33 mM. In both sets of enrichments, the naphthalene culture had As(V) reduction occur, and in Shem Creek 1 the final average As(III) concentration was 0.75 mM, while in Shem Creek 2 it was 1 mM. In the Shem Creek site three cultures, an As(V) reduction took place, but it was coupled to As(III) oxidation therefore the data was not included. Due to the potential of natural chemical oxidation/reduction, a measurable As(V) reduction took place in the sterile control cultures for both sampling sites, even though the samples had already been autoclaved twice.

The rate of As(V) reduction varied greatly based on the carbon substrate present and based on the sampling site (Table 8). For Shem Creek 1 the final average rate of

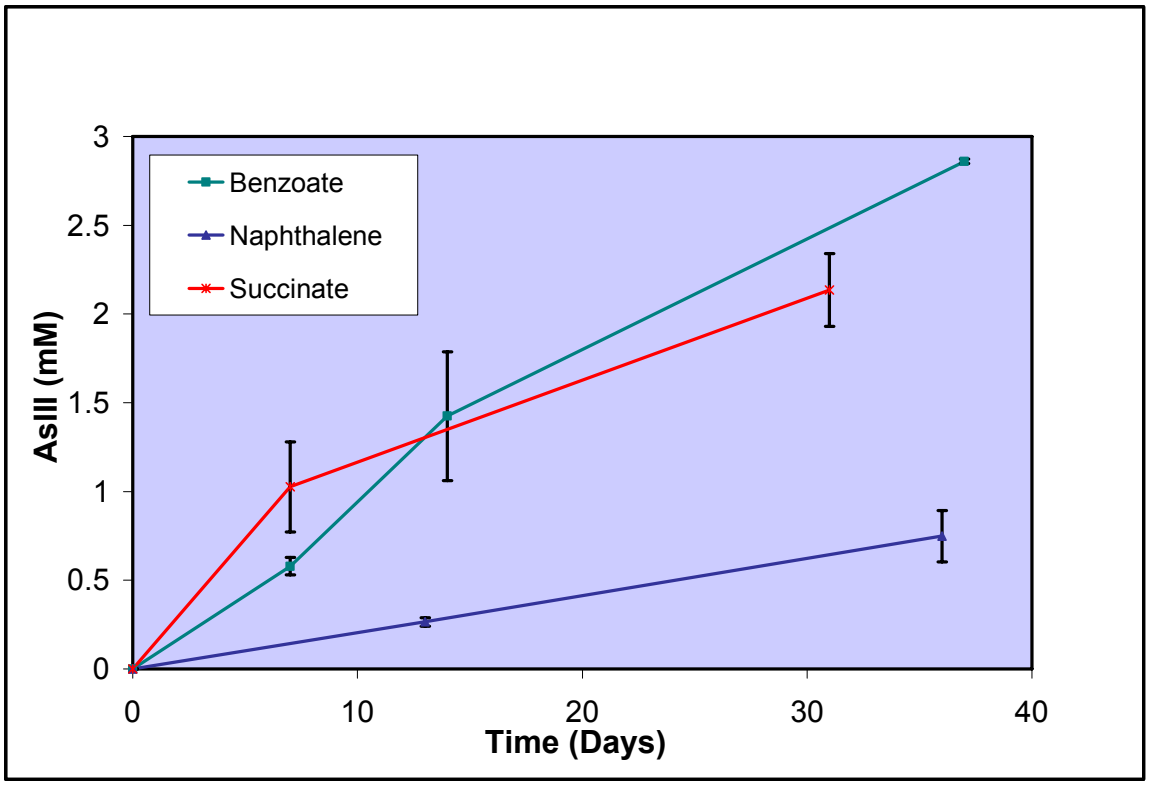

Figure 15. As(V) reduction in anaerobic enrichment cultures established with Shem Creek 1 sediment

