Microbial Reduction, Precipitation, and Mobilization of Arsenic

by

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Abstract of the Dissertation

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Dissertation Director:
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Anaerobic microbes play a critical role in the biogeochemistry of arsenic. In this Dissertation, I utilize both classical microbiological techniques and cutting edge genomics to gain a greater understanding of how arsenic reducing bacteria from both freshwater aquifers and estuarine sediments interact with As(V) as a terminal electron acceptor. The novel As(V) reducing Strain MPA-C3 was isolated from arsenic contaminated estuarine sediments in Hong Kong, and when grown in the presence of sulfide with As(V) as a terminal electron acceptor, removes arsenic from solution by precipitating it as alacranite (As_8S_9). Sequencing of both the 16S rRNA gene as well as the entire genome of Strain MPA-C3 places it among the *Deferribacteres.* Strain MPA-C3 is more metabolically versatile than any of the described *Deferribacteres,* and is able to utilize NO_3^-, Se(VI), Se(IV), fumarate, Fe(III) and mixed oxidation state polysulfide as electron acceptors, and acetate, pyruvate, fructose and benzoate as sources of carbon and energy. The draft genome of Strain MPA-C3 has allowed for the elucidation of the diverse pathways this organism uses for the metabolism of carbon sources, as well as those used for the reduction of both nitrate and sulfur. The role of microbes in the mobilization of arsenic into groundwater was studied at three sites in New Jersey:

Crosswicks Creek in Upper Freehold, and Six Mile Run and Pike Run along the Millstone River in Somerset County. At Crosswicks Creek, we determined that microbial As(V) reduction, driven by inputs of organic carbon, resulted in the mobilization of arsenic from iron-rich glauconitic sediments into the groundwater. The role of the redox status of the aquifer was studied by comparing the anoxic subsurface of Six Mile Run with the oxic subsurface at Pike Run. These two sites were found to have remarkably similar mineralogy and groundwater chemistry, and we demonstrated that the differing redox conditions resulted in the development of different microbial communities at each site. As(V) reducing organisms were found at Six Mile Run by both cultivation based and molecular methods, while these organisms were absent at Pike Run. These findings demonstrate that microbial As(V) reduction and mobilization is facilitated by the reducing conditions present at Six Mile Run, while it is inhibited by the oxidizing conditions present at Pike Run. Combined, these studies demonstrate how As(V) reducing microorganisms play a critical role in the biogeochemical cycling of arsenic.

Dedication

This dissertation is dedicated to Florence Mumford and Peggy Marcus

You each inspired me in your own way

And everyone else who has in some way contributed to this Journey

You know who you are, but you cannot know how much you've done for me

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Chapter 1

Introduction and Literature Review

1.1 Introduction

Over the course of history, arsenic has gained infamy as a poison. It has only been in recent years that research has demonstrated that arsenic can also play a vital role in the microbial world. Current research has demonstrated the ability of microbes to utilize arsenic ions as both electron donors and acceptors. This research seeks to extend these discoveries by placing As(V) reducing microorganisms into an environmental and geochemical context.

1.2 Microbial Respiratory Arsenate Reduction

Microbes generate energy for both heterotrophic and autotrophic growth by coupling the oxidation of organic carbon to the respiratory reduction of As(V) to As(III) (3), and by fixing inorganic carbon by coupling the reduction of CO_2 to the oxidation of As(III) to As(V) (105). Microbial respiratory reduction of As(V) to As(III) was first described in 1994 by Ahmann *et al*. in the ε-proteobacterium MIT-13 (3, 137). This organism was found to utilize As(V) as a sole electron acceptor coupled to the oxidation of lactate, and is reported to release arsenic from ferrous arsenate by the reductive dissolution of As(V) (3).

Dissimilatory reduction of arsenate to arsenite proceeds through the arsenate respiratory reductase (ArrAB) system (124). Under standard conditions, the respiratory reduction of As(V) to As(III) is exergonic, with a $\Delta G0'$ of -175 kJ mol^{-1} of carbon

oxidized (65). When acetate is used as an electron donor, the stoichiometry of this reaction is $CH_3COO^- + 4HAsO_4^{2-} + 7H^+ \rightarrow 2HCO_3^- + 4H_3AsO_3$. The terminal transfer of electrons to As(V) is catalyzed by the As(V) respiratory reductase (ArrAB) system, which was first purified from *Chrysiogenes arsenatis* by Krafft and Macy (66), and has since also been purified from *Bacillus selenitireducens* (1) and *Shewanella* sp. Strain ANA-3 (82). Arsenate respiratory reductase is reported to be a periplasmic enzyme, although the ArrAB from *B. selenitireducens* was reported to be associated with the external side of the cell membrane (1, 66, 82). In all cases, it appears that the enzyme is positioned to ensure that arsenate is not brought into the cell (82). The α-subunit of the enzyme is described to have a molecular mass of 87 kDa (66), 110 kDa (1), or 95 kDa (82), and the smaller β-subunit is reported to have a molecular mass of 29 kDa (66), 34 kDa (1), or 27 kDa (82). The αβ holoenzyme has a molecular mass reported to be 123 kDa (29), 150 kDa (1), or 131 kDa (35). The ArrA subunit is reported to contain the molybdopterin catalytic center, as well as at least one $[Fe_4S_4]$ cluster (1, 66, 82). ArrB coordinates an iron-sulfur cluster, and is suggested to play a role in electron transfer to the catalytic α subunit(1). Notably, in 2009, Richey *et al*. proposed that ArrAB can function as an arsenite oxidase in *Alkalimnicola ehrlichii* (119), and this finding has since been expanded with the description of the *arxA* clade of arsenite oxidases (152, 153). This finding, when taken together with the limited number of arsenic reducing microorganisms which have been isolated, suggests that many new microbial mechanisms of arsenic metabolism remain to be discovered by both traditional and molecular techniques.

1.3 Diversity of Arsenate Reducing Microbes

Dissimilatory As(V) reducing prokaryotes (DARPs) have been reported from 17 (92, 104) bacteria phyla, as well as 1 archeal phylum (55). A phylogenetic tree of these organisms demonstrating their diversity based on the 16S rRNA gene is presented in Figure 1. In Table 1 I summarize 14 characterized As(V) reducing isolates along with two members of the *Deferribacteres* as is presented in Mumford et al. (92). A review of terminal electron acceptors utilized by these isolates allows for the division of these organisms into two broad groups based on their ability or inability to utilize SO_4^{2-} and/or $S_2O_3^{2-}$. Among the organisms incapable of utilizing SO_4^{2-} or $S_2O_3^{2-}$ we note that three organisms, *G. ferrireducens, D. desulfuricans,* and *Halarsenitibacter silvermanii* SLAS-1 are reported to reduce elemental sulfur and/or polysulfide to S^{2-}, a trait shared by strain MPA-C3, which I describe in Chapter 2 (25, 34, 92, 143). Of the As(V) respiring organisms in Table 1 which are unable to utilize SO_4^{2-} or $S_2O_3^{2-}$, all except *Bacillus selenitireducens* MLS-10 (141) are able to respire NO_3^{2-} and/or Fe(III), suggesting an adaptation for life at lesser reducing conditions in comparison to the SO_4^{2-} and/or $S_2O_3^{2-}$ reducing organisms.

At the time of this writing only limited information is available about the range of organic substrates utilized by the dissimilatory As(V) reducing bacteria. In Chapter 2, I discuss my isolation of an interesting new organism, Strain MPA-C3, which utilizes a wider range of organic compounds as a source of carbon and energy than any of the currently described *Deferribacteres,* and at least as wide a range as any of the other described As(V) respiring organisms (Table 1). The ability to utilize pyruvate as a source

of carbon and energy appears to be conserved among the described DARPs and, indeed, only a single isolate among the described DARPs is incapable of utilizing pyruvate (26, 34, 46, 64, 76, 79, 87, 95, 100, 101, 113, 114, 123, 137, 141, 143, 144). Among the As(V) respiring bacteria, only *Bacillus arsenicoselenatis* E1H is reported to be incapable of utilizing pyruvate (141), and only *Desulfosporosinus* sp. Y5 is reported to utilize aromatic substrates, including benzoate and toluene (76).

1.4 *arrA* As a Biomarker for Microbial Arsenate Reduction

The *arrA* gene has been found in all dissimilatory arsenic reducing bacteria (DARBs) described to date (1, 79, 82, 83, 100, 104, 107, 124, 136, 138). The use of *arrA* as a biomarker for microbial As(V) respiration was first described by Malasarn *et al.* in 2004, and the gene has been successfully used in a number of studies since (73, 83, 90, 134). While *arrA* appears to be conserved among the arsenate respiring bacteria, enough difference appears to exist to determine evolutionary relatedness (73, 83, 134). Figure two displays a phylogenetic tree based on the *arrA* genes recovered from freshwater aquifers, marine, and hypersaline environments, and shows that samples from groundwater aquifers cluster together (Six Mile Run, Pike Run, and Crosswicks Creek (90, 91)), distinct from the cluster formed by samples taken from hypersaline lakes (Mono and Searles Lakes(67)), and marine/estuarine sites (Chesapeake Bay (134) and the Hackensack River (unpublished data)). This suggests that environmental conditions play a stronger role in the development of DARP communities than geographical location.

1.5 Microbial Precipitation of Arsenic Sulfides

Arsenic sulfide minerals present a major sink of arsenic to the environment. Arsenic has been found to be closely associated with sulfur, both as arsenic sulfide minerals (eg orpiment, realgar) as well as iron-sulfide-arsenic minerals, particularly arsenopyrite (9, 47, 131). Arsenic sulfide minerals have been described as forming geochemically under the high temperatures and pressures found within the Earth's crust, and also under hydrothermal conditions (9, 47, 88, 131). The common arsenic sulfide mineral, realgar (As_4S_4), has been described as forming in the condensation zone of hydrothermal fluids at temperatures of 50-75^oC and particularly in veins of silver, lead, and gold ores (36, 88). Several polymorphs of realgar, including alacranite, uzonite, and pararealgar have also been described as forming under similar conditions (23, 28, 109, 110). Alacranite, a polymorph of realgar with the formula As_8S_9 has been identified at several locations worldwide, as parts of hydrothermal and volcanic deposits (28, 33, 35, 88, 110), and uzonite (As_4S_5) has been both synthesized (150) and described in nature (23, 109). To date, microbial activity has never been directly linked to the formation of realgar or any of its polymorphs under mesophilic conditions, although the microbial formation of realgar has been described under hyperthermophilic conditions by the archea *Pyrobaculum arsenicum* (55), and both O'Day et al.(102) and Demergasso et al.(39) suggest the potential for microbial formation of realgar under mesophilic conditions.

The precipitation of arsenic sulfides by microbial activity has been described (39, 63, 74, 100, 120). Rittle *et al.* (1995) reported the potential for a microbial influence on

the precipitation of arsenic sulfides, and the first direct evidence of microbial orpiment (As_2S_3) precipitation by the SO_4^{2-} and As(V) respiring organism *Desulfosporosinus auripigmentum* was shown by Newman *et al.* (1997). Additionally, Demergasso *et al.* (2007) suggest that arsenic sulfide deposits comprised of realgar and orpiment in Andean salt flats are potentially microbial in origin. In Chapter 2, I describe the formation of alacranite by a novel, As(V) reducing isolate, Strain MPA-C3. Growth of this organism with As(V) as a terminal electron acceptor and in the presence of sulfide resulted in the precipitation of alacranite, a mineral previously reported to form only under hydrothermal conditions. Strain MPA-C3 was isolated from arsenic contaminated sediments from Mai Po estuary in Hong Kong, where sulfide would be plentiful as a result of the activity of sulfate reducing microbes. This mineral formed by Strain MPA-C3 provides an interesting route by which arsenic could be immobilized in the shallow subsurface of estuarine environments.

1.6 Microbial Arsenic Mobilization

1.6.1 Sources of arsenic in the environment

Arsenic occurs as a major constituent in a wide variety of minerals, however, only a limited number of these appear to act as major sources of arsenic to groundwater. Arsenic sulfides comprise a significant source of arsenic in the environment, and of these, the most common arsenic sulfide mineral is arsenopyrite, FeAsS, which is found predominantly in mineral veins (131). The sulfide minerals realgar, AsS, and orpiment, As_2O_3, have been predominantly found in hydrothermal and volcanic deposits (131). In

pyrites, arsenic replaces sulfur within the crystal structure, allowing for very high concentrations of arsenic to be incorporated (131). For example, pyrites with arsenic concentrations of up to 77,000 mg/kg have been reported, and concentrations up to 126,000 mg/kg have been observed the iron-sulfur mineral marcasite (131). Iron oxide minerals also have the potential to contain high levels of arsenic, with concentrations up to 76,000 mg/kg seen in Fe(III) oxyhydroxides (131). As such, it is not surprising that sedimentary rocks composed of these minerals have been found to have elevated concentrations of arsenic. Arsenic concentrations of up to 490 mg/kg have been found in shales, and iron formations and iron rich sediments are reported to have arsenic concentrations up to 2900mg/kg (131).

In Southeast Asia arsenic is associated with iron oxide minerals in alluvial deposits of sediments weathered and transported from the Himalayas, and the mobilization of this arsenic into drinking water has become a major public health crisis in Southeast Asia, most notably in Bangladesh, (10), the Bengal Delta of India(56, 133), Cambodia (73) and Vietnam (19). In Bangladesh elevated levels of arsenic in groundwater has been implicated in 1 out of every 5 deaths and has been called ”the world’s greatest mass poisoning”(10). In Southeast Asia, microbial reduction of iron oxides releases bound arsenic into groundwater, which is then withdrawn from the aquifer for human consumption and agriculture(6, 20, 44, 51, 52, 81, 111, 140). Harvey *et al*. (51) in particular demonstrate that this microbial activity is stimulated by organic carbon, which has been introduced to the subsurface as a result of intensive abstraction of groundwater.

In New Jersey, black shale underlying the Newark Basin of New Jersey has been found to have arsenic concentrations up to 240 mg/kg, potentially providing a source of arsenic to groundwater (130). Arsenopyrite minerals within these shales have been found to be very highly enriched in arsenic, with arsenic comprising 4% by weight of the pyrites (130). Wells drawing water from fractured shale bedrock of the Piedmont Physiographic Province of New Jersey have been found to have the highest concentrations of arsenic, with nearly 25% of the wells tested having arsenic levels in excess of 5µg/L (93). The glauconitic sediments of the Coastal Plain of New Jersey have been found to have levels of arsenic ranging from 7.1-131 mg/kg (41, 90). To date, however, only a limited number of wells in this region have been tested. While many thousands of tons of arsenical pesticides have been applied to the agricultural soils of New Jersey, they are not believed to provide a major source of arsenic to groundwater (94). It is believed that the arsenic constituent of these pesticides is immobilized on Al and Fe minerals in the topsoil, and is not leaching into the groundwater (94).

1.6.2 Microbial Mobilization of Arsenic into Groundwater

Microbes have been implicated in the mobilization of arsenic into groundwater under a variety of conditions. Arsenate in groundwater is bound much more strongly to iron oxide minerals than arsenite, suggesting that microbial arsenate reduction may play a role in arsenic mobility (42). The ability of As(V) reducing microbes to release arsenic into solution was first described by Ahmann et al in 1994 concurrently with the discovery of the first As(V) reducing microbe, *Sulfurospirillum arsenophilum* MIT-13 (3). This

organism was found to dissolve ferrous arsenate via the reduction of As(V) to As(III), with the As(III) being released into the mobile phase (3). Further experimentation by Ahmann et al. demonstrated that *S. arsenophilum* strain MIT-13 was capable of mobilizing both As(III) and Fe(II) from arsenic contaminated aquifer sediments, indicating that iron reduction may also play a role in arsenic mobilization (2). These findings have since been replicated with the As(V) and Fe(III) reducing organism *Shewanella* sp. Strain ANA-3 (56, 140). This organism was demonstrated to reduce As(V) which had been sorbed to ferrihydrite as well as the ferrihydrite itself, resulting in the mobilization of arsenic into the mobile phase (56, 140). In addition to the mobilization of arsenic from iron oxide minerals, Zhu *et al.* report that sulfide produced by bacterial sulfate reduction may mobilize arsenic via an arsenide/sulfide exchange (154). This mechanism has been suggested as a microbial route to arsenic release from arsenopyrites in black shale aquifers (154). A separate mechanism for mobilization from arsenopyrites in black shales has been described by Rhine et al., who demonstrated that under oxidizing conditions, the sulfur oxidizing Strain WAO mobilized arsenic from arsenopyrite following the oxidation of sulfur (117). In Chapter 3, I present the work published in Mumford et al. (90). I describe how microbial activity, stimulated by inputs of organic carbon, drives the mobilization of arsenic from iron-rich glauconitic sediments into the shallow groundwater beneath Crosswicks Creek in Upper Freehold, NJ. By spiking sediments from this site with As(V) and utilizing them as inocula for microcosms, we were able to demonstrate the importance of microbial action in the mobilization of arsenic. This was demonstrated by spiking microcosms prepared with

site sediment, and measuring both the concentration and speciation of arsenic released. We found that while the sediments had a high capacity to bind As(V), As(III) was released only by microbial activity, and was present in the mobile phase at a concentration similar to that observed at the site (~25 μg/L).

In Chapter 4, I describe how, again in collaboration with the USGS, I examined the potential for microbial As mobilization at two sites along the Millstone River, in Somerset County, NJ. This research has been submitted to Water Research as "Groundwater redox controls microbial release of arsenic from minerals to groundwater in a fractured rock terrain, New Jersey, USA" and is currently under review. The two sites, Six Mile Run and Pike Run, displayed very similar mineralogy and geochemistry, with the notable difference being that the groundwater at Six Mile Run was anoxic and reducing, and the groundwater at Pike Run was oxic and oxidizing. We hypothesized that these differing redox conditions would lead to the development of differing microbial communities, and that this would in turn explain why the levels of As present at Six Mile Run were an order of magnitude higher than those at Pike Run. By following a similar research methodology as described in Chapter 3, I determined through culture-independent molecular techniques that the microbial communities at each site were in fact different, and these findings were supported by the results of microcosm experiments. In these experiments, I found that microcosms inoculated with groundwater taken from Six Mile Run were capable of As(V) reduction, while those inoculated with groundwater from Pike Run were not. This finding was particularly interesting in that the

arrA gene was successfully amplified from the Pike Run site, a finding which I suggest may be due to the very similar As(III) oxidase gene (*arxA*).

The findings from these two studies, combined with the wealth of information available from the ongoing studies in Southeast Asia and particularly the Bengal Delta region, allow for the construction of a conceptual model of arsenic release and transport in the shallow subsurface. In Figure 3, I present this conceptual model. Both previous reports and my own work convincingly show that geogenic arsenic is frequently associated with iron oxide minerals in subsurface aquifers. Arsenic is released into groundwater by several mechanisms, including direct reduction of As(V) sorbed to mineral surfaces (155), and release of arsenic following reductive dissolution of the iron mineral by iron reducing microorganisms (56, 145). As shown in Mumford et al. (90), released As(V) is reduced to As(III) by As(V) reducing microbes, and this As(III) remains mobile in the groundwater. On gaining reaches beneath a river, As(III) would then be brought to the oxic sediment water interface by groundwater flow, at which point it is oxidized to As(V) either abiotically or by As(III) oxidizing microbes (153), and then re-sorbs to newly precipitated iron oxides in the streambed (91). During periods of high flow and disturbance, this streambed material may be carried far downstream and buried, moving the arsenic far from its original source, and allowing this microbial arsenic cycle to begin anew.

Table 1: Summary of Electron Donors and Acceptors used by Characterized Respiratory As(V) Reducing Bacteria

Strains	Terminal Electron Acceptors											Electron Donors						
	O_2	As(V)	Se(VI)	Se(IV)	NO_3^-	NO_2^-	SO_4^{2-}	$S_2O_3^{2-}$	S_0/ $^-S\text{-}S_n\text{-}S^-$	Fumarate	Fe(III) (Chelated)	Fe(III) (O)OH	Acetate	Pyruvate	Fructose	Benzoate	Toluene	Palmitate
Strain MPA-C3	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-
Denitrovibrio acetiphilus[1]	-	-	N/T	N/T	+	-	-	-	N/T	N/T	+	N/T	+	N/T	-	-	N/T	-
Geovibrio ferrireducens[2]	-	N/T	N/T	N/T	-	-	-	-	+	-	+	+	+	N/T	N/T	-	-	-
Deferribacter desulfuricans[3]	-	+	-	-	+	N/T	-	-	+	-	-	-	+	N/T	N/T	N/T	N/T	N/T
Halarsensibacter silvermanii SLAS-1[7]	-	+	-	N/T	-	N/T	-	-	+	-	+	N/T	-	+	+	N/T	N/T	N/T
Chrysiogenes arsenatis[4]	-	+	-	N/T	+	+	-	-	N/T	-	-	-	+	+	-	-	N/T	N/T
Desulfurispirillum indicum S5[5]	-	+	+	+	+	+	-	-	-	-	-	-	+	+	N/T	N/T	N/T	N/T
Bacillus arsenicoselenatis E1H[12]	-	+	+	+	-	N/T	-	-	N/T	-	+	N/T	-	-	+	N/T	N/T	N/T
Bacillus selenitireducens MLS-10[12]	-	+	+	+	-	N/T	-	-	N/T	-	-	N/T	-	+	+	N/T	N/T	N/T
Natranaerobius thermophilus[6]	-	+	N/T	N/T	+	N/T	-	+	N/T	+	+	N/T	+	+	+	N/T	N/T	N/T
Desulfitobacterium strain GBFH[8]	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	N/T	N/T	N/T
Desulfosporosinus strain Y5[9]	-	+	N/T	N/T	+	-	+	-	N/T	N/T	+	+	-	N/T	N/T	+	+	N/T
Desulfosporosinus auripigmentum[10]	-	+	N/T	N/T	-	N/T	+	+	N/T	+	-	-	-	+	N/T	-	-	-
Alkaliphilus oremlandii[11]	-	+	-	-	-	N/T	-	+	N/T	-	N/T	N/T	+	+	+	N/T	N/T	N/T
Shewanella strain ANA-3[13]	+	+	-	N/T	+	N/T	-	+	N/T	+	N/T	+	-	+	N/T	N/T	N/T	N/T
Sulfurospirillum barnesii SES-3[14]	micro	+	+	N/T	+	+	N/T	+	+	+	N/T	+	N/T	+	N/T	N/T	N/T	N/T
Sulfurospirillum arsenophilum MIT-13[14]	micro	+	-	N/T	+	+	N/T	+	+	+	N/T	N/T	N/T	+	N/T	N/T	N/T	N/T

Table 1: N/T: Not Tested. References: [1](64, 95), [2](34), [3](142, 144), [4](79), [5](113, 114), [6](87), [7](25), [8](101), [9](76), [10](99), [11](46), [12](141), [13](123), [14](137)

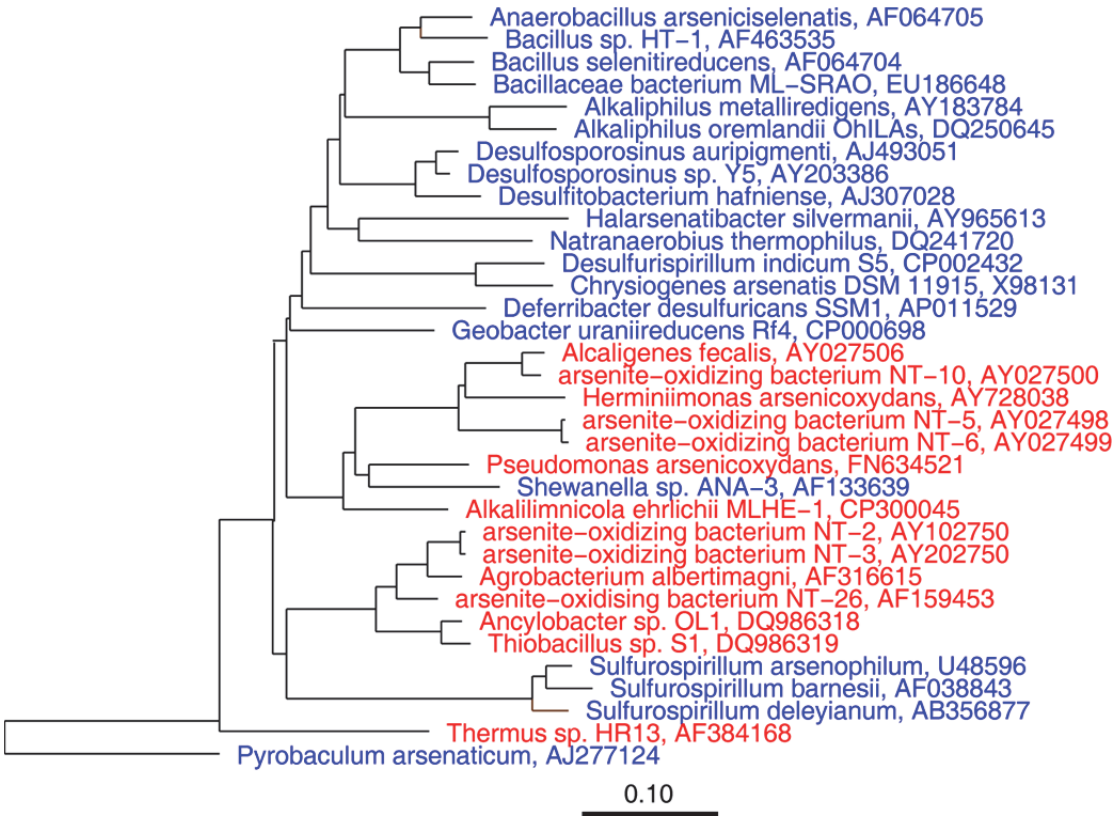

Figure 1:

Phylogenetic tree of arsenic respiring organisms based on the 16S rRNA gene. As(V) reducing organisms are in blue, As(III) oxidizing organisms are in red.

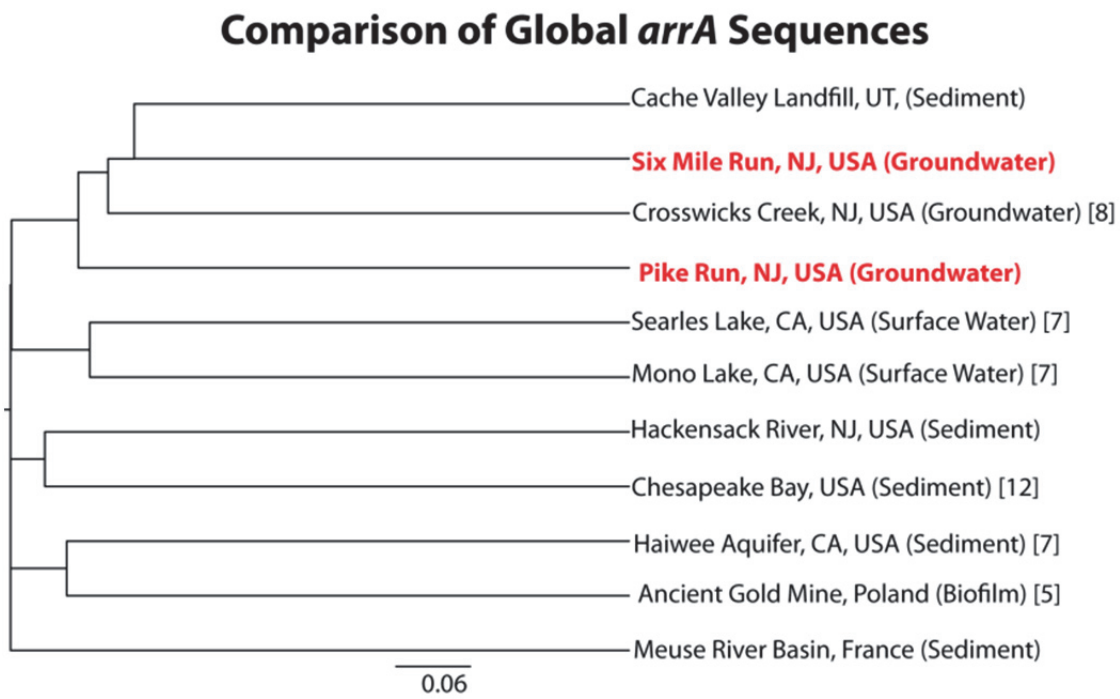

Figure 2:

Cluster analysis of *arrA* sequences from diverse worldwide sites. Freshwater sites cluster separately from marine and hypersaline sites.

