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Isolation and Characterization of a Microorganism from Groundwater that Reduces Arsenate

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ISOLATION AND CHARACTERIZATION OF A MICROORGANISM FROM GROUNDWATER THAT REDUCES ARSENATE

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An Abstract of the Thesis Presented
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This is an investigation into the microbially mediated processes involved in the transformation of arsenic. With the recent change in the Federal Maximum Contaminant Level for arsenic in drinking water, an increasing amount of resources are being devoted to understanding the mechanisms involved in the movement of arsenic. Arsenic in drinking water typically comes from natural sources, but the triggers that result in increased release of arsenic from parent material are poorly understood. Knowledge of these processes is necessary in order to make sound engineering decisions regarding drinking water management practices.

Recent years have brought forth the idea that bacteria play a significant role in arsenic cycling. Groundwater is a major source of potable water in this and many other countries. To date, no reports have been made indicating the presence and activity of arsenate reducing bacteria in groundwater settings, which may increase dissolved arsenic concentrations. This research was designed to address this question and has shown that these bacteria are present in Maine groundwater.

Two Maine wells were sampled in order to culture resident bacteria that are capable of dissimilatory arsenate reduction. Samples were collected using anaerobic techniques from wells in Northport and Green Lake. These samples were amended with specific compounds to enrich the resident population of arsenate utilizing bacteria. These cultures were monitored over time to establish rates of arsenate reduction.

Cultures from both sites exhibited arsenate reduction in initial enrichment cultures. Isolates obtained from the Green Lake enrichments, however, did not reduce arsenate. This indicates either that a symbiotic relationship was required for the observed arsenate reduction or that fast-growing fermentative organisms that could survive in high arsenate media were picked in the isolation procedure. The Northport cultures exhibited continued arsenate reduction after isolation and successive transfers into fresh media. The cultured bacteria reduced the majority of 1 mM arsenate solutions in less than one week, accompanied by a corresponding oxidation of lactate. The 16S rRNA from the isolate was amplified and sequenced. The results of the DNA sequence analysis indicate that the rRNA sequence of the bacteria isolated at the Northport site is unique. This means that this strain of bacteria has not been reported before. It is in the same taxonomic subgroup as two previously described arsenate respirers.

The implications of this study are significant. The fact that resident bacteria are capable of reducing arsenate has implications for water management practices. Reduction of arsenate to arsenite increases the mobility of the compound, as well as the toxicity. An understanding of the activity of these types of organisms is necessary in order to understand the contribution they are making to arsenic concentrations in drinking

water. The next step in this work would be to quantify the actual loading of dissolved arsenic present in aquifers because of these organisms.

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1 INTRODUCTION

This thesis is an investigation into the microbially mediated processes involved in the transformation of arsenic. The Federal Maximum Contaminant Level (MCL) for arsenic in drinking water is currently set at 50 μg/L. This will be lowered to 10 μg/L beginning in 2003. With the recent change in the Federal MCL for arsenic in drinking water, an increasing amount of resources are being devoted to understanding the mechanisms involved in the movement of arsenic. Information such as the location and concentration of arsenic is readily available, but a fundamental understanding of the origins of this displacement is not. This is necessary to make sound engineering decisions regarding drinking water management practices.

Recent years have brought forth the idea that bacteria play a significant role in arsenic cycling. In the subsurface, bacterial transformations that affect the form or solubility of arsenic in the system may either increase or decrease the concentration of arsenic in the water. Groundwater is a major source of potable water in this and many other countries. To date, no reports have been made indicating the presence and activity of arsenate reducing bacteria in groundwater settings.

This research was designed to determine if bacteria that reduce arsenate are present in groundwater. The underlying premise is that the reduced form is more mobile in soils, and thus reduction would increase the soluble arsenic concentration. Two sites with high arsenic in Maine were chosen. The objectives of the research were:

- To obtain arsenate-reducing bacterial isolates or enrichment cultures from arsenic-contaminated wells.

- To identify and characterize the bacterial cultures obtained.

This research has shown that there are bacteria present in Maine groundwater that can effectively catalyze the reduction of arsenate, with a complementary oxidation of lactate. One of the two wells tested yielded an isolate that has not been described previously. Further study of this isolate and its prevalence in the environment will improve our understanding of its role in arsenic cycling and contribution to elevated arsenic in groundwater.

2 LITERATURE REVIEW

2.1 Physical and Chemical Properties

Arsenic is a silver-gray crystalline solid[1] that may also exist as a black or yellow amorphous phase. The crystalline solid has a specific gravity of 5.73, with a boiling point of 614°C and a melting point of 817°C. The amorphous phase has a specific gravity of 1.97. Arsenic is a group 15 element with an atomic weight of 74.92 and an atomic number of 33. It is classified as a metalloid, although arsenic has an elemental structure similar to non-metals.[2] In the vapor state it occurs as a tetrameric molecule (As_4).

Arsenic is stable in five different valence states, possessing a charge of -3 (arsine), 0 (elemental arsenic), +1 (arsonium metal), +3 (arsenite), or +5 (arsenate). The higher oxidation states exhibit covalent bonding tendencies, while the lower oxidation states tend to possess ionic bonding properties.[3] Elemental arsenic, valence state of 0, is rarely found under natural conditions. The valence state is an indicator of the relative

toxicity, with arsine gas (-3) usually the most toxic, followed in order of decreasing toxicity by arsenite, arsenate, arsonium metals, and elemental arsenic.

The prevalent types of arsenic compounds may be divided into organic and inorganic categories, with inorganic compounds comprising the majority of the compounds found in water. The inorganic arsenic species usually observed are arsenious acid and arsenic acid. At relevant environmental pH values, arsenious acid or arsenite would be present as H_3AsO_3, while arsenic acid or arsenate would be present as either $H_2AsO_4^-$ or $HAsO_4^{2-}$. Both species are also commonly observed in inorganic compounds such as oxides (As_2O_3 or As_2O_5) or sulfides (AsS or As_2S_3). Organic arsenic compounds that may be observed could include species such as monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), trimethylarsine (TMA), or trimethylarsine oxide (TMAO). Organic arsenic species are usually associated with terrestrial settings but some may be found in aquatic environments, and more often in surface water than groundwater. Organic arsenic may be found in natural gas as ethylmethylarsines, in shale oil, in water when biota metabolizes inorganic arsenic, and in the livers of humans.[2]

2.2 Natural Sources

Arsenic is the 20th most abundant element in the earth's crust.[4] It is widely distributed through the environment in a variety of locations, such as the rocks of the earth's crust and the associated soil, while it may also be found to a lesser extent in water, air, and organisms.

2.2.1 Rocks and Minerals

More than 99% of the arsenic in the environment is located in rocks.[4] This disposition is a result of the ease with which arsenic can substitute for minerals such as silicon, aluminum, or iron in the crystal lattices of silicate minerals.[5] The concentration found in the crust is obviously variable, but is typically reported to range from 1.5 to 5 mg/kg.[6,7,8]

Rock type is a factor in the amount of arsenic found in a given geologic situation. Among rocks of igneous origin, the basalts tend to have a relatively higher arsenic content. Sedimentary rocks, specifically iron and manganese ores, usually contain average arsenic concentrations higher than rocks from an igneous origin.[2] Some minerals that contain relatively high portions of the available arsenic in the crust are arsenopyrite (FeAsS), realgar (AsS), and orpiment (As_2S_3). Arsenic is also commonly found in the sulfide ores of other metals, such as copper, lead, silver, and gold. It may be released from these ores to impact surface water[9], soil[10], and ground water.[11]

Various investigators have defined specific correlations between arsenic content and geologic setting. Welch *et al.*,[11] observed a strong relationship between sedimentary deposits derived from volcanic rock and high arsenic concentrations in groundwater (>50μg/L). The researchers theorized that the volcanic rock weathered to release arsenic, which then concentrated onto ferric oxyhydroxides and was deposited with the sediments.

Alternatively, in parts of Michigan and Wisconsin elevated arsenic concentrations have been shown to be associated with sulfide mineral deposits in sedimentary rocks.[12,13] Moderate to high (10 to 50μg/L) concentrations have also been associated with sulfide

minerals in bedrock aquifers of the Northeastern United States, from Massachusetts to Maine.[14]

2.2.2 Soils

Because of the arsenic concentrations found in parent material, certain soils are bound to have large amounts of arsenic present. Bedrock type is a major factor for arsenic concentrations in soils, but climate, organic content, and redox potential also have a significant effect on arsenic availability. Peterson[15] defined typical levels of soil arsenic concentrations to be in the range of 0.1 to 40 mg/kg, with an average of 5 to 6 mg/kg. Soils derived from the weathering of basalts tend to have higher arsenic concentrations than those derived from granites. Typical arsenic concentrations in soil are highly variable, with soils derived from sedimentary rocks and areas with volcanism history exhibiting levels of approximately 20 mg/kg,[2] and while soils that overlay deposits of sulfidic ores have had observed concentrations of 8000 mg/kg.[8]

2.2.3 Geothermal Waters

A major source of arsenic is also found in geothermal waters, which are most prevalent in the western U.S. Welch *et al.*[11] described 14 areas in the western U.S. where arsenic concentrations in water are greater than 50 μg/L, as a result of known or suspected geothermal sources. Dissolved concentrations range from 80 to 15,000 μg/L in these areas. When Welch *et al.* compared mean arsenic concentrations in non-thermal ground waters with those related to thermal sources; they found that dissolved concentrations of arsenic in waters derived from geothermal regions are significantly greater in any of the physiographic provinces of the United States. With concentrations

higher in these settings, it is feasible that they will impact surface waters that are hydraulically connected. In Yellowstone National Park, for example, the arsenic concentration in hot springs and geysers is in the range of 900 to 3560 μg/L.[16] It has been shown that waters from these areas of the park are correlated to elevated arsenic levels in the Missouri and Madison Rivers,[17] which must be treated for use as a municipal water supply.

2.2.4 Volcanic Activity

The last major source of arsenic from a natural standpoint arises from volcanic emissions. The specific loading of arsenic from volcanic activity is not widely agreed upon, but it is the largest natural source of arsenic emissions to the atmosphere.[6] A study by Tamaki and Frankenburger[18] suggests that natural arsenic emissions are greater than industrial sources, while other investigators[19,20] have concluded that industrial emissions are significantly greater than natural sources.

2.3 Anthropogenic Sources

Arsenic is used extensively in various sectors of the industrial and manufacturing flow of materials. Its use has declined in certain sectors, but is still a prevalent raw material for many processes. The major manufacturing uses for arsenic are wood preservation, agriculture, and industrial processes such as semiconductors and glasss manufacturing. It is also a primary and secondary product of mining and smelting operations.

2.3.1 Wood Preservatives

Approximately 90% of the arsenic that is consumed in this country on an annual basis is used in the manufacture of CCA or Chromated Copper Arsenate. CCA is an inorganic arsenic compound added to lumber in order to prolong its useful life, by defending against insect and fungal attack. Copper is used to protect against fungus, arsenic kills any insects that try to eat the wood, and the chromium complexes with the other two chemicals to "fix" the entire compound into the wood. There are different varieties of CCA available in the commercial marketplace, with different proportions of arsenic in each. Different arsenic compounds are used to produce CCA, such as arsenic acid (H_3AsO_4), arsenic pentoxide (As_2O_5), and sodium arsenate (Na_2HAsO_4). Arsenic trioxide (As_2O_3) is not used to manufacture CCA directly, although it is used to make the ingredients just mentioned. CCA is used to pressure treat lumber for decks, fences, playgrounds, and other applications that are subject to outside conditions where decay is an issue. This type of use is directly linked to the performance of the housing industry.

The EPA classifies CCA as a restricted use pesticide. On February 12, 2002, the EPA announced that the industry involved in the manufacture of CCA had voluntarily adopted a measure to move consumer use of treated lumber products away from those that contain arsenic prior to December 31, 2003. This transition applies to residential uses of the treated lumber. The EPA has mandated that CCA will not be used in any residential applications beginning January 2004.[21] Policy such as this is usually founded on scientific evidence, which in this case is not yet available. Although definitive evidence linking CCA exposure to adverse human health effects is not available, the

mandate was made based on the principle that it is better to err on the conservative side than to expose the population to potential toxicants while evidence is amassed.

2.3.2 Agriculture

Arsenic has an extensive history in the agricultural sector of this country. It has been used to make a variety of compounds, namely pesticides, herbicides, insecticides, defoliants, soil sterilizers, animal dips, feed additives, and disease preventatives.[22]

The most widely applied organic pesticide made from arsenic is the organoarsenical pesticide monosodium methanearsonate (MSMA), which is used to control broadleaf weeds, primarily in cotton crops.[23] In 1995, it was the 22nd most applied conventional pesticide in the United States with a total use of approximately 6 million pounds.[24] Disodium methanearsonate (DSMA) and cacodylic acid are also applied to cotton fields as herbicides, as well as for postemergence control of crabgrass, dallisgrass, and other weeds in turf growing operations.[8] In soil, organoarsenicals are metabolized to alkylarsines and arsenate by resident bacteria.[25]

Although organic arsenic compounds are currently used for the manufacture of agricultural compounds, the use of inorganic arsenic for this purpose has been dramatically reduced in recent years. The last inorganic arsenic compound that was used for agricultural purposes was arsenic acid, a pesticide for cotton, which was voluntarily removed from production in 1993.[26] A variety of other pesticides have been produced using inorganic arsenic, such as lead arsenate, sodium arsenate, calcium arsenate, copper acetoarsenate, copper arsenate, and magnesium arsenate. These pesticides were used to inhibit potato beetles, boll weevils, grasshoppers, budworms, moths, and other insects in the United States.[27] Sodium arsenite was used as a cattle and sheep dip, but its use has

been banned in the U.S., along with calcium arsenate, copper arsenate, and lead arsenate.[28]

The widespread use of inorganic arsenic to control insect populations has led to an undesirable consequence, the contamination of soil. Acid lead arsenate was the most abundantly used insecticide in deciduous tree fruit orchards prior to the introduction of dichlorodiphenyltrichloroethane (DDT) in 1947.[29] The high rate of application of acid lead arsenate has led to soil contamination in orchard regions of Australia, Canada, New Zealand, and the United States.[30] The arsenic present in this situation is not very mobile, partly since it possesses a negative charge and will largely be bound to the soil. However, if a phosphate fertilizer is applied to the contaminated area, ion exchange processes are possible. Phosphate and arsenate are very similar in molecular structure, having the same configuration of electrons in their outer orbital. This allows the two to exchange positions on a given bonding site. Historically, the use of phosphate fertilizers in orchard settings has been limited due to the lack of positive response by the trees after treatment.[30] Recently their use has increased because of evidence showing a favorable response when newly planted trees are treated with fertilizers containing phosphate, such as monoammonium phosphate (MAP).[31] Another scenario is the development of lands that were once used for orchards into public parks, home gardens, or golf courses which all receive applications of phosphate rich fertilizers on an annual basis.[30] Regardless of the situation where the application of phosphate fertilizer is taking place, if arsenic is present it is subject to competitive ion exchange and subsequent mobilization.

Another use in the agricultural realm is the addition of organic arsenic compounds to poultry and swine feed. Compounds such as roxarsone and arsanilic acid are added to

feed stocks in order to increase weight gain rate, improve feed efficiency, improve pigmentation, and to treat and prevent diseases such as swine dysentery or chronic respiratory diseases.[2] Aschbacher and Feil[32] found that there was little or no degradation of arsanilic acid before excretion from poultry or swine and that as much as 85% of the compound was present in the animals' feces and urine. Concentrations of arsenic in animal wastes are reported to be from 4 to 40 mg/kg.[33] A common practice in agriculture is to use the waste from the animals as a fertilizer on crops, which may eventually be eaten by these same animals. It is possible that arsenic could be involved in a localized cycle if such a situation persists, accumulating in the soil and animals that inhabit these farming operations.

2.3.3 Industry

Arsenic is used extensively on a regular basis to manufacture certain products in the industrial sector. Arsenic metal is used in the production of posts and grids in lead-acid storage batteries.[34] These non-sealed, maintainable batteries are currently being phased out of production. As these are replaced by maintenance-free automotive batteries, which contain little or no arsenic, the demand for this type of arsenic is expected to decline.[2] Arsenic metal is also used in the manufacture of crystalline gallium arsenide, a semiconducting material used in electronic devices such as computers or optoelectronic devices and circuits. Other industrial processes that may result in the release of arsenic to the environment could include the burning of fossil fuels, combustion of hazardous and non-hazardous wastes, mining and smelting, pulp and paper production, glass manufacturing, and cement manufacturing.[25]

2.3.4 Mining and Smelting

A significant source for arsenic to enter the environment occurs during mining and smelting. This refers to the procedures undertaken to obtain the arsenic used in the previously mentioned uses, as well as the release of arsenic associated with the mining and smelting of other minerals. Arsenic is obtained by smelting arsenopyrite or lollingite in the presence of air at a temperature of 650 to 700°C.[35] Arsenic trioxide (As_2O_3) can be obtained by capturing the flue dust released during the extraction of copper or lead.[3] The ASARCO smelter in Tacoma, Washington commercially produced arsenic trioxide until 1985.[6] According to the USEPA Office of Air Quality Planning and Standards, primary and secondary lead smelters, and primary copper smelters are all possible sources of arsenic emissions. Currently there are three primary lead smelters, nineteen secondary lead smelters, and seven primary copper smelters operating in the U.S.[25] Increased arsenic concentrations have been observed in precipitation downwind of a copper smelter in Washington.[36]

Mining activities may also produce locally elevated arsenic concentrations if the conditions are right. Sulfide bearing rocks are often mined for gold, lead, zinc, and copper; with arsenic being a common impurity in these ores.[2] The type of arsenic mineral present in these ores is usually arsenopyrite, orpiment, realgar, or arsenic-rich iron oxides.[11] In the process of obtaining the element of interest, arsenic becomes part of the mine tailings at the site. Mine tailings are usually stored in large piles on the perimeter of mining sites and are subject to the ambient environmental conditions, specifically precipitation. The drainage from abandoned mines and mine tailings may

have dissolved arsenic concentrations as high as 48,000 μg/L such as in Iron Mountain, California,[11] which could significantly impact local water quality.