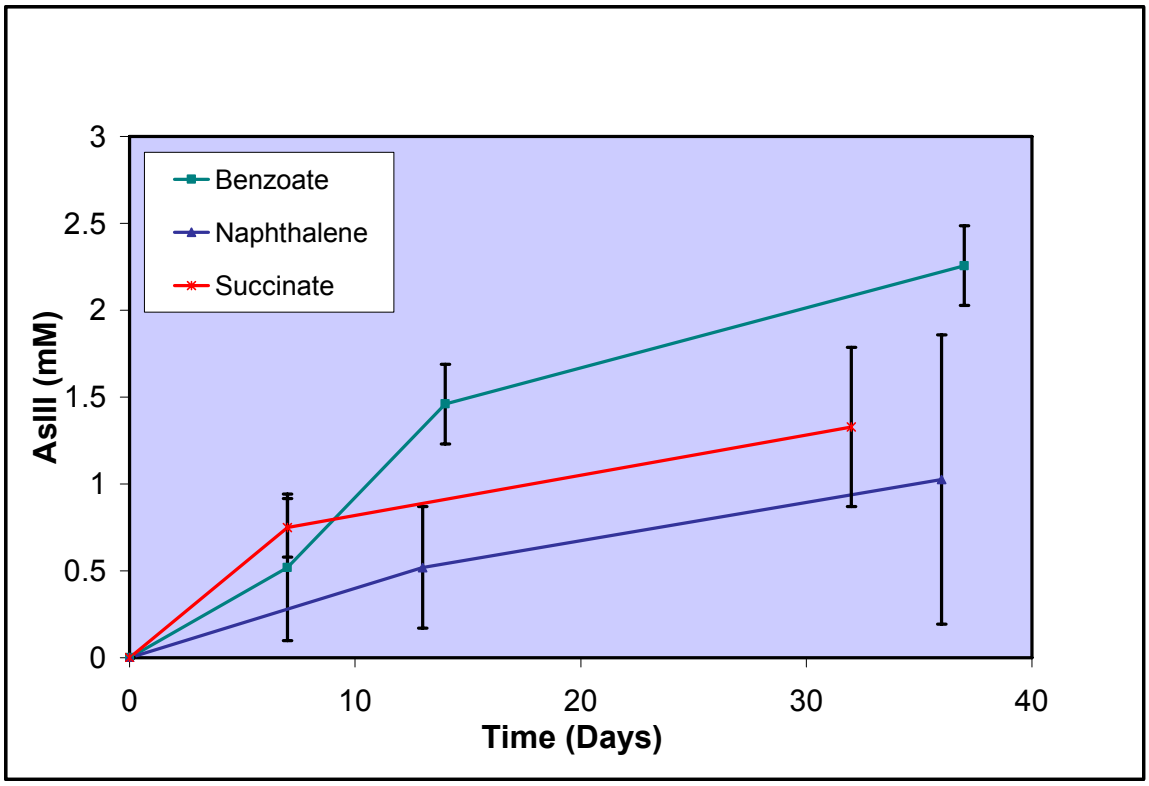

Figure 16. As(V) reduction in anaerobic enrichment cultures established with Shem Creek 2 sediment

Table 8. Rates of As(V) reduction in different enrichment cultures

Sample Name: amendments	Average Rate (μM/day)
SH1: 100μM Benzoate + 5mM As(V)	101.7[1]
SH1: 100μM Naphthalene + 5mM As(V)	20.4[1]
SH1: 100μM Succinate + 5mM As(V)	146.5[2]
SH2: 100μM Benzoate + 5mM As(V)	104.3[1]
SH2: 100μM Naphthalene + 5mM As(V)	39.9[1]
SH2: 100μM Succinate + 5mM As(V)	106.9[2]
SH2, Set 2: 500μM Benzoate + 5mM As(V)	6.8[3]
SH2, Set 2: 200μM Naphthalene + 5mM As(V)	2.7[3]
SH2, Set 2: 500μM Succinate + 5mM As(V)	4.9[3]
SH2, Set 2: Sterile Control	2.0[3]

[1] Rate of As(V) reduction was measured within 14 days.
[2] Rate of As(V) reduction was measured within 7 days.
[3] Rate of As(V) reduction was measured within 16 days.