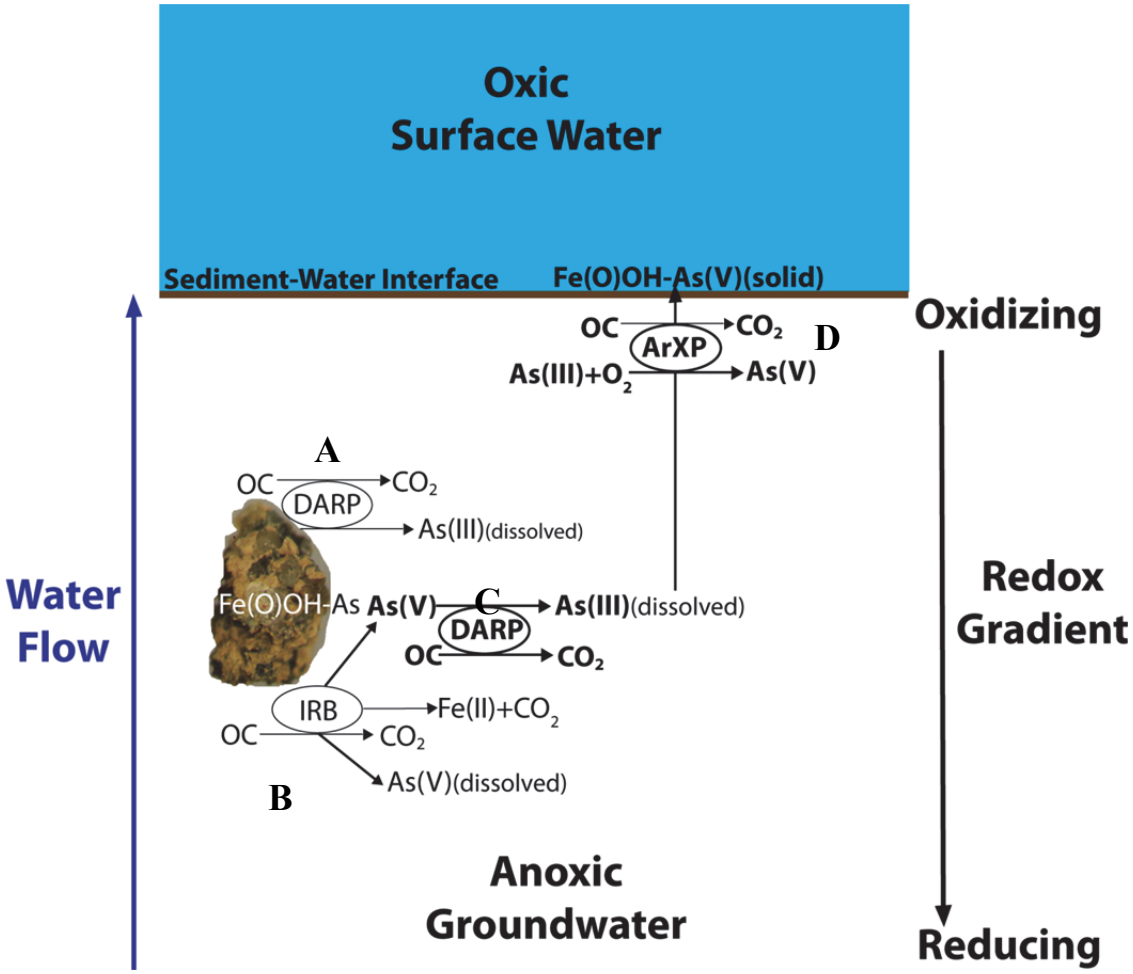

Figure 3:

Conceptual Model of arsenic mobilization and transport. Arsenic can be mobilized by either (A) direct reduction of As(V) sorbed to mineral surfaces, or (B), reductive dissolution of iron oxides by iron reducing bacteria. Once released, As(V) is reduced to As(III) by As(V) reducing microbes (C), and carried to the oxic sediment water interface by groundwater flow. At the sediment water interface, As(III) is re-oxidized, and binds to freshly precipitated iron oxides (D).

Chapter 2

Precipitation of Alacranite (As_8S_9) by a Novel As(V)-Respiring Anaerobe Strain MPA-C3

Abstract

Strain MPA-C3 was isolated by incubating arsenic-bearing sediments under anaerobic, mesophilic conditions in minimal media with acetate as the sole source of energy and carbon, and As(V) as the sole electron acceptor. Following growth and the respiratory reduction of As(V) to As(III), a yellow precipitate formed in active cultures, while no precipitate was observed in autoclaved controls, or in uninoculated media supplemented with As(III). The precipitate was identified by X-ray diffraction as alacranite, As_8S_9, a mineral previously only identified in hydrothermal environments. Sequencing of the 16S rRNA gene indicated that strain MPA-C3 is a member of the *Deferribacteres* family, with relatively low (90%) identity to *Denitrovibrio acetiphilus* DSM 12809. The arsenate respiratory reductase gene, *arrA*, was sequenced, showing high homology to the *arrA* gene of *Desulfitobacterium halfniense*. In addition to As(V), Strain MPA-C3 utilizes NO_3^-, Se(VI), Se(IV), fumarate, and Fe(III) as electron acceptors, and acetate, pyruvate, fructose and benzoate as sources of carbon and energy. Analysis of a draft genome sequence revealed multiple pathways for respiration and carbon utilization. The results of this work demonstrate that alacranite, a mineral previously thought to be formed only chemically under hydrothermal conditions, is precipitated under mesophilic conditions by the metabolically versatile Strain MPA-C3.

This chapter was co-authored with Professor Lily Y. Young and Professor Nathan Yee, has been submitted to Environmental Microbiology, where it has been accepted for publication.

2.1 Introduction

Arsenic sulfide minerals are widespread in the environment, and are found in diverse geologic settings, including continental margins, river sediments, and hydrothermal deposits (9, 47, 131). The common arsenic sulfide minerals realgar (As_4S_4) and orpiment (As_2S_3) are reported to form in the condensation zone at temperatures of 50-75^oC in hydrothermal fluids and ore deposits, and particularly in veins of silver, lead, and gold ores (36, 88). Additionally, the formation of realgar has been reported in mesophilic, estuary sediments in San Francisco Bay (102). These minerals, along with iron sulfide minerals such as arsenopyrite and marcasite, are thought to comprise much of the 2-8 mg/kg of arsenic found in continental margin and river sediments (131). Several polymorphs of realgar, including alacranite, uzonite, and pararealgar have also been characterized. Alacranite (As_8S_9) has been identified at as a component of hydrothermal and volcanic deposits as several locations (28, 33, 35, 88, 110), and uzonite (As_4S_5) has been both synthesized (150) and described in natural samples (23, 109). Although the microbial formation of realgar has been found to occur under hyperthermophilic conditions (55), the role of microorganisms in the formation of realgar and its polymorphs under mesophilic conditions remain poorly understood.

A growing list of anaerobic prokaryotes have been reported to be capable of generating energy via the respiratory reduction of arsenate [As(V)] to arsenite [As(III)], (55, 76, 79, 80, 98, 99, 104, 124, 137, 142), and As(V) reduction has been implicated as an important microbial process in the biologic formation of arsenic sulfide minerals (55, 99). The first direct evidence of microbial orpiment (As_2S_3) precipitation by the SO_4^{2-}

and As(V) respiring organism *Desulfosporosinus auripigmentum* was shown by Newman et al. (1997). Previous studies have also reported the precipitation of arsenic sulfides by the reaction of biogenic sulfide with dissolved arsenic oxyanions in laboratory incubations of contaminated aquifer sediments (Rittle et al., 1995; Kirk et al., 2010). Additionally, Demergasso et al. (2007) suggested that arsenic sulfide deposits comprised of realgar and orpiment in Andean salt flats have a potential microbial origin. Finally, recent work has shown that *Shewanella* species can form arsenic sulfide nanotubes during As(V) and thiosulfate reduction (58, 74).

While diverse prokaryotes are able to reduce As(V) for growth, this respiratory process remains poorly understood in the *Deferribacteres*, a distinct phylum within the *Bacteria* consisting of anaerobic organotrophs. Previous studies have shown that *Deferribacteres* can utilize terminal electron acceptors such as NO_3^-, Fe(III), Mn(IV), Co(III), and S^0 (48). To date, a total of 26 isolates have been described, comprising six genera within the *Deferribacteres* (96). The majority of these organisms have been isolated from high-temperature environments, but several strains have been isolated under more moderate conditions (48). These organisms are reported to utilize acetate and lactate, however, their ability to utilize more complex carbon sources has not been fully assessed (34, 48, 95, 143). The type strain of the *Deferribacteres, Geovibrio ferrireducens*, is characterized by the ability to respire ferrous iron and elemental sulfur. It is unable, however, to respire NO_3^-, and its ability to respire As(V) has not been determined. Among the described *Deferribacteria,* only *Deferribacter desulfuricans* has been reported to grow at the expense of dissimilatory reduction of As(V) to As(III) (143).

In this study, we report the isolation of a novel As(V)-respiring bacterium designated as strain MPA-C3 from arsenic-bearing sediments collected at Mai Po Marsh, Hong Kong. Phylogenetic analysis of the 16S rRNA gene indicates that strain MPA-C3 is a novel member of the *Deferribacteres* family. We show that during As(V) respiration, strain MPA-C3 forms a yellow precipitate in close association with the cells. We further demonstrate that this yellow precipitate formed under circumneutral, mesophilic conditions by strain MPA-C3 is alacranite, a mineral previously only known to be formed under hydrothermal conditions. Finally, metabolic and genomic characterization of strain MPA-C3 reveals that this organism has a wider range of electron acceptors than any previously described *Deferribacteres* isolates. The results of this work provide new insights in the route of microbial arsenic sulfide precipitation under mesophilic conditions, and elucidate a potential geochemical role for the *Deferribacteres* in sedimentary arsenic sulfide biomineralization.

2.2 Materials and Methods

2.2.1 Enrichment and Isolation

Strain MPA-C3 was isolated from sediments sampled from the Mai Po Marsh, Kowloon, Hong Kong. The marsh is part of the Pearl River Estuary system, and the sediment at this site contains arsenic concentrations of approximately 20mg/kg (Li Meng, Personal Communication, 2008). Sediment enrichment cultures from Mai Po site A were prepared by inoculating 40mL of defined freshwater anaerobic medium (ABF) with approximately 4 g of composited sediment. The composited sediment was incubated in

an anaerobic basal medium composed of 1 g/L NaCl, 400 mg/L $MgCl_2 \cdot 2H_2O$, 100 mg/L $CaCl_2 \cdot 2H_2O$, 200 mg/L KH_2PO_4, 500 mg/L KCl, 190 mg/L Na_2HPO_4, 60 mg/L NaH_2PO_4, 270 mg/L NH_4Cl, 1.05 g/L MOPS buffer (pH 7.2), 31mg/L Na_2S, 1.5 mg/L nitrilotriacetic acid, 0.8 mg/L $Fe(NH_4)_2(SO_4)_2$, 0.2 mg/L Na_2SeO_3, 0.1 mg/L $CoCl_2 \cdot 6H_2O$, 0.1 mg/L $MnSO_4 \cdot H_2O$, 0.1 mg/L $Na_2MoO_4 \cdot H_2O$, 0.1 mg/L $NaWO_4 \cdot H_2O$, 0.1 mg/L $NiCl_2 \cdot 6H_2O$, 0.01 mg/L H_3BO_3, 0.01 mg/L $CuSO_4 \cdot 5H_2O$, 0.1 µg/L nicotinic acid, 0.1 µg/L calcium pantothenate. 0.1 µg/L pyroxidine HCl, 0.1 µg/L riboflavin, 0.05 µg/L biotin, 0.05 µg/L folic acid, 0.05 µg/L α-lipoic acid, 0.05 µg/L vitamin B-12, 48.5 mg/L cysteine. The pH of the media was adjusted to 7.2. Triplicate active enrichment cultures were inoculated under anaerobic conditions with 4g of Mai Po Marsh sediment, and amended with 2mM NaH_2AsO_4 as a terminal electron acceptor and 2mM sodium acetate as a source of carbon and energy. Duplicate sterile controls were prepared in the same manner to the active enrichment cultures prior to sterilization by autoclaving (3 times over 3 consecutive days). A background control was prepared by inoculating sediment into media which was not supplemented with As(V) or acetate. All cultures were incubated in the dark at 25^{o}C. The reduction of As(V) to As(III) was monitored by HPLC as described by Perez-Jimenez et al. (2005). Following the observation of complete reduction of As(V) to As(III), the enrichment cultures were diluted 1:10 in ABF media amended with 2mM $NaH_2AsO_4$and 2mM sodium acetate. Following six 1:10 dilutions, the enrichment culture was dominated by a single morphology comprised of bent rods, and a yellow precipitate was noted. The culture was plated under anaerobic conditions onto ABF media solidified with 1% noble agar and amended with 5mM

NaH_2AsO_4 and 5mM sodium acetate. After 21 days of incubation at 25°C, isolated yellow colonies were observed, and were transferred to 3mL ABF amended with 2mM NaH_2AsO_4 and 2mM sodium acetate.

2.2.2 Survey of electron donors and terminal electron acceptors

The ability of strain MPA-C3 to utilize the following carbon sources was tested: Acetate (5mM) Benzoate (5mM), fructose (5mM), pyruvate (5mM), palmitate (5mM), lactate (5mM), toluene (1mM), hexadecane (2mM), and syringic acid (5mM). The following terminal electron acceptors were tested: As(V), Se(VI), Se(IV), Fe(III) citrate, amorphous Fe(III)(O)OH, NO_3^-, NO_2^-, SO_4^{2-}, $S_2O_3^{2-}$ and mixed oxidation state polysulfide. All terminal electron acceptors were supplied at a concentration of 5mM. Growth on alternative carbon sources was considered positive if reduction of As(V) to As(III) was observed. Growth on Fe(III) citrate and Fe(O)OH was determined by a modified ferrozine assay (147). Growth on Se(VI) and Se(IV) was measured by HPLC as described below. Growth on NO_3^-, NO_2^-, SO_4^{2-}, $S_2O_3^{2-}$, and polysulfide was determined by an increase in optical density at 580 nm as measured on a Spectronic Instruments Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific, Waltham MA) relative to sterile controls.

Reagents:

All reagents used were of reagent grade and used without further purification. Stock solutions were prepared by dissolution in degassed MilliQ reagent grade water (Millipore, Billerica MA) and stored under argon until use.

2.2.3 Chemical Analyses

Liquid samples of 600 µL were removed from culture bottles for analysis with an argon-flushed 1mL syringe with a 22 gauge needle. Particulates were filtered from samples with Costar SpinX 0.22 µM nylon centrifuge filters (Corning Life Sciences, Pittston PA), centrifuged at 14,000 x g for 2 minutes.

Arsenic and selenium ions were analyzed by HPLC (Shimadzu, Columbia MD and Beckman Coulter, Brea CA) using a Hamilton PRP-X100 anion exchange column (Hamilton, Reno NV). Arsenic ions were eluted with 40mM NaH_2PO_4 at pH 5.0 flowing at 1 mL min^{-1} and detected at 195 nm. Selenium ions were eluted with 12.5mM $(NH_4)_3PO_4$ at pH 8.5 flowing at 1 mL min^{-1} and detected at 201 nm in a modification of the method reported by Guérin et al. (1997). Acetate was analyzed by HPLC (Beckman Coulter, Brea CA) using a Resex ROA organic acid column. (Phenomenex, Torrance CA). Acetate was eluted with 5 µM sulfuric acid flowing at 0.5 mL min^{-1} and detected at 210 nm.

Soluble ferrous and ferric iron concentrations were analyzed by the modified ferrozine method described by Viollier et al. (2000).

2.2.4 Molecular Analysis

Genomic DNA was extracted from 1 mL of log phase culture with the MoBio Ultraclean DNA extraction kit, according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene was amplified using the primers 27F and 1492R, as described by Lane et al. (70). The As(V) respiratory reductase gene, *arrA*,

was amplified using the primers As1F and As1R as described by Lear et al. (73). Sequencing of 16S rRNA and *arrA* amplicons was performed by Genewiz, Inc. (Genewiz, Inc., South Plainfield NJ). Phylogenetic analysis of the 16S gene was performed using ARB with the SILVA 106 NR database, and analysis of the *arrA* gene was performed using ARB with a ClustalW aligned database of 16 *arrA* amino acid sequences from known As(V) reducing organisms downloaded from GenBank (78, 112).

The 16S rRNA gene sequence of strain MPA-C3 has been deposited into GenBank under accession number JX049127, and the *arrA* gene sequence has been deposited into GenBank under accession number JX049128.

2.2.5 Genomic Sequencing

DNA was extracted from strain MPA-C3 using the phenol-chloroform extraction protocol as described by Wilson (151). A paired end library was constructed using an Illumina Nextera kit, and sequencing was performed using an Illumina Genome Analyzer IIX (Illumina Inc., San Diego, CA). Sequence assembly was performed using CLC Genomics Workbench 5.1 (CLC Bio, Cambridge, MA). Genome annotation and metabolic pathway reconstruction was performed using RAST (11) and Pathway Tools (61), and potential rRNA genes were identified with RNAmmr (69). Additional visualization, analysis and annotation was performed using Geneious (62). Genomic data has been submitted to NCBI as Bioproject 176465.

2.2.6 Mineral Analyses

The mineral precipitate was collected by filtration onto 25 mm Millipore type HA filters with a nominal pore size of 0.45 μm. (Millipore, Billerica, MA). The filters were dissolved along with organic debris by washing with acetone followed by centrifugation at 2,000 x *g* three times. The mineral precipitate was dried under a stream of argon and stored under argon prior to analysis.

For X-ray diffraction, samples were dried under an argon stream, then tightly packed in capillary tubes (outer diameter – 0.5 mm) and sealed under strict anaerobic conditions. Powder X-ray diffraction patterns were obtained with a Rigaku MicroMaxTM-007HF with a Cr source operating at 35 KV and 25 mA. The JADE+ V5 (Materials Data Inc., Livermore, CA) software package was used for data analysis.

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) was performed to determine the molar content of arsenic and sulfur in the recovered mineral. 5 mg of alacranite was dissolved in 1 mL of 1 mol L^{-1} sodium carbonate, and a 50 μL aliquot of the dissolved mineral solution was then added to 4950 μL of trace metal grade 2% nitric acid and analyzed on a Varian Vista Pro ICP-OES (Agilent Technologies, Santa Clara, CA) at the Rutgers Inorganic Analytical Laboratory. Data analysis was performed using Varian ICP-Expert software (Agilent Technologies, Santa Clara, CA).

2.3 Results

2.3.1 Characterization of Strain MPA-C3

Sediment enrichment cultures resulted in the isolation of an anaerobic bacterium with cells 2-4 μm in length and a curved rod morphology. The isolate was designated strain MPA-C3. Figure 1 demonstrates that strain MPA-C3 is capable of the near-stoichiometric reduction of 5 mM L^{-1} As(V) to As(III) in 56 hours. Strain MPA-C3 did not grow in ABF media supplemented with acetate but without As(V), thus indicating that As(V) is being used as a terminal electron acceptor for respiration and without which no growth takes place.

Strain MPA-C3 was found to have a wide range of alternate terminal electron acceptors (TEAs). Table 1 shows a comparison of the TEAs and electron donors utilized by strain MPA-C3 with the TEAs and electron donors used by other characterized As(V) reducing isolates. In addition to As(V), strain MPA-C3 was able to grow by reducing NO_3^- to NO_2^-, Se(VI) and Se(IV) to elemental selenium, fumarate, a mixed oxidation state polysulfide, and Fe(III) when supplied as both Fe(III) citrate and amorphous $Fe(III)(OH)_3$. Strain MPA-C3 was unable to utilize oxygen, SO_4^{2-}, or $S_2O_3^{2-}$ as an electron acceptor. For electron donors, strain MPA-C3 utilizes acetate, pyruvate, fructose and benzoate as sources of carbon and energy, but is unable to utilize palmitate, hexadecane, or toluene

Figure 2A shows the results of the analysis of the 16S rRNA gene from the isolated As(V) respiring strain MPA-C3 in relation to its closest relative and other known respiratory As(V) reducers. Strain MPA-C3 has 90% sequence similarity with

Denitrovibrio acetiphilus (AF146526) as calculated by NCBI BLAST (5). Because of its relatively low sequence similarity to its nearest neighbor, strain MPA-C3 appears to be a novel member of the *Deferribactereres,* and may represent a new genus. In addition, strain MPA-C3 joins *Deferribacter desulfuricans* as the second member of the *Deferribacteres* able to generate energy from the respiratory reduction of As(V) to As(III) (142). Furthermore, based upon the 2,987,753 bp of sequence obtained from strain MPA-C3, its genome was found to be most similar to that of *Denitrovibrio acetiphilus*, as calculated by RAST (11).

The As(V) respiratory reductase gene, *arrA,* is recognized as a biomarker for As(V) respiration (73, 83), and was sequenced to confirm both its presence in strain MPA-C3 and its homology to other reported As(V) respiring organisms. Figure 2B illustrates the phylogenetic relationship of the inferred amino acid sequence of the *arrA* gene of strain MPA-C3 with the amino acid sequences *arrA* gene of other known As(V) reducing organisms. As shown in Figure 2B, the *arrA* gene of strain MPA-C3 displays the highest homology (99% BLAST identity) with the *arrA* gene of a member of the *Clostridia, Desulfitobacterium halfniense* Y51 (NC_007907), rather than with the putative *arrA* genes identified within the *Deferribacteres*. This finding suggests that strain MPA-C3 may have acquired this gene through a horizontal gene transfer event.

2.3.2 Characterization of Mineral Precipitate

A yellow mineral precipitate was observed in cultures of strain MPA-C3 after approximately one week of growth. Figure 3A shows both this precipitate, and

demonstrates that no precipitation occurred in ABF media supplemented with 5 mM As(III) in the absence of strain MPA-C3, thus demonstrating that an active culture is necessary for the precipitate to form. In addition, no precipitate occurred in autoclaved cultures with strain MPA-C3. Inspection of the precipitate under phase contrast microscopy revealed a yellow mineral closely associated with cells, and similar observations were made with scanning electron microscopy (Figure 3B). The curved rod morphology of strain MPA-C3 is shown in Figure 3C.

The mineral precipitate was identified as alacranite, As_8S_9, by powder X-Ray diffraction (XRD). The XRD pattern presented in Figure 3 displays 9 well defined diffraction peaks with corresponding d-spacings of 2.88, 3.03, 3.21, 3.96, 4.86, 5.07, 5.77, and 6.82 Å. The sample X-ray diffractogram exhibited a strong diffraction peak at 23^o 2θ (CrKα) that was absent in the orpiment reference pattern. Additionally, well defined diffraction peak at 26^o, 27^o, 34^o and 47^o 2θ were not present in the reference pattern of realgar. The best match for the sample X-ray diffractogram was the reference pattern of alacranite reported by Bonazzi et al. (2003). The molar ratio of arsenic to sulfur in the precipitated alacranite collected from the culture was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) to be approximately 1:1 (data not shown). This is consistent with the 1:1.1 As:S ratio reported for alacranite (27).

Assuming all of the 800 μM sulfide present in the media as 400 μM Na_2S and 400 μM cysteine reacts with 727 μM of the arsenite which is present in the media in excess, a maximum of 800 μMoles (710 mg) alacranite can be formed per liter of active strain

MPA-C3 culture medium. Given that the molecular weight of alacranite is 887.952 g mol^{-1} and that 1.2 L of culture was filtered, this would be a maximum theoretical yield of 852mg. The actual yield of 54 mg of alacranite from 1.2 L of active MPA-C3 culture medium is 6.3% of the maximum theoretical yield.

2.3.3 Genomic Characterization of Strain MPA-C3

Genomic sequencing of strain MPA-C3 allowed for the assembly of 33 contiguous sequences (contigs) containing a total of 2,985,604 base pairs. The draft genome has a G+C content of 47%, and 2816 open reading frames (ORFs) have been identified. Phylogenetic analysis of both the 16S rRNA gene and the entire genome place strain MPA-C3 among the *Deferribacteres*. Comparative analysis of the draft genome with sequenced members of the *Deferribacteres* and other As(V) reducing bacteria has allowed for more detailed insight into the metabolic pathways utilized by strain MPA-C3.

Figure 5 provides a schematic overview of some of the pathways identified within the genome, and connects those pathways with the electron donors and terminal acceptors identified in culture studies. We identified the complete TCA cycle in strain MPA-C3, which allows the bacterium to respire simple organic acids such as pyruvate and acetate. Many of the genes involved in the TCA cycle cluster together on the genome of strain MPA-C3, including the α, β, γ, and δ subunits of 2-oxoglutarate oxidoreductase (MPAc_gene_2410-2413), the α and β subunits of succinyl-CoA synthetase (MPAc_gene_2408-2409), malate dehydrogenase (MPAc_gene_2407), isocitrate dehydrogenase (MPAc_gene_2406), aconitate hydratase (MPAc_gene_2403), and

fumarate hydratase (MPAc_gene_2398-2400) and succinate dehydrogenase (MPAc_gene_2672-73). Additionally, genes for glycolysis (data not shown) were also found, allowing for the utilization of more complex carbon sources. This underscores the physiological data reported in Table 1 that shows fructose can serve as a carbon source for growth. The aromatic compound benzoate can also be used as a carbon source (Table 1), and genes encoding for a partial benzoyl-CoA reductase (MPAc_gene_0433-4) were identified in the genome.

Strain MPA-C3 can use a wide range of terminal electron acceptors (TEAs), similar to other characterized members of the *Deferribacteres*. Table 1 compares the TEAs and electron donors utilized by strain MPA-C3 with the TEAs and electron donors used by the other characterized members of the *Deferribacteres* as well as the other characterized non-members of this group that reduce As(V) for respiration. Strain MPA-C3 generate energy by the reduction of As(V) to As(III), a process catalyzed by the enzyme ArrAB (66, 124), and this process is included in the schematic view presented in Figure 5. While the *arrAB* genes were not identified on the draft genome of strain MPA-C3, as described above, they were identified by PCR amplification and found to be most similar to those found in the organism *Desulfitobacterium hafniense* Y51, which suggests that they may have been introduced into strain MPA-C3 via horizontal gene transfer. We suggest that the *arrAB* genes found by PCR in strain MPA-C3 lie within one of the unsequenced gaps in the draft genome, and will be found upon completion of the genome.