2.3.5 Medicine

Some of the earliest uses of arsenic have been in the medical field as a therapeutic agent. Arsenical compounds were used in medicine 2000-3000 years ago in the Orient. An early use of inorganic arsenic in the West was in Fowler's solution in 1786, which contained 1% arsenic trioxide.[37] Arsenic trioxide is currently being researched as a treatment for acute promyelocytic leukemia. Inorganic arsenic has a history of being used for conditions such as eczema, psoriasis, malaria, rheumatism, asthma, pernicious anemia, leukemia, and Hodgkin's disease.[2] Due to the toxic side effects of inorganic arsenic, its use was dramatically reduced in the 1970's. Prior to the advent of penicillin in the 1940's, organic arsenic was a constituent of drugs used to combat the parasites that cause syphilis, yaws, relapsing fever, trichomal vaginitis, trypanosomiasis and amebic dysentary.[8] Although organic arsenic is seldom used today, it is still in some drugs such as melarsoprol, which is used against trypanosomiasis and filariasis with occasionally fatal side effects.[8]

2.4 Toxicology

Arsenic compounds are absorbed through the gastrointestinal tract, lungs, and skin. After absorption, these compounds are transported to other areas of the body and accumulate in various organs. In humans, the highest concentration of arsenic is in hair and nails, followed by skin and lung tissue.[38] Arsenate is reduced *in vivo* to arsenite, and the trivalent form is active in animal tissues.[44] Arsenite binds to sulfhydryl groups of

globulins and other large molecules, accumulating in mitochondria inhibiting energy linked functions.[39] Arsenate does not react strongly with sulfhydryl groups.[73] Trivalent arsenic activates nuclear oncogenes, which encourage and promote the normal growth and division of cells, via its ability to bind to vicinal dithiols within a protein, or to bridge two thiols between neighboring proteins. The number of proteins with vicinal dithiols is relatively small, but these structures are common in DNA binding proteins and transcription factors, and in some DNA repair proteins.[40] Following reduction, methylation occurs via a nonenzymatic mechanism producing monomethylarsonic acid (MMAA), which is enzymatically methylated to dimethylarsinic acid (DMAA)[41] for excretion.[39] Excess arsenite can inhibit the initial nonenzymatic step and saturate the second enzymatic pathway.[42] In mammals, reduction takes place in the blood while methylation occurs primarily in the liver,[43] with methylation in humans being less efficient than in other species implying an increased sensitivity to arsenic.[39]

Arsenic excretion occurs mainly through the urinary tract. Pentavalent arsenic is excreted at a faster rate than the trivalent form, and organic arsenic compounds are removed faster than inorganics.[44] Arsenic concentration in urine is the best indicator of current or recent exposure.[45] Determination of arsenic levels in blood has little practical use, due to the short half-life of arsenic in blood.[44] Normal concentrations of arsenic in blood and urine average 100 and 15 μg/L, respectively.[46] The urinary concentration of inorganic arsenic plus MMA and DMA would be a more accurate biological marker of the absorbed dose of inorganic arsenic than the total urinary concentration[47] because it excludes other arsenic compounds, in particular arsenobetaine, which is abundant in

shellfish and some fish.[48] Arsenobetaine is relatively nontoxic and is rapidly excreted in the urine with no metabolism necessary.[49]

Occupational exposure to arsenic is mainly of concern for workers involved in the processing of copper, gold, lead, and antimony ores, producing and using arsenicals and arsenic-containing pesticides, burning of arsenic-containing coal in power plants, and treating wood with arsenic preservatives and working with such treated wood.[44] As early as 1820, Ayrton Paris described the high incidence of scrotal carcinoma among copper smelter workers in Cornwall, England as a consequence of exposure to arsenic fumes. In 1879, it was suggested that inhaled arsenic might be the cause of high rates of lung cancer in German miners.[44] Studies of smelter workers, insecticide-manufacturing workers, and sheep dip workers in many countries around the world have all found an association between occupational arsenic exposure and lung cancer.[44]

An association between skin cancer and arsenic ingestion has been confirmed by numerous studies in Taiwan, Chile, Argentina, and Mexico,[50,51,44,52] where drinking water supplies are contaminated by arsenic. Results showing no excess skin cancer among U.S. residents using drinking water contaminated by arsenic[53,54] appear to be of inadequate statistical power because of small sample size and therefore are not necessarily inconsistent with the positive results obtained abroad.[44] Although it has been more than 100 years since the carcinogenic properties of arsenic were first suggested, it is still unclear to what extent inorganic arsenic contributes to the formation of cancers other than skin and lung. A follow up study of the population living in the area of Taiwan, where a high prevalence of skin cancer was associated with arsenic contamination in the water supply, also found significantly elevated standard mortality ratios for cancers of the

bladder, lung, liver, kidney, and colon. Like skin cancer, the incidence of bladder, liver, and lung cancer cases in this study showed a dose related increase with arsenic exposure. Effects other than cancer were also noted in this population such as influences on the peripheral vasculature leading to Blackfoot's disease and noncancerous skin lesions such as altered pigmentation and skin thickening (hyperkeratosis).[50]

2.5 Geochemistry

Arsenic is ultimately controlled by the other elements that it interacts with, on or near the surface of the earth. The original source of all arsenic on the planet is the earth, and therefore an understanding of how it interacts with this parent material is essential. In order to understand the mineral-water interactions of arsenic compounds, knowledge of the oxidation-reduction processes and speciation/adsorption behavior of arsenic in water is necessary. This basic framework of water-arsenic interactions will be addressed first, followed by a summary of kinetic considerations for oxidation-reduction reactions. Finally, solid phase interactions will be presented, focusing primarily on adsorption and precipitation/dissolution processes.

2.6 E_h-pH Characteristics

Arsenic exists in both inorganic and organic states in nature. For work focusing primarily on groundwater and mineral interactions, the inorganic forms are the most relevant due to their abundance in these types of systems. Inorganic arsenic occurs in multiple valence states, in groundwater the most common would be the pentavalent compound arsenate and the trivalent form arsenite. Figure 1 is a diagram of the dominant

arsenic species that would be expected at given pH and E_h values, given sufficient time to reach equilibrium.

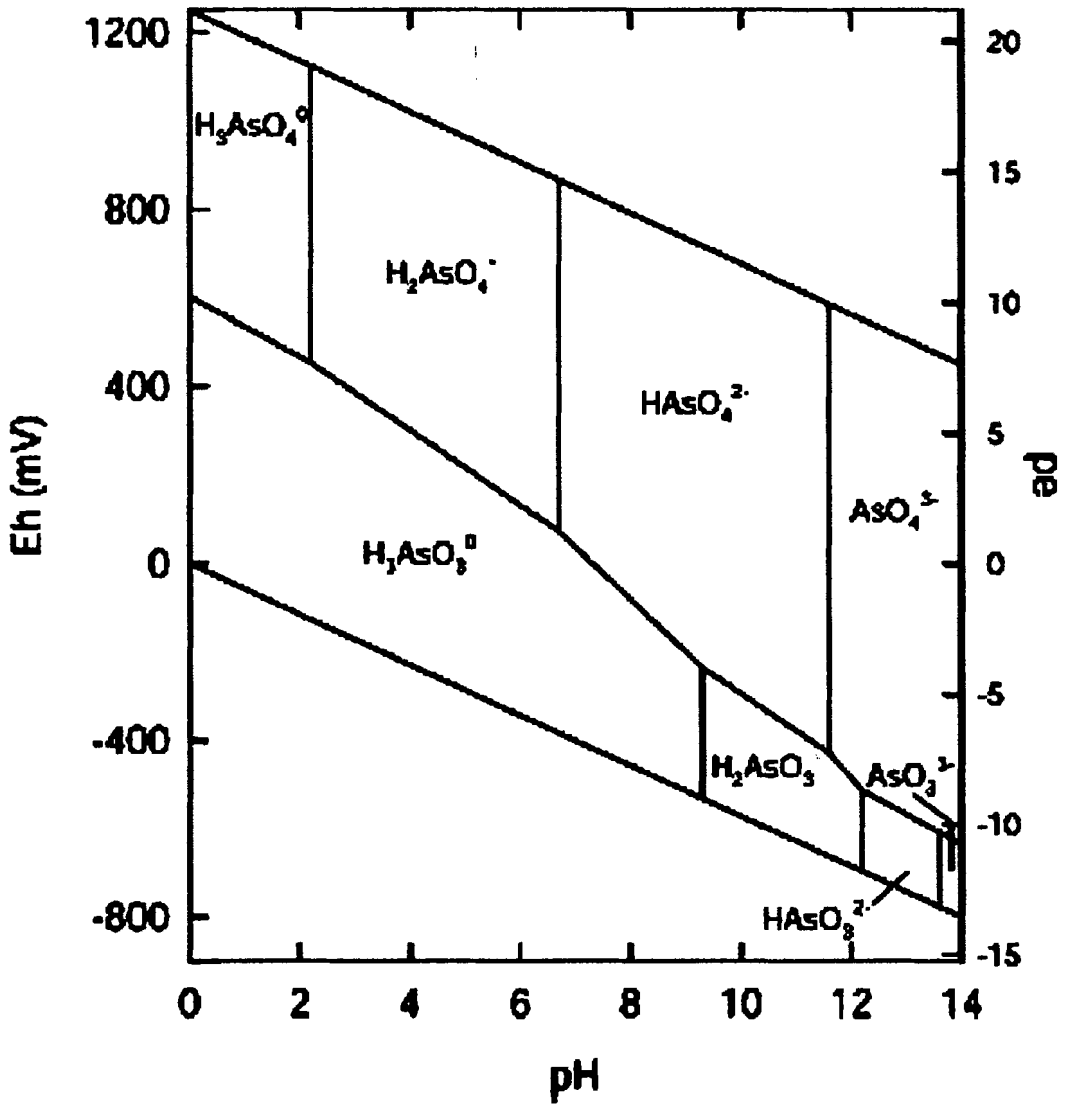

Figure 1. Eh-pH diagram for aqueous As species in the system As-O_2-H_2O at 25° and 1 bar total pressure.[55]

Several concepts may be inferred from this diagram. In well aerated settings, the dominant arsenic compounds will be in the form of arsenic acid, with a valence state of +5. In reduced settings, arsenious acid species will dominate with a valence state of +3. As pH increases, both compounds will deprotonate. At a pH level characteristic of natural waters, arsenate will have one or two hydrogen atoms in its structure with a corresponding charge of -2 or -1 respectively. Arsenite's first pK_a is at 9.2, so it will be uncharged and fully protonated below a pH of approximately 8.5. This lack of charge at neutral pH values accounts for arsenite's ability to move through soil and membranes.

Various investigators have studied the effects of pH and E_h upon the mobilization of arsenic from contaminated river sediments, muds, and soils.[56,57,58] These studies provide insight towards the mechanisms in operation with respect to acidity and oxygen levels. In pH ranging from 2 to 11, the release of arsenic from sediments to overlying waters follows a pattern of substantial release with decreasing pH, with a sharp increase in release of arsenic also noted at high pH.[9] Overall, the investigators also found that there was an increase in mobility once reducing conditions were present.

2.7 Redox Kinetics

The focus of this investigation is on the microbial reduction of arsenate, therefore an understanding of the abiotic reduction and oxidation pathways is necessary. Various compounds are capable of either reducing arsenate to arsenite, or oxidizing arsenite to arsenate. A major reductant that can effectively reduce arsenate is dissolved sulfide. Aqueous sulfide (H_2S or HS^-) is a strong reductant that is usually found in considerable concentrations in reduced environments. Based on standard state redox conditions,

hydrogen sulfide should be capable of reducing arsenate to arsenite via the following reaction:

(1) $$H_2AsO_4^- + H_2S + H^+ \Leftrightarrow H_3AsO_3 + \tfrac{1}{8}S_8 + H_2O$$

Relatively few studies have been done evaluating the kinetics and elementary steps of this reaction, with little agreement between the various results. It has been shown that the rate of arsenate reduction is highly pH dependent with differences of up to three hundred times when results for pH values of 6.8 and 4 are compared. Newman *et al.*[59] found that the reduction process was relatively slow at a near neutral pH of 6.8, calculating a second-order rate constant of 1.04 $\underline{M}^{-1}$ hr^{-1}. At a pH of 4.0, Rochette *et al.*[60] approximated a rate constant equal to 3.2×10^2 $\underline{M}^{-1}$ hr^{-1}, citing the conversion of sulfide from HS^- to H_2S below pH 7 as the reason for the large difference. Cherry *et al.*[61] observed a similar trend of increasing reduction rate as pH declined, yet did not provide rate constants. A solution of 50 ppb arsenate, 50 ppb arsenite, and a total sulfide concentration of 10^{-2} $\underline{M}$ was allowed to react for 32 hours.[61] At a pH of 7, approximately 30% of the arsenate was reduced, 90% of the arsenate was reduced at a pH of 5, while at pH 4 virtually all of the arsenic present at the end of 32 hours was in the form of arsenite. Obviously, the protonation of arsenate and/or sulfide has a substantial effect on the reduction rate, but a detailed mechanism has yet to be determined. It should be understood that the rates of reduction observed by Cherry *et al.* are much faster than one would expect in nature, since the concentration of sulfide provided was much higher than the amount normally present in natural systems.

It would seem that the most likely candidate for the oxidation of arsenite would be dissolved oxygen; however, this is not necessarily true. Studies investigating the

oxidation of arsenite by dissolved oxygen show that it is able to oxidize arsenite, although it is a fairly slow and incomplete conversion. Evidence pertaining to this is available from direct studies on the topic, as well as investigators primarily interested in sample preservation. Cherry *et al.*[61] also investigated the effect of dissolved oxygen on arsenic speciation. After 3 to 4 hours of purging distilled water with pure oxygen, 50 ppb of arsenate and 50 ppb of arsenite was allowed to react for 10 days in a closed vessel. Solubility calculations estimated the concentration of oxygen to be $10^{-1.66}$ M. After the ten day period, approximately 30% of the arsenite had been oxidized to arsenate.

Johnson and Pilson[62] give half-lives for the oxygenation of arsenite in seawater ranging from several months to years. Eary and Schramke[63] reported a half-time of one year for arsenic oxidation by oxygen. Tallman and Shaikh[64] reported that the oxidation of arsenite to arsenate in groundwater samples was relatively slow, with no oxidation occurring after 3 weeks. Elkhatib *et al.*[65] reported no oxidation of arsenite in soil extracts containing 5 to 500 µg/L of arsenite. Seyler and Martin[66] reported that 5 µg/L arsenite was not oxidized at 4°C and pH 7 after 10 days. In contrast, Andreae[67] reported that at concentrations of 0.05 µg/L, loss of arsenite was detectable after one week and that acidification increased the rate of oxidation. Feldman[68] reported that unpreserved samples, containing 1 to 10 µg/L of arsenite were oxidized completely within 4 days and Haswell *et al.*[69] reported that arsenite was oxidized in 5 hours when present at the microgram per liter level in soil pore water. Since there is little agreement between studies on the stabilization of arsenic species in samples, it implies that on site speciation of arsenic species is the most reliable method for sample preservation.

Samples of this nature have an unknown composition and the identity of the dominant oxidant can only be speculative. Another compound with oxidative effects on arsenic is manganese, which could have been the responsible oxidant in some of the previous examples. Mn[IV] and Fe[III] oxides are both thermodynamically capable of oxidizing arsenite by the following:[70]

(2) $MnO_2 + HAsO_2 + 2H^+ \Leftrightarrow Mn^{2+} + H_3AsO_4$ $E^o = 0.67$ V

(3) $2Fe^{3+} + HAsO_2 + 2H_2O \Leftrightarrow 2Fe^{2+} + H_3AsO_4 + 2H^+$ $E^o = 0.21$ V

The positive E^o values of these reactions indicate that they are spontaneous, but research has shown that iron is a fairly poor oxidant with respect to arsenite while manganese is quite effective. Cherry *et al.*[61] found that Fe[III] was capable of oxidizing arsenite with an increase in rate at lower pH values. At neutral pH, negligible oxidation occurred in a solution of 50 ppb arsenite and 50 ppb arsenate, with a ferric iron concentration of 10^{-6} M. The same amount of arsenic at pH 5 with trivalent iron at a concentration of 10^{-5} M resulted in about a 30% oxidation of arsenite. When the pH was lowered to 2 and the Fe[III] concentration was raised to $10^{-2.3}$ M, over 95% of the arsenite was oxidized. These three experiments were all allowed to react for a period of five days. According to the investigators, this behavior was most likely due to a decrease of ferric ions at higher pH, because of the low solubility of the ferric oxy-hydroxide phase. This indicates that trivalent iron is capable of oxidizing arsenite under very acidic conditions when the iron is present in solution, although iron present as a solid at near neutral pH would be a more realistic setting.

Oscarson *et al.*[71] found that oxidation of arsenite to arsenate in the presence of a synthetic Fe-oxide did not occur within 72 hours at neutral pH values, and deemed it to

be of minor significance in the oxidation of arsenite. They did find that synthetic Mn[IV] oxide was a very effective oxidant with respect to arsenite. When 1000 μg/mL of As[III] was added to a suspension of 0.1 g of MnO_2, 216 μg/mL of arsenate was formed within 72 hours. According to the authors, the results indicate that approximately 19% of the Mn[IV] present in the 0.1 gram sample of manganese dioxide was reduced to Mn[II] when 1000 μg/mL of arsenite was added to the oxide. The formation of arsenate leveled off with increasing additions of arsenite; the investigators hypothesized that the Mn[IV] at or near the surface of the oxide and accessible by arsenite was reduced to Mn[II]. This Mn[II] accumulated at the surface of MnO_2 creating a barrier at the surface of the solid, which prevented further reduction of the Mn[IV] present in the inner part of the oxide. This theory has since been substantiated by another investigation into the role of manganese dioxide as an oxidizing agent for arsenite.[72]

Scott and Morgan[72] sought to investigate these processes further by examining the influence of synthetic birnessite on the oxidation of arsenite with variables such as pH, temperature, arsenite concentration, and the presence of oxygen taken into account. When 100 μM As[III] was added to a 0.21 g/L suspension of δ-MnO_2 at pH 4, 25°C, and ionic strength of 0.1 M, depletion of arsenite from solution was quite rapid. Fifty percent of the arsenite was oxidized within ten minutes, and after 90 minutes, the arsenite concentration was below the analytical detection limit signifying greater than 99% oxidation. Arsenate appeared in solution as rapidly as arsenite was depleted, and the total dissolved arsenic concentration was almost constant over the duration of the experiment. This led to the conclusions that electron transfer followed by release of arsenate into solution was relatively fast when compared to the adsorption of arsenite; and that

adsorption of the released product (arsenate) on the birnessite surface was limited. The authors also noted that release of the reduced product Mn^{2+} was slower and to a smaller extent than the release of the oxidized product As[V]. This may be explained by the adsorption phenomenon, where Mn^{2+} has a stronger affinity for the surface of the birnessite causing it to be released later in time. An overall reaction mechanism was postulated by the investigators as follows: The first step is assumed to be the formation of an inner-sphere complex, where the arsenite displaces surface bound hydroxides and water molecules by ligand substitution and binds directly to the oxidized metal ion. This is followed by a transfer of two electrons from the anion to the metal ion, the breaking of two Mn-O bonds, and the addition of an oxygen from water to the arsenic atom. Finally, release of the surface bound oxidized anion occurs followed by release of the reduced metal ion. The authors also investigated varying the amount of arsenite in solution and found that the time of reaction was slower for the experiments with greater amounts of arsenite. Also noted was a maximum As[III] removal rate at pH 4, but increasing the pH up to 8.2 only doubled the half-life of each sorption process. There was no difference in the rate of arsenate release to solution at pH 4 and 25°C whether dissolved oxygen was present or not. This observation confirms that the surface Mn[IV] is the oxidant in the reaction and is not just a catalyst in the oxygenation of arsenite.