As(V) reduction was 101.7 μM/day for the benzoate cultures, 20.4 μM/day for the naphthalene cultures, and 146.5 μM/day for the succinate cultures. For Shem Creek 2 the rates were 104.3 μM/day for the benzoate cultures, 39.9 μM/day for the naphthalene cultures, and 106.9 μM/day for the succinate cultures. The rates were calculated based on the day 14 concentration with the exception of succinate which was calculated at day 7 because most of the reduction had already taken place during that time. The rates were higher for succinate than for any of the other cultures, but the rates were also higher for the site 1 enrichments when compared to site 2, with the exception of naphthalene. When looking at the rate of degredation of carbon substrates in the enrichment cultures, the rate of the benzoate culture was higher than that of the naphthalene culture because naphthalene is more difficult to degrade. The benzoate was utilized by sediment communities in Shem Creek 1 at the rate of 15.7 μM/day and in Shem Creek 2 was used at a rate of 14.1 μM/day (Figure 17). For the rate of naphthalene degradation, Shem Creek 1 was 3.8 μM/day while Shem Creek 2 was 2.5 μM/day (Figure 18). The rates were based on the day 14 concentration.

For the 1:10 diluted Shem Creek enrichment cultures, which were made from Shem Creek 2 inocula, samples were again taken over the course of one month. The HPLC analysis for this set revealed that no As(V) reduction took place in the no cell control or the no substrate control, but again the sterile control had small amounts of As(V) reduction due to the natural chemical oxication and reduction processes which occur due to the high organic concentrations in the sediment. As in the first sets of enrichments, reduction took place in the benzoate, naphthalene, and succinate cultures (Figure 19). The benzoate cultures and the naphthalene cultures both had a final

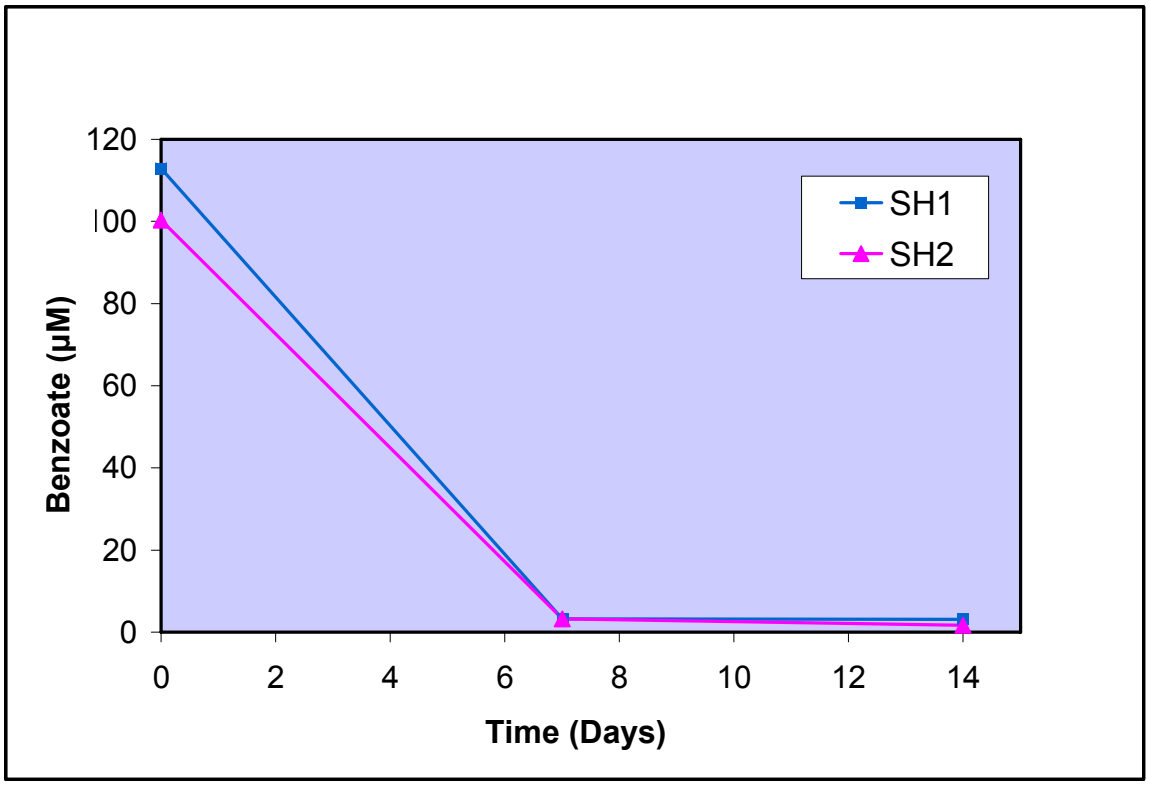

Figure 17. Benzoate degradation in As(V) reducing enrichment cultures from Shem Creek 1 and 2