The reduction of NO_3^- to NO_2^-, but not to N_2, was observed in strain MPA-C3 (Table 1). This is similar to the incomplete reduction of NO_3^- as observed in both *D. acetiphilus* and *D. desulfuricans* (64, 95, 143, 144). In these latter two organisms, the respiratory reduction of NO_3^- to NO_2^- is catalyzed by the periplasmic NO_3^- reductase (Nap) genes (64, 84, 144). A schematic view of this system is provided in Figure 5. In Figure 6A, we display a cluster of genes from the draft genome of strain MPA-C3 (MPAc_0585-0589) which is homologous to the cluster of *Nap* genes of *D. desulfuricans,* thus supporting the presence of the periplasmic NO_3^- reductase system in strain MPA-C3 and the experimental data noted in Table 1. As is consistent with the experimental data for strain MPA-C3, the *Nar* genes, which catalyze the reduction of NO_3^- to N_2 (24), were not found on the genome.

The respiration of fumarate, Se(VI) and Se(IV) are depicted in Figure 5, as we have demonstrated that strain MPA-C3 generates energy for growth by reducing fumarate as well as both Se(VI) and Se(IV). Respiratory fumarate reduction is catalyzed by the FrdABC genes (72), and a homolog of the A subunit of this enzyme was identified near the end of contig 8 of the draft genome (MPAc_2609). The remaining genes for fumarate respiration were not found in the preliminary genome sequence, and likely reside in the gaps within the draft genome. No respiratory selenium reduction genes were found in the draft genome. Genes with high homology to the selenate reductases SerA (Schröder et al. (1997), YnfE (49), or SrdA (68) were not identified on the strain MPA-C3 genome. The selenate reductase genes may also reside in the gaps within the draft genome, or

conversely, Se(VI) reduction in this organism is proceeding via a novel, unknown pathway.

Growth experiments demonstrated that strain MPA-C3 is capable of generating energy by the dissimilatory reduction of elemental sulfur (Table 1), and this pathway is represented schematically in Figure 5. Dissimilatory sulfur reduction has also been reported for two members of the *Deferribacteres*, *Deferribacter desulfuricans* and *Geovibrio ferrireducens* (34, 143), and is reported to be catalyzed by the polysulfide reductase (Psr) genes (144). Figure 6B demonstrates the homologs of the polysulfide reductase (Psr) genes of *Deferribacter desulfuricans* are found in the genome of strain MPA-C3. *D. desulfuricans* utilizes polysulfides produced by the reduction of elemental sulfur by sulfide (144), and we believe this is also occurring with Strain MPA-C3.

Strain MPA-C3 is capable of growth on both soluble Fe(III) citrate as well as $Fe(III)(OH)_3$ (Table 1). Fe(III) reduction has been described as a characteristic of the *Deferribacteres* (48), and has been reported for *Denitrovibrio acetiphilus* and *Geovibrio ferrireducens* (34, 95). Soluble c-type cytochromes have been suggested as a mechanism of microbial iron reduction (77), and the genome of strain MPA-C3 contains a cluster of genes encoding the Ccm c-type cytochrome biogenesis proteins (MPAc_2111-2115). Figure 5 includes a suite of cytochrome c oxidase genes (MPAc_1085-8), as a similar mechanism may be involved in the Fe(III) reduction activity observed in strain MPA-C3.

2.4 Discussion:

The precipitation of alacranite by strain MPA-C3 is to our knowledge is the first reported instance of the biogenic formation of this mineral. Alacranite, along with a similar realgar-like mineral, uzonite (As_4S_5), was orginally described by Popova et al. in the Uzon Caldera in Kamchatka, Russia (109, 110). It has since been identified at Conical Seamount near Lihir Island, Papua New Guinea (33) and as a part of a mineral deposit formed by the emissions of a burning coal mine in the Czech Republic (27). In each of these cases, alacranite is described as forming under condition of high temperatures, and to date, the formation of alacranite has not been described under mesophilic conditions. While the microbial formation of arsenic sulfide minerals, particularly orpiment, has been previously reported, (17, 39, 55, 74, 99, 120), this is the first report of the microbial precipitation of alacranite at any temperature. The formation of realgar in low temperature arsenic contaminated aquifer sediments was reported by O'Day et al. (2004), however, the mechanism of realgar formation in this system was not discussed.

Arsenic sulfide mineral precipitation did not occur when As(III) was added to sterile media with no microbes, clearly demonstrating that strain MPA-C3 is required for the formation of alacranite. In contrast to the microbial formation of orpiment by *D. auripigmentum* (99), the realgar precipitation by *Pyrobaculum arsenaticum* (55) or the precipitation of arsenic-sulfide nanotubes by *Shewanella* HN41 (74), strain MPA-C3 is unable to reduce SO_4^{2-} or $S_2O_3^{2-}$, and requires the presence of S^{2-} in the media for the precipitation of alacranite. In its native estuarine environment, S^{2-} would be supplied to strain MPA-C3 via the activity of SO_4^{2-}-reducing bacteria of the surrounding microbial

community. As shown in figure 2A, the crystals were observed to be larger than the cells of strain MPA-C3, and appeared to form extracellularly. Mineral precipitation was not observed in the absence of cells, and we propose that mineral formation is mediated by cellular metabolism and occurs only in close proximity to the cell, as has been suggested by Newman et al. (1997) and Lee et al. (2007) for the formation of orpiment and arsenic-sulfide nanotubes, respectively. The possible role of cell wall nucleation in the formation of alacranite merits further investigation.

16S rRNA gene and genomic sequencing suggest that strain MPA-C3 is a novel member of the *Deferribacteres*. Table 1 summarizes 14 characterized As(V) reducing isolates along with two members of the *Deferribacteres*. A review of these isolates allows for the division of these organisms into two broad groups based on their ability or inability to utilize SO_4^{2-} and/or $S_2O_3^{2-}$. Among the organisms incapable of utilizing SO_4^{2-} or $S_2O_3^{2-}$, we note that three organisms, *G. ferrireducens, D. desulfuricans,* and *Halarsenitibacter silvermanii* SLAS-1 are reported to reduce elemental sulfur and/or mixed oxidation state polysulfide to to S^{2-}, a trait shared by strain MPA-C3 (25, 34, 143). Of the As(V) respiring organisms in Table 1 which are unable to utilize SO_4^{2-} or $S_2O_3^{2-}$, all except *Bacillus selenitireducens* MLS-10 (141) are able to respire NO_3^{2-} and/or Fe(III), suggesting an adaptation for life under lesser reducing conditions in comparison to the SO_4^{2-} and/or $S_2O_3^{2-}$ reducing organisms. It should be noted that the reduction of elemental sulfur to S^{2-} provides a source of S^{2-} which can react with As(III) and precipitate as alacranite.

Interestingly, strain MPA-C3 utilizes a wider range of organic compounds as a source of carbon and energy than any of the currently described *Deferribacteres,* and at least as wide a range as any of the other described As(V) respiring organisms (Table 1). At the time of this writing only limited information is available about the range of organic substrates utilized by the dissimilatory As(V) reducing bacteria. The ability to utilize acetate and pyruvate as a source of carbon and energy appears to be conserved among the *Deferribacteres* (34, 48, 95, 143). On the other hand, strain MPA-C3 appears to be unique in this phylum for its ability to utilize the more complex organic compounds such as fructose and benzoate. Among the other As(V) respiring bacteria, only *Bacillus arsenicoselenatis* E1H is reported to be incapable of utilizing pyruvate (141), and only *Desulfosporosinus* sp. Y5 has been described as capable of utilizing aromatic substrates, including benzoate and toluene (76).

The metabolic capabilities of strain MPA-C3 suggest that it lives in the less-reducing shallow subsurface in its native estuarine environment. Under these conditions it would be supplied with NO_3^- from the surface waters, as well as elemental sulfur via the activity of S^{2-} oxidizing bacteria. Additional sulfide would be present due to the activity of sulfate reducing bacteria in the deeper, more reducing regions of the subsurface. This sulfide would allow for the precipitation of alacranite by the As(V) reducing strain MPA-C3 in the moderate conditions of the shallow subsurface.

2.5 Conclusions

We demonstrate that a metabolically flexible anaerobic, As(V) reducing bacterium, Strain MPA-C3, precipitates alacranite under mesophilic, circumneutral conditions. This is the first report of this mineral being formed outside of a hydrothermal system, and supports earlier findings that arsenic sulfide minerals may be formed biotically. In the environment, therefore, such processes may serve as additional sinks for arsenic in neutral, reducing ground waters. The evidence presented in this study indicates that strain MPA-C3 is a novel strain within the phylum *Deferribacteres,* as shown by both its metabolic versatility and by the differences between this organism and the currently described *Deferribacteres.* We base this conclusion on the physiological differences, the 16S rRNA gene phylogeny, and the draft genome. We anticipate that completion of the genome will allow for much greater insights into the metabolism of this new isolate, as well as a better understanding of its mechanism of alacranite precipitation.

2.6 Supplemental Material

Supplemental material for this chapter is presented in Appendix 1.

2.7 Acknowledgements

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Table 1: Summary of Electron Donors and Acceptors used by Characterized Respiratory As(V) Reducing Bacteria

Strains	Terminal Electron Acceptors												Electron Donors						
	O_2	As(V)	Se(VI)	Se(IV)	NO_3^-	NO_2^-	SO_4^{2-}	$S_2O_3^{2-}$	S_0/ $^*S\text{-}S_n\text{-}S^*$	Fumarate	Fe(III) (Chelated)	Fe(III) (O)OH	Acetate	Pyruvate	Fructose	Benzoate	Toluene	Palmitate
Strain MPA-C3	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-
Denitrovibrio acetiphilus[1]	-	-	N/T	N/T	+	-	-	-	N/T	N/T	+	N/T	+	N/T	-	-	N/T	-
Geovibrio ferrireducens[2]	-	N/T	N/T	N/T	-	-	-	-	+	-	+	+	+	N/T	N/T	-	-	-
Deferribacter desulfuricans[3]	-	+	-	-	+	N/T	-	-	+	-	-	-	+	N/T	N/T	N/T	N/T	N/T
Halarsensibacter silvermanii SLAS-1[7]	-	+	-	N/T	-	N/T	-	-	+	-	+	N/T	-	+	+	N/T	N/T	N/T
Chrysiogenes arsenatis[4]	-	+	-	N/T	+	+	-	-	N/T	-	-	-	+	+	-	-	N/T	N/T
Desulfurispirillum indicum S5[5]	-	+	+	+	+	+	-	-	-	-	-	-	+	+	N/T	N/T	N/T	N/T
Bacillus arsenicoselenatis E1H[12]	-	+	+	+	-	N/T	-	-	N/T	-	+	N/T	-	-	+	N/T	N/T	N/T
Bacillus selenitireducens MLS-10[12]	-	+	+	+	-	N/T	-	-	N/T	-	-	N/T	-	+	+	N/T	N/T	N/T
Natranaerobius thermophilus[6]	-	+	N/T	N/T	+	N/T	-	+	N/T	+	+	N/T	+	+	+	N/T	N/T	N/T
Desulfitobacterium strain GBFH[8]	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	N/T	N/T	N/T
Desulfosporosinus strain Y5[9]	-	+	N/T	N/T	+	-	+	-	N/T	N/T	+	+	-	N/T	N/T	+	+	N/T
Desulfosporosinus auripigmentum[10]	-	+	N/T	N/T	-	N/T	+	+	N/T	+	-	-	-	+	N/T	-	-	-
Alkaliphilus oremlandii[11]	-	+	-	-	-	N/T	-	+	N/T	-	N/T	N/T	+	+	+	N/T	N/T	N/T
Shewanella strain ANA-3[13]	+	+	-	N/T	+	N/T	-	+	N/T	+	N/T	+	-	+	N/T	N/T	N/T	N/T
Sulfurospirillum barnesii SES-3[14]	micro	+	+	N/T	+	+	N/T	+	+	+	N/T	+	N/T	+	N/T	N/T	N/T	N/T
Sulfurospirillum arsenophilum MIT-13[14]	micro	+	-	N/T	+	+	N/T	+	+	+	N/T	N/T	N/T	+	N/T	N/T	N/T	N/T

Table 1: N/T: Not Tested. References: [1](64, 95), [2](34), [3](142, 144), [4](79), [5](113, 114), [6](87), [7](25), [8](101), [9](76), [10](99), [11](46), [12](141), [13](123), [14](137)

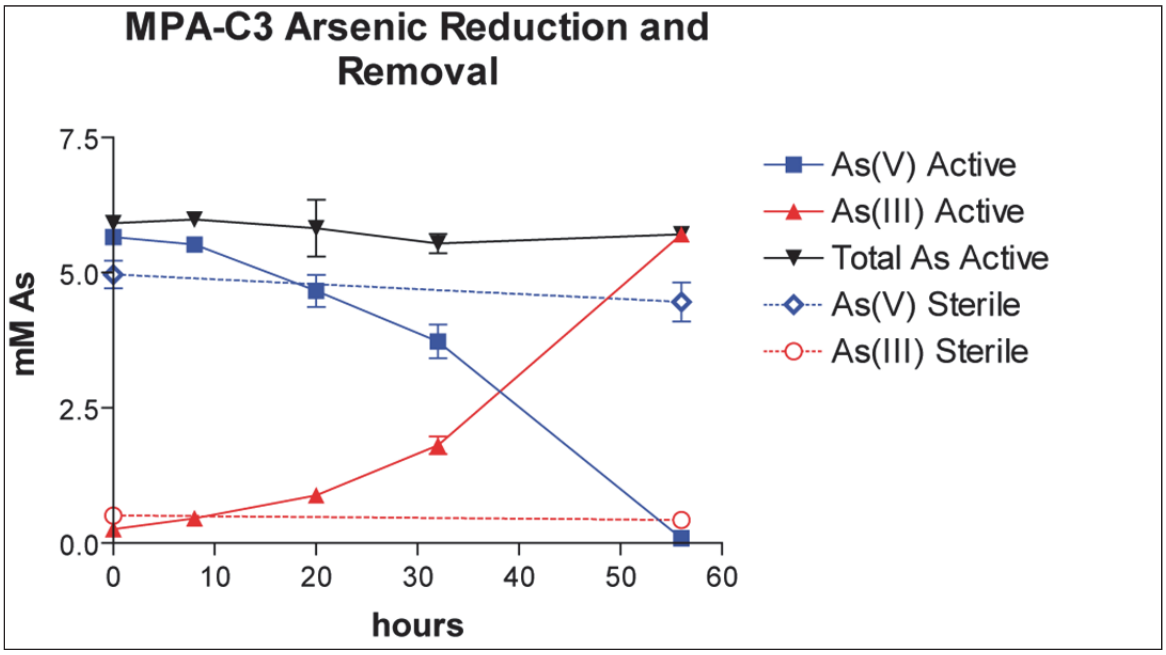

Figure 1:

Stoichiometric reduction of As(V) to As(III) by strain MPA-C3. No As(V) reduction was observed in sterile (autoclaved) controls.

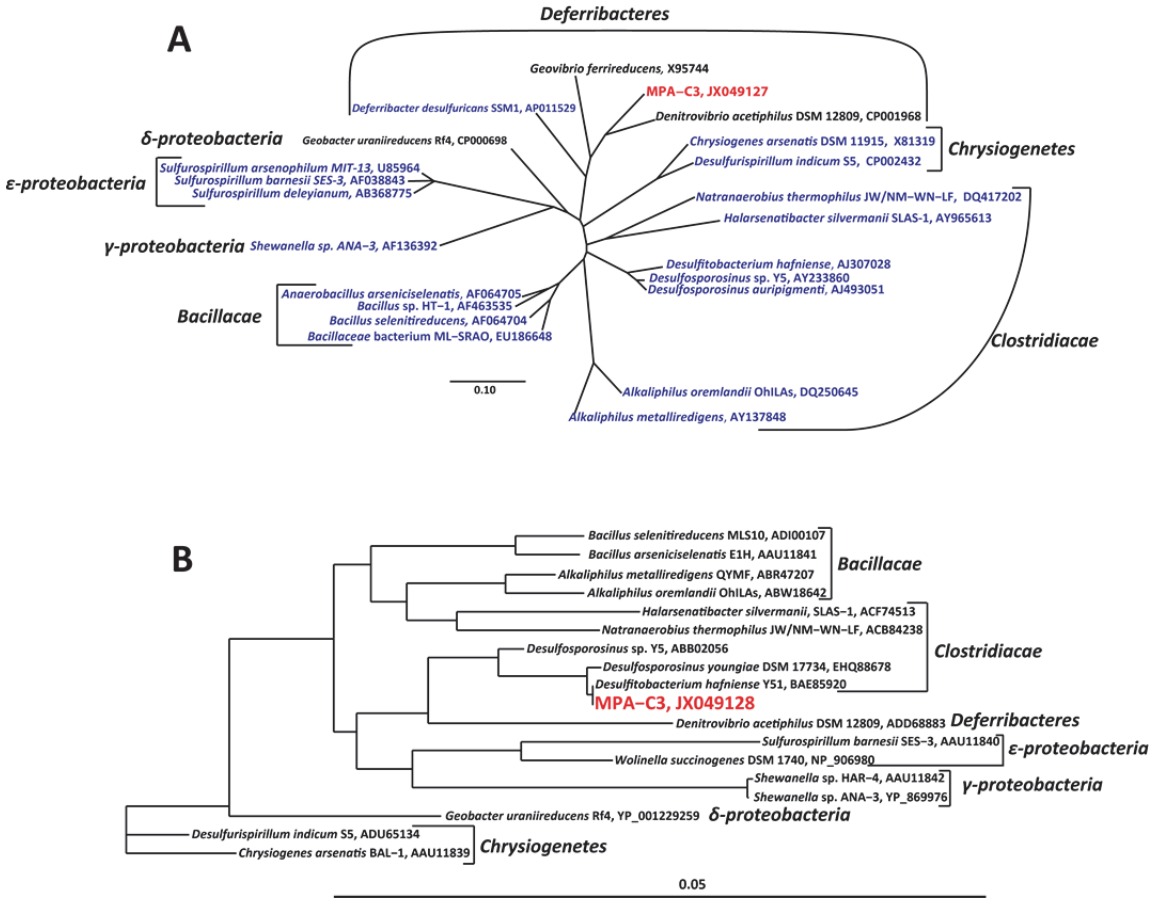

Figure 2A:

rAXML Maximum likelihood tree showing phylogeny of MPA-C3 based on the 16S rRNA gene. Strain MPA-C3 is highlighted in red, organisms which have been demonstrated to reduce As(V) are shown in blue, and those which do not reduce As(V) are shown in black.

Figure 2B:

rAXML Maximum likelihood tree showing phylogeny of MPA-C3 based on the published *arrA* amino acid sequences. Strain MPA-C3 is highlighted in red.

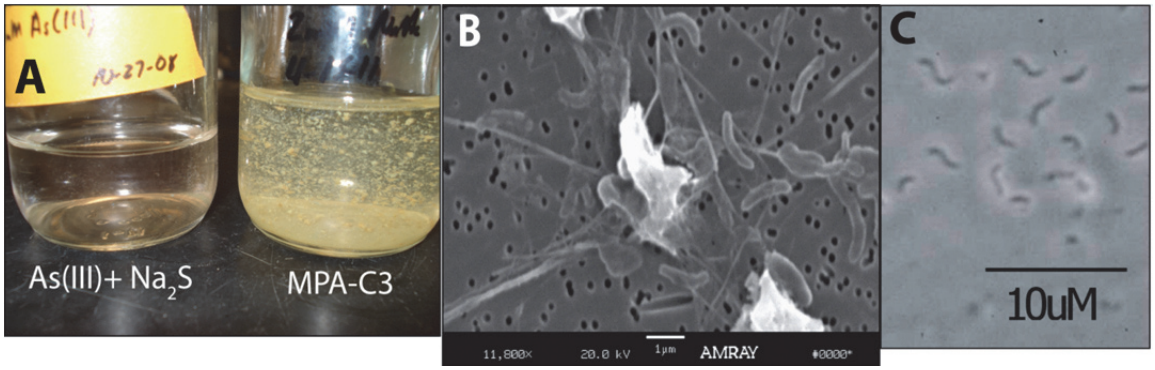

Figure 3:

Microscopic analysis of precipitate formed by MPA-C3.

A: Visible yellow precipitate was observed in the bottle containing MPA-C3 (right). No precipitate was observed in sterile ABF media supplemented with As(III) and sodium sulfide (left).

B: Electron micrograph (11,800x) of precipitate with associated cells.

C: Light micrograph (1000x) of MPA-C3 cells without precipitate

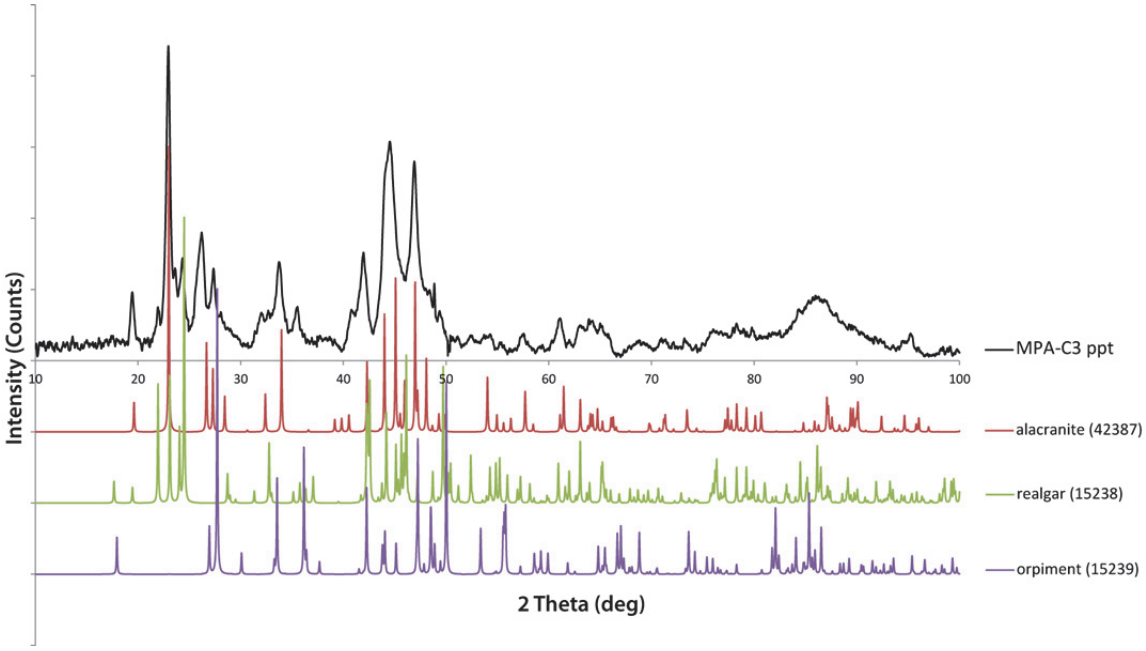

Figure 4:

Powder X-Ray diffractogram (CrKα) of the yellow mineral precipitated by MPA-C3 (top - black line). Below are reference XRD patterns for, alacranite (red), realgar (green), and orpiment (purple). ISCD reference numbers are in parentheses.

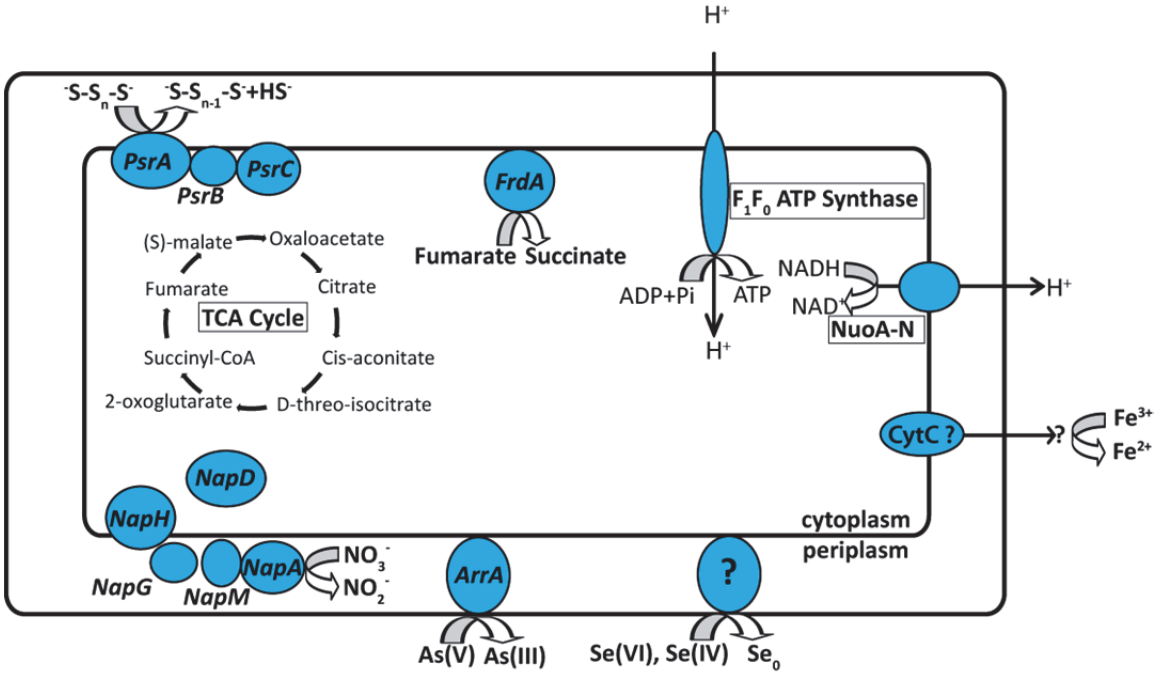

Figure 5:

Schematic representation of metabolic pathways present in MPA-C3. Pathways *in italics* represent pathways observed experimentally, and pathways in boxes are those inferred from genomic analysis with RAST. Nap, periplasmic nitrate reductase; Arr, arsenate respiratory reductase; CytC, cytochrome C; Nuo, NADH dehydrogenase; Psr, Polysulfide reductase.