2.8 Solid Phase Interactions

The hydrous oxides of iron, manganese, and aluminum, particularly the redox sensitive manganese and iron hydroxides, are significant sinks for arsenic in aquatic systems. In sediments, these hydrous oxides exist as coatings on silicate minerals rather than as discrete, well-crystallized minerals. This allows oxides to display chemical

activities far out of proportion to their physical activity.[9] Under reducing conditions, the adsorbed arsenic from hydrous iron and manganese oxides is released and mobile. In the presence of sulfide, with no oxygen present, iron sulfide minerals can form. Arsenic has a tendency to coprecipitate with iron sulfides as arsenopyrite (FeAsS) or form arsenic sulfides such as realgar (AsS) or orpiment (As_2S_3).[73] Under aerobic conditions, these sulfides are subject to oxidation, which would release arsenic into solution.[74] The role of sulfides and hydrous iron, manganese, and aluminum oxides as controls on arsenic cycling is environmentally significant. Changes in redox potential, acidity, and complexing agents can lead to formation and dissolution of these sulfides and colloidal hydrous oxides, directly influencing the mobilization of arsenic in an aquatic environment.[9]

2.8.1 Iron

A very important mechanism for the retention of arsenic in sediments and aquifers is adsorption to and coprecipitation with hydrous iron oxides.[75,76] Arsenate and arsenite are easily adsorbed onto hydrous iron oxides since they have a high pH_{PZC} (point of zero charge) of approximately 8.6.[77] This leads to a net positive charge in most geological environments and preferential adsorption of anions. Adsorption under the combined influence of ionic and nonionic bonding is called specific adsorption and can occur even when the surface has no net charge.[79] The oxidation state of the arsenic species is a factor in adsorption affinity, where arsenate is adsorbed to a greater extent than arsenite.[78,79] Arsenite is therefore more mobile than arsenate in the vicinity of iron oxides. Arsenate may be immobilized by coprecipitation with hydrous iron oxides,

eventually leading to the formation of the mineral scorodite ($FeAsO_4 \cdot 2H_2O$)[80,81] by the following reaction:

(4) $$Fe(OH)_3 + H_3AsO_4 \Leftrightarrow FeAsO_4 \cdot 2H_2O + H_2O$$

The influence of iron hydroxides on arsenic movement can be witnessed in the vicinity of mining operations. Weathering of sulfide ores from mines and tailings releases large quantities of sulfuric acid, arsenic, and other metals (Cu, Zn, Mn, and Al) into the West Squaw Creek, a stream in California that drains igneous rocks of low acid-neutralizing capacity.[82] A similar situation was observed by Moore *et al.*[83] in their study of the mobilization of arsenic and metals (Cu, Zn, Cd, and Ni) through 215 km of the Blackfoot River in Montana which has been impacted by headwater input of acid mine effluent. In both river systems, it was obvious through visual inspection that the sediments were associated with iron hydroxides. An amorphous red-orange precipitate consistent with hydrous ferric oxide was present in both streambeds. As acid mine drainage flows from the anaerobic subsurface into the oxygenated surface waters, oxyhydroxides precipitate from solution along with other hydroxide and sulfate minerals. In the upper streams, the acid water (pH 2-6.5) keeps metals in solution rather than adsorbed on to or precipitated as solid phases, yet arsenic is almost completely scavenged from solution in the upper reaches of the Blackfoot River and West Squaw Creek.[9] The iron hydroxide particulate matter becomes more positively charged as pH decreases, which lowers its ability to scavenge positively charged metal ions.[84] Since arsenate exists as an anion above pH 2, it is scavenged very effectively by the sediments and associated particulate matter. This leads to very low concentrations of dissolved arsenic at a relatively short distance from its source.

2.8.2 Manganese

Arsenic species may also be immobilized by, adsorbed to, or coprecipitated with hydrous manganese oxides. Hydrous manganese oxides have a pH_{PZC} of about 2.3[85] leading to a net negative surface charge in most situations. This would indicate that hydrous manganese oxides would not be able to adsorb arsenic anions. However, the overall surface charge of hydrous manganese oxides can be changed by adsorption of divalent cations,[86,87] leading to a decrease in the negative surface charge and eventually to a positive surface charge.[88] This enhances the ability for manganese oxides to remove arsenate and arsenite from solution, with arsenite initially oxidized by the manganese solid as discussed previously. Arsenate may also be incorporated into sediments by coprecipitation with hydrous manganese oxides by the following reaction:[58]

(5) $$3MnOOH + 2HAsO_4^{2-} + 7H^+ + 3e^- \Leftrightarrow Mn_3(AsO_4)_2 + 6H_2O$$

The effect of hydrous iron and manganese oxides on the mobility of arsenic is apparent in a study of arsenic and antimony in Idaho's Coeur d'Alene River, which also has been impacted by mining activities. Arsenic is retained to a greater extent than antimony in these river sediments, through stronger adsorption to existing oxides and incorporation into newly formed oxides.[57] These processes lead to higher arsenic concentrations in the sediments and higher antimony concentrations in the river water. Arsenite was the dominant arsenic species observed in the river, with a higher affinity between oxides and arsenate being cited as the cause. The strong interaction between arsenic and manganese hydrous oxides is also apparent in the surface sediments at the mouth of the Coeur d'Alene River. Very high concentrations (197 ppm) are present in the surface sediments, with concentrations declining to a few ppm as depth increases.

The arsenic concentrations exhibited a depth profile very similar to manganese with a correlation coefficient of 0.99, implying that precipitation and dissolution of these oxides are controlled the dissolved arsenic concentration.[57]

2.8.3 Aluminum

Aluminum compounds, in addition to iron and manganese, are also a sink for arsenic and thereby affect its mobility. The pH_{PZC} for amorphous $Al(OH)_3$ is 8.5.[89] Amorphous hydroxides with such high isoelectric points are extremely efficient adsorbers.[90] At low pH, the surface of the aluminum hydroxide is positive and adsorption of arsenic anions is through coulombic attraction. At higher pH, the surface of the hydroxide is negative and specific adsorption competes with coulombic repulsion.[9] The very reactive $Al(OH)_x$ adsorbed onto the clay minerals accounts for its high adsorption capacity for arsenic, primarily as a result of the exposed octahedral cation on the broken clay particle edges.[91] A study by Manning and Goldberg[92] investigated the adsorption characteristics of arsenite on amorphous aluminum hydroxide, kaolinite, illite, and montmorillonite. Clays are primarily aluminum and silicon, so the characteristics of aluminum should have some effect on their overall adsorption properties. Batch adsorption of arsenite was examined by shaking suspensions in 40 mL centrifuge tubes containing 500 mg of clay in 20 mL of 0.4 μM arsenite or 50 mg of solid amorphous $Al(OH)_3$ in 20 mL of 0.4 μM arsenite. The authors found that in general, adsorption was weakest at low pH and maximum arsenite adsorption occurred between pH 7.5 and 9.5. The amorphous aluminum hydroxide adsorbed a maximum of 0.142 μmol As[III] per gram of solid at a pH of 8. The greatest adsorption value among the clays was on montmorillonite, which had a maximum adsorption of 0.128 μmol arsenite per gram of

clay at pH 9. The lower adsorption values for the clays led to the conclusion that the SiO_4 tetrahedral sites are less reactive towards arsenite than Al-OH functional groups. If the value for arsenite adsorbed per gram of aluminum hydroxide were presented as a percentage of the total arsenic in solution, it would be slightly less than two percent.[92] This means that arsenite is adsorbed to aluminum hydroxides to a very limited extent. In comparison, arsenate is adsorbed much more effectively by aluminum hydroxides. Anderson *et al.*[89] found that nearly 100% of arsenate adsorbed to amorphous $Al(OH)_3$.

2.8.4 Sulfide

Sulfides also play an important role in controlling the mobility of arsenic, either sequestering it as a solid phase or releasing it upon oxidation. In an aquifer system where an upper oxidized zone overlays a reduced portion below, the concentration and partitioning of arsenic is controlled by the redox boundary. In the presence of sulfide in the reduced zone, arsenic changes from a predominantly oxyhydroxide phase to a sulfide phase.[93] In this type of reduced zone with a low redox potential, iron minerals are partially reduced to ferrous iron, while manganese is partly reduced to the manganous state. When reduction occurs, the adsorbed and coprecipitated arsenic is released to the porewater.[58,93] Sulfides in a reduced environment will scavenge the arsenic by formation of arsenic sulfide precipitates.[75] The formation of sulfides also accompanies the reduction of arsenate to arsenite as follows, and arsenic will dominate in the porewater as As[III]:[93]

(6) $$H_2AsO_4^- + 3H^+ + 2e^- \Leftrightarrow H_3AsO_3 + H_2O$$

(7) $$2H_3AsO_3 + 6H^+ + 3S^{2-} \Leftrightarrow As_2S_3 + 6H_2O$$

A study by Moore *et al.*[93] provides clear evidence for the retention of arsenic by sulfide minerals in reduced settings. As a consequence of sulfide ore mining that began in the mid 1800's, Milltown Reservoir sediment in western Montana has elevated levels of arsenic and heavy metals such as copper, cadmium, lead, zinc and iron. High arsenic in community water supply wells led to a series of investigations into the source of contamination for the aquifer. Upstream from the Milltown dam at the confluence of the Clark Fork and Blackfoot Rivers, sediments up to 30 feet thick have accumulated. A sediment core was taken in order to determine the likely controls for arsenate mobilization through a combination of pore water analysis and selective extractions of the sediment. The core was from a location that has a known oxic zone overlying reduced sediments. The core was divided into ten-centimeter sections and all sections underwent porewater analysis and selective extractions. The results of this study showed that there was a redox boundary approximately 70 cm below the surface, where dissolved oxygen levels fell below the analytical detection limit. Above this boundary, arsenic concentration in the sediment extracts was relatively constant at about 100 μg/g sediment. Below 60 cm, total arsenic concentration steadily increases to over 800 μg/g sediment at 105 cm below the surface. This trend was mirrored by sulfide extractable arsenic, which had a value less than 20 μg/g sediment in the upper 40 cm of the core, but once below 60 cm it increased to over 500 μg/g sediment. The authors state that in the oxidized zone, the sediments are dominated by hydroxylamine hydrochloride extractable arsenic with noticeable fractions in organics and sulfides, but in the reduced zone below, arsenic resides dominantly in the sulfides. The pore water concentrations of arsenic were relatively low in the oxidized zone, at less than 20 μg/L primarily as arsenate. Below the

redox boundary, the concentration rose to greater than 550 μg/L and was almost all arsenite. At approximately 115 cm below the surface, the dissolved arsenic concentration declined sharply to a value of about 70 μg/L. Dissolved concentrations of iron and manganese, as the reduced species Fe^{2+} and Mn^{2+}, also increased significantly as depth below the surface increased. This situation leads to a window of high dissolved arsenic concentration below the oxic zone and above the sulfidic zone. In fully oxic conditions, arsenic is sequestered by oxide phases while in anaerobic settings sulfide minerals control arsenic speciation. The transition area between these two settings would have the greatest amount of mobilized dissolved arsenic species.

2.9 Microbiology

It is now apparent that the sequestering of inorganic arsenic species on or within solid phases is very significant in the arsenic cycle, along with their subsequent release from a solid state into solution given the right conditions. A crucial part of dissolution and precipitation reactions is that in most cases the chemicals need to be present in a certain form or valence state. This valence state is normally associated with the ambient conditions of the setting within which the reaction occurs. In low oxygen settings, the reduced form of a compound usually dominates; and in atmospheric, oxygen rich environments the higher valence states tend to dominate. What is possibly even more important when considering processes that may impact drinking water supplies, is not just the form of compounds present but the rate at which the conversions between them occur. Oxidized compounds will gain electrons to become reduced, given that a suitable electron donor is present and enough time elapses for the reaction to proceed. Certain

bacteria facilitate this electron transfer and can significantly increase the rate at which these transformations take place. The mechanism differs by species, but some organisms gain useable energy by the process, while others facilitate reactions in order to transport compounds through their membranes. These reactions may occur completely in solution, where one dissolved species is reduced or oxidized to its complementary form; or a solid phase may be colonized by bacteria eventually leading to the dissolution of the substrate. Bacteria and their associated enzymes can increase the rate of reduction for solid iron oxyhydroxide phases, manganese oxides, and iron-arsenic compounds.[94,95] Other groups of microbes oxidize certain solid arsenic-containing compounds, such as orpiment or arsenopyrite.[96,97]

The overall process involving bacteria is the use of arsenate as an electron acceptor during the process of respiration. This may accompany the dissolution of a compound or it may occur entirely in solution. Bacteria oxidize organic carbon or a reduced sulfur compound while reducing a complementary electron acceptor. This electron flow is harnessed in order to build biomass and perform basic metabolic functions. The most widely used electron acceptor is oxygen, but in its absence other compounds are utilized. Once oxygen is depleted, organisms turn to the next best alternative, in terms of the energy gained per mole of electron transfer. Some common electron acceptors that are utilized by microbes when oxygen is not present are oxidized forms of nitrogen, manganese, iron, and sulfur. The significance of these compounds has been known and studied for some time. The use of other chemicals, arsenic and selenium for example, as electron acceptors has also been added to this list in recent years. A presentation of the current understanding of these processes will be presented with an

obvious bias concerning arsenic's role in the overall sequence of events. This discussion will address the reduction of a variety of compounds that are used as electron acceptors, the reduction of arsenic for dissimilatory growth or detoxification, and the oxidation of arsenic species in both a dissolved and solid state.

2.10 Reduction Sequence

The reduction of any compound is accompanied by the oxidation of another chemical species, which loses electrons. In most cases involving groundwater or sediments, the electron donor is a reduced carbon compound. For the purposes of comparison, a generic carbohydrate (CH_2O) will be used to establish the order in which compounds are used as a terminal electron acceptor. Free energy of formation calculations give an indication of the relative energy involved in a certain reaction and whether it proceeds spontaneously. If a common carbon compound is assumed to react with different electron acceptors, resulting in the production of carbon dioxide, water, and the reduced electron acceptor, a sequence of electron acceptors can be constructed. Mentioned previously, compounds that may be used as electron acceptors are forms of nitrogen, manganese, selenium, iron, arsenic, and sulfur. It can be shown through thermodynamic calculations using available constants[98] that the order in which electron acceptors are utilized is as follows:

(8) $CH_2O + O_2 \leftrightarrow CO_2 + H_2O$ ΔG = -121.87 kcal/mole C

(9) $5CH_2O + 4NO_3^- + 4H^+ \leftrightarrow 5CO_2 + 2N_2 + 7H_2O$ ΔG = -118.64 kcal/mole C

(10) $CH_2O + 2MnO_2 + 4H^+ \leftrightarrow CO_2 + 2Mn_2^+ + 3H_2O$ ΔG = -118.13 kcal/mole C

(11) $CH_2O + SeO_4^{2-} \leftrightarrow CO_2 + SeO_3^{2-} + H_2O$ ΔG = -101.87 kcal/mole C

(12) $CH_2O + 2HAsO_4^{2-} + 4H^+ \leftrightarrow CO_2 + 2H_3AsO_3 + H_2O$ ΔG = -82.12 kcal/mole C

(13) $CH_2O + 4FeOOH + 8H^+ \leftrightarrow CO_2 + 4Fe_2^+ + 7H_2O$ ΔG = -66.40 kcal/mole C

(14) $CH_2O + 2H_2AsO_4^- + 2H^+ \leftrightarrow CO_2 + 2H_3AsO_3 + H_2O$ ΔG = -63.67 kcal/mole C

(15) $2CH_2O + SO_4^{2-} + H^+ \leftrightarrow 2CO_2 + HS^- + 2H_2O$ ΔG = -27.62 kcal/mole C

The above sequence is based purely on thermodynamically derived equilibrium constants and assumes unlimited time. The negative sign indicates that energy is released when these reactions occur. The most negative values release relatively more energy indicating their value as electron acceptors. This sequence of utilization has been observed in environmental settings; although not all of the above compounds may be present manganese, iron, arsenic, and sulfur tend to be available especially in mining impacted areas. It is interesting to note that the diprotic and monoprotic arsenate species fall on either side of the iron value. With a dissociation constant of 6.9, this suggests that equal amounts of the diprotic and monoprotic species will be present at this pH. Based on thermodynamic predictions, the monoprotic arsenate could effectively compete with iron for status as the preferred electron acceptor at this level of anoxia.

2.11 Arsenic Microbiology

The role of arsenic in bacteriological processes is beginning to be accepted as a significant pathway for arsenic reduction and oxidation reactions. Although normally considered an unlikely compound for life sustaining reactions due to its toxic properties, arsenic is increasingly being observed as an effective electron acceptor and donor. This

section will describe the current knowledge of arsenic in regards to bacterially mediated chemical reactions.

2.11.1 Oxidation

It has been known for some time that arsenic is effectively oxidized in the presence of certain bacteria. In 1964, it was shown that bacteria are capable of oxidizing the arsenic solid phases orpiment and arsenopyrite inducing dissolution.[96,97] In 1976, oxidation of a solution containing 10 mM arsenite occurred after 6 hours when it was inoculated with *Alcaligenes faecalis*.[99] This organism was isolated from raw sewage taken from a grit chamber in Ithaca, NY. When the organisms were grown in chemically defined media, an enzyme was released either late in the exponential growth phase or once the stationary phase of growth was reached that was capable of oxidizing arsenite to arsenate. More recently, a study investigating the oxidation kinetics of arsenite in source waters of the Los Angeles aqueduct, specifically Hot Creek, implicated bacteria in the rapid production of arsenate.[100] The investigators found that aquatic macrophytes and their associated surface bacteria were responsible for the oxidation of large quantities of arsenite released from subsurface geothermal inputs. Sterilized controls in batch experiments, both filter sterilized and killed with antibiotics, showed no oxidation of arsenite. Macrophytes and surface material taken from the plants were capable of oxidizing micromolar concentrations of arsenite within two hours. Although no specific strains of organism were isolated, the fact that lab batch kinetics matched field observations indicates that in this setting, these organisms are responsible for the oxidation state of arsenic.

2.11.2 Detoxifying Reduction

As stated previously, the reduction of arsenate to arsenite increases the solubility and relative mobility of arsenic in solution. Some bacteria utilize this process to remove the toxic compound from their system. Arsenic detoxification has been documented in *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus xylosis*, where an arsenate reductase reduces As[V] and an efflux pump removes the toxic As[III] from the cell.[18] Reduction of both aqueous and adsorbed arsenic has been observed in cultures isolated from the upper 20 cm of a fine loamy, frigid Typic Calciaquoll, an agricultural soil that contained naturally elevated concentrations of both dissolved and adsorbed arsenic.[101] The investigators found that the fermentative bacteria were capable of reducing dissolved arsenate or arsenate adsorbed to the surface of hydrous ferric oxides.[102] The organism did not use the arsenic as an electron acceptor, supporting the idea that arsenic was reduced for detoxification purposes.

2.11.3 Dissimilatory Reduction

Within the last ten years, it has become increasingly apparent that bacteria use arsenic as an electron acceptor in energy generating reactions. A brief history of the reported state of this topic will be provided with a focus on the site the organisms were isolated from, the reduction rates and concentrations of arsenic, and any other electron acceptors or donors that the organism has the ability to utilize.

An organism originally isolated with selenate as the electron acceptor was obtained from Massie Slough, a freshwater marsh in the Stillwater Wildlife Management Area of western Nevada.[103] After subsequent experiments, it was found that this organism (designated SES-3) could utilize other electron acceptors including Fe[III],

thiosulfate, arsenate, elemental sulfur, Mn[IV], and nitrite.[104] This isolate could reduce 10 mM solutions of arsenate to arsenite in less than one week with corresponding oxidation of lactate to carbon dioxide.

Sediments taken from a San Francisco Bay salt marsh located in Palo Alto, California and Lahontan Reservoir, a man-made freshwater lake in eastern Nevada were documented to reduce 10 mM arsenate when lactate or acetate was present.[105] When nitrate was provided along with arsenate, nitrate was reduced before arsenite was observed. When sulfate was provided, it was used as an electron acceptor but only after the arsenate was consumed. This supports the order of electron acceptor utilization mentioned previously.

In 1994, an isolate known as MIT-13 was described that could use 10 mM arsenate as an electron acceptor with lactate as the carbon source and electron donor.[106] This species was isolated from the Halls Brook Storage Area, a reservoir near the headwaters of the Aberjona watershed in Massachusetts. The lactate was presumably oxidized to acetate, but not completely to carbon dioxide, since acetate could not be used by the organism as a growth substrate. The doubling time for this organism when grown on 10 mM arsenate was approximately 14 hours. This organism could also use sulfate as an electron acceptor when lactate was the carbon source. It was also proven that this organism influenced the dissolution of an iron arsenate solid phase by reduction of the arsenate.[107]

An organism was isolated from a reed bed at Ballarat Goldfields in Australia that is capable of growing with 10 mM arsenate and 10 mM lactate.[108] This species, *Chrysiogenes arsenatis* was able to use nitrate as an electron acceptor but not sulfate. It

was also able to utilize pyruvate, lactate, succinate, malate, and fumarate as electron donor and carbon source. Subsequent culturing research by this same group of Australian scientists has yielded two more bacterial strains.[109] They were both cultured on sulfate initially, and both would produce significant amounts of arsenite when arsenate was added to the culture. One of the strains would reduce arsenate by dissimilatory means, while the other reduced by a detoxifying mechanism.