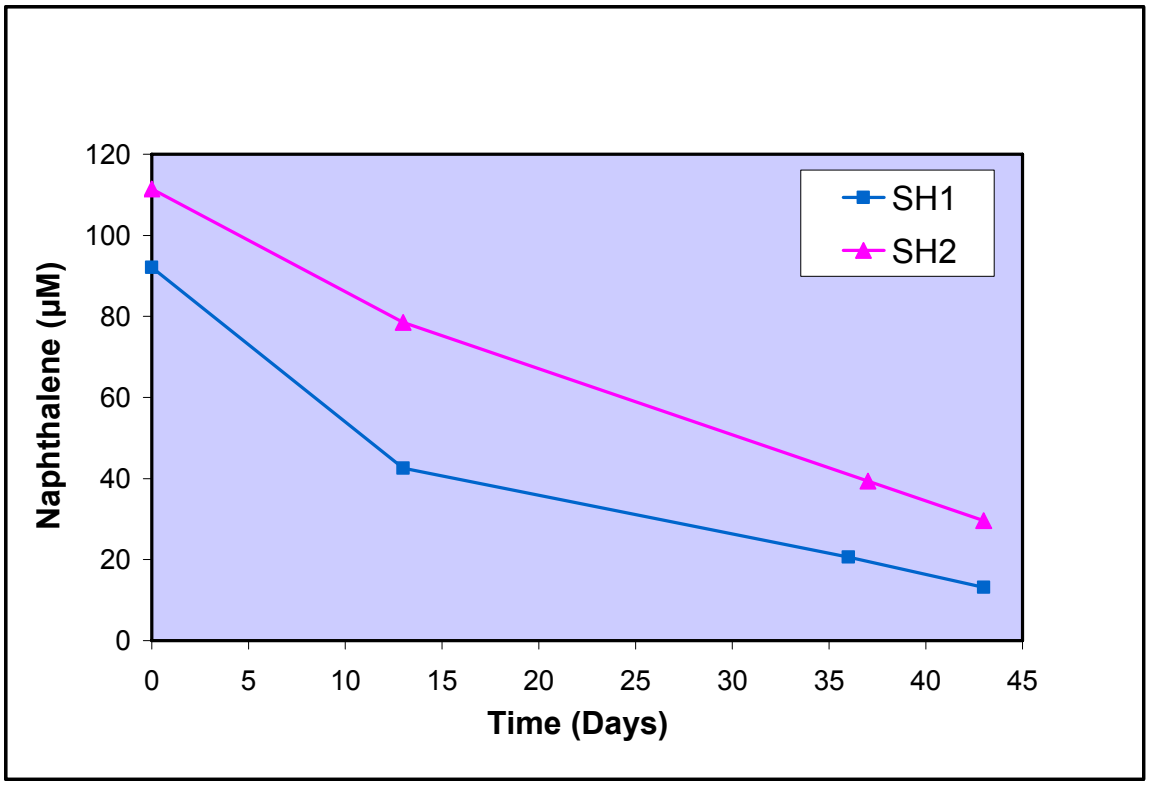

Figure 18. Naphthalene degradation in As(V) reducing enrichment cultures from Shem Creek 1 and 2