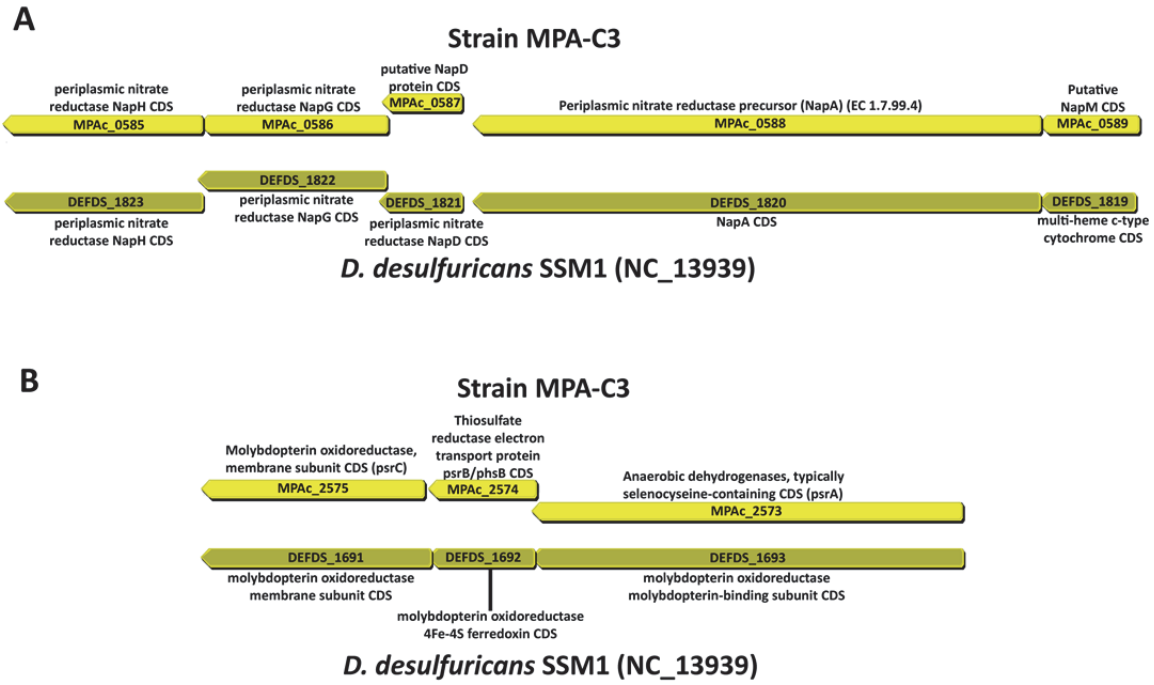

Figure 6A-B:

Alignments between the (A) periplasmic nitrate reductase (Nap) and (B) polysulfide reductase (Psr) genes on the genomes of MPA-C3 and *Deferribacter desulfuricans* (144).

Chapter 3

Microbial Transformations of Arsenic: Mobilization from Glauconitic Sediments to Water

Abstract

We hypothesize that microbes play an active role in the mobilization of arsenic from glauconitic subsurface sediments into groundwater in the Inner Coastal Plain of New Jersey. We have examined the potential impact of microbial activity on the mobilization of arsenic from subsurface sediments into the groundwater at a site on Crosswicks Creek in southern New Jersey. The As contents of sediments 33-90 cm below the streambed were found to range from 15 to 26.4 mg/kg, with siderite forming at depth. Groundwater beneath the streambed contains arsenic at concentrations up to 89 µg/L. Microcosms developed from site sediments released 23µg/L of total arsenic, and active microbial reduction of As(V) was observed in microcosms developed from site groundwater. DNA extracted from site sediments was amplified with primers for the 16S rRNA gene and the arsenate respiratory reductase gene, *arrA,* and indicated the presence of a diverse anaerobic microbial community, as well as the presence of potential arsenic reducing bacteria. In addition, high iron concentrations in groundwater and the presence of iron reducing microbial genera suggests that iron reduction in minerals may provide an additional mechanism for release of associated As, while arsenic-reducing microorganisms may serve to enhance the mobility of arsenic in groundwater at this site.

This Chapter was co-authored with Julia L. Barringer, William M. Benzel, Pamela A. Reilly, and L. Y. Young. It has been published in Water Research volume 46, pages 2859-2868 (2012).

3.1 Introduction

Arsenic (As) is toxic and carcinogenic (37). The effects of chronic exposure to arsenic contaminated groundwater on populations in Asian and Latin American countries (21, 32) illustrate the potential for negative human health effects including cancers of the lung and bladder (132). The arsenic that contaminates groundwater in these countries is geogenic, and, in the extensively studied Ganges delta area, is thought to be released from geologic materials by microbial activity (56, 126).

Naturally occurring microorganisms can affect arsenic availability in several different ways. Microbes can reduce Fe(III) in minerals, promoting mineral solubilization and subsequent release of sorbed As (60, 116, 122). Anaerobic bacteria containing the arsenate respiratory reductase gene *arrA* can use As(V) as a terminal electron acceptor in respiration (107, 124). These dissimilatory As(V)-reducing prokaryotes generate energy from the reduction of arsenate (As(V)) to arsenite (As(III)) (76, 117); with the latter being more mobile in groundwater (131). Other microorganisms may release methyl-arsenic compounds or reduce As(V) to As(III) via the non-respiratory *ars* pathway as mechanisms for detoxifying assimilated As(V) (104).

In southern New Jersey's Inner Coastal Plain Province, geogenic-sourced arsenic is responsible for high dissolved concentrations in streamwater, shallow groundwater, and, possibly, deeper groundwater (14, 15). The primary source of arsenic in this province appears to be glauconite present in the Tertiary and Cretaceous age sediments of marine and marginal marine origin, which underlie much of the Inner Coastal Plain (14, 15, 40, 41). Other phyllosilicates (illite, micas) in these sediments may contain arsenic,

and apatite-rich phosphorite deposits that occur along several formation boundaries are enriched in As (15).

A cooperative study in 2007-09 by the US Geological Survey (USGS) and the New Jersey Department of Environmental Protection (NJDEP) and a companion study at Rutgers University determined that concentrations of arsenic in excess of the NJDEP Maximum Contaminant Level (MCL) of 5 μg/L were present in shallow groundwater discharging to two Inner Coastal Plain streams, and that the release appeared to be microbially mediated (14). This elevated concentration of arsenic required the calculation of the Total Maximum Daily Load (TMDL) for As in Crosswicks Creek. The arsenate respiratory reductase gene (*arrA*), which is considered a marker for arsenic-respiring bacteria (83), was identified in groundwater beneath a site (C6) on Crosswicks Creek (CRO), a major Coastal Plain stream (14) (Fig. 1). Only shallow (< 20-m depth) groundwater discharges to the CRO at C6, which is underlain by clay layers of local extent.

The sediment underlying CRO at C6 includes material accumulated during two centuries of human activity (e.g., gristmill, saw mill, farming), and historic records show the stream was once navigable to C6 from the Delaware River. The accumulated streambed sediments derive from farm soils and weathered outcrops of clay- and glauconite-rich sediments and phosphorite deposits upstream. Elevated levels of DOC, Cl^- and NH_4^+ in the shallow groundwater indicate fertilizers and human and animal wastes leaching to groundwater during the long span of human activities at the site (14,

15); a groundwater NH_3 $\delta^{15}N$ of 5.96 ‰, which is within the range for soil organics (7) and for animal wastes (148), supports this interpretation.

Groundwater discharge at CRO-C6 and CRO-C7 (approximately 3km downstream of CRO-C6) contains high concentrations of Fe and As that precipitate as As-bearing Fe hydroxides upon reaching the stream (14, 15). The oxidation process results in a dichotomy between dissolved concentrations in groundwater (16- 89 µg/L) and those in the stream (generally ≤ 1 µg/L) during low flow (12, 14, 15). Further, speciation of arsenic in the groundwater in 2007 indicated dimethylarsinate (DMA) at 0.24 and 0.36 µg/L and monomethylarsonate (MMA) at 0.24 and 0.28 µg/L, another possible indication of microbial activity (18). At CRO site C6, we initiated a pilot investigation of the microbiological, geochemical, and mineralogical environments beneath the stream to identify those processes involved in the release of As to groundwater, streambed sediments, and stream water.

3.2 Methods

3.2.1 Sample collection

In August, 2009, a set of aqueous and streambed samples was collected at C6 on the CRO. The ultra-clean sampling techniques for stream water and groundwater (sampled with 1.3-cm inner dia. polyvinyl chloride piezometers) in a gaining reach are described in detail in (14, 15)). For the microbiological investigations, 300 mL of groundwater and 200 ml of stream water was filtered (0.45 µm pore-size) aseptically, the

filters were then transferred to dry ice for transport and stored at -20^{o} C prior to DNA extraction, as described in (14).

Two adjacent sediment cores from the streambed at C6 on the CRO were retrieved by driving 1-m-long stainless steel corers with clean butyl acetate liners into the sediments about 1 m from the right stream bank. Cleaning and preparation of the corers is described in detail by (16). The first streambed sediment core was retrieved for microbiological analysis and frozen on dry ice. Sediments retrieved from the second, adjacent core were frozen for mineralogical and chemical analysis.

3.2.2 Microbiological analysis

In the laboratory, the organic layer of the top 15 cm of the core was removed and stored. Sediments from the inner diameter of the core were sectioned, composited by segment and well mixed under sterile conditions. DNA extractions from sediments began with the segment from 15 to 30 cm, just above where the screened interval of the piezometer was positioned in the streambed. DNA also was extracted from the membranes used to filter groundwater and streamwater using a modified Mo-Bio Ultraclean protocol (Mo Bio Laboratories, Carlsbad, CA); these extractions, amplification of the 16S small subunit rRNA gene in sediment and water extracts, and identification of the arsenic respiratory reductase gene (*arrA*) are described in detail by (14). The 16S rRNA gene was amplified with the primers 27F and 1492R (Lane, 1991) and the *arrA* gene was amplified with the primers As1F and As1R. 16S and *arrA* amplicons were cloned using the Invitrogen TOPO-TA kit (Life Technologies, Grand

Island, NY) according to the manufacturer's instructions, and sequencing was performed by Genewiz Inc. (South Plainfield, NJ).

All sequence data was initially screened using the NCBI VecScreen tool to remove vector sequence prior to analysis (97). From the groundwater, 51 16S sequences were recovered, 44 16S sequences were recovered from sediment, and 16 sequences from a groundwater microcosm enriched under As(V) reducing conditions were aligned using the SINA webaligner (112). Results were imported into the ARB software package for phylogenetic analysis (78, 112). Nearest neighbors were picked from the SILVA 102 Ref Nonredundant database (112). A ClustalW aligned reference database of 93 *arrA* sequences deposited in Genbank was constructed in ARB (71, 112). Thirteen *arrA* sequences obtained from groundwater were aligned to the reference *arrA* sequences using ClustalW within ARB (71, 112). The phylogenetic tree shown earlier in (14) was expanded to include all the sequences by using the rAXML maximum likelihood algorithm within ARB (78). This updated tree can be found in the Supplementary Material. An ARBNJ distance matrix was constructed within ARB to allow for further analysis with the mothur software package (128). Sequences were separated into operational taxonomic units (OTUs) based on a cutoff of 85% similarity using mothur (128).

We inoculated microcosms using either site sediment or groundwater supplemented with As(V) (as sodium arsenate) and acetate (as sodium acetate) to enrich for microorganisms capable of respiration using As(V) as a terminal electron acceptor. Active microcosms were prepared in triplicate by inoculating 10 g of composited

sediment from the 15-33 cm depth or 10 mL of groundwater into 30 mL of defined anaerobic media (see table S-1 in Supplementary Material for composition) in 120 mL serum bottles flushed with argon and sealed with butyl rubber stoppers. The microcosms were supplemented with 2.5 mM As(V) (as sodium arsenate) as an electron acceptor and 2.5 mM acetate (as sodium acetate) as a carbon and energy source to enrich for arsenate reducing microorganisms. An additional set of sediment microcosms was supplemented with 2.5 mM acetate as a carbon source but no additional As(V) in order to test for arsenic mobilization from the sediments in the microcosms. Duplicate sterile controls for each set of microcosms were prepared by autoclaving inoculated microcosms three times on three consecutive days. Background controls for each set of microcosms were prepared by inoculating media which did not contain As(V) or acetate. The reduction of As(V) to As(III) as well as the utilization of acetate was monitored by high-performance liquid chromatography (HPLC). After more than 50% of the As(V) was reduced, new microcosms were established in triplicate, with duplicate autoclaved controls, by anaerobically transferring 5 mL of the culture to 45 mL of fresh medium.

All microcosms were incubated in the dark under anaerobic conditions at T = 25 ^{o}C (about the same temperature (26.5 ^{o}C) as the groundwater). Each week, 600 μL was sampled using an argon-flushed syringe, filtered through a Costar 0.22 μM nylon centrifuge tube filter (Corning Inc., Corning, NY) and analyzed for arsenic species until complete reduction of As(V) to As(III) was observed. Culture supernatant samples from the sediment microcosm supplemented with acetate only were obtained on days 0 and 23, and analyzed for As by inductively coupled plasma-optical emission spectrometry (ICP-

OES). 50 μL aliquots of the sample were diluted in 950 mL of 1% nitric acid, and ICP-OES analysis was performed at the Rutgers Inorganic Analytical Laboratory using a Varian Vista Pro Inductively Coupled Plasma Optical Emissions Spectrometer. ICP-OES data analysis was performed using Varian ICP-Expert software.

The reduction of arsenic in the microcosms was measured using a Beckman System Gold HPLC (Beckman Coulter, Brea CA) equipped with a Hamilton PRP X-100 anion exchange column (Hamilton, Reno NV) as described by Perez-Jimenez et al. (107). As(V) and As(III) ions were eluted with 40 mM sodium phosphate flowing at 1 mL/min, and detected at 195 nm. Acetate was measured using a Beckman System Gold HPLC equipped with a Phenomenex ROA Organic Acids Column (Phenomenex, Torrance CA). Acetate was eluted with 5 mM sulfuric acid flowing at 0.5 mL/min, and detected at 205 nm.

3.2.3 Chemical and mineralogical analysis of core sediments

The chemical composition and mineralogy of the second sediment core was determined at the USGS Geologic Discipline Research Laboratory (GDL), Denver, CO. The frozen core was sectioned into 2 to 4-cm segments; these were retrieved from 33 to 36 cm; 43 to 46 cm; 56 to 58cm; 63 to 66 cm, 71 to 74 cm, and 86 to 90 cm in order to assess changes in chemistry and mineralogy reflective of changes in redox conditions in the sediments at and below the interval selected for microbial analysis.

Samples were prepared by removing the outer rim (~0.5 cm) of each sample plug; the remaining material was freeze-dried, loosely consolidated, and disaggregated. Each bulk

sample was riffle split into three 50-g aliquots. One aliquot was ground and chemically analyzed at the GDL. Analyses for carbon were done by titration and LECO analyzer (30, 31) at the GDL. Major- and trace-element analysis was done by inductively coupled plasma/mass spectrometry (ICP/MS), following total acid digestion (29).

Another fraction of the bulk sediment was analyzed for mineralogy by X-ray diffraction (XRD) to determine mineral abundances. Samples were crushed by hand to <250 μm, micronized in a McCrone mill (McCrone Microscopes and Accessories, Westmont IL) with isopropanol, randomly packed into holders, and analyzed on a Scintag X-1 diffractometer (Scintag Inc, Sunnyvale, CA). The RockJock program (43), used to quantify the mineralogy, yielded semi-quantitative mineral contents because the analysis did not consider amorphous and poorly crystalline material. Heavy mineral concentrates of the five deepest samples were examined optically.

Polished grain mounts were made from aliquots of the five deepest samples. Unconsolidated sediment was stirred into slow-set epoxy and centrifuged to create graded grain sizes. The epoxy plugs were cut on an angle, yielding a section through the graduated grains, and polished. A Scanning Electron Microscope (SEM) scanned several hundred particles in each mount for minerals with substantial arsenic content. Select areas and individual grains also were studied by SEM to document texture and growth habits of the grains. Glauconite and siderite separates were analyzed for total As content by ICP.

3.3 Results and Discussion

3.3.1 Sediment chemistry and mineralogy

The sediment beneath the streambed at CRO-C6 is composed primarily of quartz and phyllosilicates—mainly glauconite, but also micas (biotite, phlogopite, muscovite, illite)—with minor amounts of feldspar and siderite. The arsenic content of the sampled sediments ranged from 15 to 26.4 mg/kg (Table 1), with the highest arsenic and calcium contents at 33- 36 cm, and at 71-74 cm. Analysis of mineral separates indicates that arsenic content of the glauconite and siderite was 33.7 mg/kg, and 184 mg/kg, respectively (Table 1). Fines (< 250 μm illite and Fe hydroxide) contained 46 mg/kg of arsenic, similar to the precipitate formed at a CRO groundwater seep (15). Regression analysis indicated the arsenic contents of the sediment were linearly and strongly related to iron and calcium contents (R^2 = 0.91 and 0.95, respectively; $p < 0.05$), indicating that iron minerals form the primary source of arsenic at this site. The relation of As with Ca may be indicative of precipitation of a Ca-As phase; however, speciation calculations indicated that the groundwater was undersaturated with respect to calcite (Barringer et al., 2011). The high arsenic and calcium contents of the 33-36 and 71-74-cm intervals also are distinguished by relatively high contents of micas other than glauconite (Table 1). SEM scans of selected bulk sediment samples did not detect pyrite or amorphous sulfide, indicating that arsenical sulfides do not form a significant source of arsenic at this site.

Although siderite was not detected in surficial streambed sediments, speciation calculations indicated groundwater supersaturation (15). Siderite as grains and cement was detected in the deeper sediments, along with glauconite in iron hydroxide cement

(Figure S3.2). Siderite is a secondary mineral that could be precipitated through activity of iron-reducing microbes, as described by Anawar et al. (2006) and Islam et al.(2005). Such biogenic siderite has been proposed as a sink for As(V), but not As(III) (6, 59); As(III) has been shown (59) to bind weakly to siderite at pH values below 7. Analysis of the mineral separates from the CRO C6 site shows that siderite is a sink for arsenic (presumably As (V)) in the New Jersey sediments at slightly acidic pH values (groundwater pH 6.4-6.9 (15)). Therefore, in addition to primary glauconite in the accumulated sediments of the streambed, arsenic is present in at least two secondary phases; these include iron hydroxides that formed in what were once the upper portions of the streambed sediments, and siderite that precipitates as individual grains and as cement for other grains.

3.3.2 Microbes in streambed sediments and the aquifer

As illustrated in Figures 2A-2C, As(V) reduction, arising from arsenic respiration, was observed in both groundwater and sediment microcosms supplemented with acetate and As(V). The reduction of As(V) to As(III) was observed in active groundwater microcosms, while no reduction was observed in sterile controls (Fig. 3A). Unlike the groundwater microcosms, the sediment microcosms (Fig. 2B) sorbed the supplied 2mM As(V) in the time between preparation of the microcosm, and the initial sampling (<10 minutes). This loss was observed upon analysis of the Day 0 sample, and an additional spike of 5mM As(V) was added on day 7 (Fig. 2B). As(V) reduction was observed following the addition of As(V) into the microcosms, Figure 2B also shows that no

production of As(III) from As(V) reduction was observed in sterile controls, clearly demonstrating that reduction was microbially mediated.

In both the groundwater and the sediment microcosms, the product of As(V) reduction, As(III), persisted in solution, demonstrating that microbial reduction can play a role in enhancing arsenic mobility in groundwater. Jönsson and Sherman (59) report that As(III) binds only weakly to siderite, a primary arsenic sink in this system, compared to the strong binding exhibited by As(V). Although no As(V) reduction occurred in the sterile controls, As(V) concentrations decreased over time in the sterile sediment microcosms (Fig. 3B), suggesting continued binding of As(V) to the sediment. Arsenic mobilization was not detected in background controls (containing sediment inoculum but no added As(V) or acetate), indicating the requirement for additional organic carbon to stimulate the native microbial community (data not shown). Following subsequent transfer of 5 mL of culture to a fresh medium, rapid As(V) reduction was observed, a clear indicator of an enriched community of arsenic respiring microbes (Fig. 2C). Furthermore, As(V) loss due to binding to sediment was greatly reduced insofar as very little sediment was transferred to these subcultures.

Figure 3 shows total arsenic released (mobilized) from native sediment in the sediment microcosms supplemented with acetate. By day 23, the total soluble arsenic concentration was 27 µg/L, with no mobilization of soluble arsenic observed in the sterile control (Fig. 3A). The analyses by ICP-OES do not speciate; nonetheless, it is not unreasonable to assume that the released arsenic is in the reduced As(III) form since a.) the microcosms are anoxic and reducing, and b.) our data (Figs. 2A-C) and others (59,

131) show very little sorption of As(III). The microbial utilization of acetate was observed in active microcosms, with acetate concentrations decreasing over the 20 day period, while no loss of acetate was seen in the sterile microcosm controls (Fig. 3B). Note that the only available arsenic in these bottles is that which is bound to the sediments. The fact that this arsenic is mobilized and released into solution when acetate is available is evidence for microbially mediated release. This indicates that an influx of organic carbon enhances microbial growth and metabolism, leading to an increase in microbiologically mediated arsenic release from the CRO-C6 sediments under anaerobic conditions. It is noteworthy that our observed concentrations of arsenic released in these microcosms is consistent with the concentration of arsenic in CRO-C6 groundwater reported by Barringer et al. (2010), and further reinforcing the relevance of microbial processes to arsenic mobilization at this site.

3.3.3 Molecular characterization of groundwater and sediment microbes

The 16S rRNA gene sequences recovered from the sediment and groundwater of CRO site C6 suggest the presence of diverse anaerobic bacterial communities in both groundwater and sediment (Figs. 4A,B). Analysis of 51 cloned sequences from the groundwater microbial community allowed for the identification of 49 unique OTUs, based on a 97% cutoff (Fig. 4A). These sequences have been submitted to Genbank under accession numbers JQ041420 through JQ041470. Sixteen clones were sequenced from a microcosm inoculated with a 1:1000 dilution of C6 groundwater. Fifteen of these clones showed 97+% identity to the Fe-reducing bacterium *Geobacter* sp. Ply1

(EF527233), while one showed 99 % identity to *Alkaliphilus oremlandii* (CP000453) (formerly Clostridium sp. strain OhlLAs), an organism that has been demonstrated to respire As(V) (54, 139). The presence of organisms highly similar to *Geobacter* and *Clostridia* suggests that the iron reduction inferred from the groundwater chemistry and sediment mineralogy is occurring in the CRO sediments, and is one likely mechanism for release of arsenic (38, 56, 106, 139). The sequences from this enrichment culture have been submitted to Genbank under accession numbers JQ062862 through JQ062877.

From the sediment core, 44 clones were sequenced, leading to the identification of 11 unique OTUs, suggesting a lower diversity in the sediment than in the groundwater (Figure 5B). All 16S rRNA gene sequences have been submitted to Genbank, with accession numbers JQ041471 to JQ041514. Others have also reported on higher microbial diversity in groundwater than in adjoining sediment (Kilb et al., 1998; Alfreider et al., 2002), and we believe that is the case here.

The PCR amplification of *arrA* from DNA extracted from sediment and groundwater as well as a preliminary analysis of the *arrA* sequences recovered is described in detail in Barringer et al. (2010). The 13 partial *arrA* sequences obtained were aligned to 93 full length and partial *arrA* sequences obtained from GenBank using ClustalW within ARB (71, 78). Following construction of an ARBNJ distance matrix within ARB, sequences were clustered using mothur (Schloss et al., 2009) into three OTUs based on a 15% cutoff (85% similarity). As suggested by earlier results (14), two of the three OTUs showed 75-77% homology to a putative *arrA* gene found on the genome of *Geobacter uraniireducens* Rf4, while the third OTU displayed 71-72%

homology to the *arxA* gene of *Alkalilimnicola erlichii* MLHE-1, which has been reported to function as an arsenic oxidase (103). The presence of sequences similar to the *arxA* gene of the arsenic oxidizing bacteria *A. erlichii* MLHE-1, which uses *arxA* as an As(III) oxidase under nitrate reducing conditions (103, 119), indicates the potential for microbial arsenic oxidation at site CRO-C6 as well, particularly at the sediment-water interface where nitrate may be supplied by surface water. All recovered *arrA* gene sequences have been submitted to Genbank under accession numbers JQ041515 through JQ041527.

Successful amplification of the *arrA* gene, a known biomarker for arsenic respiration (83) combined with the enrichment of As(V)-reducing microorganisms provides strong evidence for the presence of arsenic reducing microbes at the CRO C6 site. As suggested by (118), microbial arsenic oxidation can also serve as a mechanism of mobilization. The presence of an arsenic oxidizing microbe would fit well with the shallow groundwater chemistry, insofar as As species measured in the water discharging to the stream in 2007 showed roughly equal concentrations of As(V) and As(III) (15).

3.4 Conclusions

Based upon our findings, we can conclude that the release and transport of arsenic from geologic materials at the CRO C6 site is mediated and enhanced by microbial metabolism. Our data indicate that the release of arsenic from stream bed sediment is strongly enhanced by the addition of organic carbon (Figure 3A,B), indicating that microbial metabolism plays an important role in arsenic mobilization. Notably, our finding that reduced As (as As(III)) persists in solution allows us to conclude that the

solubility and therefore the transport of arsenic in shallow groundwater at the CRO C6 site is influenced by microbial respiratory reduction of As(V) to As(III) and is consistent with reports from other studies (59, 131). While siderite was noted as a major sink for arsenic at this site, it binds As(III) poorly (59), further suggesting that microbially reduced As(III) will remain in solution at this site.

A complex series of interactions between microbes, primary and secondary geologic materials, and water is envisioned to result in the levels of arsenic found in sediments, groundwater and stream water. Figure 5 displays our conceptual model, with the processes confirmed in this study in black, and major contributing processes suggested by other reports in gray. Our overall conceptual model of arsenic release at CRO-C6 includes microbial reduction of Fe(III) enhanced by inputs of organic carbon in both crystallized and amorphous Fe-bearing materials and subsequent release of As to groundwater as reported by Islam et al. (2004) and Tufano et al. (2008); direct release of arsenic from Fe minerals through microbial respiratory arsenic reduction (155) coupled to the oxidation of organic carbon; microbial respiratory reduction of released As(V) to As(III) enhanced by inputs of organic carbon as demonstrated in this study; and the binding of released As(V) to microbially precipitated siderite (59) within the accumulated streambed sediments. Thus, microbially reduced arsenic will persist in groundwater, and present a hazard if the groundwater is used for consumption. Insofar as the kinetics of abiotic oxidation of As(III) in oxic water have been reported to be extremely slow (131), microbial oxidation of As(III) to As(V) (117, 125) may enhance the co-precipitation of As and Fe at the oxic/anoxic interface in the upper streambed as described by (15). Based

upon the results of our study, we believe that microbial activity plays a significant role in arsenic cycling at CRO-C6, and that the further elucidation of microbially mediated geochemical processes is important for understanding water quality in areas with As-enriched geological materials and agricultural and residential development.

3.5 Supporting Information

Supporting information including XRD data for the sediment core, photograph of glauconite grains, SEM results showing high P in amorphous material, and the composition of anaerobic medium can be found in Appendix 2.

3.6 Acknowledgments

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Table 1:

Contents of selected major and trace elements in a streambed sediment core and mineral separates from Crosswicks Creek at site C6, August, 2009.

Sample name	Depth below streambed (cm)	Carbonate C (%)	Organic C (%)	Al (mg/kg)	As (mg/kg)	Be (mg/kg)	Ca (mg/kg)	Fe (mg/kg)	K (mg/kg)	P (mg/kg)
01464485A	33-36	0.15	7.5	38,100	26.1	3	4,020	111,000	20,700	4,010
01464485B	43-46	0.03	0.86	19,700	15	2.4	1,240	66,700	21,800	889
01464485C	56-58	0.21	2.25	38,100	20.4	3.3	2,530	94,300	26,000	2,460
01464485D	63-66	0.41	0.54	19,300	22.8	3.4	3,810	110,000	29,000	2,200
01464485E	71-74	0.08	0.27	18,800	26.4	3.2	4,170	110,000	32,700	2,500
01464485F	86-90	0.27	2.22	30,900	20	3.2	2,740	90,300	25,800	2,000
Glauconite Separate	Composite depths	NA	NA	30,600	33.7	5.3	14,000	189,000	62, 800	6, 760
Siderite Separate[a]	Composite depths	NA	NA	24, 700	184	3.7	5, 850	310,000	11, 100	6, 540
Fines[b]	Composite depths	NA	NA	66, 600	46	5.4	5,100	181,000	44,700	2, 290

[a] The siderite separates contained about 50% quartz and minor glauconite; because of quartz dilution, the As value is probably higher than shown here. [b] Fines include illite and Fe hydroxides. NA = not analyzed.