Desulfotomaculum auripigmentum is a species that was isolated from surface sediments of Upper Mystic Lake, located in Woburn, Massachusetts.[110] This species was able to reduce 5 mM of arsenate to arsenite in less than a week with lactate provided as the carbon source and electron donor. This species was also able to reduce sulfate, but only after the arsenate was utilized. A precipitate was observed in lab cultures that had the chemical composition of orpiment (As_2S_3) when both arsenate and sulfate were provided.[59] This was a result of both reduced arsenic and reduced sulfur being produced in amounts sufficient to reach saturation.

Another group of organisms was isolated from the hypersaline sediments of Mono Lake, California. This is a high pH, atypical setting which apparently fosters somewhat extreme organisms. Two bacteria were isolated from the anoxic sediments of the lake that were capable of dissimilatory reduction of arsenate to arsenite accompanied by oxidation of lactate to acetate and carbon dioxide.[111] These organisms were able to use nitrate, iron, and certain forms of selenium as electron acceptors. Both strains had limited growth on electron donors other than lactate.

A strictly anaerobic, hyperthermophilic archaeon was isolated from a hot spring in Italy.[112] The organism, *Pyrobaculum arsenaticum*, was capable of growing

chemolithoautotrophically with carbon dioxide as a carbon source, hydrogen as an electron donor, and arsenate, thiosulfate, or elemental sulfur as an electron acceptor.

Modern lab methods and techniques combined with the willingness to understand arsenic behavior has led to the discovery of this group of organisms. What has for quite some time been recognized as a powerful poison is a life-sustaining compound for these microorganisms. There are some differences in the characteristics of each of these strains, such as the type of carbon substrate and electron acceptors that each can use, reduction rates and morphology. They are also widely distributed phylogenetically, as discussed below. Although they are distinctly different from one another, a similar niche is occupied by these organisms. Among the arsenate-respiring organisms that have been characterized, all can use lactate as an electron and carbon source, with others being utilized by some. Most of the investigations were focused on arsenic, obviously leading to use of this as an electron acceptor. Other electron acceptors were utilized by many of these organisms, mainly nitrate, selenate, and sulfate. Organisms that have the ability to use multiple acceptors are at an advantage when resources are scarce, and have undoubtedly evolved to cope with such scarcity.

In addition to morphological and physiological methods, microorganisms can be characterized by examining their genetic code. The mechanism for DNA replication is imperfect, and as a result mutations occur with a low but measurable frequency. The evolutionary relationship of organisms can therefore be determined by comparing the degree of similarity of sequences of DNA. Organisms containing similar sequences are more closely related than organisms with a greater degree of difference. This implies that the former diverged more recently than the latter.

The ribosomal RNA genes (rRNA) are particularly good indicators of relatedness because all cell-based organisms on Earth contain ribosomes, which are required for protein synthesis. The small subunit rRNA gene is small enough to sequence easily and large enough to carry enough information to distinguish evolutionary relationships. Furthermore, a large database of rRNA genes exists, facilitating the detection of relationships among a broad range of organisms. Phylogenetic trees can be constructed by comparing sequence data for an organism of interest with sequences contained in the database. This kind of analysis of arsenate-reducing microorganisms has revealed that they are very widely spread, with some belonging in archaeal taxa, some in the gram-positive bacterial lines and others in a variety of gram-negative taxa. This indicates that arsenic related mechanisms are widespread and not related to a single lineage.

The sites where these organisms were discovered are highly variable. They have been isolated in marine and fresh water sediments, anthropogenically contaminated areas, and a hypersaline lake. What has not been found to date is an organism of this type that inhabits aquifer settings. Groundwater has a tendency to be devoid of oxygen, and is a likely place for anaerobic bacteria to live if enough utilizable carbon is present. The electron acceptor would most likely be present as a byproduct of the parent material, but organic carbon may not be available in sufficient quantities. This study was designed to determine if arsenate-reducing organisms are active in groundwater aquifers at two sites in Maine with elevated arsenic concentrations. Since microorganisms significantly speed up reactions, and the reduced form of arsenic may be more mobile as well as more toxic, the presence of these arsenate-reducing microorganisms could contribute to the elevated arsenic levels observed at the sites.

3 METHODS

3.1 Sampling

Two wells were sampled in this study to determine if arsenate-reducing bacteria are present and active in influencing the cycling of arsenic in groundwater. Both locations have a known history of high arsenic levels in the groundwater, as well as ties to the university that allow for access.

3.1.1 Green Lake

The Green Lake property is owned by the University of Maine Foundation. There is an operational well that supplies one house with drinking water after treatment via a reverse osmosis unit located in the basement. Three locations in the water system were sampled for culturing on June 1, 2001. The treatment unit and pump were shut down and all electrical current was stopped prior to sampling. The well has a submersible pump with black polyvinylchloride piping, which was pulled from the well by hand. Approximately every ten feet, samples were scraped with a sterile spatula from the surface of the piping and placed in 30 mL sterile serum bottles containing liquid media. The serum bottles were opened momentarily while the sample was added; nitrogen gas was used to purge the headspace during inoculation through a sterile cannula. The bottles were then stoppered, crimp sealed, and placed in a closed box to limit exposure to light. Thirteen scrapings were taken from the PVC and two samples were obtained from the pump casing itself, for a total of fifteen samples from the well. The pump was then brought back on-line and allowed to run for several minutes. Four samples were

collected from the spigot on the exterior of the house. An initial sample was taken immediately after the system was pressurized, with the other three obtained at approximately five minute intervals. These samples were collected in sterile 125 mL serum bottles, purged with nitrogen gas and crimp sealed with sterile rubber stoppers. These samples were amended with specific compounds that will be discussed in the culturing section. At this stage, water was also collected for laboratory chemical analysis and ion exchange columns were used to speciate arsenic on site (see section 3.4.1). Three samples were taken from the particulate filter that is a pre-treatment stage in the reverse osmosis system. The pump was shut off briefly and the pre-treatment cartridge was scraped in three different areas to obtain solids for inoculation of media. These three samples were individually placed into 30 mL serum bottles containing sterile media, degassed with nitrogen, and crimp sealed with rubber stoppers.

3.1.2 Northport

The University of Maine Geological Sciences department has several research wells located in the Northport area. These wells do not provide potable water and therefore have no operational pumps in place. What is known as the Baker well was sampled on June 19, 2001. Tygon tubing fed through a peristaltic pump was lowered to 30 meters below the land surface and was run for approximately twenty minutes. At this time, 25 mL samples were taken by pumping the effluent water into sterile 30 mL serum bottles while they were being purged with nitrogen, followed by crimp sealing with butyl rubber stoppers. Five serum bottles were filled in this manner, and then amended with compounds once they were returned to the laboratory.

3.2 Culturing

3.2.1 Sample Amendment

The four serum bottles from Green Lake that contain only source water were amended with the following compounds: potassium phosphate; 1.5 mM, ammonium chloride; 28 mM, sodium acetate; 10 mM, sodium arsenate; 10 mM, vitamin and trace elements; 100 μL from the three vitamin/mineral stock solutions. The vitamin and element stocks consist of three different solutions: trace elements, vitamins, and vitamin B_{12}. These stocks are 1000X stocks; therefore one milliliter would be added to one liter of media. All three of these solutions are filter sterilized (0.2 μm) and the two vitamin solutions are stored in a freezer until used. The trace element solution contains deionized water (987 mL), EDTA (5200 mg), $FeSO_4 \cdot 7H_2O$ (2100 mg), H_3BO_3 (30 mg), $MnCl_2 \cdot H_2O$ (100 mg), $CoCl_2 \cdot 6H_2O$ (190 mg), $NiCl_2 \cdot 6H_2O$ (24 mg), $CuCl_2 \cdot 2H_2O$ (2 mg), $ZnSO_4 \cdot 7H_2O$ (144 mg), $Na_2MoO_4 \cdot 2H_2O$ (36 mg), sodium vanadate (25 mg), $Na_2SeO_3 \cdot 5H_2O$ (6 mg), $Na_2WO_4 \cdot 2H_2O$ (8 mg). The vitamin solution contains 100 mL of 10 mM phosphate buffer (pH 7.2), 10 mg of riboflavin, and 100 mg of the following: thiamine-HCl, L-ascorbic Acid, D-Ca-pantothenate, folic acid, niacinamide, nicotinic acid, 4-aminobenzoic acid, pyridoxine-HCl, lipoic acid, NAD, thiamine pyrophosphate. The vitamin B_{12} solution contains 100 mL of deionized water and 100 mg of cyanocobalomine.

All five serum bottles from the Northport site were amended with the following compounds: sodium chloride; 70 mM, ammonium chloride; 23.4 mM, potassium phosphate; 1.25 mM, calcium chloride; 0.7 mM, potassium chloride; 1.1 mM, and 25 μL

from each of the three vitamin/mineral stocks. The carbon source and electron acceptor concentration were varied in order to select for different organisms. NP1 contains only the compounds listed above. NP2 contains 10 mM sodium lactate and 1 mM sodium arsenate. NP3 contains 10 mM sodium lactate and 10 mM sodium arsenate. NP4 contains 10 mM sodium acetate and 1 mM sodium arsenate. NP5 contains 10 mM sodium acetate and 10 mM sodium arsenate.

3.2.2 Media Preparation

The media used in this experiment was designed to select for arsenate respiring organisms. The basic requirements were that it be free of oxygen, and provide the basic constituents necessary for cell growth. The compounds present in the media will be presented followed by the procedure used to make and dispense the media. An initial review of the literature available indicated that the following compounds were necessary: pH buffer, sodium chloride, ammonium chloride, potassium phosphate, calcium chloride, potassium chloride, vitamins, minerals, a red-ox indicator, a reducing agent, a carbon source, and an electron acceptor. Initially, media was formulated in deionized water with these compounds: PIPES buffer (piperazine-N,N'-bis[2-ethanesulfonic acid]); 20 mM, NaCl; 85 mM, NH_4Cl; 28 mM, K_2HPO_4; 1.15 mM, $CaCl_2$; 0.7 mM, KCl; 1.3 mM, vitamin/mineral stock; 1 mL, resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide); 0.2 μM, L-cysteine; 2 mM, sodium lactate; 10 mM, and sodium arsenate; 10 mM. Due to limited culture performance, salt levels were decreased and the following media recipe was adopted for use in all but the first few rounds of transfers: PIPES buffer; 5 mM, NaCl; 5 mM, NH_4Cl; 1 mM, K_2HPO_4; 0.5 mM, $CaCl_2$; 0.5 mM, $MgCl_2$; 0.5 mM,

vitamin/mineral stocks; 1 mL, resazurin; 0.2 μM, L-cysteine; 2 mM, sodium lactate; 1 mM, and sodium arsenate; 2 mM.

Preparation of anaerobic, sterile media was accomplished through a series of steps. A one liter vacuum flask was filled with approximately 950 mL of deionized water and the pH buffer was added. This was magnetically stirred while sodium hydroxide was added dropwise until the buffer was completely dissolved and the pH stabilized at 7.00. The salts were then added individually and allowed to dissolve. After the salts were in solution, the carbon source was added, followed by the resazurin. The flask was filled to one liter, covered with aluminum foil, and set aside to await sterilization. Two solutions were prepared for addition to the media after bulk sterilization. The first consisted of 0.242 grams of cysteine and 10 mL of deionized water, degassed with nitrogen and crimp sealed in a clean 30 mL serum bottle. The second consisted of 0.6240 grams of sodium arsenate and 30 mL of deionized water also degassed with nitrogen and crimp sealed in a clean 30 mL serum bottle. The liter of media (with the magnetic stir bar), the two solutions, foil covered serum bottles to be filled, butyl rubber stoppers in a beaker covered with foil, and a gassing manifold (wrapped in foil) were all placed in the autoclave and sterilized for 22 minutes at a temperature of 121°C and a pressure of 1 kg/cm^2, with slow exhaust. The gassing manifold consisted of a three-holed #8 rubber stopper, fitted with three sections of glass tubing (see Figures C.1 and C.2 in Appendix C). Plastic Nalgene tubing was attached to the three sections to yield nitrogen-in, heat-labile-in, and media-out ports. The media-out piece was fitted with an autoclaved stainless steel cannula which was flame sterilized prior to dispensing each container of media. Another tube was split off the gas line and fitted with a cannula to provide

nitrogen to flush the serum bottles. Once the media had cooled in the autoclave to approximately 60°C, the filter flask was placed on the stirrer and the gassing manifold was inserted into the top. The gas-in line was attached to the regulator and the pressure was set to 15-25 psi. The nitrogen gas pressurized the headspace and the filter opening was used to relieve pressure by feathering with a valve. A media out line to dispense media originated at the bottom of the interior of the flask and ended with a cannula. The final port remaining on the stopper was used to add heat labile constituents and the reducing agent, and one nitrogen line with a cannula for degassing serum bottles.

The three vitamin solutions were added with an auto pipet via the injection port and allowed to mix completely. The cysteine was added through the same port with a sterile 10 mL Becton-Dickinson syringe fitted with a 1.5", 25 gauge needle. This was allowed to mix until the solution was colorless, signifying the attainment of anaerobic conditions. The gassing cannula was dipped in ethanol, flame sterilized, and inserted into a serum bottle. After sufficient time had passed to purge the vessel of atmospheric gases (1-2 minutes), the media-out cannula was dipped in ethanol, flame sterilized, and inserted into the serum bottle. The filter valve was closed to initiate media flow, and was reopened once the container was full. The media cannula was withdrawn and a sterile butyl rubber stopper was placed on the container using flame-sterilized forceps. The gassing cannula was withdrawn as the stopper was seated firmly into position. The serum bottle was crimp sealed with an aluminum seal and the remaining bottles were filled in the same manner. Once all the media was dispensed, arsenate was added to the serum bottles through the stopper using sterile 1 mL B-D syringes fitted with 1.5", 25

gauge needles. Each syringe was used only once to inject one mL of 30X arsenate stock per serum bottle.

3.2.3 Inoculation/Transfer

As discussed briefly in the sampling section, the fifteen "residue" samples from the PVC piping and the three scrapings from the particulate filter were inoculated into sterile media. These will be denoted as GL1-15 and F1-3 respectively. The four water samples from Green Lake and the five samples from Northport were amended with specific compounds. These will be denoted as 1-4W and NP1-5. This gives an initial culture bank of 27 vessels. These enrichments were transferred several times into fresh media containing varying amounts of electron acceptor and varying amounts and type of carbon source. All transfers were performed using sterile B-D syringes and needles, used only once before disposal. Only the cultures that yielded data that is presented in the results will be described here, for a complete history and nomenclature description see Appendix A.

Let day zero be the day samples were acquired from the well in Northport. On day 169, three milliliters from the original NP4 enrichment culture was inoculated into 30 mL serum bottles containing media with 1 mM arsenate and 10 mM lactate or acetate. On day 187, 1 mL of the lactate based culture was transferred to an agar tube for isolation (see next section for isolation procedure). On day 238, 3 mL of this isolated culture was transferred into a 30 mL serum bottle containing media with 1 mM arsenate and 10 mM lactate. On day 322, 10 mL of this enrichment culture was inoculated into 125 mL serum bottles containing 100 mL of media with 0.7 mM arsenate and 1.2 mM lactate. On day 354, ten mL of this culture was transferred into 125 mL serum bottles containing 100 mL

of media with either 1 mM of sulfate, selenate, nitrate, or arsenate and 10 mM lactate; or 10 mM acetate, glucose, benzoate or lactate and 1 mM arsenate.

Let day zero be the day samples were acquired from the well in Green Lake. On day 187, three milliliters from the original GL11 enrichment culture was inoculated into 30 mL serum bottles containing media with 1 mM arsenate and 10 mM lactate or acetate. On day 205, 1 mL of the lactate based culture was transferred to an agar tube for isolation (see next section for isolation procedure). On day 256, 3 mL of this isolated culture was transferred into a 30 mL serum bottle containing media with 1 mM arsenate and 10 mM lactate. On day 340, 10 mL of this enrichment culture was inoculated into 125 mL serum bottles containing 100 mL of media with 0.7 mM arsenate and 1.2 mM lactate. On day 372, ten mL of this culture was transferred into 125 mL serum bottles containing 100 mL of media with either 1 mM of sulfate, selenate, nitrate, or arsenate and 10 mM lactate; or 10 mM acetate, glucose, benzoate or lactate and 1 mM arsenate.

3.2.4 Isolation

As stated previously, selected cultures were transferred into an agar based media in order to divide the mixed culture into single species inoculants. This was performed by making media with the same components as stated earlier, with 2% agar added to solidify the media. The media was made and sterilized in a similar fashion to the liquid media; the major difference was that the flask was kept in a hot water bath at 65°C to keep the agar in a molten state until inoculation and dilutions were performed. Nine mL of the media was dispensed into 25 mL Balch test tubes which were degassed and crimp sealed. Once the arsenate was added by syringe, the inoculant of choice was added via a one mL syringe and a three to five step serial dilution was performed to select for

populations with different reproductive strategies. The syringe used to inoculate the tube was left pierced through the butyl rubber stopper while the tube was rocked slightly to induce mixing. One mL of culture was then withdrawn and injected into the next tube of the dilution sequence. The mixing, withdrawal, and injection sequence was repeated until all the desired tubes were inoculated. The tubes were then placed in ice to solidify the agar. Once cooled to room temperature the tubes were covered with foil and placed in a dark area. Once growth was present, individual colonies were picked from these tubes with a sterile Pasteur pipet under a stream of N_2, and inoculated into fresh liquid media. Prior to sterilization, the pipet was heated over a Bunsen burner and stretched with forceps to provide a needle like point in order to theoretically pick single colonies. The method was then repeated to further purify the culture. For a detailed history of the isolation process, see Appendix B.

3.3 Nucleic Acid Techniques

For comparison against known strains of bacteria, DNA sequencing of organisms was performed by Dr. Katherine Boettcher in the Department of Biochemistry, Microbiology and Molecular Biology at the University of Maine. To amplify the sequence of interest into a useable amount, the polymerase chain reaction was used. The primers used for PCR and sequencing were: EUBACT 27F: 5'-AGAGTTTGATCATGGCTCAG – 3' and EUBACT 1492R: 5'-GGTTACCTTGTTACGACTT – 3'. The reaction solution consisted of the following in a total of 50 microliters: 1 X Taq buffer (Gibco BRL), 200 μM dNTPs, 1 μM 27F, 1 μM 1492R, 1 mM MgCl2, 2.5 units Taq polymerase (Gibco), and 1 μl of template (pelleted cells). The cycle times were as follows: an initial 4 minute denaturation step at 94°C,

then 31 cycles of: 45 second denaturation at 94°C, 30 second annealing at 55°C, 90 second elongation at 72°C, and a final 10 minute elongation step at 72°C. The PCR products were analyzed on a 1.5% agarose gel, and then purified with a Microcon PCR centrifugal device (Millipore, Bedford MA). These amplified products were then sequenced at the University of Maine DNA Sequencing Facility to give an identifying strand of nucleic acids. This sequence was compared to other known small subunit RNA sequences using the BLASTN program. A phylogenetic tree was constructed using the PHYLIP program by the maximum likelihood method.