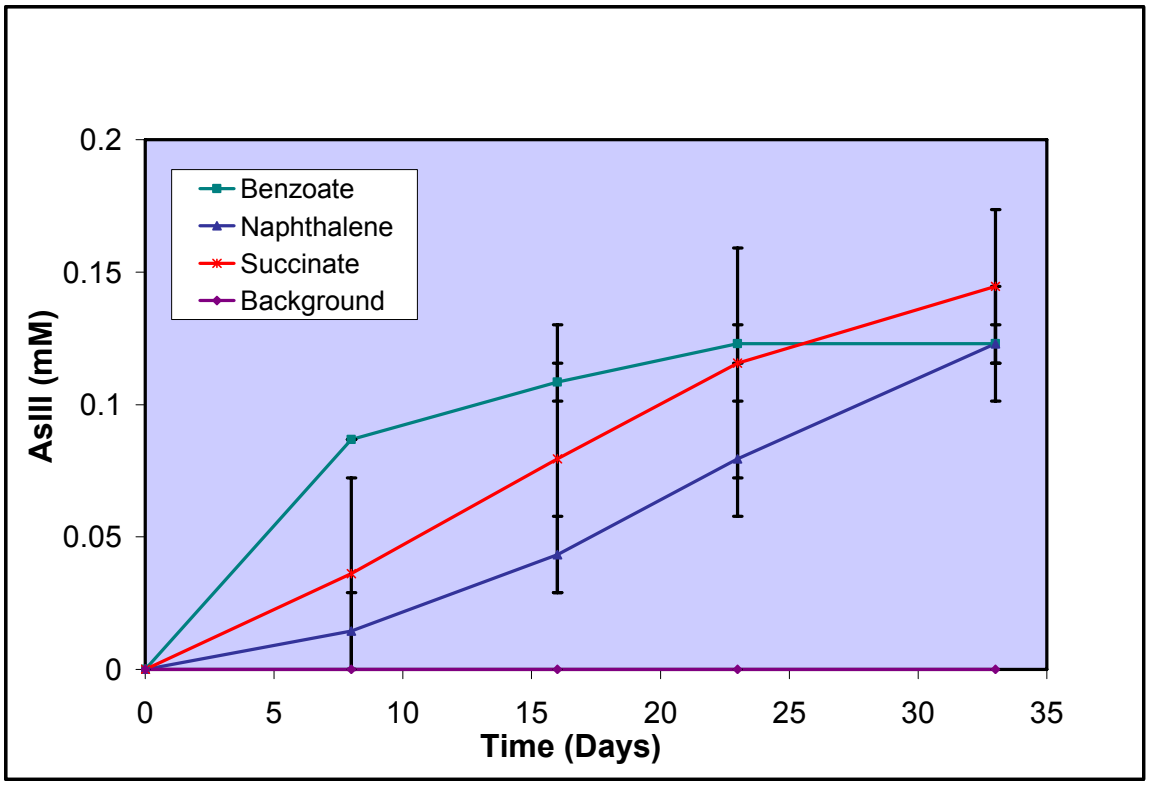

Figure 19. As(V) reduction in 1:10 diluted enrichment cultures from Shem Creek 2

average As(III) concentration of 123 µM, while the succinate cultures' average was 145 µM. The rate of As(V) reduction varies based on the culture condition (Table 8). The benzoate culture had the highest rate of As(V) reduction with an average of 6.8 µM/day. The succinate cultures average rate was 4.9 µM/day and the naphthalene cultures had the lowest rate of As(V) reduction with 2.7 µM/day. All of these rates were calculated for day 16 of this experiment.

Isolation of Arsenate Reducing Bacteria

The enrichment Shem Creek Set 2, naphthalene 1 was used as the inocula for the anaerobic shake tubes. The tube with 1:10,000 dilution had good sized colonies with enough separation to be able to take them out of the tube without disturbing the other colonies. Four colonies were taken from the Shem Creek shake tubes and injected into liquid media. The optical density measurements that were taken from these isolates growth were very low, samples were taken from the isolate on day 28 ran on the HPLC. This showed that the second isolate, SH2-1 #2, was capable of reducing As(V) to As(III) (Table 9). The concentration of As(III) measured at day 28 was 0.53 mM.