Table 2:

Semi-quantitative mineralogy reported in wt% in selected intervals of a streambed sediment core, Crosswicks Creek, New Jersey, August 2009.

		Sample number and depth below streambed sediment surface					
		01464485A 33 - 36 cm	01464485B 43 - 46 cm	01464485C 56 - 58 cm	01464485D 63 - 66 cm	01464485E 71 - 74 cm	01464485F 86 - 90 cm
Mineral	Chemical formula	Mineral (wt. %) or presence					
quartz	SiO_2	43	64	63	60	45	52
glauconite	$K(Fe,Al)_2(Si,Al)_4O_{10}(OH)_2$	15	14	15	18	19	18
mica (total)[a]	$(K,Na)2(Al,Mg,Fe)_6$ $(Si,Al,Ti)_8O_{20}(OH,F)_4$	29	18	17	19	27	19
plagioclase	$Na(AlSi_3O_8)$	3	2	2	1		1
K-feldspar	$KAlSi_3O_8$	6	3	2	1	3	4
rutile	TiO_2	2	<1	<1			<1
ilmenite	$FeTiO_3$	<1	<1	<1		1	<1
goethite	$FeO(OH)$	2			<1	<1	<1
hematite	Fe_2O_3		<1				
siderite	$Ca_{0.1}Mg_{0.33}Fe_{0.57}(CO_3)$				2	<1	5
pyrite	FeS_2		pc[b]	absent[c]	absent	absent	absent
Total wt. %		100	101	99	101	97	99

[a]Mica (total) includes biotite, muscovite, phlogopite and illite. [b] pc - observed in pan concentrate. [c] absent - not observed in pan concentrate.

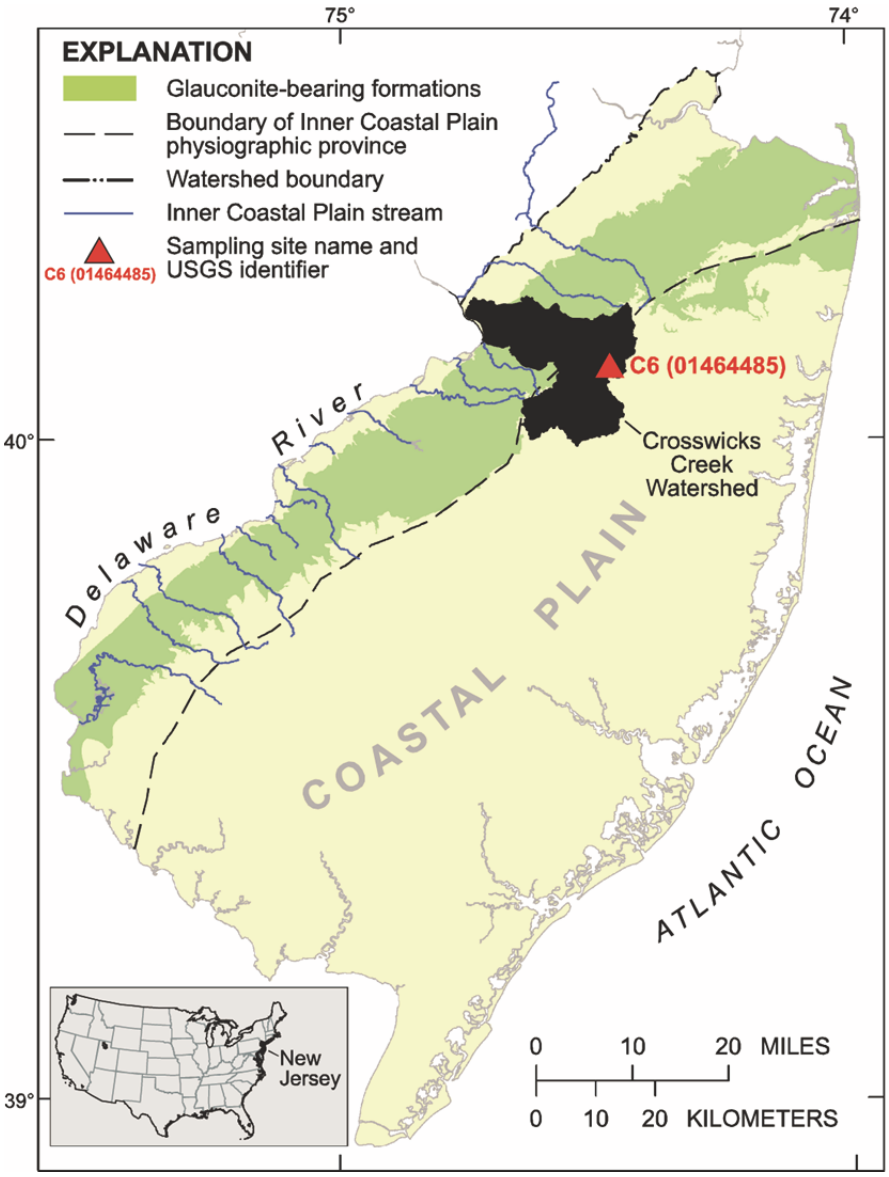

Figure 1:

Map of New Jersey Coastal Plain showing outcrop area of glauconite-bearing marine sediments, Crosswicks Creek watershed, and sampling site C6.

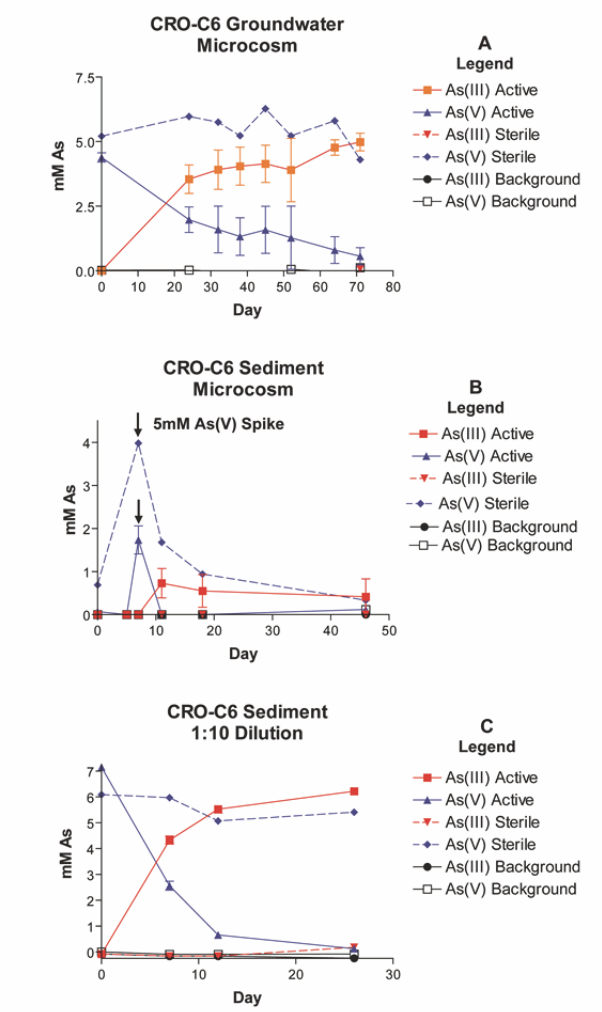

Figure 2:

Microcosm results showing As(V) reduction in (a) groundwater microcosms amended with arsenic and acetate; (b) streambed sediment microcosms amended with arsenic and acetate (15-30 cm depth); (c) streambed sediment microcosms diluted 1:10 and amended with arsenic and acetate (15-30 cm depth). As(V) reduction was not noted in sterile controls. Sterile controls were amended with arsenic and acetate prior to sterilization by autoclaving, while background controls contain media and sediment, but were not supplemented with As(V) or acetate.

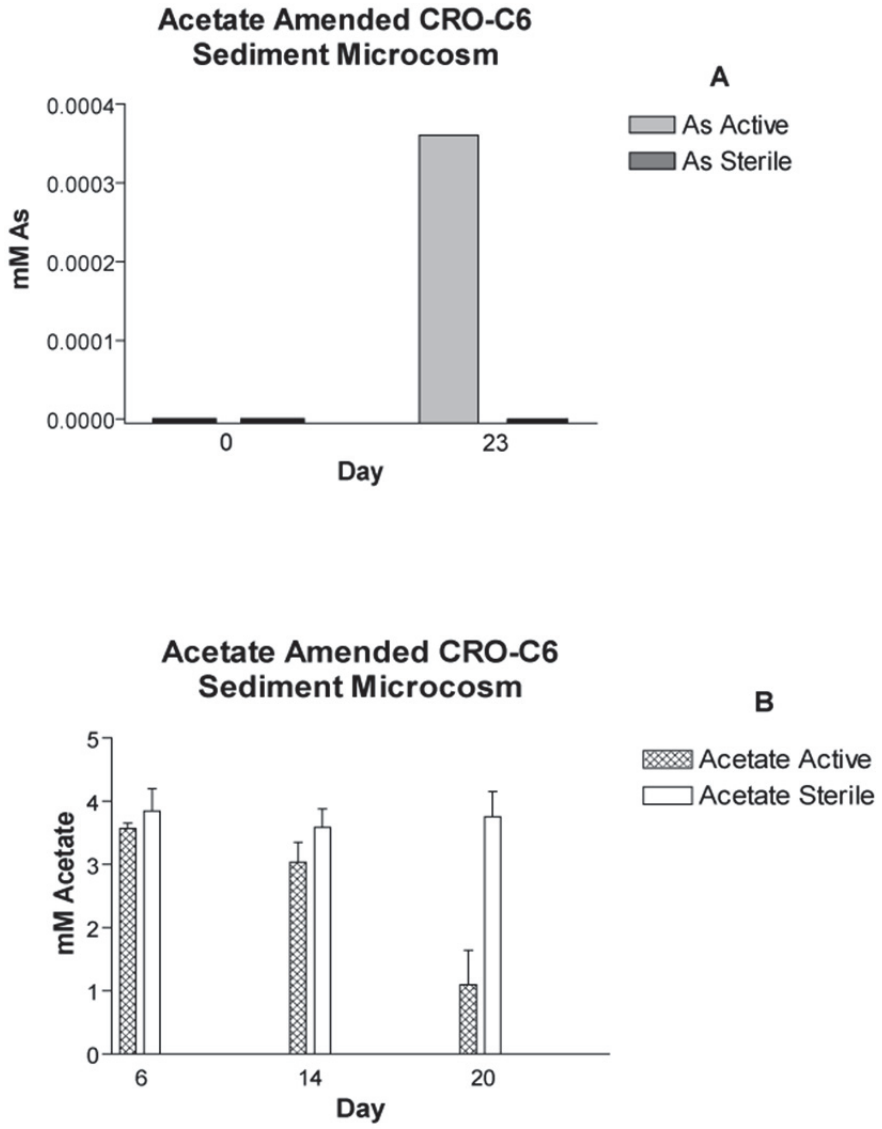

Figure 3:

Total arsenic release from streambed sediments amended with acetate (A), coupled to the utilization of acetate (B). Arsenic was not detected on day 0 or in control microcosms sterilized by autoclaving.

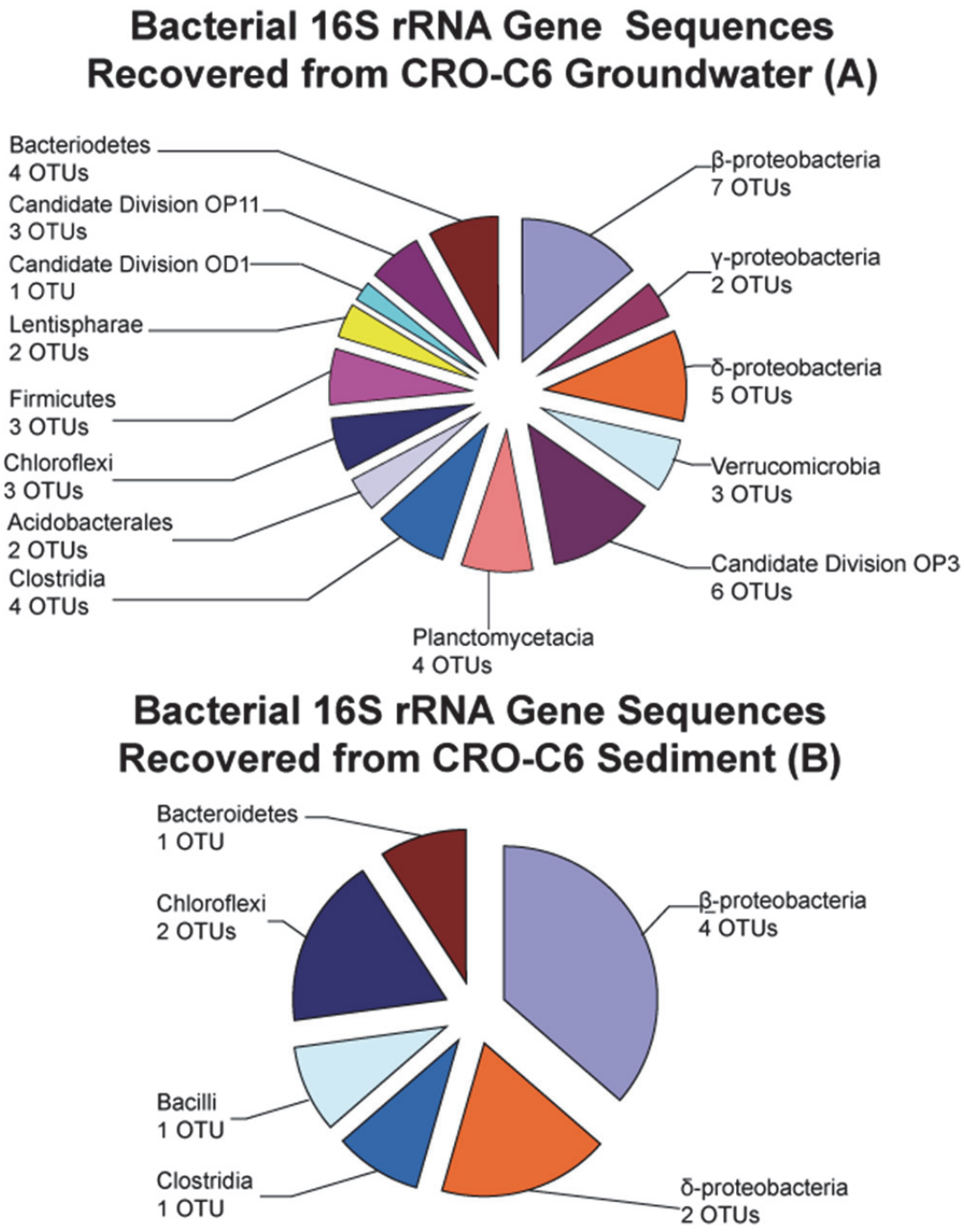

Figure 4:

Microbial communities in found in groundwater (A) (51 clones) and a streambed sediment core (B) (44 Clones), Site CRO-C6 August, 2009.

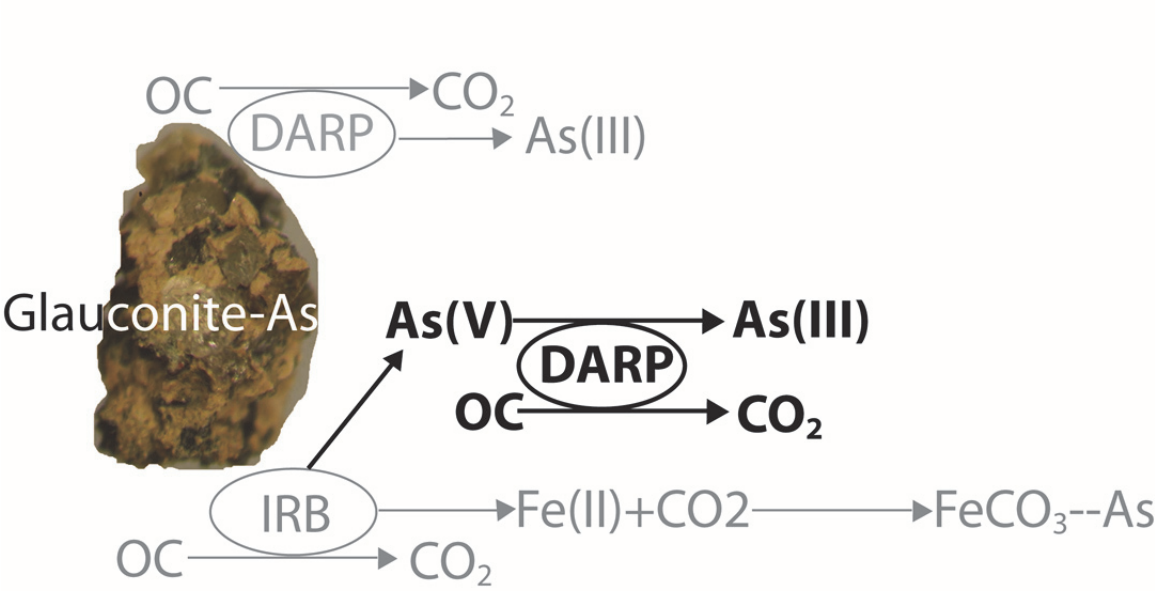

Figure 5:

Conceptual diagram of pathways for microbially mediated arsenic release from glauconite ($(K,Na)(Mg,Fe^{+2})_{0.33}(Fe^{+3},Al)_{1.67}[(Si,Al)_4O_{10}](OH_2)$). Processes observed in this study are in black, those observed in other studies and potentially also occurring at this site are in gray. Microbial activity may be enhanced by inputs of organic carbon, leading to an increase in the reduction of iron and mobilization of arsenic. As(V) in particular may be re-precipitated with, or sorbed to, siderite.

OC: Organic Carbon

IRB: Iron Reducing Bacteria

DARP: Dissimilatory Arsenate Respiring Prokaryotes

Chapter 4

Groundwater redox controls microbial release of arsenic from minerals to groundwater in a fractured rock terrain, New Jersey, USA

Abstract

The mobilization of arsenic bound to aquifer sediments is a source of contamination in groundwater in New Jersey. Deep Coastal Plain sediments have average arsenic concentrations of 15-23 mg/kg, and arsenic concentrations of 26 mg/kg are found in shallow glauconitic sediments. In this study we investigate microbial As(V) mobilization in the shallow aquifers beneath the streambeds at two tributaries of the Millstone River, Six Mile Run and Pike Run, in the black and red shales of the New Jersey Piedmont Physiographic Province. These two sites offer a distinctive comparison, namely a similar mineralogy and geochemistry, with the notable difference being that reducing conditions are present at Six Mile Run (E_H=-39.5mV), and oxidizing conditions are present at Pike Run (E_H=+144mv). Oxygen-depleted groundwater at the Six Mile Run site has an arsenic concentration of 27 µg/L, exceeding the New Jersey Department of Environmental Protection maximum drinking water contaminant level of 5 µg/L, whereas the arsenic concentration in more oxidizing groundwater at the Pike Run site is 2.1 µg/L. Groundwater sampled on gaining reaches of these rivers was inoculated into anaerobic microcosms with acetate as a carbon source and As(V) as an electron acceptor.

Microcosms developed from Six Mile Run groundwater reduced 1 mM of As(V) to As(III) in 30 days, while microcosms from Pike Run did not reduce As(V). The 16S rRNA gene and the As(V) respiratory reductase gene, *arrA*, were amplified from DNA extracted from groundwater. Analysis of the 16S rRNA gene by Denaturing Gradient Gel Electrophoresis (DGGE) indicated that distinct bacterial communities are present at each site, a finding supported by cloning and sequencing. Cloning and sequencing of the As(V) respiratory reductase gene, *arrA,* revealed distinct arsenic respiring communities at each site, with 8 unique OTUs found at Six Mile Run and 11 unique OTUs at Pike Run, which may be representative of the arsenite oxidase gene *arxA*. Our findings demonstrate that the microbial As(V) reduction and mobilization is facilitated by the reducing conditions present at Six Mile Run, while it is inhibited by the oxidizing conditions present at Pike Run.

This Chapter was co-authored with Julia L. Barringer, Pamela A. Reilly, D.D. Eberl, A.E. Blum, and L.Y. Young. It has been submitted for publication in Water Research, and is currently in review.

4.1 Introduction

Arsenic is contained in numerous geologic materials that, in parts of the world, have contributed As to groundwater used as a potable water source. The result has been exposure of millions of people to hazardous levels of As (up to hundreds of micrograms per liter) in drinking water, creating illnesses, including cancer (133). The role of microbes in the release of As into groundwater from the alluvial sediments of the river deltas of Southeast Asia to groundwater has been studied, particularly in several Asian countries with widespread As contamination in drinking water(4, 6, 20, 21, 56, 73, 135)

In glauconitic sediments in the Coastal Plain of New Jersey, shallow groundwater discharging to streams contains As at concentrations that range up to 89 µg/L (14, 90). These As concentrations have been linked to the microbially mediated release of As from minerals such as glauconite, other phyllosilicates, apatite, and siderite in the sediments (Barringer et al., 2010; Mumford et al., 2012). Additionally, similar sediments in the Atlantic Coastal Plain of the USA have been reported to contain levels of As in groundwater that exceed the Federal drinking-water maximum contaminant level (MCL) of 10 µg/L (50, 146).

The Piedmont Physiographic Province of New Jersey is composed of sedimentary rocks with minor igneous intrusives and extrusives of the Newark Basin and borders the Coastal Plain to the northwest as shown in Figure 1. The Newark Basin rocks of the Piedmont Physiographic Province contain As-bearing minerals identified as pyrite, hematite, and clays (130). Groundwater in various parts of the sedimentary and igneous fractured-rock aquifers is contaminated with As, with concentrations ranging from 5 µg/L

(the State maximum drinking water contaminant level (MCL)) to 215 μg/L (130). Processes (e.g., oxidation of pyrite in black shales, desorption from red shales) that release As from bedrock to groundwater have been investigated (108, 118, 130, 154). Microbially mediated oxidation of S^{2-} in pyrite-containing bedrock by an autotrophic inorganic-S and As(III) oxidizing bacterium was demonstrated by Rhine *et al.* (2008). Although high concentrations of As tend to coincide with low oxygen levels in Piedmont groundwater (130), the role of microbes in As release under reducing conditions has, to the authors' knowledge, not been explored, particularly in shallow groundwater discharging to the region's streams.

There are streams and rivers in the New Jersey Piedmont Physiographic Province in which the stream water contains As at levels exceeding the State Surface Water Quality Standard (SWQS) that, owing to the carcinogenic nature of As, is a stringent 0.017 μg/L (N.J.A.C. 7:9b). Among these rivers is the Millstone River, which rises in the glauconitic sediments of the Coastal Plain and continues through the Piedmont's Newark Basin fractured rock terrain to its confluence with the Raritan River near Raritan Bay as shown in Figure 1.

As part of a river-wide study of the Millstone River, two tributaries were targeted for a combined geochemical/microbiological study to determine if microbially mediated As release, as seen in the NJ Coastal Plain (14, 90), is occurring in the fractured bedrock aquifer characteristic to the NJ Piedmont. The tributaries—Six Mile Run and Pike Run—are underlain by bedrock of the Passaic Formation, which is composed primarily of red mudstones and siltstones interspersed with black shale and gray mudstone. The dark

strata are the richest in As mainly because of the presence of pyrite (Serfes, 2005). Arsenic contents of the more abundant red strata were found to range from 4.5 to 14.8 mg/kg (Serfes, 2005), and it is over these (presumably) less enriched strata that the channels of the two tributaries pass at the chosen sampling sites.

Our study at the two sites included determining the mineralogy and chemistry of streambed sediments, the chemistry of shallow groundwater and streamwater, and the microbial communities of the shallow aquifer and their abilities to reduce As(V). Results of the study and discussion of the biogeochemical processes that affect As mobility are presented herein.

4.2 Methods

4.2.1 Groundwater and streamwater sample collection

The Six Mile Run and Pike Run sites were selected in the Piedmont portion of the Millstone Basin due to the presence of sufficient weathered material to allow for the insertion of piezometers into streambed sediments during a period of low flow conditions. Acid-washed PVC piezometers (1.3-cm inner diam. with a 15-cm-long slotted screen) were driven into the streambeds in gaining reaches for measurements of shallow groundwater chemistry (13-15, 90). Production of groundwater from the piezometers was confirmed by an increase in conductance prior to sample collection, and samples were obtained using a peristaltic pump through Teflon^{TM}-lined tubing that extended down through the piezometer to the screen. NJDEP monitoring well MW114, located approximately 60m south of the Six Mile Run stream sampling site, is

hydraulically connected to the aquifer beneath Six Mile Run, and was sampled with the Six Mile Run site in order to study the processes and conditions deeper beneath Six Mile Run. MW114 is screened from 3.3 to 6.4 meters below land surface, and samples were obtained using a Grundfos submersible pump with acid-washed tubing.

Tributary streamwater samples and groundwater samples were collected at the same time during a period of low-flow conditions. Streamwater was collected as grab samples using an equal-width-interval technique and composited in an acid-washed churn, as in previous studies (Barringer et al., 2010a, 2011). Processing and filtering of each composited sample was done in a new polyethylene bag on a PVC frame (bags were changed between samples). Ultraclean techniques were followed throughout (Wilde et al., 2004). All equipment was acid washed (5 percent HCl) and copiously rinsed with de-ionized water prior to use. De-ionized water (DI) and field-rinsed 0.45-µm-pore-size capsule filters were used to collect filtered samples for analysis of As and other trace elements. Disc filters (0.45-µm-pore-size) were used to collect dissolved organic carbon (DOC) samples. Sample bottles, except the baked glass bottles used for DOC and total organic carbon (TOC) samples, were DI- rinsed before sampling and field rinsed before sample collection.

Field parameters (temperature, dissolved oxygen, pH, specific conductance, turbidity) were measured at all sites using a YSI data sonde (YSI Inc., Yellow Springs, OH). Filtered and unfiltered samples were collected for analysis of As, metals, DOC and TOC.

4.2.2 Sampling For Microbiological Analysis

Groundwater samples were collected using sterile equipment and filtered with sterile Pall GN6 (0.45 μm pore-size) filters (Pall Corp. Ann Arbor, Michigan). The filter membranes were immediately stored on dry ice for transportation and stored at -80^{o}C until processing. Groundwater samples were transported on ice and stored at 4^{o} C prior to use as inoculum for microcosms.

4.2.3 Streambed-Sediment Sampling

Streambed-sediment samples were collected under low-flow conditions. Streambed-sediment grab samples were collected in areas of deposition using an acid-washed plastic scoop. The sediments were sieved (<2-mm grain size) using an acid-washed plastic sieve and placed in field-rinsed jars. Attempts to collect cores of streambed sediments with a hand-driven stainless steel corer were unsuccessful.

4.2.4 Sample Analysis

Streamwater and groundwater samples were analyzed for major ions, nutrients, acid-neutralizing capacity (ANC), DOC and TOC, As, and other selected trace elements at the U.S. Geological Survey National Water Quality laboratory (NWQL) in Denver, CO. The samples included filtered and unfiltered water. Major and trace elements were determined by inductively coupled plasma optical emission spectroscopy (ICP/OES) and inductively coupled plasma mass spectrometry (ICP/MS) and collision cell ICP/MS

Nutrients were analyzed by colorimetry or photometry, DOC and TOC by infrared spectrometry, anions by ion chromatography, and ANC by titration at NWQL.