3.4 Analytical Techniques

3.4.1 Speciation

Arsenate and arsenite are separated for analysis by use of anion exchange resins in chromatographic columns. The resin (AG 1-X8, Bio-Rad Laboratories), has a dry mesh size of 50 to 100 μm and a wet bead size of 180 to 425 μm. It was purchased in the chloride ionic form and had to be converted to an acetate ionic form prior to use. Removal of the chloride ions and their subsequent replacement with acetate ions was accomplished through a series of steps. First, 125 grams of the resin was mixed with 125 mL of a 1 M sodium hydroxide solution, stirred for five minutes, filtered through a Buchner funnel with a scintered disc, and repeated twice. This removed the chloride ions and replaced them with hydroxides. The resin was then stirred with 125 mL of deionized water for five minutes, filtered, and repeated once. This washed out any residual chloride. The resin was stirred for five minutes with 125 mL of a 1 N acetic acid solution, filtered through the Buchner funnel, and repeated three times. This replaced the

hydroxides with acetate ions. The resin was then mixed with 125 mL of deionized water for five minutes, filtered, and repeated twice to remove any residual hydroxide ions. The resin was resuspended in 125 mL of deionized water and transferred to a 250 mL Teflon separatory funnel. This was used to dispense approximately 3 to 4 mL of the slurry into each of the chromatographic columns. After settling, approximately two mL of resin was packed in the bottom of the columns with 1 to 2 mL of overlying water to keep the resin moist until use. The columns (Poly-Prep 0.8 x 4 cm, Bio-Rad Laboratories) were kept upright at 5 to 10°C until used. These columns have a shelf life of about six months.

These columns were used for the separation of arsenate and arsenite, which can be quantified by instruments that measure total arsenic. In this experiment, columns were used to speciate arsenic in the field at the Green Lake site and in the lab to monitor conversion rates in cultures. Field samples were run through the columns at full strength, while lab samples withdrawn from cultures were diluted prior to separation to a concentration dependent on the instrumentation used for analysis. A graphite furnace atomic absorption spectrometer was used in this case, so a 1:7500 dilution of a culture amended with 1 mM arsenate yielded a concentration of approximately 10 μg/L, which is within the 1 to 100 μg/L operational range of the instrument. The soluble portion of the sample was obtained by filtration through a 0.45 μm filter. After filtration, the sample was acidified using 0.5 mL concentrated H_2SO_4 (ultra-pure) per liter of sample (0.05% v/v) to lower the pH of the sample to approximately pH 2 - 3. At pH 2, the negatively charged As(V) species adsorbed to the column resin while the neutral As(III) species passed through the column. The acidified sample was split, with one split analyzed separately for total (soluble) arsenic, while the other split was passed through the anion

exchange column and the effluent analyzed for total arsenic. The separated samples were preserved with 1 % acid until analyzed. As(III) concentration is reflected by the total arsenic concentration of the resin-treated sample. As(V) concentration is determined by the difference of the total arsenic concentration of the acidified sample and the total arsenic concentration of the resin-treated sample (i.e. inlet $[As]_T$ - effluent [As(III)]).

3.4.2 Inductively Coupled Plasma-Atomic Emission Spectrometry

The overall water chemistry for the two wells was provided by the ICP-AES at the University of Maine Environmental Chemistry Laboratory. This instrument measures light emission at a specific wavelength for each element of interest. These numbers were provided, although a brief description of the instrument will still be given. The instrument is a Perkin-Elmer Optima 3300XL axial view ICP-AES. The liquid sample is nebulized into a fine mist or vapor, then introduced into a plasma (which is about 10,000°C) where the entire sample is atomized. Every element emits electromagnetic radiation (light energy) at a specific wavelength (down to 1/1000 of a nanometer) and many of these emissions fall in the UV/visible range, so they can be measured with a photomultiplier detector. Since everything is atomized at once, many elements may be quantified simultaneously. The limiting factors are sample size, detection limits, and how many elements to calibrate for at one time. The methods are set up in a similar way to the atomic absorption (AA) spectrometer, there is a set of multi-element standards, and each element gets a calibration curve and quality control.[113]

3.4.3 Graphite Furnace Atomic Absorption

Atomic absorption (AA) spectrometry utilizes the absorbance of a compound at a specific wavelength to measure its concentration. Graphite furnace AA uses high temperatures to volatilize the compound of interest into a gaseous state, then an electrodeless discharge lamp illuminates the pathway above a graphite platform and a spectrometer measures the absorbance at the wavelength of interest. For this work, a Perkin Elmer Zeeman Atomic Absorption Spectrometer model 4100ZL was used equipped with an AS-71 autosampler. This instrument is located at the University of Maine Environmental Chemistry Laboratory. The electrodeless discharge lamp is run with a current of 350 mA. The carrying gas is argon, at a flow rate of 250 mL/min except during the atomization stage when the flow of gas is stopped for four seconds. The instrument analyzes a sample volume of 30 μL at 20°C, combined with a 15 μL matrix modifier consisting of 5 μg/L Pd and 3 μg/L of $Mg(NO_3)_2$, which selectively retains the arsenic while the other compounds are removed by pyrolysis. The furnace is elevated to different temperature values for specific periods of time to remove specific compounds after the sample is injected. Stage 1 raises the temperature of the column to 110°C for 20 seconds; this heats the graphite platform to a temperature slightly above the boiling point of the matrix. Stage 2 raises the temperature to 130°C for 35 seconds, this second drying step heats the platform 20-30 degrees above the matrix boiling point to remove solvent and associated vapors. A two step drying process prevents rapid heating and spattering of the solvent. Stage three raises the platform to 1100°C for 50 seconds, this is called pyrolysis and removes as much of the matrix as possible prior to atomization. Stage four is when the actual volatilization of the arsenic occurs. The platform is raised to 2300°C

for four seconds and the spectrometer measures light intensity for the entire duration. Stage five is the clean out stage, when the platform is raised to 2500°C to remove all residual sample from the graphite platform. The spectrometer uses background corrected atomic absorption at a wavelength of 193.7 nm and a slit width of 0.7 nm. Peak area is integrated to yield concentration values. Standard curves are obtained by running samples with 0, 5, 10, and 25 μg/L arsenate, which is the useable detection range. The detection limit was 0.1 μg/L. Quality control is obtained by running a sample of known concentration along with the samples of interest.

3.4.4 High Performance Liquid Chromatography

Liquid chromatography utilizes the fact that different chemical structures are retained for different times when run through a column with specific packing. The absorbance of each specific compound is plotted versus time and peak area is integrated to yield concentrations. In this case, a column packed with polystyrene-divinylbenzene sulfonic acid resin was selected since it would selectively retain arsenate, arsenite, lactate, and acetate. This allowed for multiple data acquisition in a single step. The apparatus, located in the Russell Laboratory in Holmes Hall, consists of a Hewlett Packard model 1050 HPLC fitted with a Bio-Rad HPX-87H organic acid analysis ion-exclusion column preceded by a Phenomenex security guard column with carbo-H+ inserts. The mobile phase is 5 mM sulfuric acid at a flow rate of 0.20 mL/min, which results in a column pressure of approximately 300 psi. Nitrogen at a pressure of 60 psi injects a sample volume of 35 μL. The diode array detector is set to monitor absorbance at wavelengths of 191 and 220 nanometers. Each sample has a total run time of 80 minutes, with an order of elution of arsenate, lactate, arsenite, and acetate. The arsenic

species are monitored at 191 nm, while the carbon species are monitored at 220 nm. The detection limit for this configuration was approximately 0.1 mM.

A run time of this length was required to separate the peaks of arsenite and lactate. They were monitored at different wavelengths but with a short run time, they would interfere with each other leading to inaccurate readings. With this low flow rate, the elution times were far enough apart to cause the interference to be less than one percent. Two example chromatograms are included in Appendix E for reference. The first, Figure E.1, is a chromatogram with an elution time of 25 minutes. It is obvious that the times of elution are too close to distinguish the lactate and arsenite peaks reliably. Figure E.2 shows the output from the HPLC when an elution time of 80 minutes was used. In this case, the lactate and arsenite peaks are approximately one minute apart so distinguishing between them is possible. Sample preparation consists of withdrawing 2 mL of solution from a culture and adding 25 μL of concentrated sulfuric acid, followed by filtration through a 0.2 μm syringe filter into a chromatography vial. The vials are sealed with a silicon-lined cap and analyzed within a few hours.

Periodic column cleaning was accomplished by backwashing for 4 hours with 5 mM sulfuric acid containing 5% acetonitrile, followed by 12 hours of backwashing with 5 mM sulfuric acid containing 30% acetonitrile. This is followed by an additional backwash phase with the mobile phase for 4 hours. The column is then returned to normal operation with the mobile phase until a steady base line is attained.

4 Results

4.1 Preliminary Data

The water chemistry data available for each site is summarized in Table 1. Listed are the analytes of interest and their associated concentration at each site. The laboratory analyses of the Green Lake sample were conducted by the staff at the University of Maine Environmental Chemistry Laboratory. Information on the Northport site was provided by Dr. Andrew Reeve of the University of Maine Geological Sciences Department. Anions and metals were quantified by the ICP-AES, while pH and DO were measured in the field with electrodes. A few overall trends should be observed in this table. Green Lake, at the time of sampling, had a higher pH. This site also had nitrate present when there was none detected in the Northport well. The arsenic concentrations at both sites are significant, but the Northport site has an extremely high concentration of dissolved arsenic.

Table 1. Chemical parameters available for each sampling site.

Analyte	Green Lake	Northport
DO (mg/L)	3.3	
pH	8.08	7.37
DOC (mg/L)	0.2	
ANC (μeq/L)	1102	1256
NO_3^- (μeq/L)	2.2	0
SO_4^{2-} (μeq/L)	22.5	327
Cl^- (μeq/L)	85.4	
PO_4^{3-} (μg/L)	118.9	
Fe (μg/L)	1740	1515
Mn (μg/L)	38.8	193.1
total As (μg/L)	274	1446
As[III] (μg/L)	2.56	

The initial enrichment cultures that were inoculated early in the research period were checked for active arsenate reduction in order to select cultures to take through the isolation procedure. Table 2 presents the results from two rounds of samples that were analyzed using the GFAAS along with ion exchange resins. GL11 (on acetate or lactate), GLF3, NP4 (on acetate or lactate) and NP2 all caused a significant fraction of the arsenate to be reduced to arsenite. Reduction by GLF3 was either significantly slower than the other enrichments or had been arrested when less than half of the arsenate was converted. More arsenate was converted in NP4 enrichments grown on lactate than those incubated with acetate. GL11 enrichments converted arsenate to the same degree, regardless of the carbon source.

Table 2. Percent As[III] in initial enrichment cultures.

Sample ID	1/30/02			2/6/02		
	Total As	As [III]	%As [III]	Total As	As [III]	%As [III]
blank	0.0	-	-	0.0	-	-
5 mM standard [V]	4.3	-	-	4.9	-	-
10 mM standard [V]	10.3	-	-	9.9	-	-
25 mM standard [V]	25.0	-	-	25.1	-	-
10 mM standard [III] (1)	13.4	11.7	87%	11.4	11.8	104%
10 mM standard [III] (2)	11.8	11.4	97%	12.6	11.8	94%
control	-	-	-	9.6	-0.5	0%
GL 6 (8.1)	48.0	4.0	8%	-	-	-
GL 9 (dilution) (7.3)	70.7	5.1	7%	-	-	-
GL 11 (8.1)	14.9	14.0	**94%**	15.2	14.6	**96%**
GL 11 (10 mM acetate) (7.5)	-	-	-	14.8	14.3	**97%**
TGL 11 D (4.2)	109.1	3.8	3%	-	-	-
GLF 3 (8.1)	11.1	4.7	42%	10.8	4.8	45%
GLF 3 (10 mM acetate) (7.5)	-	-	-	3.3	1.5	47%
DGL 3W (5.5)	16.3	1.5	9%	-	-	-
NP 4 (7.5)	10.5	10.2	**98%**	10.5	9.4	**89%**
NP 4 (10 mM acetate) (7.0)	-	-	-	8.9	6.6	**74%**
TNP 1D (4.4)	113.2	3.2	3%	-	-	-
NP 2 (7.5)	-	-	-	9.3	9.4	**101%**

Note:

All concentrations are in mM. Arsenate standards were used to calibrate the instrument, and the arsenite standards verified the efficacy of the ion exchange resins. The carbon source was 10 mM lactate unless otherwise noted. The value reported for arsenite is an average of two replicates. Those cultures exhibiting a significant degree of reduction were reanalyzed on the second day for verification. The control consisted of media with arsenate and lactate, but no inoculant. "GL" signifies the culture was derived from Green Lake samples while "NP" stands for Northport. A "D" preceding the name indicates that it is a 1 mL transfer and "T" signifies that 3 mL of culture were inoculated into the media (10%). A "D" following the culture represents a picked colony from solid media. The age of the cultures (in months) is also presented with the sample identification.

4.2 Rate Experiments

From the results of the initial probe across various cultures, actively reducing microcosms were selected from each site in order to establish rates of arsenate reduction. Before the next round of culturing experiments, individual colonies were isolated by transferring liquid culture to agar media and picking individual colonies from the solid media. After growth of the picked colonies in liquid media, the cultures were used in rate experiments. At this stage, arsenic measurement was being done by the graphite furnace method. This lead to samples being obtained and quantified every five to seven days since ion exchange columns had to be converted and poured between successive sampling days. The culture that reduced arsenate most rapidly during this period was from the Northport site with the designation NP4. This enrichment was initially provided with 1 mM arsenate and 10 mM acetate. It had since been grown on lactate and arsenic, and based on a higher proportion of reduction when grown on lactate was kept on this carbon source. Figure 2 represents the amount of arsenic converted from arsenate to arsenite over a period of approximately 280 hours or 12 days. Within the first five days, essentially all of the arsenate was reduced to arsenite and remained fairly constant from this time on. The control which contained everything except organisms, showed a small amount of reduction presumably from abiotic transfer of electrons. From these data, reduction rates were estimated. For the inoculated culture, the rate was at least 6.6×10^{-3} mM/hour and reduction in the control system was 8.9×10^{-4} mM/hour, demonstrating that the culture increased the rate of reduction by at least 7.4 times over control. Tabular data for the concentrations measured on each sampling day is available in Appendix D as

Table D.1. All figures presented have accompanying tables in Appendix D, which provide the values used to construct each figure.

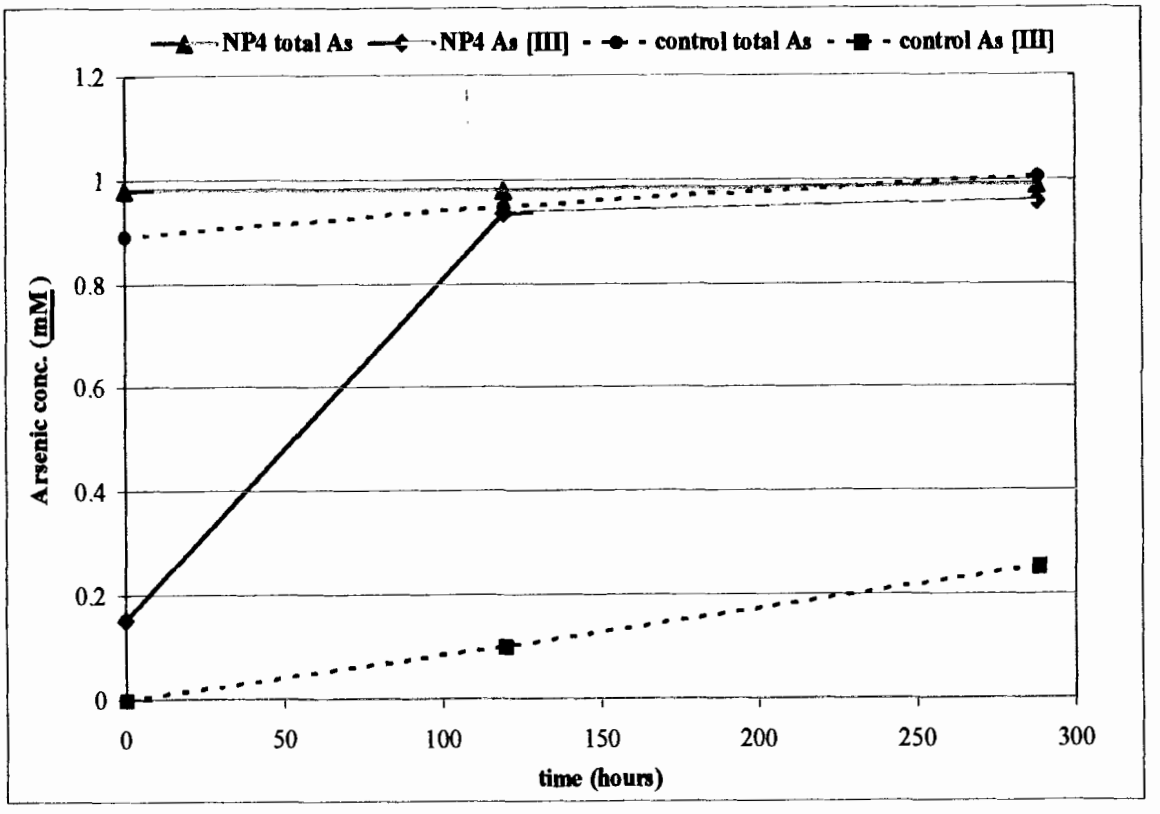

Figure 2. Arsenic concentrations in culture media for duplicate NP4 cultures incubated with 1 mM arsenate and 10 mM lactate.

Some of the Green Lake cultures also exhibited arsenate reduction during the initial probe across enrichment cultures. The culture designated GL11 was selected for rate experiments due to the proportion of the arsenate that was reduced by initial enrichment cultures (see Table 2, page 53), as well as significant visible signs of growth. Figure 3 represents the amount of arsenic converted from arsenate to arsenite over a period of approximately 280 hours or 12 days. There was no arsenic reduction in this

culture when a colony was picked and inoculated into fresh media with 1 mM arsenate and 10 mM lactate. Arsenite was produced at the same rate as in the control. The control, which contained everything except organisms, showed a small amount of reduction presumably from abiotic transfer of electrons.

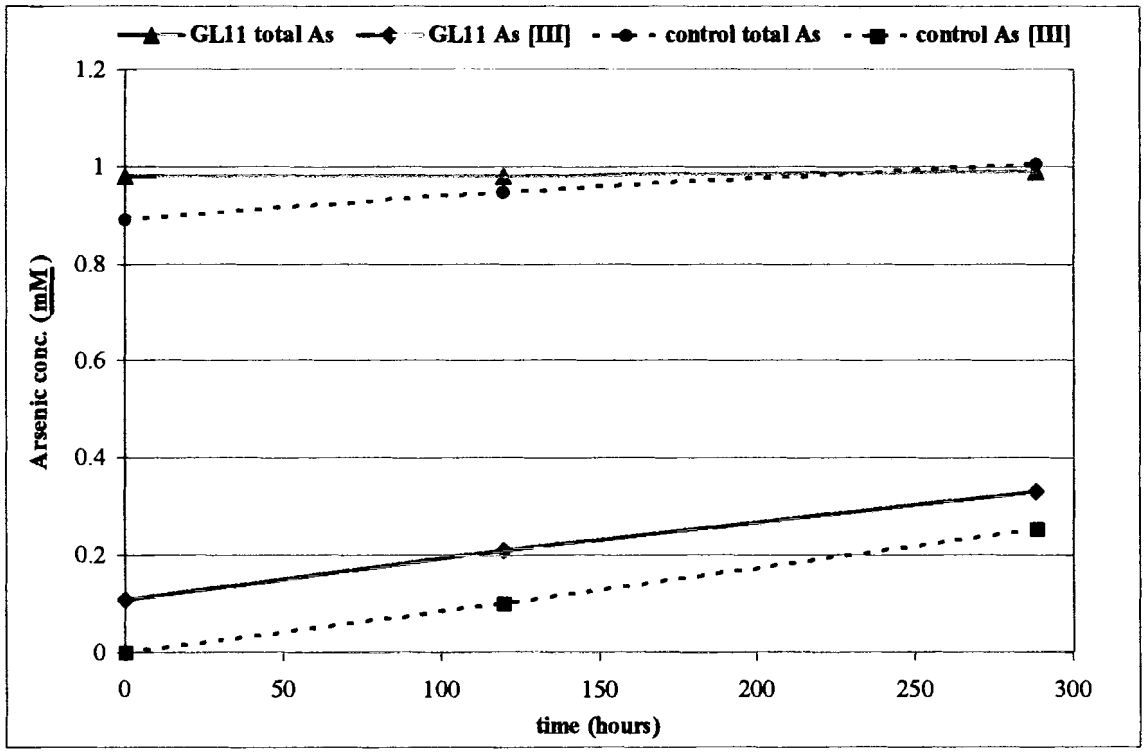

Figure 3. Arsenic concentrations in culture media for duplicate GL11 cultures incubated with 1 mM arsenate and 10 mM lactate.