Table 9. Test for As(V) reduction in the isolates from Shem Creek

Isolate	As(V) Reduction
SH2-1 #1	-
SH2-1 #2	+
SH2-1 #3	-
SH2-1 #4	-

DISCUSSION

The molecular data from this project was successful in providing information about As(V) reducing bacteria in Shem Creek sediments. The nested PCR amplified the *arr*A genes present in all three sampling sites. Even though the sequencing data showed that the DARB in Shem Creek has a monophyletic diversity, interestingly enough, most of the DARB were closely related to *Desulfosporosinus* Y5. *Desulfosporosinus* Y5 is a known As(V) reducer and a gram negative spore-former (Liu et al., 2004). It is capable of As(V) reduction coupled to the degradation of aromatic compounds such as benzoate and toluene. It is strictly anaerobic and was originally isolated from the sediment of Onondaga Lake, a superfund site in Syracuse, New York (Liu et al., 2004). It makes sense that Shem Creek has such a low diversity of As(V) reducers present with the majority of the DARB being closely related to *Desulfosporosinus* Y5. The microbial communities at Shem Creek and Onondaga Lake have been exposed to similar environmental stresses such as large quantities of contaminants, including arsenic and PAHs. These stresses could limit the number of DARB capable of surviving and growing there. The interesting aspect about *Desulfosporosinus* Y5 is that is has only been shown to grow in the presence of As(V) (Liu et al., 2004), so the high concentrations of As(V) in Shem Creek would lead to a higher growth of the bacteria, which would lead to the dominant presence of *Desulfosporosinus* Y5.

The enrichment culture studies also confirmed the presence of As(V) reducing bacteria in the Shem Creek sediments. However, the final concentration of As(III) and the reduction rate of As(V) varied depending on the sampling site and the carbon

substrate provided. The slight difference in the As(V) reduction activities for Shem Creek 1 and 2 could be explained based on the T-RFLP analysis (Figure 14), which shows the lowest diversity of As(V) reducing bacteria present in Shem Creek 2 when compared to the other two sampling sites. Low diversity of As(V) reducers could be correlated to a lower activity, as the rates and final As(III) concentrations for Shem Creek 2 were lower than those of Shem Creek 1. As(V) reduction activities also varied depending on the carbon substrate that was present. The enrichment cultures amended with succinate had the fastest initial rate of As(V) reduction, but ended up having the second highest final concentration and rate over the 33 day time course when compared to those with benzoate. This could be related to the amount of carbon available in the cultures and since succinate is a four-carbon chain, it can be broken down more easily by the bacteria, but it is quickly consumed. Benzoate, however, is more difficult to break down initially due to its benzene ring structure, which leads to a slower rate of As(V) reduction during the first week. But, due to the fact that it has seven carbons total, the bacteria are capable of using the carbons to reduce more As(V) than the cultures with succinate. The cultures amended with naphthalene had the slowest overall rate and the lowest final concentration, but the increase of As(III) was more steady than in any of the other cultures. Due to the double ring structure, naphthalene is more complex than the other substrates, which makes its utilization by the DARB more difficult. It takes much longer for the naphthalene to be consumed, so the rate is steadier and lower than that of the other carbon substrates provided.

With all of the organic compounds in the sediments obtained from Shem Creek, it was important to make sure that the As(V) reducing bacteria were utilizing the carbon

substrates provided. The degradation of benzoate and naphthalene coupled to the As(V) reduction were measured using HPLC analysis. The benzoate was degraded quicker than naphthalene with a rate of 15.7 μM/day in Shem Creek 1 and 14.1 μM/day in Shem Creek 2. For the rate of naphthalene degradation, Shem Creek 1 was 3.8 μM/day while Shem Creek 2 was 2.5 μM/day. This shows that just like with the As(V) reduction, the Shem Creek 1 cultures are more active than the Shem Creek 2 cultures due to higher diversity. The naphthalene degradation is also slower because it is more difficult for the bacteria to consume. The degradation of naphthalene coupled to As (V) reduction in Shem Creek sediments was demonstrated for the first time in this study. Due to the high levels of PAHs in the estuary (Sanger et al., 1999b), the coupling of arsenic reduction to the naphthalene degradation would support the natural attenuation of PAHs in this estuarine environment.

The diluted set of Shem Creek 2 enrichment cultures were not as active in reducing As(V) as the first cultures. This could be related to the dilution factor because just as the sediment slurry was diluted ten fold, so was the cell density. This ten fold reduction in cells causes a ten fold lower reduction of As(V). This can be seen in the final As(III) concentrations and in the rate of As(V) reduction. For example, in the first set of Shem Creek 2 enrichments, the average final concentration of As(III) in the benzoate enrichment was 2.26 mM. In the diluted, second set, the concentration was more than ten fold less with 123 μM. This same pattern occurred in the naphthalene and succinate cultures as well. The rate was also reduced by a large amount as is shown by the first benzoate enrichment reducing 104.3 μM/day, while the second set

only reduced 6.8 μM/day. The naphthalene and succinate cultures also follow this pattern.