Splits of the sediment samples were analyzed for recoverable (HCl leachable) metals by ICP/OES and ICP/MS at the NWQL and for total metals (total sediment digestion by HNO_3, HCl, HF, $HClO_4$) by ICP/MS (8, 29), for Hg by cold vapor atomic fluorescence spectroscopy (CVAFS), and for total carbon and carbonate carbon by titration and LECO analyzer (30, 31) at the USGS Geologic Discipline (GD) research laboratory in Denver, CO. Organic carbon (OC) was determined by difference between total and carbonate carbon. Splits of selected samples were analyzed by X-ray diffraction for mineralogy, using a Siemens D500 X-ray Diffraction System (Bruker AXS Inc., Madison WI) at the USGS Research Laboratory at Boulder, CO. Details of analytical methods, references, and reporting limits are presented in table S1 in Supplementary Material.

4.2.5 DNA Extraction and Amplification

DNA was extracted from cells trapped on filter membranes using a Mo Bio Ultraclean Soil kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, with modifications as described in Mumford *et al.* (2012). The 16S small subunit rRNA gene was amplified from extracted DNA using primers 27F and 1492R and the As(V) respiratory reductase gene, *arrA,* was amplified using primers As1F and As1R, as described by Lane *et al.* (70), and Lear *et al.* (73), respectively. The primer sequences are reported in supplementary table S2. PCR results were visualized by

agarose gel electrophoresis and products cloned into a pCR4 vector and transformed into *Escherichia coli* TOP10 as described in Barringer *et al.* (2010a). Positive clones were sequenced by Genewiz, Inc. (South Plainfield, NJ). A total of 14 16S rRNA gene clones and 9 *arrA* gene clones were recovered from Six Mile Run, and a total of 16 16S rRNA gene clones and 7 *arrA* gene clones were recovered from Pike Run. Taxonomic classification was performed based on the 16S rRNA gene using the RDP classifier (149). Amplification of DNA from filters recovered from MW114 was unsuccessful; DNA was instead extracted from a microcosm prepared with groundwater from MW114, which allowed for the recovery of 15 16S rRNA gene clones and 13 *arrA* gene clones. All sequence data were screened with the NCBI VecScreen tool to eliminate vector sequence prior to analysis. 16S rRNA gene sequences have been submitted to Genbank under accession numbers JX440972 through JX441016. As(V) respiratory reductase (*arrA)* gene sequences have been submitted to Genbank under accession numbers JX845248-JX845276.

4.2.6 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed to detect differences between the microbial communities present in the groundwater at Six Mile Run and Pike Run. Extracted DNA was amplified with the universal 16S primers 27F and 519R in order to generate a 497bp amplicon for separation by DGGE. The amplicons were separated using an 8% polyacrylamide gel with a denaturing gradient of 40-80% urea-formamide. Electrophoresis was performed at 55V for 16 hours at 60^{o} using

a Bio-Rad DCode Universal Mutation Detection System according to the manufacturer's instructions (Bio-Rad Inc., Hercules, CA). The gel was stained for 45 minutes in an aqueous solution of 10% ethanol, 0.5% acetic acid and 0.1 μL/mL SYBR Green (Life Technologies, Grand Island, NY). The gel was imaged using a Storm 860 (GE Healthcare Life Sciences, Piscataway, NJ), scanner with excitation at 450 nm and emission at 525 nm in high sensitivity mode with the photomultiplier tube set at 900V. Major bands were excised from the gel and eluted in 100 μL of sterile MilliQ water. The eluted DNA was amplified using the 27F forward primer and 519R reverse primer, and the product of this amplification was purified using ExoSap-IT (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions prior to sequencing by Genewiz, Inc (South Plainfield, NJ). Sequences recovered from DGGE bands can be found in supplementary table S2.

4.2.7 Sequence Analysis

16S rRNA gene sequences obtained from cloning and DGGE were aligned to the SILVA 111NR database using the SINA webaligner (112). These aligned 16S rRNA gene sequences were then imported into the ARB software package for phylogenetic analysis, and a phylogenetic tree of the sequences and nearest published related sequences from the SILVA 111NR database was constructed utilizing the RAxML maximum likelihood algorithm within ARB (78, 112). This tree can be found in the supplementary material.

The recovered *arrA* gene sequences were imported into ARB and aligned using ClustalW within ARB to a database of 392 *arrA* gene sequences retrieved from Genbank

(71, 78). A phylogenetic tree of the recovered *arrA* gene sequences combined with selected *arrA* gene sequences from Genbank was generated using the RAxML maximum likelihood algorithm within ARB (78) and can be found in the supplementary material. An ARBNJ distance matrix was constructed to allow for OTU based analysis with mothur (78, 128). *arrA* gene similarity between Six Mile Run and Pike Run was calculated by grouping OTUs by site and generating Venn diagrams within mothur (128).

4.2.8 Preparation of Groundwater Microcosms:

We inoculated microcosms using site groundwater supplemented with As(V) (as sodium arsenate) and acetate (as sodium acetate) to test for the presence of microorganisms capable of the respiratory reduction of As(V) to As(III). Active microcosms were prepared in triplicate by inoculating 10 mL of groundwater into 30 mL of defined anaerobic freshwater medium as described by Mumford *et al*. (2012). The microcosms were supplemented with 5 mM As(V) as an electron acceptor and 5 mM acetate as a source of carbon and energy. Sterile control microcosms were prepared in duplicate in the same manner as active microcosms, followed by autoclaving three times on three successive days. Background microcosms were prepared by inoculating media which had not been supplemented with As(V) or acetate. The reduction of As(V) to As(III) and the utilization of acetate was monitored by high-performance liquid chromatography (HPLC). When reduction of 25% of the As(V) to As(III) was observed, secondary microcosms were established in triplicate, with duplicate sterile controls, by

anaerobically transferring 5 mL of the primary culture to fresh medium. All microcosms were sampled and analyzed as described in Mumford *et al*. (2012).

4.3 RESULTS

4.3.1 Stream Sediment Mineralogy and Chemistry

The sampling sites of both Six Mile Run and Pike Run are underlain by shales and siltstones of the Passaic Formation. As shown in Table 1, streambed sediments at Six Mile Run contained more organic matter and were more quartz-rich (33.4 wt. percent) than those at Pike Run; phyllosilicates (mainly illite) constituted 42.8 wt. percent of the minerals at Pike Run but were only 32.6 wt. percent at Six Mile Run. Feldspars were major phases at both sites, and Fe oxides and hydroxides were minor phases. Amorphous Fe hydroxides would not have been detected, however, these probably are present and may contain As, as in the Coastal Plain site (14, 15, 90). Both total–digestion and HCl-leachable As contents at Pike Run, Six Mile Run, and 9 other sites in the Millstone Watershed were strongly and positively related with total-digestion and HCl-leachable Fe and Mn contents (USGS data not shown).

As shown in Table 2, the As content of streambed sediments at the Six Mile Run and Pike Run were 16.5 mg/kg and 14.6 mg/kg, respectively—in general agreement with the maximum As content (14.8) found by Serfes (2005) for the red beds in the Passaic Formation. The importance of clays, feldspars, and Fe oxides in the sediments is indicated by the substantial contents of Al, Fe, and K. The P content of sediments at Six

Mile Run was substantially higher than that at Pike Run, suggesting that some of the P content may result from anthropogenic inputs at the Six Mile Run site.

4.3.2 Groundwater Geochemistry

The groundwater chemistry of Six Mile Run, Pike Run, and MW114 is summarized in table 3, and shows that the Cl^- concentration in shallow groundwater discharging at Pike Run greatly exceeded both the median (18 mg/L) and maximum (100 mg/L) concentrations for Passaic formation groundwater (130). Groundwater from the Passaic Formation aquifer is mainly Ca-Mg-Na-HCO_3^- or Ca-SO_4^{2-} (Serfes, 2005); the enrichment in SO_4^{2-} with Ca^{2+} is ascribed to dissolution of gypsum, a secondary mineral that lines rock fractures. This elevated Cl- concentration, combined with elevated levels of Na^+, NO_3^-, and B suggest anthropogenic inputs to the aquifer at Pike Run. While the Na^+ concentration at Pike Run does not exceed the Passaic Formation maximum (270 mg/L) reported by Serfes (2005), it greatly exceeds the median concentration of 15 mg/L. Concentrations of these constituents were substantially lower in the shallow groundwater discharging at Six Mile Run, however, concentrations of DOC (2.6 mg/L) and P (1.37 mg/L) were higher than at Pike Run (Table 3), suggesting the potential for anthropogenic inputs to Six Mile Run.

Table 3 summarizes the groundwater chemistry of Six Mile Run, its attendant monitoring well MW114, and Pike Run. The DO concentration in the Six Mile Run piezometer was low (2.8 mg/L), and the Fe concentration (20,700 μg/L) was an order of magnitude higher than that in the well-water sample, indicating the presence of both

reducing conditions and the potential for microbial Fe reduction. All of the Fe in the filtered groundwater is assumed to be Fe(II), as the solubility of the least stable Fe(III) oxide, ferrihydrite, at a pH of 6.7 as found at Six Mile Run is less than 106 μg/L, less than 1% of the Fe measured. The E_H of the aquifer at Six Mile Run was calculated based upon the pH and the concentration of Fe(II), and found to be -39mV, a value consistent with the observed conditions. The water chemistry from well MW114, which is an input to Six Mile Run, is displayed in Table 3 and clearly indicates that conditions in the shallow aquifer near Six Mile Run are reducing. The anoxic (0.1 mg/L DO) water at MW114 contains high Fe (9010 μg/L) and low SO_4^{2-}(0.015 μg/L) concentrations and As concentrations (22.1 μg/L) that were nearly as high as the As concentrations in the Six Mile Run piezometer sample (27.6 μg/L). The E_H of the aquifer at MW114 was calculated in the same manner as Six Mile Run, and found to be a weakly oxidizing 32mV.

The chemistry of the piezometer sample from Pike Run is summarized in Table 3, and the more oxidizing conditions found there contrasted with the reducing conditions found at Six Mile Run. The more oxidizing conditions at Pike Run were reflected in the higher DO concentration (4.9 mg/L), low Fe (197 μg/L) and As (3.4 μg/L) concentrations, and higher concentrations of SO_4^{2-} and NO_3^- (Table 3). The E_H of Pike Run was calculated based upon the very low concentration of Fe(II) observed, and found to be an oxidizing 144mV. The oxidizing conditions at Pike Run were further confirmed by the observation of 6.85 μg/L of U, as U(IV) is insoluble, presumably all U observed was present as U(VI). The U concentration at Pike Run, presumably all as soluble,

oxidized U(VI), was higher than the median concentration reported for Newark Basin groundwater in Pennsylvania (129), and greatly exceeded that at Six Mile Run (0.03 µg/L) in the samples from the piezometer and MW114 (0.015 µg/L).

4.3.3 Streamwater Chemistry

Streamwater at Pike Run and Six Mile Run was sampled twice, once under high-flow conditions, and once under low-flow (base-flow) conditions, and the results of this sampling are summarized in Table 3. The higher concentrations of Na^+ and Cl^- in streamwater at both sites during base flow conditions when compared to high flow conditions points to substantial inputs of dissolved constituents from groundwater discharge. Groundwater inputs of SO_4^{2-} and NO_3^- at Pike Run, in particular, are reflected in streamwater concentrations at base flow. As shown in Table 3, the concentrations of both Fe and As in streamwater at the sites were higher during high-flow conditions than at base flow. Most of the Fe was particulate ($Fe_{unfiltered} - Fe_{filtered} = Fe_{particulate}$) at high flow, and about 25% of the As was particulate. Reduced Fe and As in groundwater discharge apparently oxidizes in the stream and Fe precipitates as a hydroxide, taking As(V) from solution either by co-precipitation or sorption, similar to the process seen in the Coastal Plain site described in Mumford *et al* (2012). Nevertheless, As(III) was detected in streamwater at both sites, as it was at nearly all Millstone mainstem sites and tributaries sampled during 2009-10 (USGS unpublished data).

4.3.4 Results of Microcosm Studies:

Figure 2A demonstrates that the microcosm inoculated with groundwater from Six Mile Run stoichiometrically reduced approximately 2.5 mM As(V) to As(III) in 20 days. Further reduction was not observed, possibly due to the toxicity of As(III). Figure 2B demonstrates that microcosms inoculated with groundwater from MW114 began reducing As(V) after 52 days of incubation, and stoichiometric reduction of 5 mM As(V) to As(III) was observed over the course of 88 days. The large error bars in figure 2B are attributed to the fact that one of the three bottles in the set did not reduce As(V). Figure 2C shows that no As(V) reduction was observed in samples from Pike Run, indicating that As(V) reducing organisms are either not active or present in the groundwater at Pike Run. As shown in figures 2A-C, no As(V) reduction was observed in the sterile controls, indicating that microbial reduction, not abiotic processes, are mediating the reduction of As(V) to As(III).

4.3.5 Molecular Characterization of Microbial Communities at Six Mile Run, and Pike Run

4.3.5.1 Characterization of the 16S rRNA gene

Figure 3 displays the diversity of the taxonomy of the differing microbial communities at Six Mile Run (including MW114) and Pike Run as determined by the RDP Classifier (149). The 11 16S rRNA gene OTUs from Six Mile Run were combined with the 2 16S rRNA gene OTUs from MW114 (13 OTUs total) and compared with the 14 16S rRNA gene OTUs from Pike Run. These sequences were also used to generate a

RAxML maximum likelihood tree with related sequences picked from the SILVA 111NR database; this tree can be found in the supplemental material as figure S1. The communities shared several OTUs with high sequence similarity to the *Actinobacteria*, a number of important differences were noted. Sequences similar to the Fe-reducing β-proteobacterium *Rhodoferax ferrireducens* (45) were identified in both Six Mile Run groundwater as well as an As(V) reducing microcosm prepared with groundwater from MW114, indicating the potential for microbial Fe reduction at the Six Mile Run site, however, no Fe-reducing OTUs were identified from Pike Run. Conversely, sequences similar to the known Fe-oxidizing β-proteobacterium *Gallionella* (75) were identified in groundwater from Pike Run, indicating the potential for microbial Fe oxidation with concomitant As sequestration (121, 131). No sequences similar to any known Fe-oxidizing organisms were found at Six Mile Run.

Figure 4 demonstrates the differences in the microbial communities between Six Mile Run and Pike Run as shown by the unique DGGE banding patterns obtained from each site. Seven unique major bands were excised and sequenced from Six Mile Run, and six unique major bands were excised and sequenced from Pike Run. The BLAST identities of the sequences recovered from the DGGE bands are summarized in table S2. As each band represents an individual microbial genus, these results indicate that the differing redox states of the two sites drive the development of different microbial communities. As such, these different microbial communities, anaerobic at Six Mile Run and aerobic at Pike Run, may be the determining factor between the elevated concentration of As at Six Mile Run and the low concentration of As at Pike Run.

4.3.5.2 Characterization of the Arsenate Respiratory Reductase (*arrA*) gene

Figure 5 demonstrates that different As(V) respiring genes, and thus potentially different As(V) respiring organisms, were present at each of the sites based on *arrA* gene sequences. Based on a 97% similarity cutoff, 9 *arrA* gene OTUs were recovered from Six Mile Run and MW114, and 6 *arrA* gene OTUs were recovered from Pike Run. A phylogenetic tree of the recovered *arrA* sequences can be found in figure S2 in the supplementary material.

4.4 Discussion

Six Mile Run and Pike Run are both tributaries of the Millstone River, and lie approximately 8 km apart within the Piedmont Physiographic Province of New Jersey. Because the shallow aquifer zones beneath streambeds can be subject to reducing conditions, the biogeochemical reactions that release As to ground water under oxidizing conditions found by Rhine et al. (2008) in the Piedmont may not pertain to reactions beneath some stream channels. Recent studies (14, 15, 90) of shallow groundwater discharge in the New Jersey Coastal Plain have supported earlier findings where microbial activity promoted release of As and Fe from aquifer materials under reducing conditions (56, 57, 85, 135). However, it was not known whether similar microbially mediated reactions occur in shallow groundwater (<10 m below the water table) in the fractured rock aquifers of the New Jersey Piedmont, nor was it known what the redox conditions might be that foster As release. Further, there was little information as to whether groundwater contributed As to Piedmont streams, although relatively deep As-

bearing groundwater was thought to eventually discharge to streams (Serfes, 2005). Both of these tributaries run over fractured bedrock comprising red shales of the Passaic Formation, and as shown in Table 1 and Table 2, display similar mineralogy and bed sediment chemistry. In particular, the As and Fe contents of these sediments are nearly identical. Despite these similarities in geology, however, the groundwater at Six Mile Run was found to be reducing, with an (E_H=-39.5mV), and the groundwater at Pike Run was found to be oxidizing, with an E_H=+144mv. As shown by our results, this difference in redox conditions is responsible for the development of differing microbial communities at each of these sites, and these anaerobic microbial communities are responsible for the release of both Fe and As into the groundwater at Six Mile Run, whereas the aerobic microbial communities active at Pike Run release neither As nor Fe into the groundwater.

The presence of distinct microbial communities at Six Mile Run and Pike Run is shown in Figure 4, and when the high degree of similarity between physical conditions found at the sites is taken into account, suggests that microbial community development is driven by the differences in redox in the groundwater. The hypothesis that the development of these different microbial communities is driven by difference in groundwater redox conditions is supported by our microcosm studies, which, as shown in Figure 2, demonstrate that anaerobic organisms capable of As(V) reduction are present and active at Six Mile Run, and are either not present or not active at Pike Run. In addition, sequences similar to the Fe reducing organism *Rhodoferax ferrireducens* were found in microcosms prepared with water from a monitoring well (MW114) in the

shallow aquifer which feeds Six Mile Run. Microbial Fe reduction has been reported as a major mechanism for As release (56, 57), and the presence of such organisms suggests that microbial Fe reduction plays a role in As release at Six Mile Run. Our previous studies have demonstrated that microbial As(V) reduction plays a major role in the mobility of As in groundwater (90), and as such, it becomes clear that microbial activity driven by reducing conditions in the groundwater is responsible for the elevated levels of As at Six Mile Run. As shown in Figure 5, distinct sequences for the As(V) respiratory reductase gene (*arrA*) were found at both Six Mile Run and Pike Run. While *arrA* has been reported as to be a biomarker for As(V) respiration (83), this was not the case for Pike Run. While *arrA* gene sequences were recovered from Pike Run groundwater, As(V) reducing activity was not noted in microcosms inoculated with groundwater from this site, suggesting that the organisms carrying these genes are inactive under the conditions found at Pike Run. An alternative explanation, however, is that these sequences represent the *arxA* gene, which catalyzes the oxidation of As(III) and shares high homology with *arrA* (152, 153). The two *arrA* OTUs shared by Six Mile Run and Pike Run showed higher similarity to the As(III) oxidase gene, *arxA,* than to any of the other described *arrA* gene sequences. If indeed the *arxA* gene is present, that would explain the lack of As(V) reduction in the Pike Run microcosms, and is consistent with the oxidizing conditions found in the Pike Run groundwater. The As(III) oxidase (*arxA)*-like sequences recovered from Six Mile Run suggest the potential for As cycling should oxidizing conditions become prevalent in the dynamic environment beneath the streambed. As reported by Rhine et al. (117), shifting redox conditions beneath the

streambed could allow for As(V) reduction and mobilization to occur under reducing conditions, and As(III) oxidation to occur as conditions become more oxidizing. Alternately, As(III) reduced in the deeper, more reducing portions of the aquifer may be oxidized in the upper, more oxidizing regions of the aquifer as the groundwater flows from the aquifer into the stream, as suggested by Mumford et al. (2012).

Our findings along the Millstone River suggest the existence of a pathway by which As can be mobilized from fractured bedrock aquifers, and then potentially be transported far from its original source. As shown at a gaining reach of Six Mile Run, reducing groundwater is enriched in As by the microbial Fe and As reduction, flows from the aquifer into the stream, and As and Fe then co-precipitate as it crosses the oxic/anoxic interface in the streambed. The precipitated Fe and As may then be transported during periods of high flow, and re-deposited far downstream of the original source, as seen in the accumulation of Himalayan As in the river deltas of Southeast Asia (4, 6, 19-22, 51, 53, 60, 73, 111, 115, 127, 135, 140).

4.5 Conclusions

Our study utilized two sites with highly similar mineralogy and geochemistry to demonstrate that the redox status of an aquifer plays a role in the microbial reduction and mobilization of As. Our data demonstrate that suboxic, reducing conditions in shallow groundwater facilitate microbial respiratory reduction of As(V) to As(III), while oxidizing conditions inhibit this process. The successful enrichment of As(V) reducing microbial communities from groundwater sampled at Six Mile Run and MW114

indicates the presence and activity of As(V) reducing organisms under the suboxic (Six Mile Run) and anoxic (MW114) conditions present at these sites. Conversely, no reduction of As(V) was observed in microcosms prepared with oxic groundwater from Pike Run, suggesting that As(V) reducing organisms are not present or not active. While As(V) reductase gene (*arrA*) sequences were recovered at each of the sites, they were not predictive of As(V) reducing activity as has been previously reported (83). However, the presence of sequences similar to the recently discovered As(III) oxidase gene, *arxA*, suggests the potential for As(III) oxidation under the oxidizing conditions found at Pike Run (119, 152, 153). In addition, the high NO_3^- concentration found at Pike Run would promote NO_3^- reduction coupled to As(III) oxidation via *arxA,* as proposed by Zargar *et al*. (2010, 2012). This finding underlines the importance of cultivation-based studies to confirm the presence of active microorganisms when assessing the potential for As(V) reduction. The differing redox conditions below the streambeds at Six Mile Run and Pike Run result in the development of differing microbial communities at the two sites, which in turn determines how microbial activity affects the mobility of As and Fe. Based on our findings at the Millstone River tributaries of Six Mile Run and Pike Run, we conclude that under similar geochemical and mineralogical conditions, the potential for microbial reduction and mobilization of As is highly dependent on the redox status of the aquifer. These findings suggest a mechanism by which microbial activity can mobilize As from fractured rock aquifers, and allow it to be transported far from its original source.

4.6 Supplemental Material

Supplemental information for this chapter may be found in Appendix 3.

4.7 Acknowledgements

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Table 1:

Mineralogy of streambed sediments at sampling sites on tributaries Six-Mile Run and Pike Run, Millstone River watershed, 2011.

[%, percent; organic-matter content is entered as peat; weight percents are normalized to total 100 percent; mineral formulas are from Eberl and Smith (2009); weight percents of varieties of oligoclase and illite are combined]

Site name and USGS number	**Six-Mile Run (01401900)**	**Pike Run (01401700)**
Mineral and approximate formula	Weight %	Weight %
Framework silicates		
Quartz (SiO_2)	33.4	22.3
Potassium feldspar (microcline) ($KAlSi_3O_8$)	4.5	3.3
Potassium feldspar (orthoclase) ($KAlSi_3O_8$)	0.9	0.0
Plagioclase (albite) ($NaAlSi_3O_8$)	9.1	11.4
Plagioclase (oligoclase) ($(Na,Ca)(AlSi)_4O_8$)	2.8	5.1
Metal oxides		
Hematite (Fe_2O_3)	2.5	2.1
Goethite ($FeO(OH)$)	0.2	0.2
Maghemite (Fe_2O_3)	0.8	0.7
Ilmenite ($FeTiO_3$)	0.2	2.8
Organic matter		
Peat ($C_4H_6O_3$)	13.0	9.3
Phyllosilicates		
Halloysite ($AlSi_2O_5(OH)_4.nH_2O$)	8.1	1.5
Kaolinite (disordered) ($AlSi_2O_5(OH)_4$)	0.0	0.0
Chlorite ($(Mg,Fe,Al_6)[(Si,Al)_4O_{10}](OH_8)$)	4.1	0.0
Muscovite ($KAl_2(Si_3Al)O_{10}(OH)_2$)	20.4	11.1
Illite ($K_{0.8}(Al,Fe,Mg)_2(Si,Al)_4O_{10}(OH)_2.nH_2O$)	0.0	24.4
Vermiculite	0.0	5.8
Total	100	100

Table 2:

Contents of total major and trace elements in streambed sediments from Pike Run and Six Mile Run, Millstone River watershed, New Jersey, USA.

Site name	Carbonate C (%)	Organic C (%)	Al (mg/kg)	As (mg/kg)	Ca (mg/kg)	Fe (mg/kg)	K (mg/kg)	Mn (mg/kg)	P (mg/kg)	Sr (mg/kg)	U (mg/kg)
Pike Run near Rocky Hill	0.02	1.14	77,700	14.6	4,170	54,400	23,800	1,280	697	107	7.3
Six Mile Run at Blackwells Mill	0.02	1.77	69,500	16.5	2,730	50,100	23,400	1,100	1,290	73.1	3.78

Table 3:

Concentrations of selected constituents in streamwater and groundwater at Pike Run and Six Mile Run, Millstone River watershed, New Jersey, 2010.

Medium/ Site name	Date	DO (mg/L)	DOC (mg/L)	pH	Na^+ (mg/L)	Cl^- (mg/L)	SO_4^{2-} (mg/L)	NH_4^+ (mg/L as N)	NO_3^- (mg/L as N)	P_u (mg/L)	As_u (µg/L)	As_f (µg/L)	Fe_u (µg/L)	Fe_f (µg/L)	B_f (µg/L)	U_u (µg/L)
Streamwater high flow																
Pike Run near Rocky Hill	6/14/10	7.5	4.3	7.5	24.6	31.1	30.4	0.064	1.41	0.16	1.9	1.4	380	64	114	N/A
Six Mile Run at Blackwells Mills	6/14/10	8	5.5	7.4	22.7	38.9	11	0.017	0.80	0.12	1.4	1.1	415	136	60	N/A
Streamwater low flow																
Pike Run near Rocky Hill	8/6/10	5	3.9	7.4	61.4	63.9	111	0.023	8.08	0.16	1.7	1.3	156	9	255	0.409
Six Mile Run at Blackwells Mills	8/5/10	7.4	2.8	7.3	30.2	56.8	28.1	0.02	0.31	0.14	1.9	1.6	132	44	86	0.288
Groundwaterbase flow																
Pike Run near Rocky Hill	8/6/10	4.9	1.3	6.9	102	366	20.9	0 .010	5.48	0.06	3.4	2.1	197	4	117	6.85
Six Mile Run at Blackwells Mills	8/5/10	2.8	2.6	6.7	62.6	138	5.54	1.76	0 .05	1.37	27.6	27.4	20,700	21,400	83	0.031
MW-114 at Six Mile Run	10/28/10	0.1	2.7	6.6	29.6	67.0	0.15	2.25	<0.020	1.19	22.1	20.2	9,010	2,501	41	0.015

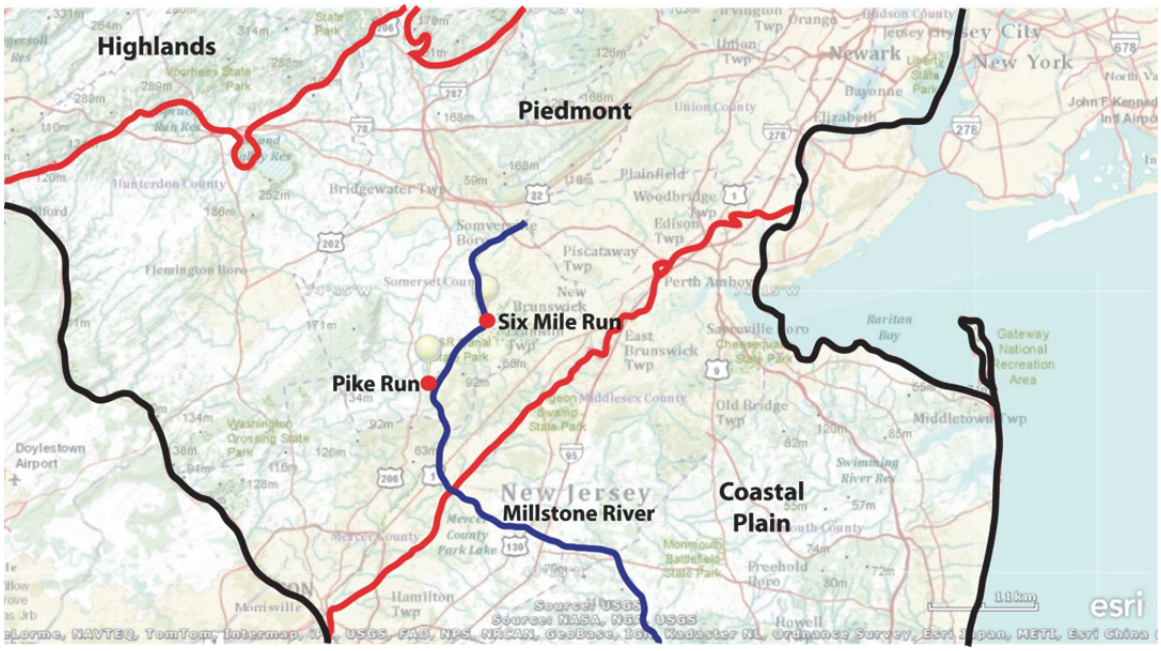


Figure 1:

Map of Central New Jersey, with sampling sites along the Millstone River marked. The Highlands, Piedmont, and Coastal Plain Physiographic provinces are delineated by red lines.