The concentration of arsenic species is shown in the preceding figures, but the carbon source cannot be monitored with the graphite furnace. In order to be certain that these organisms were using the provided carbon source while simultaneously reducing the electron acceptor, high performance liquid chromatography was used. This allowed

for simultaneous determination of arsenate, arsenite, lactate, and acetate. Figure 4 shows the results of an experiment using the same Northport culture after it was transferred to fresh media. In this case, the control was the same as the experimental bottles but it was chemically inhibited with formaldehyde. As Figure 4 shows, approximately 0.5 mM of arsenate was reduced in the cultures during the ten day experiment, in the same period about 0.4 mM arsenite was formed. Over the observation period, 0.9 mM lactate was consumed and 0.5 mM acetate was produced. The other product of lactate metabolism was probably CO_2, but this product was not measured in the system. No arsenite or acetate was observed in the formaldehyde-inhibited control samples, and the arsenate and lactate concentrations remained constant throughout the experiment.

Figure 5 shows the results of an experiment using the Green Lake culture discussed previously when it was transferred to fresh media. A significant amount of visible growth was present but as Figure 5 shows, no reduction of arsenate occurred and minor consumption of lactate was evident. A small amount of acetate was observed in the later stages of the monitoring. In control samples, the concentrations of all components were essentially constant throughout the experiment. Arsenite and acetate were not present in control media.

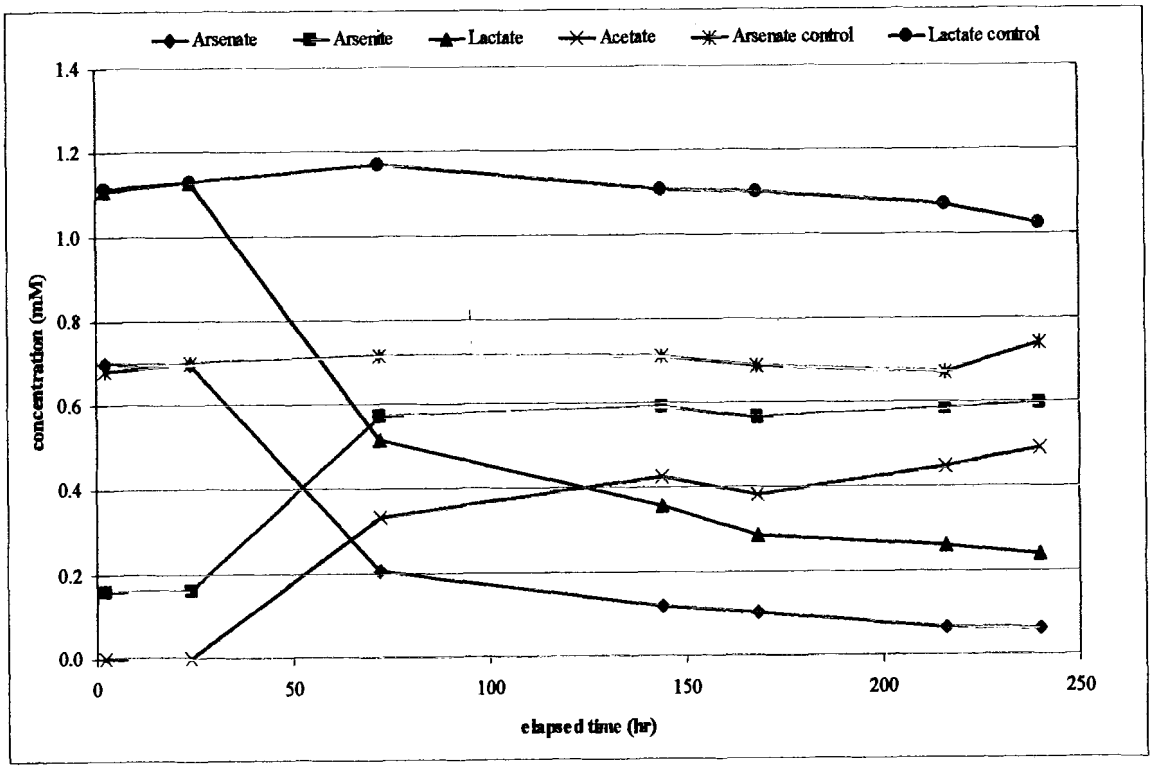

Figure 4. Arsenic species, lactate and acetate in NP4 culture medium and formaldehyde-inhibited control over time.

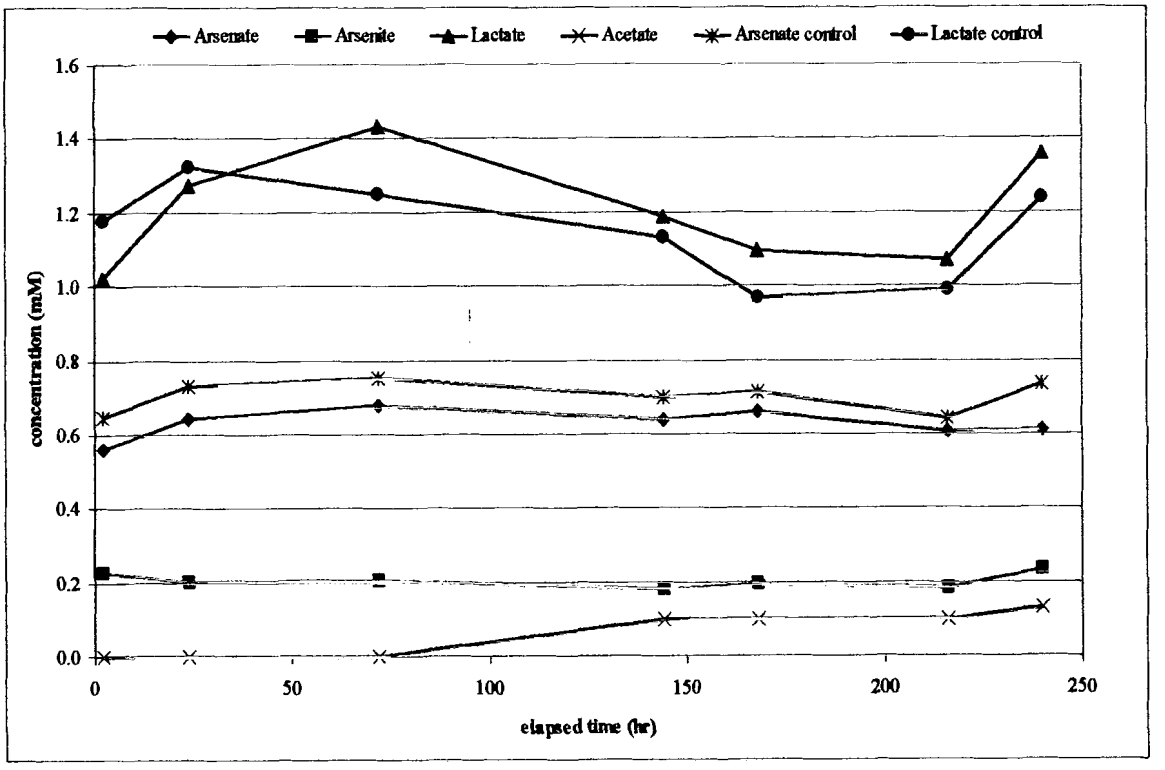

Figure 5. Arsenic species, lactate and acetate in GL11 culture medium and formaldehyde-inhibited controls over time

4.3 Alternative Donors and Acceptors

The utilization of other electron acceptors and donors was investigated for the Northport culture, NP4. As described in the methods section (page 46), active cultures were inoculated into media containing either selenate, nitrate or sulfate with lactate; or acetate, benzoate or glucose with arsenate. Figure 6 shows the lactate concentrations in media containing selenate, nitrate, and arsenate. The figure shows that there was a preferential use of arsenate, followed by nitrate, and finally selenate. The results of the experiment using sulfate as the electron acceptor are not included since usable peaks for

lactate could not be obtained on the liquid chromatograph. Although it did not show any visible signs of growth, the results with regard to sulfate should be classified as inconclusive since sulfate cannot be reliably determined with the HPLC as configured.

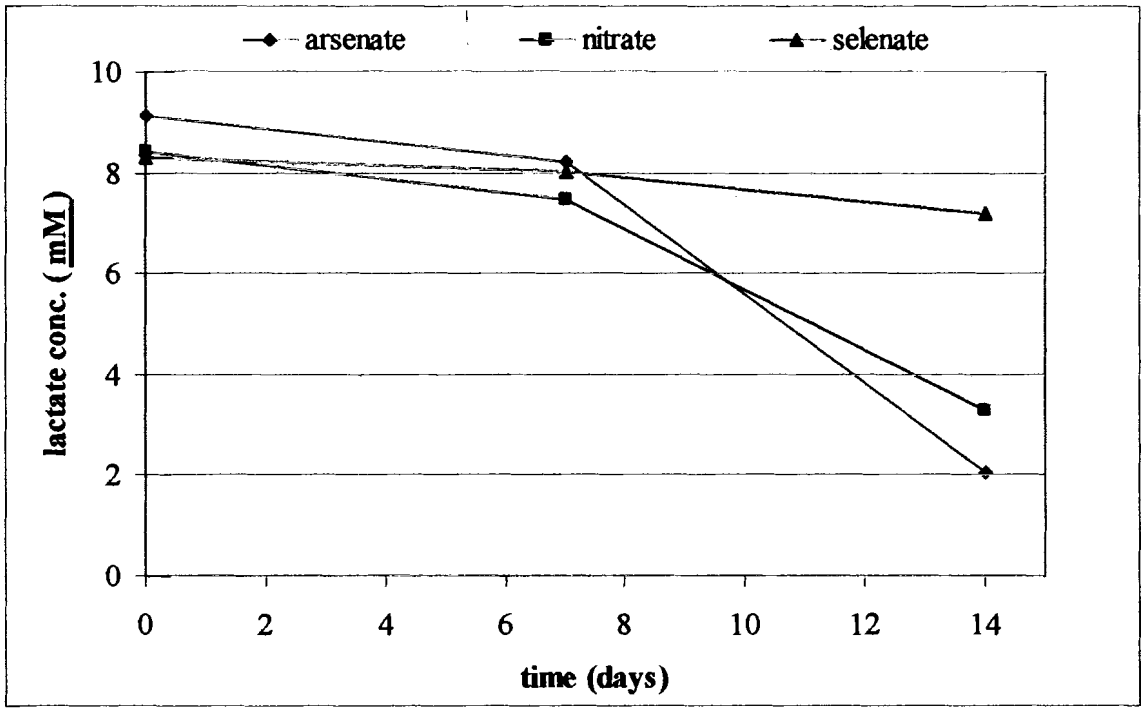

Figure 6. Utilization of alternative electron acceptors by NP4 shown as consumption of lactate.

The use of alternative carbon sources was investigated in a similar manner. Figure 7 shows the changes in arsenate concentration when provided with different carbon sources, while Figure 8 is the accompanying production of arsenite by the same cultures.

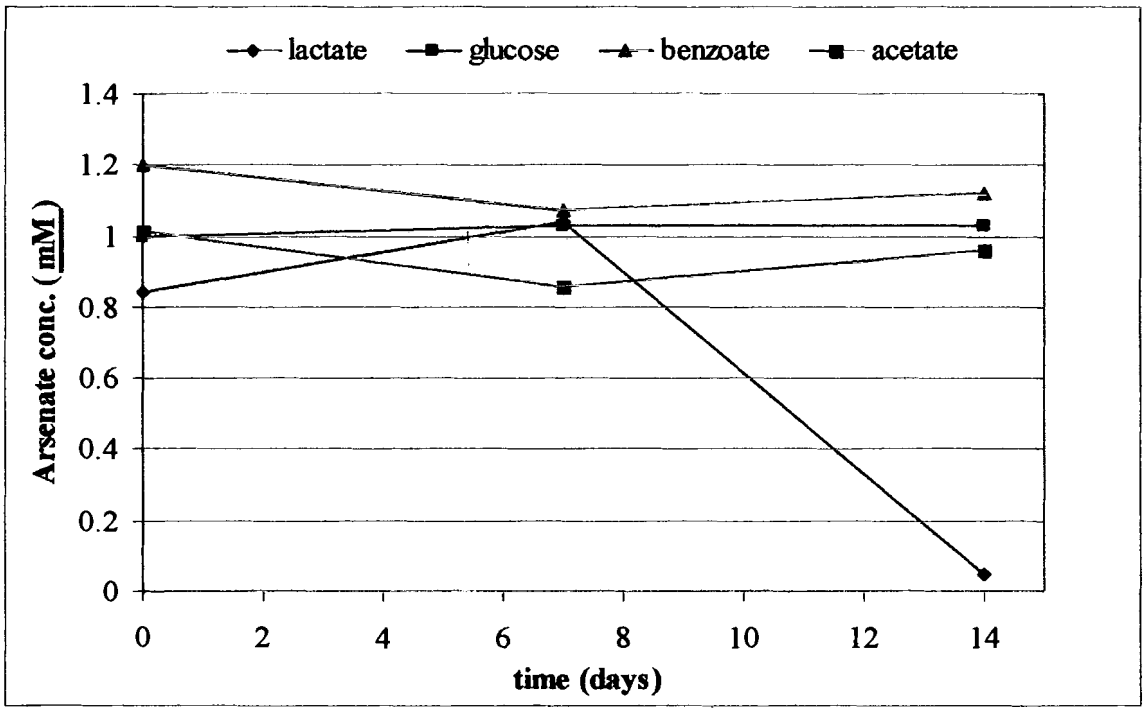

Figure 7. Arsenate concentration in NP4 cultures given alternative carbon sources.

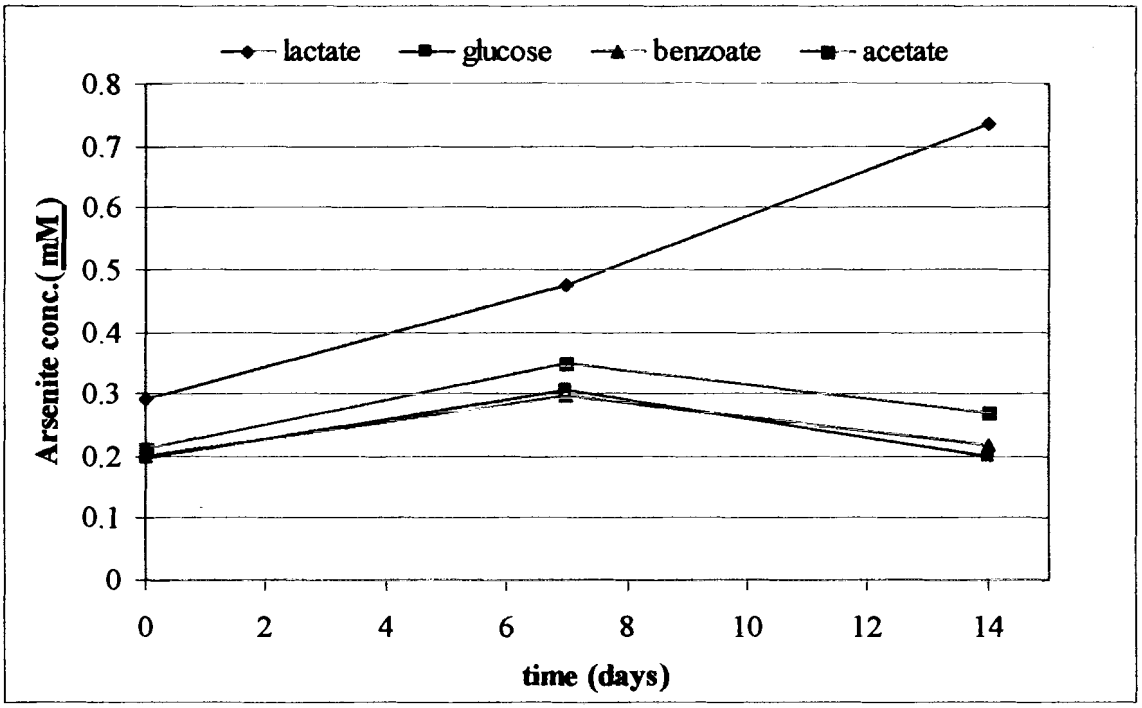

Figure 8. Arsenite concentration in NP4 cultures given alternative carbon sources.

4.4 Characterization of NP4 Isolate

A readable sequence and single band was obtained by PCR amplification using a general bacteria primer, indicating that there was only one bacterial species in the sample. This confirms the presence of an isolate assuming there were no archaeal species present. An image showing the general size and shape of the isolate is included in Appendix C as Figure C.4. Comparison of the DNA sequence to the 16S rRNA sequences of other known bacteria indicates that the bacteria isolated at the Northport site is unique. This means that this strain of bacteria has not been reported before. Figure 9 is a phylogenetic tree that shows the relative position of this isolate with respect to other arsenate respirers and close relatives. It was 97% similar to a Campylobacter species CCUG 13942 that was isolated from oilfield brine. SES-3 and MIT-13 are in the same subgroup as the NP4 isolate, which is the Sulfurospirillum barnesii subgroup. SES-3 is 94.5% identical while MIT-13 is 95.6% identical to the NP4 isolate. These organisms are all part of the epsilon subdivision of the Protobacteria in the Thiovulum group. The sequence of the NP4 strain is available in Appendix D. The horizontal distance shown by the scale bar represents a 10% difference in sequence. To estimate the degree of difference between two species, the lengths of the connecting horizontal lines are added. It is evident that the Pyrobaculum is distantly related while the Campylobacter species CCUG 13942 is the closest relative shown.

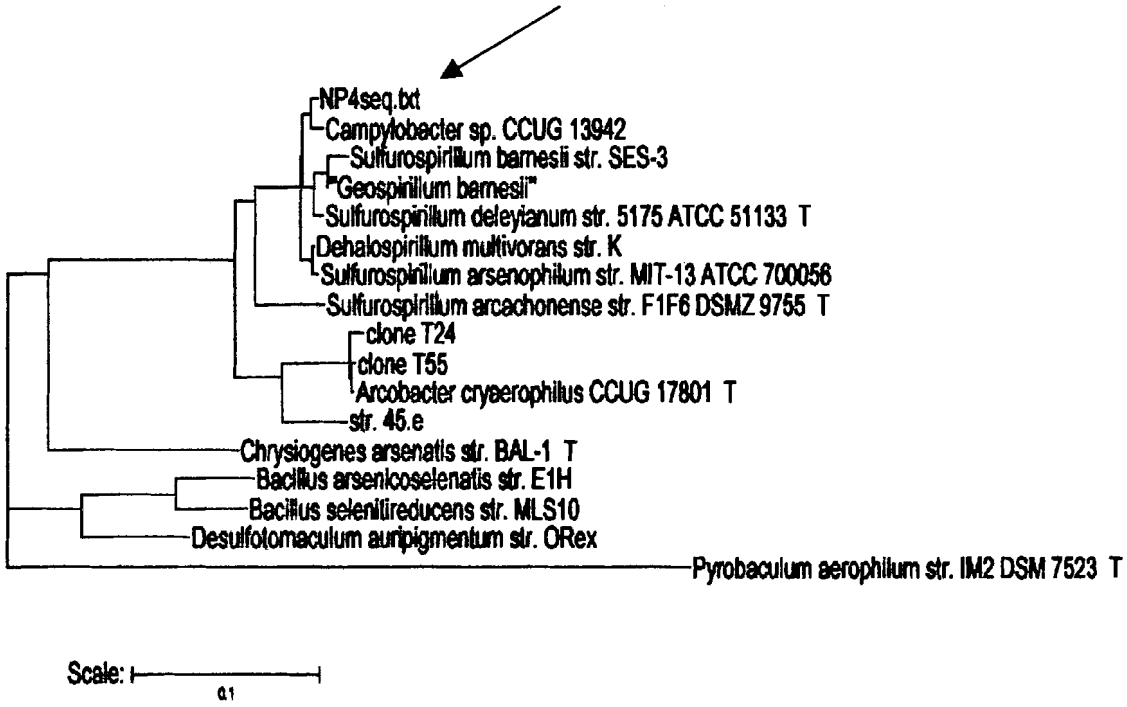

Figure 9. Phylogenetic tree for NP4.