The most exciting result of this research is the isolate that was obtained from the Shem Creek set 2 culture that contained naphthalene and As(V). This isolate has already been shown to reduce As(V) with a final As(III) concentration on day 28 of 0.53 mM, with only 1 mM added to the culture. That equals a reduction rate of 19 μM/day with y.s.t. as the carbon substrate. Since the isolate obtained, came from the naphthalene enrichment culture, more tests will be done on this isolate to see if it does in fact have the capability to use naphthalene as a carbon substrate coupled to the As(V) reduction.

In conclusion, the sequencing data reveals that there are clearly As(V) reducing bacterial communities present in Shem Creek sediments and that the majority of these communities are composed of *Desulfosporosinus* Y5. The DARB in Shem Creek actively reduce As(V) to As(III) with the presence of carbon. Also, based on the HPLC analysis, these bacteria have the ability of degrading naphthalene coupled to the As(V) reduction. The isolate obtained from the Shem Creek 2 enrichments will provide promising information about the capabilities of As(V) reducing bacteria in the creek. Based on the reduction rates seen in the enrichment cultures and the amount of As(III) that can be produced from this reduction, these bacteria have the potential to significantly impact the levels of arsenic in Shem Creek sediments.

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CONCLUSION

Overall, this project provided the evidence which shows the significance of the As(V) respiring bacteria in various environments. The areas of study were two North Carolina drinking water wells and Shem Creek, an estuary in South Carolina. Molecular techniques were employed in these studies to show the presence of DARB in these environments. Diverse DARB were found in all of the sampling sites based on the phylogenetic analysis.

Phylogenetic and DOTUR analyses showed that there is a link between the diversity of the DARB community and the arsenic concentration in the environment. The Rushing well had more diverse community of DARB than the Deese well. This might show that the diversity of the DARB in underground aquifers is directly related to the amount of arsenic present in the system and the molecular techniques are an effective way of determining this correlation.

Enrichment cultures were established as a method of measuring the As(V) reduction capabilities of the DARB communities in each sampling location. The cultures that were provided with carbon substrate were all capable of supporting growth of the bacteria while reducing As(V). The As(V) reducing activities were significantly related to the diversity of DARB and the levels of arsenic at each sampling site. In addition, depending on the available carbon substrates, the rate of As(V) reduction varies as observed in the Shem Creek studies. This showed that the enrichments provided with benzoate and succinate had a higher final As(III) concentration than the cultures provided with naphthalene. Naphthalene is more difficult to utilize than the other two substrates, which is why the final As(III) concentration is lower. But, this is the first time

that an As(V) reducing bacteria has been shown to degrade naphthalene while reducing As(V).

By utilizing various methods such as molecular and enrichment techniques, it was determined that the DARB were present in the environments. It was also found that the diversity of the DARB contribute to the amount of arsenic present in the environment, and that this had a direct impact on the activity of the As(V) reducing bacteria in the enrichment cultures. It is evident that both studies support the major hypothesis of this research that the DARB community significantly contributes to the desorption of arsenic by the reduction of As(V) to As(III) in both Shem Creek sediments and NC groundwater aquifers.

BIOGRAPHICAL SKETCH

Holly G. Oates was born on August 19, 1983, in Clinton, North Carolina. She graduated from Clinton High School in 2001 and the following fall began attending Meredith College in Raleigh, North Carolina. She completed her B.S. degree in Biology in August of 2004 and applied to the University of North Carolina Wilmington for the Masters of Science in Marine Science program. She was accepted and worked under Dr. Bongkeun Song in the microbial ecology lab at the Center for Marine Science beginning in May of 2005 She started her degree program and graduate research the following fall working on arsenic reducing bacteria and their impacts. She graduated in August 2007 and will be pursuing a job in environmental molecular and microbiology.