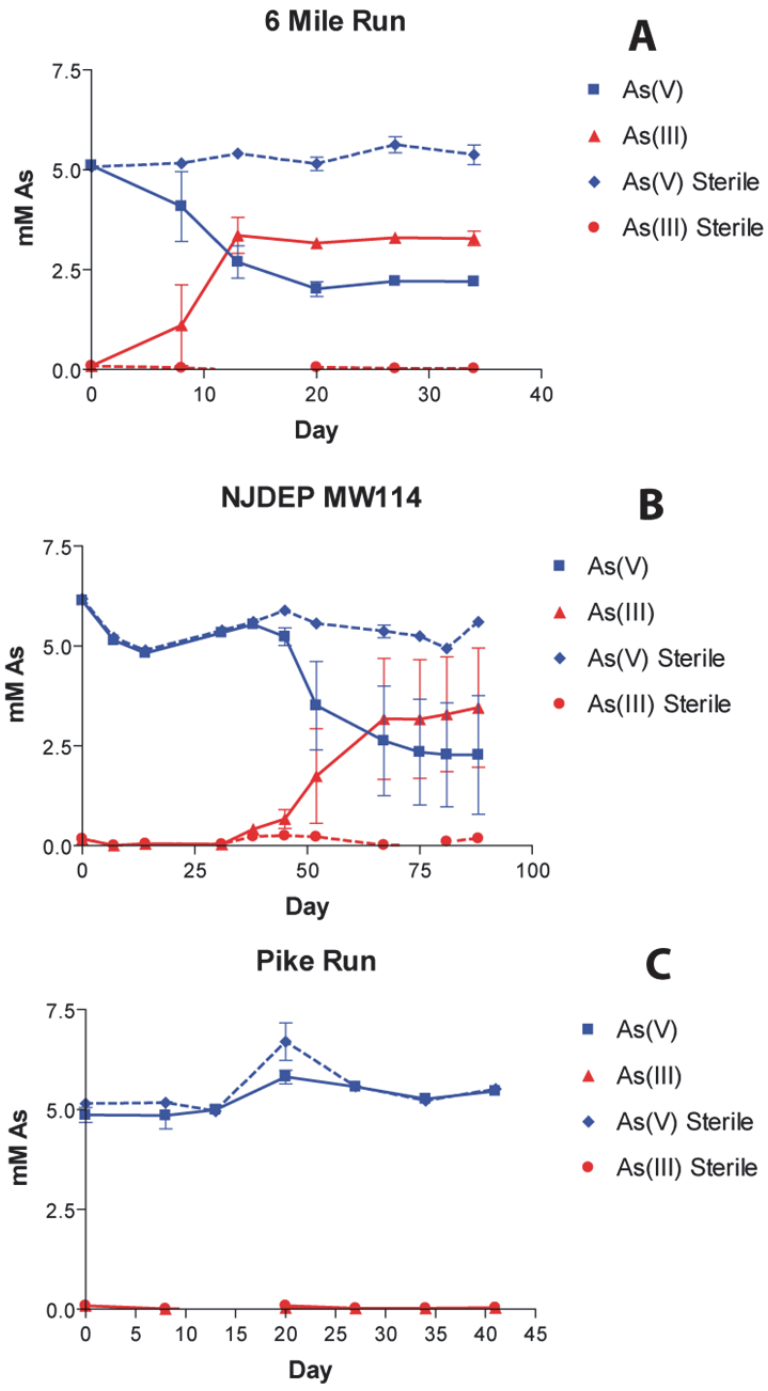


Figure 2:

Stoichiometric As(V) reduction was observed in microcosms inoculated with groundwater from Six Mile Run (A) and MW114 (B). No As(V) reduction was observed in microcosms inoculated with groundwater from Pike Run (C).

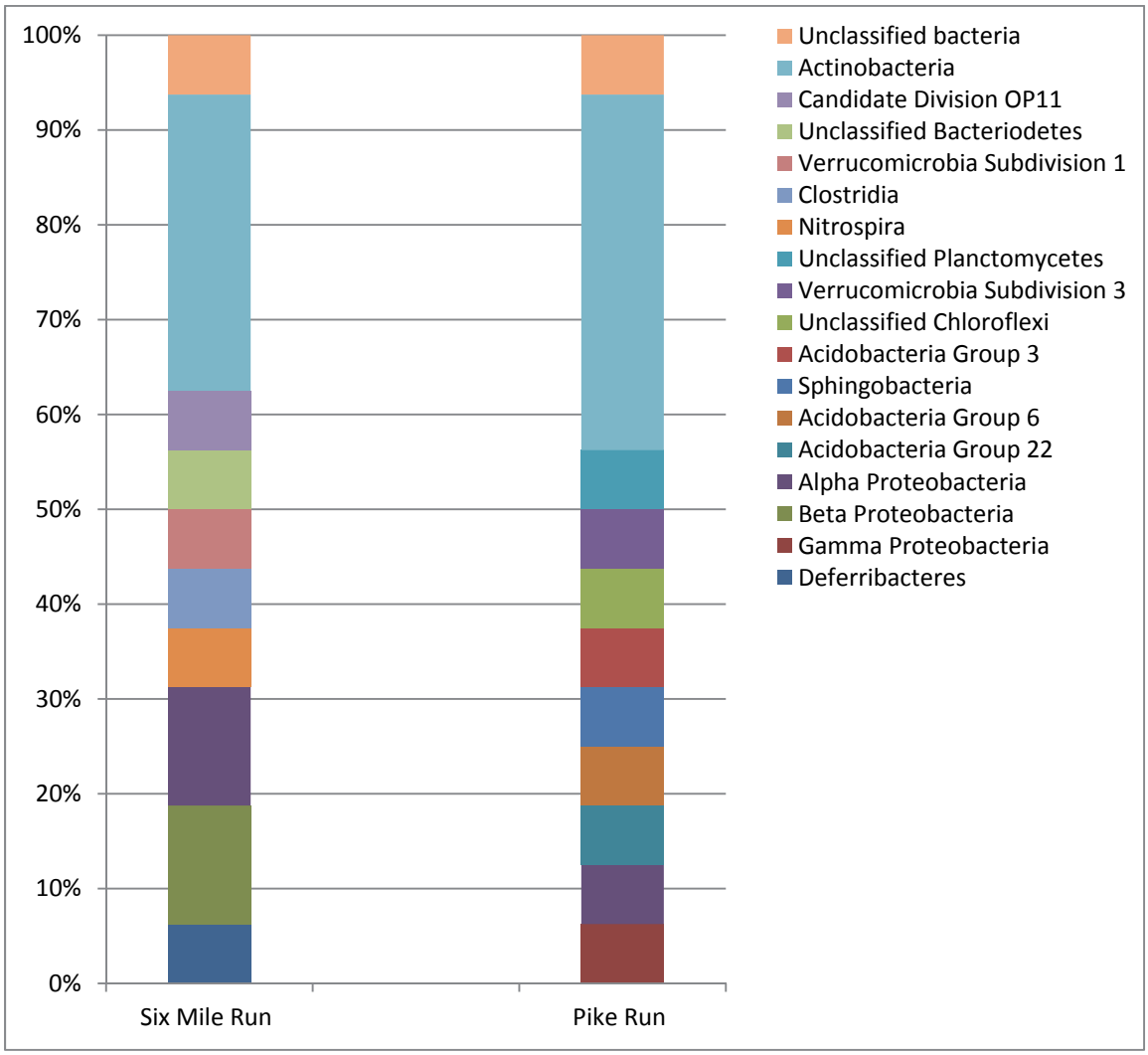


Figure 3:

Taxonomic classification of 16S rRNA gene OTUs recovered from Six Mile Run, Pike Run, and NJDEP MW114. Sequences recovered from Six Mile Run are combined with those from MW114.

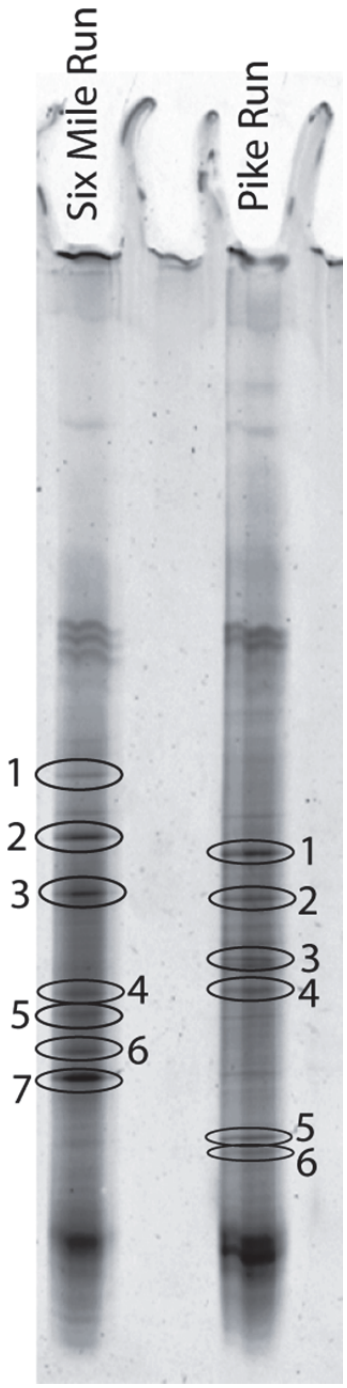


Figure 4:

Image of the 492bp 16S rRNA gene amplicons from Six Mile Run and Pike Run as separated by DGGE. Unique banding patterns were obtained from each site. Circled bands were excised from the gel and sequenced.

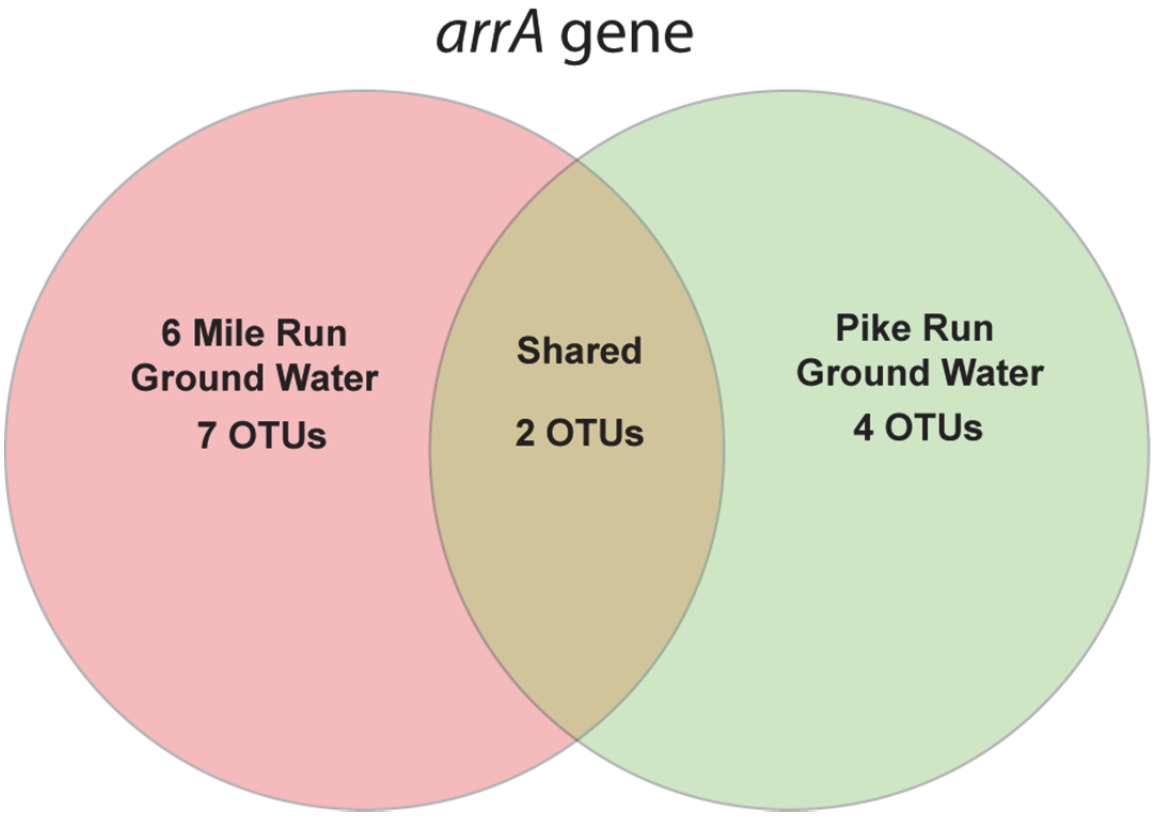


Figure 5:

Venn diagram illustrating the different microbial communities found at Six Mile Run and Pike Run based on As(V) respiratory reductase (*arrA)*.

Chapter 5

Conclusions and Future Directions

Chapter 1 describes how an As(V) reducing organism I isolated, Strain MPA-C3, is required for the precipitation of an arsenic sulfide mineral, alacranite (As_8S_9). This research has been submitted to Environmental Microbiology, and is currently in review. In this chapter, I describe how I isolated a novel, As(V) reducing strain, MPA-C3, from estuarine sediments sampled in the Mai Po Marsh in Hong Kong, China. I found that Strain MPA-C3 is metabolically diverse, and capable of using a wide range of electron donors and acceptors. Sequencing of first the 16S rRNA gene and then the entire genome demonstrated that Strain MPA-C3 is a member of the *Deferribacteres*, a genus of anaerobic heterotrophs. The genome sequence allowed for elucidation of the metabolic pathways utilized by Strain MPA-C3, including those for nitrate reduction, sulfur reduction, and the utilization of simple carbon sources. While Strain MPA-C3 has a wider metabolic range than any of the described *Deferribacteres*, its most notable trait is that, when grown with As(V) as a terminal electron acceptor, it precipitates a yellow mineral from the culture medium. Analysis of this precipitate identified it as alacranite (As_8S_9), a mineral which has previously only been described as forming under hydrothermal conditions. This finding suggests that the precipitation of a wider range of arsenic sulfides may be mediated by microbial activity than previously thought, and such activity may play an important role in the biogeochemical cycling of arsenic.

At this point in time, several aspects of this project present useful topics for further study. Foremost among these is the mechanism by which Strain MPA-C3 catalyzes the precipitation of alacranite. This process appears to occur extracellurlarly,

and we have hypothesized that it is a result of an as-yet unidentified product released from the cell. Newman et al. suggest that the precipitation of orpiment occurs both in the periplasm of *Desulfosporosinus auripigmentum* and and extracellularly, however, no mechanism for this precipitation is discussed (99). As I demonstrated in Chapter 2, neither alacranite nor any other arsenic sulfide mineral formed when As(III) and sulfide were added to the media used to grow Strain MPA-C3, thus demonstrating the conditions necessary for low-temperature formation as suggested by O'Day et al. (102) are not found in the absence of cells. We did note, however, that the mineral appeared to form either in direct contact or in the immediate vicinity of the cells, which suggests that the cells may be providing a nucleation site for crystal formation. This could in turn by studied by TEM, which would allow for a much closer look at the environment in the immediate vicinity of the cell, and could potentially show if crystal nucleation is occurring at the cell surface, in the periplasm, or simply very near to the cells.

The As(V) respiratory reductase (*arrA*) gene of Strain MPA-C3 was successfully amplified by PCR, sequenced, and found to be most similar to that of *Desulfitobacterium hafniense* Y51, an organism in a distant phylum than the *Deferribacteres*. In Chapter 2, I suggest that this is the result of the *arrA* gene of Strain MPA-C3 having been incorporated via horizontal gene transfer. However, this gene was not found on the genome of Strain MPA-C3, making determination of the surrounding genes, and perhaps confirmation of this hypothesis, much more challenging. I anticipate that completion and closure of the genome would allow for the identification of the *arrA* gene identified by PCR, as well as the elucidation of more of the metabolic pathways utilized by this fascinating isolate.

Strain MPA-C3 provides an interesting example of a microbe biologically catalyzing a process under moderate conditions which has been thought to only occur chemically at very high temperatures and pressures. In its native estuarine environment, Strain MPA-C3, and organisms like it, may in fact be responsible for the formation of minerals which would have otherwise been assumed to have formed geologically and transported to a moderate environment. This finding provides an example of the importance of biological processes in the geochemical cycling of both trace and major elements. A greater understanding of the process by which Strain MPA-C3 precipitates alacranite could potentially lead to the development of biological treatment methods for the removal of arsenic contaminated drinking water.

In Chapter 3, I present the paper "Microbial transformation of Arsenic: Mobilization from glauconitic sediments to water" (90) in which I collaborated with the United States Geological Survey (USGS) to assess the potential for microbial activity in the release of As to groundwater at Crosswicks Creek, in Upper Freehold, NJ. In this chapter, I describe how we utilized cultivation-based techniques to determine that microbial activity releases As from aquifer sediments, and combined these findings with cultivation-independent molecular characterization of the microbial community present at our study site. Through sequencing of the 16S rRNA gene, I found that the microbial communities present in the groundwater of the site were more diverse than those present on recovered sediments. In addition, I describe how sequencing of the As(V) respiratory reductase gene (*arrA*) led to the finding that As(V) reducing organisms were present at the site. In this chapter, I describe how we determined that As release from the sediments is most likely driven by Fe reducing microorganisms, and once released, As mobility in

groundwater is enhanced by the reduction of the less mobile As(V) to the more mobile As(III).

In Chapter 4, I describe how, again in collaboration with the USGS, I examined the potential for microbial As mobilization at two sites along the Millstone River, in Somerset County, NJ. This research has been submitted to Water Research as (TITLE) and is currently under review. The two sites, Six Mile Run and Pike Run, displayed very similar mineralogy and geochemistry, with the notable difference being that the groundwater at Six Mile Run was anoxic and reducing, and the groundwater at Pike Run was oxic and oxidizing. We hypothesized that these differing redox conditions would lead to the development of differing microbial communities, and that this would in turn explain why the levels of As present at Six Mile Run were an order of magnitude higher than those at Pike Run. By following a similar research methodology as described in Chapter 3, I determined through culture-independent molecular techniques that the microbial communities at each site were in fact different, and these findings were supported by the results of microcosm experiments. In these experiments, I found that microcosms inoculated with groundwater taken from Six Mile Run were capable of As(V) reduction, while those inoculated with groundwater from Pike Run were not. This finding was particularly interesting in that the *arrA* gene was successfully amplified from the Pike Run site, a finding which I suggest may be due to the very similar As(III) oxidase gene (*arxA*).

Both Chapter 3 and Chapter 4 would be enhanced and better completed by further work in several directions. First and foremost, it is likely that the incubation of sediment and groundwater from Crosswicks Creek, Six Mile Run, and Pike Run would allow for

the identification of microbial As(III) oxidation by both cultivation-based and molecular techniques. This would allow for better understanding of how arsenic is biologically cycled at this site, and would provide field confirmation for the arsenic cycling demonstrated *in vitro* by Rhine et al. (117). Additionally, the recent advances in high-throughput sequencing would allow for a more complete picture of the microbial communities present, which would in turn allow for more detailed comparisons between the sites. Furthermore, such information would allow for a more detailed model of the geomicrobiological processes occurring in the subsurface aquifer, the groundwater, and the streambed to be constructed.

A deep understanding of the microbial processes which drive arsenic release into drinking water is of critical importance, not only when one considers the highly publicized issue of arsenic contaminated drinking water in Bangladesh, but also when one considers the potential impacts of adding organic carbon to any aquifer which resides in arsenic-bearing sediments or bedrock. As I have shown in this Dissertation, microbial arsenic reduction can result in both the mobilization and immobilization of arsenic, depending both on the geochemistry of the environment, and the organisms present. In all cases, the organisms present are heterotrophs, requiring the introduction of organic carbon to survive. As I demonstrated in Chapter 3, inputs of organic carbon drove the release of arsenic into the shallow subsurface aquifer at Crosswicks Creek, NJ. Following the example, it is not impossible to imagine a scenario in the addition of organic contaminants to an otherwise carbon-limited aquifer by industrial processes might result in the release of arsenic into drinking water where arsenic contamination had never before been observed. Conversely, the ability of microbes such as Strain MPA-C3

to precipitate insoluble arsesnic-sulfide minerals such as alacranite presents great promise as a biological treatment method for drinking and irrigation waters contaminated with arsenic. Current techniques for arsenic removal are based upon sorption to iron hydroxides, which while effective, also retain the risk of re-mobilization of arsenic should redox conditions change upon disposal (86). A treatment based upon microbial arsenic sulfide precipitation would instead result in the removal of arsenic as an insoluble phase, without the volumes of arsenic-laden waste and risk of re-mobilization as present with current iron oxide based techniques.

As the evidence for the importance of microbial processes in geochemical cycles mounts, it is becoming more and more apparent just how little is known about these processes. My research fits into a growing body of evidence for the importance of microbial processes in geochemical cycles, demonstrating both how microbes can form 'hydrothermal' minerals, and drive the dissolution and mobilization of sequestered elements. Much remains to be studied on the mechanisms behind these biogeochemical cycles, and their elucidation will help to enable the rational, harmonious utilization of our limited natural resources.

Appendix 1: Supplemental Material For Chapter 2

Assembly Statistics for MPA-C3:

Organism Overview for Deferribacter sp MPA-C3 (6666666.21886)

Genome	Deferribacter sp MPA-C3
Domain	Bacteria
Taxonomy	Bacteria; Deferribacter sp MPA-C3
Neighbors	View closest neighbors
Size	2,984,305 bp
Number of Contigs (with PEGs)	33
Number of Subsystems	325
Number of Coding Sequences	2773
Number of RNAs	43

For each genome we offer a wide set of information to browse, compare and download.

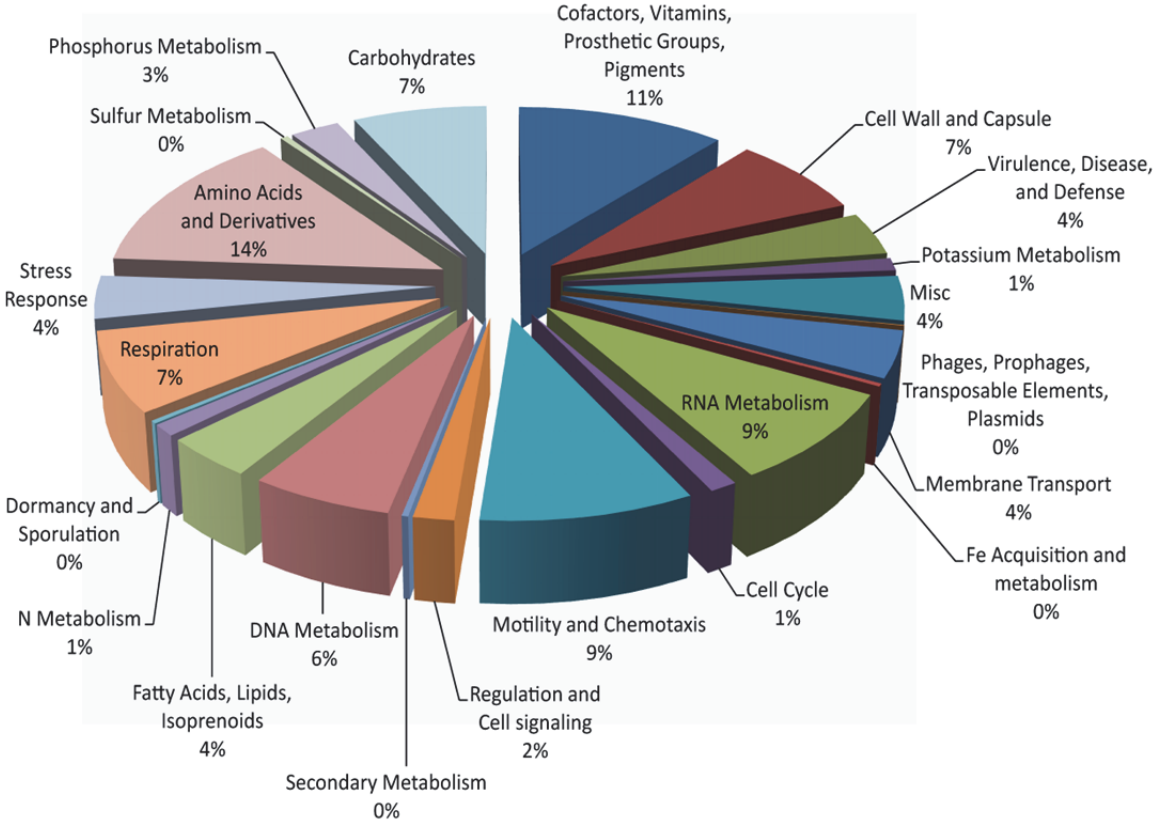
Browse | Compare | Download | Annotate

Browse through the features of Deferribacter sp MPA-C3 both graphically and through a table. Both allow quick navigation and filtering for features of your interest. Each feature is linked to its own detail page.

Click here to get to the Genome Browser

Reference count	33
Type	Reference mapping
Total reference length	2,987,753
GC contents in %	46.88
Total read count	28,517,103
Mean read length	109.35
Total read length	3,118,415,180

Subsystems Found in the Genome of Strain MPA-C3 as annotated by RAST



Appendix 2: Supplemental Material For Chapter 3

Table S-1. Composition of anaerobic medium used in microcosm experiments using Crosswicks Creek groundwater and streambed sediments.