5 DISCUSSION

If a reduced carbon compound and arsenate were allowed to react for an unlimited amount of time, electrons would slowly transfer from the carbon compound to arsenate. The fact that arsenate is reduced while a carbon compound is oxidized in these experiments does not represent a novel process. What is significant is the rate at which reduction of arsenate occurs when these bacteria are present. Figure 2 contains valuable information regarding this concept. The arsenite control has a positive slope, indicating that arsenate is slowly being reduced to arsenite in this system. If this line, the dashed line with square points on Figure 2, were extended to give an estimate of the time needed for the full one millimolar concentration of arsenic to be converted, it would take

approximately 1125 hours. In contrast, the conversion of one millimolar arsenate facilitated by the dissimilatory organisms isolated in this study occurs in less than 120 hours, as noted by the red line in Figure 2. It is reasonable to say that bacteria of this type could cause the exceedance of the Federal Maximum Contaminant Level.

The rates observed in the NP4 experiments were comparable to values that have been reported elsewhere. SES-3 reduced ten millimolar arsenate solutions in less than one week[103], while *Desulfotomaculum auripigmentum* reduced five millimolar arsenate in less than one week as well.[110] In this work, NP4 cultures reduced 1 mM arsenate solutions in approximately ten days with the majority of the reduction occurring within the first 72 hours (Figure 4). The two conversion products (arsenite and acetate) were both observed in the experimental cultures but not in the controls (Figure 4).

The results suggest that dissimilatory arsenate reduction may be occurring, because as the arsenate was being reduced lactate was being oxidized. In contrast, arsenate was not reduced and lactate was not oxidized in the formaldehyde-inhibited controls. Stoichiometrically, the ratios of the observed products to the reactants indicate that dissimilatory reduction may not be the dominant process underway. This isolate could possibly be an arsenic resistant organism that lives by fermentation. It is also possible that the fermentation products themselves may abiotically reduce the arsenate. Complete monitoring of carbon compounds (such as carbon dioxide, propionate, pyruvate, etc.) and hydrogen gas would help to resolve this.

The mixed cultures from Green Lake showed full reduction of arsenate in the initial enrichment phase. The isolates that were obtained and transferred to fresh media could not reduce arsenate within the time period of the experiment. Since the control,

which contained everything except inoculum, showed no significant abiotic reduction (Table 2), some type of bacterial influence may account for the reduction that took place. There are possible explanations for this observation:

- A detoxification reaction could have been producing As[III] in the original culture. What was isolated and transferred in later experiments could have been a fermentor that lacked this detoxifying ability.
- Cysteine may have been utilized as a fermentation substrate with sulfide groups from the cysteine accounting for the initial reduction of arsenate. In subsequent experiments, the time allowed for culturing may not have been long enough for these fermentors to develop or for the chemical reaction to occur.
- A mixed culture relationship could have been disrupted by isolation if a two-step process was in operation. Lactate was possibly being fermented to H_2 and acetate by one organism. Another organism could have been using this H_2, acetate, and arsenate to grow. Since isolation was done with lactate as a carbon source, this would have prevented the development of the second organism. The first organism may have been what was isolated since it would be the first to form colonies.
- A mixed culture may have been present inhabited by both arsenate reducing organisms and fermentors. If these were inoculated into solid media and the fermentors grew faster, they would have been picked and cultured since they would be the first to form colonies.

Although this isolate did not reduce arsenate or oxidize lactate, it is noteworthy that it could persist through the isolation process with such a substantial amount of arsenic present. The isolation procedure was only a few weeks and a longer duration may have allowed other organisms (that could reduce arsenate), to form colonies and be picked.

The NP4 cultures were capable of growth on lactate and arsenate. To help understand the niche that these organisms occupy, a portion of an active culture was inoculated into media with alternative electron acceptors and carbon sources. Figure 6 indicates that the NP4 isolates were capable of utilizing both nitrate and possibly selenate as electron acceptors. Nitrate was utilized almost as effectively as arsenate, with selenate utilized less effectively than either arsenate or nitrate. This relative "efficiency of use" does not comply with the reduction sequence described in the Literature Review. It should be noted that the straight lines connecting time 7 and time 14 of Figure 6 are not true measures of rate, since it is unknown when growth actually initiated. The actual rates could well have conformed with the hierarchy of electron acceptors if the start time was known. It should also be understood that these organisms were acclimated to arsenate and lactate as their electron acceptor and carbon source. There was obvious growth in both the nitrate and selenate-grown cultures (see Figure C.3 in Appendix C). The red precipitate in the selenate-grown culture is presumably from the saturation of elemental selenium because of its reduction. The nitrate grown organisms exhibited a high amount of turbidity. If successive transfers were performed into selenate and nitrate media, it is possible that once acclimation occurs the lag time may decrease and/or the rate of reduction will increase.

Another point to note is that the initiation of arsenate reduction took longer than the previous round of rate experiments. In the previous NP4 cultures, the majority of the arsenate was reduced within a week, where in this case the onset of reduction was delayed by at least a week. This may be a result of the stage of growth that the organisms were in at the time of inoculation. The previous round of cultures was inoculated out of active cultures that had not consumed all of the arsenate and lactate they had been provided. The cultures in this instance had been inoculated with NP4 culture that had been in the stationary phase of growth for some time. These cultures seemed to go through a lag phase.

As stated in the Dissimilatory Reduction section of the Literature Review (page 34), several other arsenate reducers described in the literature can utilize nitrate and selenate as electron acceptors. SES-3 and the isolate from Mono Lake were both capable of using selenate as an electron acceptor.[103,111] The isolates from Nevada and California[105], *Chrysiogenes arsenatis*[108], and the Mono Lake isolates[111], were able to use nitrate as an electron acceptor. The fact that NP4 can use alternative electron acceptors indicates that it has the ability to survive in a variety of settings and may be able to out-compete other bacteria that lack this characteristic.

NP4 did not use the alternative carbon sources that were tested to any extent within two weeks (Figures 7 & 8). This result could be due to an inability of the isolate to use the carbon sources provided, or the lag phase associated with their use might have been longer than two weeks. Growth and arsenate reduction had been observed on acetate during the earlier stages of the research, indicating that there may be a substantial

period of acclimation. Lactate was chosen initially due to superior performance as a carbon source in other investigations and it may simply be a more useable form.

Table 3 is a comparison of the NP4 isolate with other arsenate reducers. It shows which species have the ability to use certain electron acceptors and donors, along with their taxonomic classification. It is significant to note the relationship of some of the species shown in Figure 9. The species MIT-13 and SES-3 are in the same phylogenetic subgroup as isolate NP4. Both these species are capable of reducing arsenate when provided with lactate and anaerobic conditions. *Chrysiogenes arsenatis* and *Desulfotomaculum auripigmentum* are also arsenate reducers but they are distant in relation when compared to the NP4 sequence. To date, a major conclusion has been that arsenate reducers are not closely related. This study has resulted in an isolate that is quite closely related to two other known arsenate reducers, along with other bacteria that utilize toxic compounds such as *Dehalospirillum multivorans* which utilizes tetrachloroethene under anaerobic conditions.[114] The use of what are commonly considered to be toxic compounds may be more prevalent than previously assumed.

Table 3. Comparison of electron acceptors and donors utilized by arsenate reducers.

reference	103	106	110	111	111	108	112	
	S. barnesii	*S. arsenophilum*	*D. auripigmentum*	*B. arsenicoselenatis*	*B. selenitireducens*	*C. arsenatis*	*P. aerophilum*	
	SES-3	MIT-13	OREX-4	E1H	MLS10	BAL-1^T		NP4
Electron Acceptor								
Arsenate	+	+	+	+	+	+	+	+
Selenate	+	-	-	+	+	-	+	+
Oxygen (5%)	+a	+a	0	-	+	-	+	-
Nitrate	+	+	-	+	+	+	+	+
Fe(III)	+	0	-	+	-	-	0	-
Sulfate	-	-	+	-	-	-	0	0
Fumarate	+	+	+	+	+	0	0	-
Electron Donor								
Lactate	+	+	+	+	+	+	0	+
H_2 + Acetate	+	+	+	-	-	+	0	0
Acetate	-	-	-	-	-	+	0	-
Formate	+c	+c	-	-	-	-	0	0
Propionate	0	-	-	-	-	0	0	0
Butyrate	0	+c	+	0	0	0	0	0
Fumarate	+	+	-	0	0	+	0	0
Succinate	+	0	-	-	-	+	0	0
Citrate	+	0	-	+	-	-	0	0
Benzoate	0	-	-	0	0	-	0	-
Glucose	0	0	-	-	+b	-	0	-
Taxonomic class	epsilon proteobacteria	epsilon proteobacteria	low G+C gram positive	low G+C gram positive	low G+C gram positive	deep-branch proteobacteria	crenoarchaea	epsilon proteobacteria
+ growth - no growth 0 no data								

a - microaerobic, concentration not given
b - equivalent growth without e acceptor
c - growth only in presence of acetate

This study has shown that arsenate-reducing bacteria are present in Maine groundwater. In order to fully understand their role in the overall cycle, further studies are necessary. The role of iron and manganese oxides, as arsenic adsorbents or as electron acceptors, was not really examined in this work and may be an avenue worth exploring. As noted in the geochemistry section, oxides play a substantial role in controlling the mobility of arsenic. Iron and manganese oxides are also subject to reduction by bacteria. If there are bacteria present in groundwater that have the ability to reduce arsenic and oxides, they may have a significant role in increasing the dissolved arsenic concentration in aquifers.

Another area that warrants study is defining the actual amount and activity of these organisms in a particular setting. This work has answered the question of whether or not these creatures exist, now the extent to which these organisms affect drinking water needs to be quantified. Gene probes, which can indicate the presence of specific bacteria in the field, should be developed so environmental samples can be taken and numbers of bacteria may be estimated. This would prove particularly useful in a contaminated setting where a known arsenic problem is present. This was the case for both of the wells sampled in this study, a high concentration of dissolved arsenic was present, and the cause for this situation was under investigation. The availability of an initial assay to determine if arsenate reducers are present would help to limit the scope of an investigation to save time and resources.

The role of sulfate reducing bacteria should also be investigated. As discussed previously, sulfide is capable of reducing arsenate when present in sufficient quantities.

If sulfate-reducing bacteria are actively producing sulfide, they may indirectly be influencing the mobility of arsenic in anaerobic groundwater settings.

Seasonal variations may also need to be investigated. The activity of bacteria may possibly change during the seasons, especially in a climate such as Maine's where a large variation of temperature occurs on a yearly basis. The role of temperature on these organisms was not investigated and it should be defined. Other things change during the seasons as well, such as water consumption. In a vacation prone setting such as Northport or Green Lake, significantly more water may be drawn from wells in the summer months increasing the drawdown of aquifers. If anaerobic sulfide-arsenic phases are subjected to oxygen, it could cause them to dissolve, releasing bound arsenic. This arsenic would eventually be precipitated with iron or manganese oxides and immobilized, but in the interim would be free to travel with the groundwater. If these newly formed oxides were then resubmerged in the winter months they would be prone to dissolution once anoxic conditions are reestablished. If arsenate-reducers are present, they could catalyze the release of this bound arsenic that would once again be free to travel in solution. A localized cycle may be in action where arsenic is being exchanged between a liquid and solid state on an annual or semi-annual basis. This has implications for management practices when simply the time of year could have an effect on the dissolved concentration in a given water supply. Times of rapid drawdown may coincide with periods of substantial arsenic solubilization.

6 CONCLUSIONS

- Microbial reduction of As(V) was observed in water and sediment slurries made from samples taken from 2 arsenic contaminated wells.
- The GL11 cultures showed reduction prior to isolation of colonies from the mixed enrichment cultures.
- NP4 enrichment cultures and an isolate derived from it exhibited arsenate reduction.
- The oxidation of lactate accompanied the reduction of arsenate in NP4 cultures.
- A single 16s rRNA sequence was obtained for the NP4 culture after the isolation procedure, indicating that a single species was present. This sequence has not been reported before.
- The sequence is in the Sulfurospirillum barnesii subgroup of the epsilon Proteobacteria.

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APPENDIX A: TRANSFER HISTORY

Unless noted, all transfers were into 30 mL serum bottles. A relative dating scheme will be used, with day 0 signifying the time of initial inoculation. A D preceding the culture code signifies a one ml transfer, while a T signifies a three mL transfer. The exponential power after the D or T signifies the generation of each culture. For example, T^2GL1 would be the culture resulting from two successive transfers of 3 mL from the GL1 original culture. A description of the transfer history will now be provided, with each location being discussed separately.

On day 7, one mL of GL1-15 was transferred into fresh media containing 10 mM lactate and arsenate. On day 18, one mL of GL5, GL6, GL9, GL11, GL12, GL13, and 1W, 2W, 3W, 4W was transferred to fresh media containing 10 mM lactate and arsenate. On day 20, one mL of 4W was transferred into three serum bottles of fresh media containing 1, 5, and 10 mM arsenate and 10 mM lactate. On day 27, one mL of F1, F2, and F3 was transferred into media containing 10 mM arsenate and lactate. On day 35, one mL from all 22 Green Lake cultures was transferred into media containing 10 mM acetate and arsenate. On day 69, one mL of lactate based DGL2, DGL4, DGL5, DGL6, DGL7, DGL8, DGL9, DGL10, DGL11, DGL12, DGL13, DF1, DF2, and DF3 was transferred into fresh media containing 10 mM arsenate and lactate. Also on day 69, one mL of acetate based DGL1-DGL15 was transferred to fresh media containing 10 mM acetate and arsenate. On day 82, three mL of GL1-GL15 was inoculated into media containing 10 mM arsenate and lactate. On day 88, three mL of F1, F2, F3, 1W, 2W, 3W, and 4W was transferred into media containing 10 mM arsenate and lactate. On day

187, three mL of cultures D1W, F3, and GL11 was transferred into fresh media containing 5 mM lactate and 1 mM arsenate. On day 194, three mL of GL11, and GLF3 was transferred in triplicate to fresh media containing 5 mM lactate and 1 mM arsenate. On day 318, ten mL from cultures GLF3 and GL11 was transferred in duplicate into 125 mL serum bottles containing 100 mL of fresh media containing 1 mM lactate and 2 mM arsenate. Various other transfers were made into liquid media for isolation purposes; this will be discussed in the isolation section.

On day 7, one mL of NP1, NP2, NP3, NP4, and NP5 was transferred into media containing 10 mM lactate and arsenate. One mL of NP3 and NP5 was also transferred into media containing 10 mM lactate and 1 mM arsenate on this day. On day 17, one mL from all five Northport cultures was transferred to media with 10 mM arsenate and 10 mM acetate. On day 50, one mL from NP1, NP2, and NP3 were inoculated into media containing 10 mM arsenate and lactate. One mL from NP1, NP4, and NP5 was also transferred into media containing 10 mM arsenate and acetate. Lactate based DNP1-DNP5 were all transferred to fresh media containing 10 mM arsenate and lactate. Acetate based DNP1-DNP5 were all transferred to fresh media containing 10 mM arsenate and acetate. On day 51, one mL from lactate based DNP3 and DNP5 was inoculated into media containing 1 mM arsenate and 10 mM lactate. On day 70, three mL of cultures NP1-NP5 was transferred to media containing 10 mM arsenate and lactate. On day 169, three mL from NP1, NP2, and NP4 was inoculated into media containing 5 mM lactate and 1 mM arsenate. On day 176, three mL from culture NP4 was inoculated into media containing 5 mM lactate and 1 mM arsenate in triplicate. On day 300, ten mL from culture NP4 was transferred into two 125 mL serum bottles

containing 100 mL of fresh media containing 1 mM lactate and 2 mM arsenate. Various other transfers were made into liquid media for isolation purposes; this will be discussed in the isolation section.

APPENDIX B: ISOLATION HISTORY

The letters trailing the culture name indicate the step in the serial dilution sequence. An A signifies the first tube inoculated while an E would represent the fifth tube in a sequence. A P preceding the code for a culture indicates that it has been picked from solid media with the exponent signifying the generation of transfer. A history of each sites' inoculation into solid media will be given in a similar fashion to the transfer history. On day 90, one mL from GL8, GL13, F3, and D4W was injected into nine mL of agar media with 10 mM arsenate and lactate. A five step serial dilution sequence was carried out for all four. On day 97, one mL from GL7, TF2, TGL2, and TGL11 was injected into nine mL of agar media with 10 mM arsenate and lactate. A four step serial dilution was performed for all four. On day 109, colonies were picked from solid media and inoculated into fresh media with 10 mM lactate and arsenate. The colonies selected were from GL7C, GL7D, GL8C, GL8D, TGL2A, TGL11D, TF2C, and TF2D. On day 118, one mL of culture was withdrawn from these same 8 cultures and used to inoculate solid media with 10 mM arsenate and lactate. No serial dilution was performed in this round of transfers. On day 126, colonies were picked from the solid cultures inoculated on day 118 and used to inoculate liquid media with 10 mM arsenate and lactate. On day 147, colonies were picked from the following solid cultures: PTF2C, PTGL11D, PGL8A, P^2GL8C, PGL7B, PTGL11B, PGL7C, PTF2D, P^2GL7D, PGL7D, and PTGL2A. These were inoculated into fresh liquid media with 10 mM arsenate and lactate. On day 248, 0.4 mL of liquid culture was withdrawn from the following cultures: TGL11 (acetate based), TGL11 (lactate based), TGLF3 (acetate based), TGLF3 (lactate based),

and PTGL11D. These were all inoculated into solid media with 5 mM lactate and 1 mM arsenate.

On day 72, one mL from NP1, NP3, and NP5 was injected into nine mL of agar media with 10 mM arsenate and lactate. A five step serial dilution sequence was carried out for all four. On day 79, one mL from NP1, NP2, NP3, NP4, and NP5 was injected into nine mL of agar media with 10 mM arsenate and lactate. A four step serial dilution was performed for all four. On day 91, colonies were picked from solid media and inoculated into fresh media with 10 mM lactate and arsenate. The colonies selected were from NP1C, NP1D, NP2C, NP3D, NP4A, NP5B, and NP5C. On day 100, one mL of culture was withdrawn from these same 7 cultures and used to inoculate solid media with 10 mM arsenate and lactate. No serial dilution was performed in this round of transfers. On day 108, colonies were picked from the solid cultures PNP1C and PNP1DC and used to inoculate liquid media with 10 mM arsenate and lactate. On day 129, colonies were picked from the following solid cultures: PNP1D, PNP4C, P^2NP1DC, PNP5C, PNP3C, and P^2NP1C. These were inoculated into fresh liquid media with 10 mM arsenate and lactate. On day 230, 0.4 mL of liquid culture was withdrawn from the following cultures: TNP4 (acetate based), TNP4 (lactate based), PNP4A, and TNP2. These were all inoculated into solid media with 5 mM lactate and 1 mM arsenate.