Compound	Concentration
NaCl	1g/L
$MgCl_2 \cdot 2H_2O$	400 mg/L
$CaCl_2 \cdot 2H_2O$	100 mg/L
KH_2PO_4	200 mg/L
KCl	500 mg/L
Na_2HPO_4	0.19 g/L
NaH_2PO_4	0.06 g/L
NH_4Cl	0.27 g/L
MOPS	1.05 g/L
Nitrilotriacetic Acid	1.5 mg/L
$Fe(NH_4)_2(SO_4)_2$	0.8 mg/L
Na_2SeO_3	0.2 mg/L
$CoCl_2 \cdot 6H_2O$	0.1 mg/L
$MnSO_4 \cdot H_2O$	0.1 mg/L
$Na_2MoO_4 \cdot H_2O$	0.1 mg/L
$NaWO_4 \cdot H_2O$	0.1 mg/L
$NiCl_2 \cdot 6H_2O$	0.1 mg/L
H_3BO_3	0.01 mg/L
$CuSO_4 \cdot 5H_2O$	0.01 mg/L
nicotinic acid	0.1µg/L
calcium pantothenate	0.1µg/L
pyroxidine HCl	0.1 µg/L
riboflavin	0.1 µg/L
thiamine HCl	0.1 µg/L
biotin	0.05 µg/L
folic acid	0.05 µg/L
α-lipoic acid	0.05 µg/L
vitamin B-12	0.05 µg/L
Na_2S	31 mg/L
Cysteine	48.5 mg/L

Table S2. Primers used in this study

Primer Name	Sequence	Reference
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	Lane, 1991
1492R	5'-GGTTACCTTGTTACGACTT-3'	Lane, 1991
As1F	5'-CGAAGTTCGTCCCGATHACNTGG -3'	Song *et al.*, 2009
As1R	5'-GGGGTGCGGTCYTTNARYTC-3'	Song *et al.*, 2009

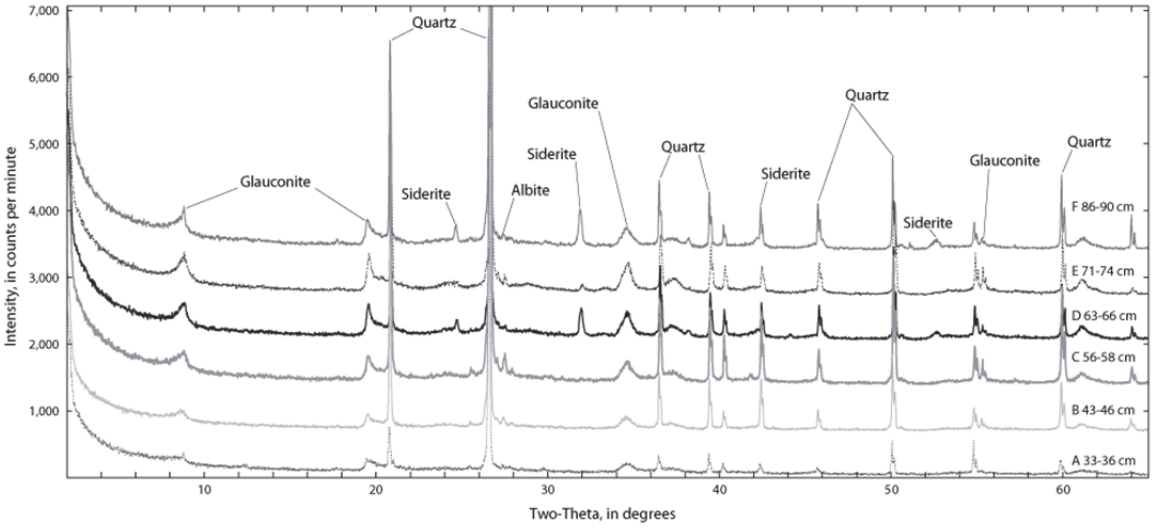
Table S3, selected groundwater chemistry from CRO-C6

Site Chemistry

Constituent	pH	DO (mg/L)	Nitrite (mg/L as N)	Nitrate (mg/L as N)	DOC mg/L	Sulfide (mg/L)	Sulfate (mg/L)	Arsenic (µg/L)	Sediment Arsenic (mg/kg)	Water Fe (µg/L)	Sediment Fe (mg/Kg)	As(III) (µg/L)	As(V) (µg/L)
Surface Water (8/18/09)	7	7.3	0.006	0.35	7.3	no data	19.7	1.4	n/a	3200	n/a	no data	no data
Shallow Groundwater (7/13/07)	6.8	3.6	0.005	<0.060	14.6	no data	<0.90	82.9	n/a	51200	n/a	no data	no data
Shallow Groundwater (10/17/07)	6.6	1.7	0.004	<0.04	14	0.027	0.22	55.7	n/a	44100	n/a	25	36.4
Shallow Groundwater (8/18/09)	6.5	2.2	<0.002	<0.04	16.5	0.5	1.23	16.6	n/a	48100	n/a	no data	no data
Bed Sediment (4/11/07)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	25	n/a	87000	n/a	no data

HTTP://NWIS.WATERDATA.USGS.GOV/USA/NWIS/QWDATA/?SITE_NO=01464485

Supplementary Material



XRD scans of the analyzed intervals of a sediment core from Crosswicks Creek site C6 (01464485), Inner Coastal Plain, New Jersey, August 2009.

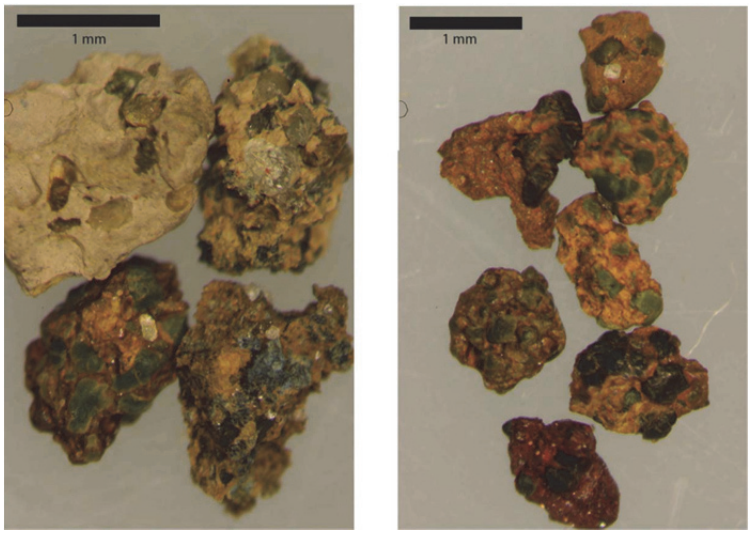


Figure S3.2. Photograph of glauconite grains from CRO-C6

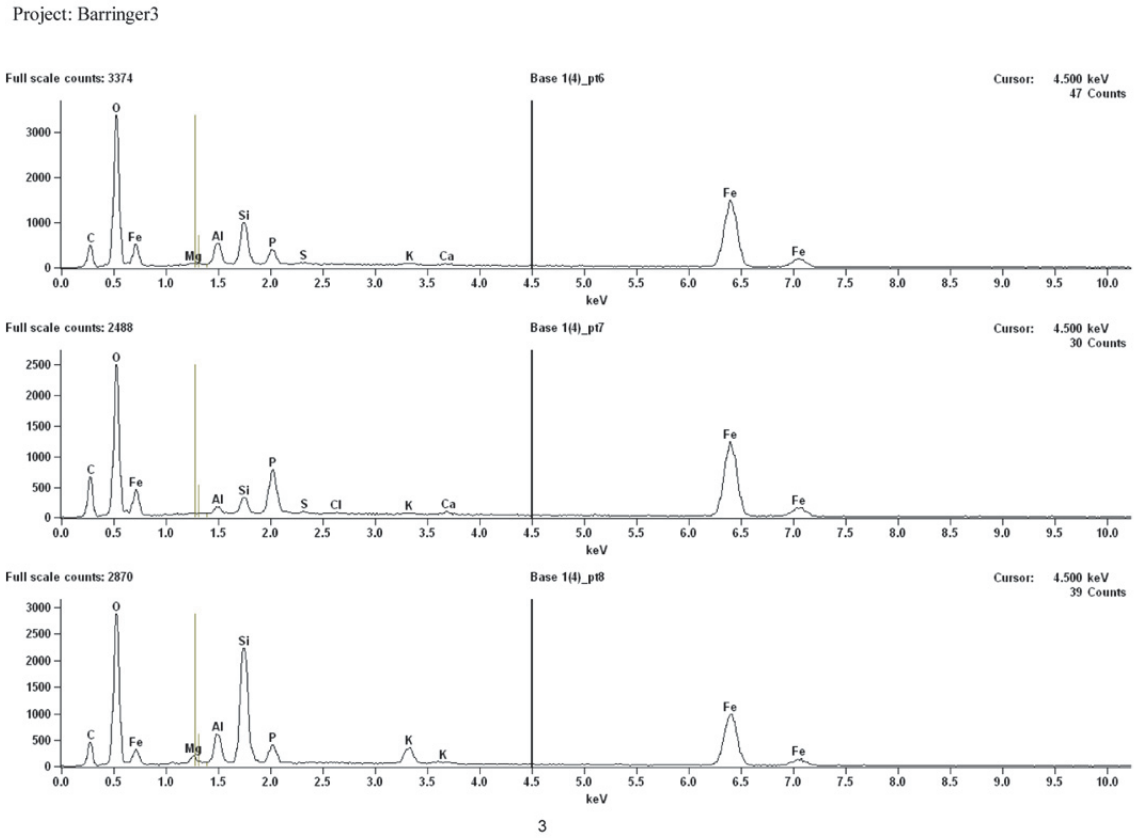


Figure S3.3 SEM scans of sediment particles from streambed sediment core from Crosswicks Creek, site C6 (01464485), Inner Coastal Plain, New Jersey, August 2009.

Note P content of cement surrounding grains. The SEM scans indicated that amorphous phases likely are composed of Fe hydroxide; Fe-phosphate cement also may be present. Such cement is reported elsewhere, formed though the activity of nitrate-reducing, iron-oxidizing microbes(89).

Appendix 3: Supplemental Material for Chapter 4

Table S4.1 Primers used in Chapter 4

Primer Name	Primer Sequence	Reference
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	(70)
519R	5'-GTATTACCGCGGCAGCTGGCAC-3'	(70)
1492R	5'-GGTTACCTTGTTACGACTT-3'	(70)
As1F	5'-CGAAGTTCGTCCCGATHACNTGG-3'	(73)
As1R	5'-GGGGTGCGGTCYTTNARYTC-3'	(73)

Table S4.2 Sequences amplified from DGGE Gel Bands

Six Mile Run Band 1	gctTAcCaTGCAGTCGACGGNAGCACGGACTTCGGTCTGGTGGCGAGTGG CGAACGGGTGAGTAATATATCGGAACGTGCCCAGTCGTGGGGGATAACGT AgTGAAAATTACGCTAATACCGCATACGATCTACGGATGAAAGCGGGGGA TCGCAAGACCTCGCGCGATTGGAGCGGCCGATATCAGATTAGGTAGTTGG TGGGGTAAAGGCTCACCAAGCCAACGATCTGTAGCTGGTCTGAGAGGACA ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC AGTGGGGAATTTTGGACAATGGACGCAAGTCTGATCCAGCCATTCCGCGT GCAGGACGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACAGAACGAAAAG GTTTCTATTAATACTAGGAGCTCATGACGGTACTGTAAGAATAAGCACCG GCTAACTACGTGCCAGCCGCCGCGGNAATTCAAAAAA
Six Mile Run Band 2	CTaCCaTGCAGTCGACGGCAgCACGGACTTCGGTCTGGTGGCGAGTGGCG AACGGGTGAGTAATATATCGGAACGTGCCCACTCGTGGGGGATAACGTAT TGAAAATTACGCTAATACCGCATACGATCTACGGATGAAAGCGGGGGATC GCAAGACCTCGCGCGATTGGAGCGGCCGATATCAGATTAGGTAGTTGGGG AGGTGAAgGCTCACCAAgGCAACGATATGTttcTGGTCTCAAAAGACaAA CAGgCaCCCTGGGNATGcgAAACNgGGAagAAATCCCCTGGGGCGGtT
Six Mile Run Band 3	CTAcaaTGCAGTCGACGGTGTCTTCGGAGATAGCGGGGGACGGGGGCGGA ACACGTATGGACCTACCTTACATTGGGGGACAGCCTTGCGAAAGGGAGAT TAATACCGCATAATACCGTAGCTGGGCATCCAGCAGCTGTTAAAGATTTA TCGATGTACTCTGGGGATGGGTCCAATTAatTAcTTGGTGAGGAAACGGC TCACCAAGGCTATGATTGGTAGGGGAACTGAgAGGGCAATCCCCCACACT GGCACTGAgATACGGGCCAGACTCCTACGGGAGGCAGCAGTAAGGAATAT TGGGCAATGGACGCAAGTCTGACCCAGCCATGCCGCGTGCAGGATGAAGG CGTTATGCGTTGTAAACTGCTTTTATACAGGAATAAACGACTCTTGCGAG AGGCATTGACGGTACTGTATGAATAAGCACCGGCTAACTCCGTGCCAGCC GCCGCGGAtAAATtCAAAAA
Six Mile Run Band 4	gCtACctTGCAGTCGAACGGgattGGtGTTTCCGGTGGCGGACGGGTGAG TAACGCGTAAGAACCTGCCCTTGGGAGGGGAACAACCGGTGGAAACGGCT GCTAATACCCCATAAGCTGAGGAACAAAAGGAGGAATCCGCCCAAGGAGG GGCTCGCGTCTGATTAGTTAGTTGGTGAGGCAATGGCTTACCAAGGCGAC GATCAGTANCTGGTCCGAGAGGATGATCAGCCACACTGGGACTGAGACAC GGCCCANACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCG AAAGCCTGACGGAGCAATGCCGCGTGAAGGAAGAAGGCCCACGGGTCGTG AACTTCTTTTCTCGGAGAAGAAACAATGACGGTATCTGAGGAATAANCAT CGGCTAACTCTGTGCCAGCCGCCGCGGTAATTCaAAAAa
Six Mile Run Band 5	CTAccaTGCAGTCGAGCGATGAgCGCCTTCTGGCGTGAtTAGCGGCGAAC GGGTGAGGAACACGTGAGAAATCTGCCTTCTCTCTGGGATAACTCCGGGA AAACGGGGGTAATACCGGATATGAAATCTACAGGCATCTGTGGATCTGGA AAGTTTTTCGGTTGAANATGATCTCGCGGCCTATCAACTTGTTGGTGAGG TAATGGCTCACCAAGGCGACGACGGGatTCCCGGCTGAaAAGgctTcCgG

	CCACACTGGGACTGAGACACGGcCCAGACTCCTATTTGGGagcTCTTCGG GGTCCATTgNACTGTGGCgCTGCACTCGAGCCTTCGaCTGCCCGCGNGGG AATCAACTCACTCaCAGGCGTAAATACggTTcTCCGCGGAATCaNNCGAT GGGCGGGATNTAACATGTGAgCAAAAATCCANTTATgTGCCAgCCGCCCT
Six Mile Run Band 6	gcgCTAcTGCAGTCACGtgacTGGTCTTGTgGGGGGataGGGGANGGGGG AGATaTACCTGACGGTCTGCCCTTgGCTCTGGGACCCTGCGGgAGCTGTA ATACCTGCCtTCAACctNGgCTCaAGCGGGCGAGGGCTGGACTTCTTTTG TGAAGAGTTCGCTCTCGATTTATTTGCTGGTTGGGGAAGGAATGGCCCAC CCgACGAACtGTAGGTGGGCTGACAGGACGACCGACCACCCTGGAACTGA GACACAGcCCAgACTCCTACCGGAGGGAGGAGTGGGGAATTTTGTTTAAT GGGNGAAACTCTGACCCACCCATGCCgCGTGCGGGATGAAAGcCTTCCTT TTGTTTACTACTTTTTCTTCAGGAAAAAAAGaGAATAAAgTTTNAGTAAc CACgcAANCAAAAcaCCaCTATATGTCCCGCGCGCCGCCAAN
Six Mile Run Band 7	tAccaTGCAGTCGAGCGATGAAGCACCTTCGGGTGTGAATTAgCGGCGAA CGGGTGAGGAACACGTGAGAAATCTGCCTTCAACTCTGGGATAACTCCGG GAAACCGGGGCTAATACCGGATATGAGCCTTGATCGCATGATCGGGGCTG GAAAGTTTTTCGGTTGAAGATGATTTCGCGGCCTATCAGCTTGTTGGTGA GGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACC GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT GGGGAATATTGGGCAATGGAGGAAACTCTGACCCAGCGACGCCGCGTGAG GGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAA GTGACGGTACCTACAGAAGAAGCACCGGCTAACTATGTGCCAGCCGCCGC GGGTAATTCAaaaa
Pike Run Band 1	Sequencing unsuccessful
Pike Run Band 2	TGCaccgAGGaCNaAGgatGcCCCCAGcGCtgTCCNggNGcTacCcNTaN aTTCCCGTTTGNTGNCgGGggaGGGGGGGAATACCCCGCTCGGGCcNANa ACAAAAAACTCGTTGAttTGTTTGgTTCGgcCCGAANTGTGaTTcGgANg CTaaATaCtTtNATTcTTTGGgTCGaGACCgcTTaCcAAGCcgATATCAC TNNGGNNCTNAANAATGAAcCGccAACTGGAGGtGAAAaCGGcTAtNAtt ccAgrGcTCgAAcaATTGAAcAtatttcTGgACAATCTgcATTCgCCaTC CaAaaac
Pike Run Band 3	agaTacTGcagTCgACGgAtTActTTcgTAAgAAACGGGGNaGANGNTGN aCTCTTTCTCaTcTGTATTATTCGGGNGAAAAGGAcAaTAATACCTAATA CTTCCTAATGAAATCCTGGGGTTAtCTTCTGAGCTCTCATGATCATGCTC TGCCAGcATCGCTATATCGAGTTGGTGGGGCAGCGTNCCATAAAATCAAA GAGACGGAACTGTCTCTGACAGGGTGATGATGNACNATCCCCCCGGATAG CgCTGAgNACCCCcNCNGGAGcCTAACAAGGTgAGTACCCCTACgTTGGc GAAAAATTGCGCGATCTTTgCANTACCNGACGGGAAATGAACTCNTNNNA CNAAAAAAAAGNNTN
Pike Run Band 4	TaccatGCAGTCgAACGGtattGtGTNCGggcGCTGACGGGaGGCGAACG GGTGAgAAATTGTTATTGcgTaCCTTTAGtGGGGGATAACGcTGCAataC CCAATAAATACCGaATTAAAACAAAGGATCTGCCTGGCGAGGGGCTCGCG NGTGATTTGTTAGATGGCGATATGTTGGATTACCTAGGTGGCGATCGAAA AGCGGaCCGAGGGGAAGATCAGTCTCCCTGGGAATGAGACGATGCCCcaA CTCGGACGGGAGGCACGGCTGGGACATTTTCCGCAgTGGGCAAaGGCCTG ATTtActGATGGCGCGTGAGCCTGGAaCCCCaTGGGTCGCGACCTTCTTT TCTCTGACANTTATAATGACCTTTTgTGAGaANTaAACATCCTGTAACTC TGTACCACCGCCGCGgTAATTCAaCAACAATAGtgCCTTCTcaCaAAgTG gCtCCCGCCCAGAACtTTCAAAcagNCAcCgNtAATacAAcAAN
Pike Run Band 5	gctAcACATGCAGTCGAACGAgAagTTCGGGTGTAGCAATACATTCGGGC GAGTAAAGTGGCGTCCGGGTGAGTAACACGTANCTAACCTACCCTCGAGC GGGGGATAACCTGGGGAAACTCGGGCTAATACCGCATAACGTCCAGGCTA CATATGTAGCTTGGATCAAAGGCCCGCAAGGGCCACTGGAGGAGGGGGCT GCGGCCGATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGATGATCG GTACCCGGCCTGAGAGGGCGCACGGCCACACTGGCACTGAAATACGGGCC AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCACAATGCCCGAAAGG GTGATGCAGCAACGCCGCGTGAGGGATGAANGtCTTCGGGTCGTAAACCT CTTTCGACCGGGAAGAATGGCTCCGTGAACAAAGGACCCTGACAGTCCCG GNGAAAAAAAACCCGGCTAaCTACgTGCCtgCCNCCN

Pike Run Band 6	GCtAccaTGCAGTCGACGAgAagtGcGGGTGggcatGCATTCGGGCGAGA ACGGGGGAGTCCGGGTGAGAAACACGTACTTAAgGTACCCTCGAGCGGGG GATAAcccGGGCACACTTCCACACAACCCGcGAGGCGGAAAGGTAAAGTG TGAAGCCTGGATCGCCTAATGAGTGANCTAACTCACATTAATTGCGTTGC GCTCACTGCCCGCTTTCCAGTCAGGAAACCTGTCGTGCCAGCTGCATTAA TGAaTCGGCCAACGCGCGGGGAGAGGCGGTTTGCgTATTGGGCGCTCTTC CGCTTCCTCGCTCANTGNNTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAG CGGtATCAaCTCACTCAaAGGCNgTAATACGGTTATCCACAaAATCAaGg GAtAaCgcaGGAAaga

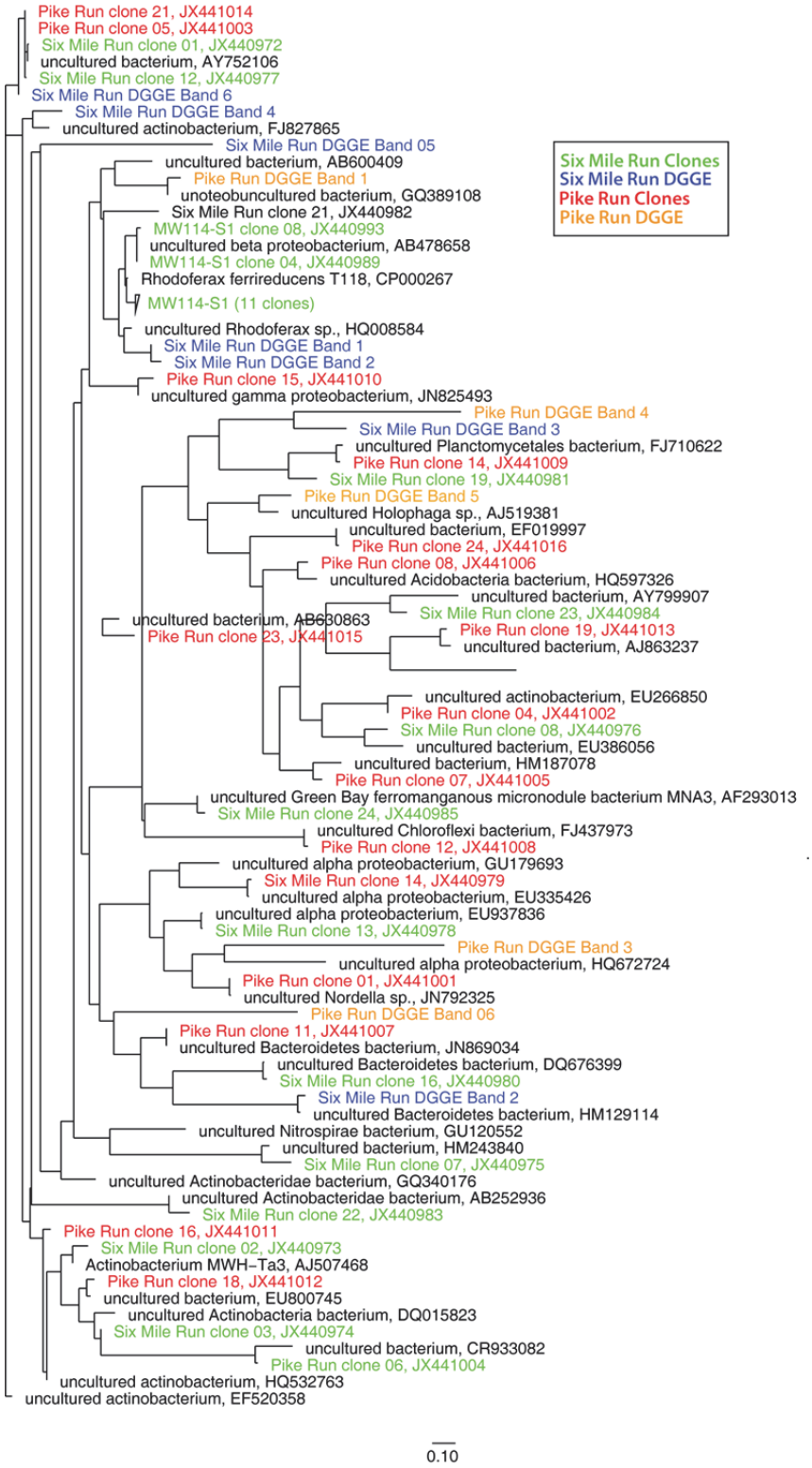


Figure S4.1 Annotated rAXML tree of 16S rRNA sequences from Six Mile Run and Pike Run

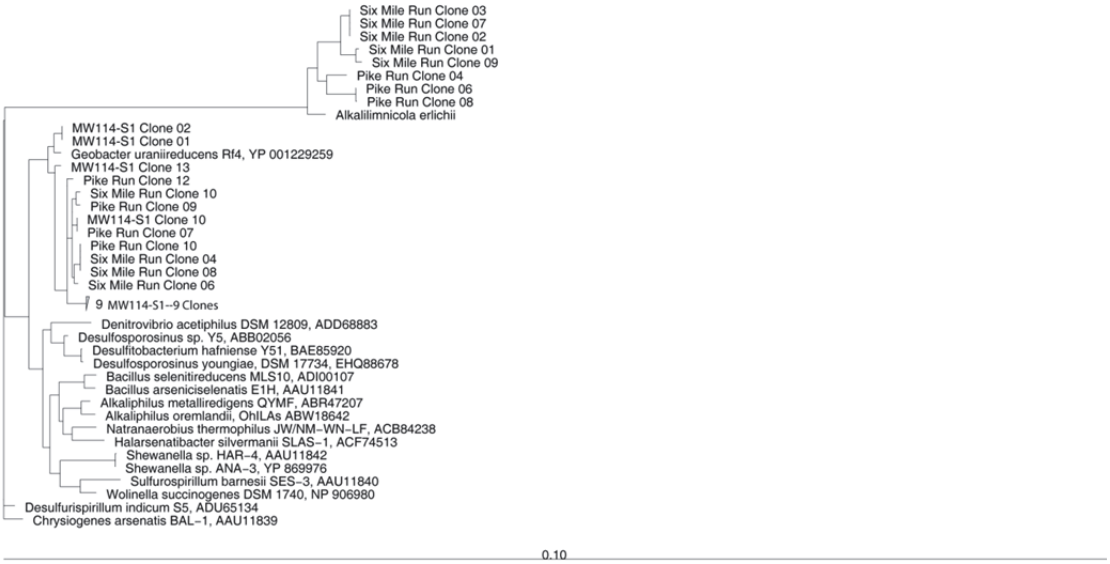


Figure S4.2 Annotated rAXML tree of *arrA* sequences obtained from Six Mile Run and Pike Run.

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