APPENDIX C: IMAGES

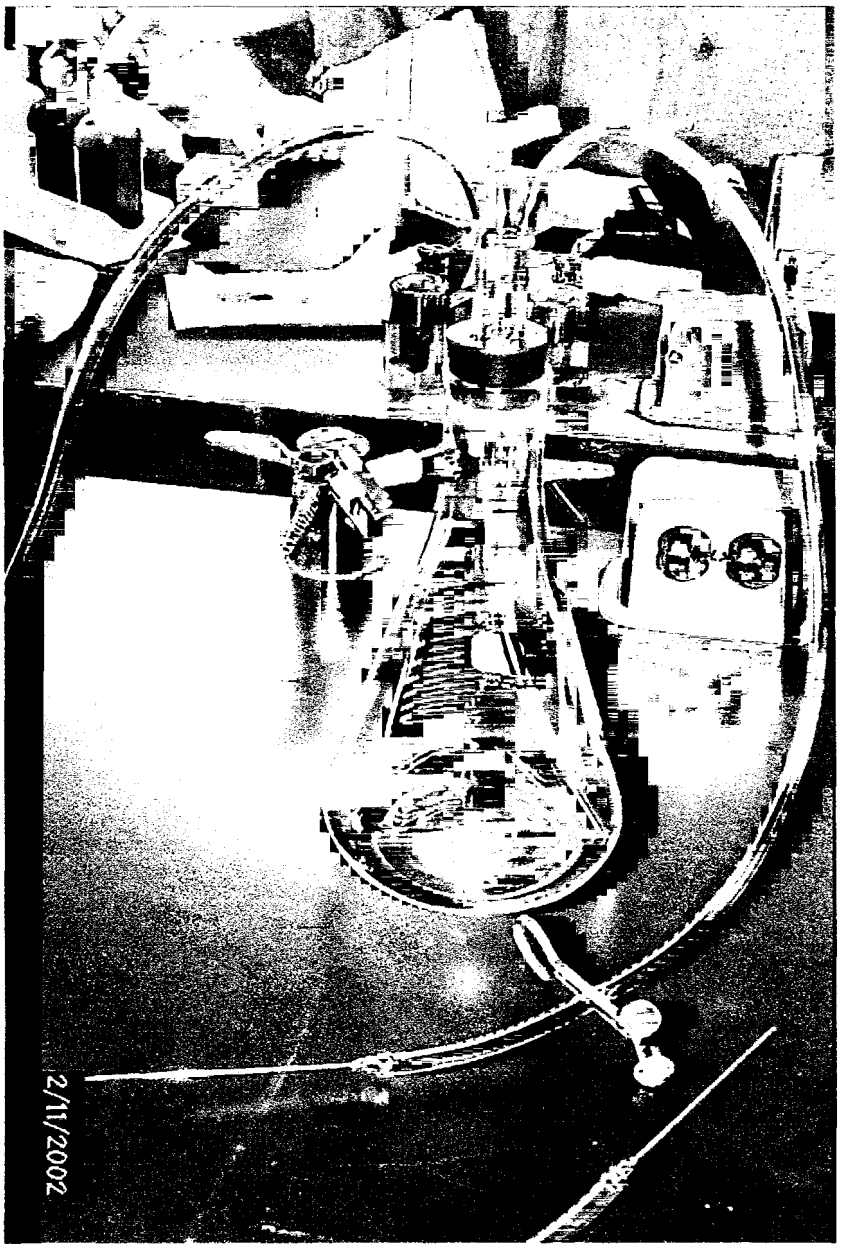

Figure C.1 Anaerobic Media Preparation Flask

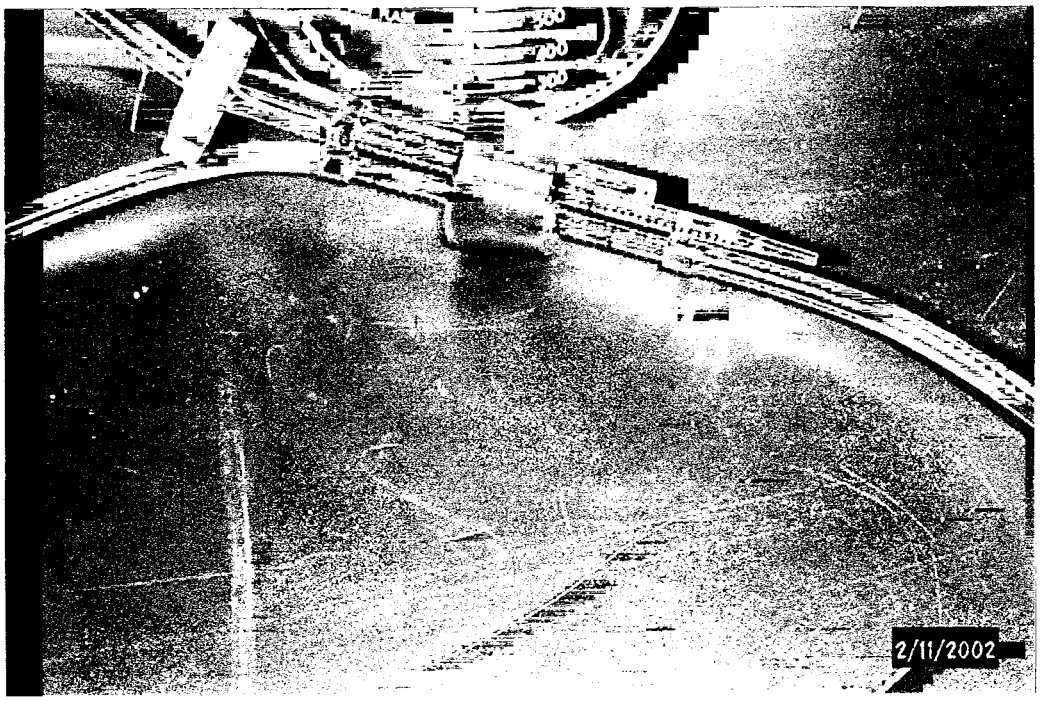

Figure C.2 Detailed view of stopper configuration.

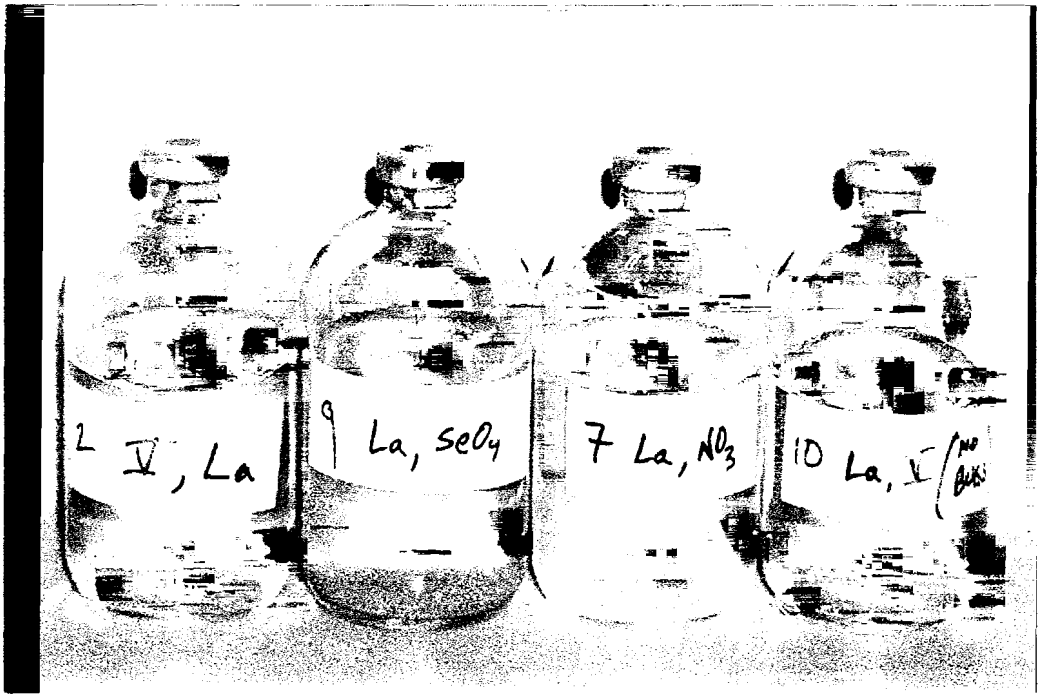

Figure C.3 NP4 inoculated with alternative electron acceptors.

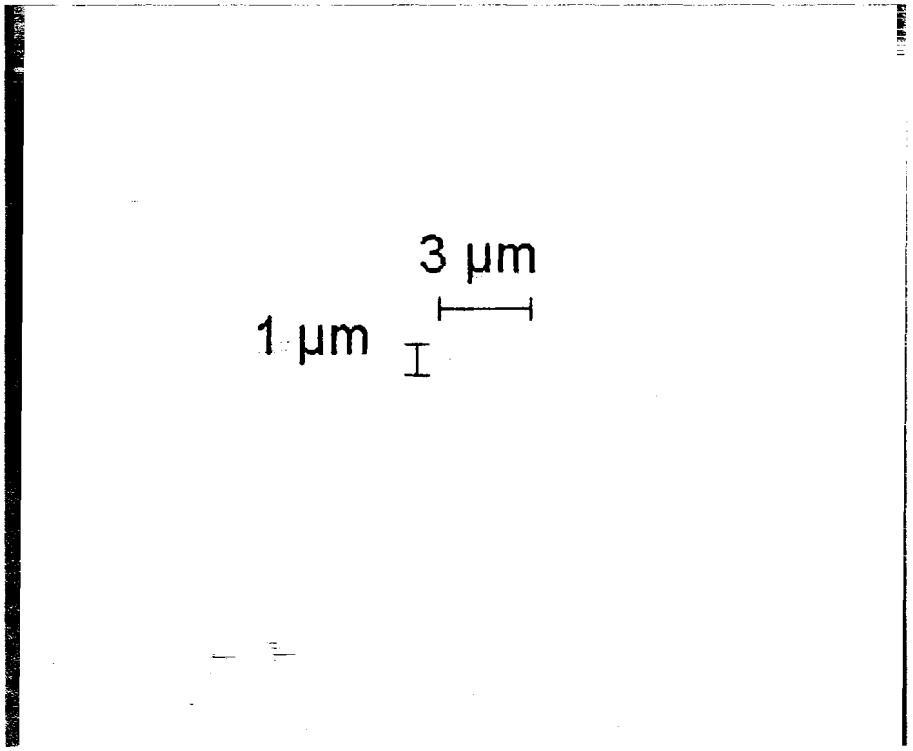

Figure C.4. Image of Northport culture at 40X objective.

APPENDIX D: SUPPORTING DATA

Table D.1. Arsenic concentrations in NP4 culture medium, initially given 1 mM arsenate and 10 mM lactate.

	As concentration (mM)		
time (hrs)	0	120	288
NP4 total As	0.98	0.98	0.99
NP4 As [III]	0.15	0.94	0.96
control total As	0.89	0.95	1.01
control As [III]	0.00	0.10	0.25

Table D.2. Arsenic concentrations in GL11 culture medium, initially given 1 mM arsenate and 10 mM lactate.

	As concentration (mM)		
time (hrs)	0	120	288
GL11 total As	1.03	1.00	1.07
GL11 As [III]	0.11	0.21	0.33
control total As	0.89	0.95	1.01
control As [III]	0.00	0.10	0.25

Table D.3. Values of arsenate reduction and lactate oxidation for NP4.

time (hrs)	2	24	72	144	168	216	240
NP4							
arsenate	0.70	0.70	0.21	0.12	0.10	0.06	0.06
arsenite	0.16	0.16	0.57	0.59	0.57	0.58	0.60
lactate	1.11	1.13	0.52	0.36	0.29	0.26	0.24
acetate	0.00	0.00	0.33	0.43	0.38	0.45	0.49
NP4 w/formaldehyde							
arsenate	0.68	0.70	0.71	0.71	0.69	0.67	0.74
arsenite	0.00	0.00	0.00	0.00	0.00	0.00	0.00
lactate	1.11	1.13	1.17	1.11	1.10	1.07	1.02
acetate	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(concentration in mM)							

Table D.4. Arsenic and carbon compound concentrations in media inoculated with GL11.

time (hrs)	2	24	72	144	168	216	240
GL11							
arsenate	0.56	0.64	0.68	0.64	0.66	0.61	0.61
arsenite	0.23	0.20	0.21	0.18	0.20	0.18	0.24
lactate	1.02	1.27	1.43	1.19	1.09	1.07	1.36
acetate	0.00	0.00	0.00	0.10	0.10	0.10	0.13
GL11 w/formaldehyde							
arsenate	0.65	0.73	0.75	0.70	0.71	0.64	0.74
arsenite	0.00	0.00	0.00	0.00	0.00	0.00	0.00
lactate	1.18	1.32	1.25	1.13	0.97	0.99	1.24
acetate	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(concentration in mM)							

Table D.5. Utilization of alternative electron acceptors by NP4.

Lactate concentration (mM)			
electron acceptor	**Sampling Date**		
	8-Jun	**15-Jun**	**21-Jun**
arsenate	**9.12**	**8.22**	**2.04**
nitrate	**8.42**	**7.46**	**3.28**
selenate	**8.30**	**8.01**	**7.20**

Table D.6. Utilization of alternative carbon sources by NP4.

C source	[As] (mM)	Sampling Date 8-Jun	15-Jun	21-Jun
lactate	V	0.84	1.04	0.05
	III	0.29	0.47	0.74
glucose	V	1.00	1.03	1.03
	III	0.19	0.31	0.20
benzoate	V	1.20	1.08	1.12
	III	0.20	0.30	0.22
acetate	V	1.02	0.86	0.96
	III	0.21	0.35	0.27

Figure D.1. DNA sequence for NP4.

TTTCGTTAGTGGCGCACGGGTGAGTAATGTATAGCTAACCTGCCCTTTAGTGG
GGGACAACAGTTGGAAACGACTGCTAATACCCCATATTCCTTCTTATCACAA
GGTAAGTTGGGAAAGATTTATTGCTAAAGGATGGGGCTTTATGGTATCAGCT
AGTTGGTGGGGTAACGGCCTACCAAGGCTATGACGCCTACCTGGTCTGAGAG
GATGATCAGGCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCAACGCCGC
GTGGAGGATGACGCATTTCGGTGTGTAAACTCCTTTTATAAGGGAAGATAAT
GACGGTACCTTATGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAAGGATGCGTAG
GCTGGAAATCAAGTCGAGAGTGAAATCCAACGGCTCAACCGTTGAACTGCTC
TCGAAACTGGTTACCTAGAATATGGGAGAGGTAGATGGAATTGGTGGTGTAG
GGGTAAAATCCGTAGATATCACCAGGAATACCGATTGCGAAGGCGATCTACT
GGAACATTATTGACGCTGAGGCATGAAAGCGTGGGGAGCAAACAGGATTAG
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CGCAAGATTAAAACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGATACACGAAGAACCTTACCTGGGCTTGATATCCT
AAGAATCCTGTAGAGATATGGGAGTGCTAGTTTACTAGAACTTAGAGACAGG
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AGACTGCCTTCGCAAGGAGGAGGAAGGTGAGGNCGACGTCAAGTCATCATG
GCCCTTATGCCCAGGGCTACACACGTGCTACAATGGCTAGGACAAAGAGAAG
CGATANTGNGAAGTGGAGCAAATCTTAAAACCTAGTNTCAGTTCGGATTGGA
GTCTGCAACTCGACTCCATGAAGCTGGAATCGCTAGTAATCGTAGATCAGAT
ATGNTACGGTGAATACGTTCCCGGGTCTTGTACTCAC

APPENDIX E: SAMPLE CHROMATOGRAMS

Figure E.1. Example chromatogram with 25-minute elution time.

NP4 culture media, time = 9 days.

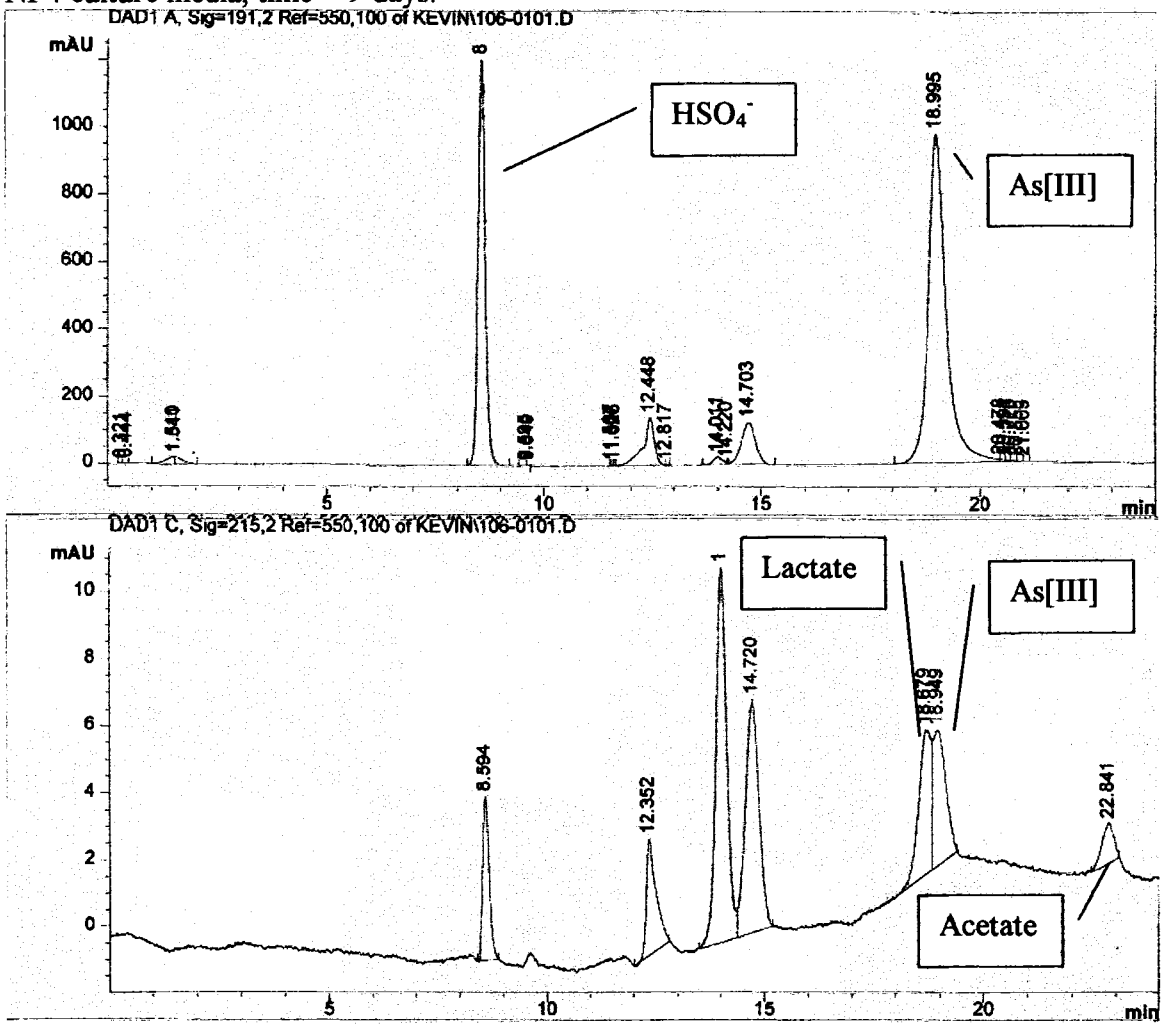
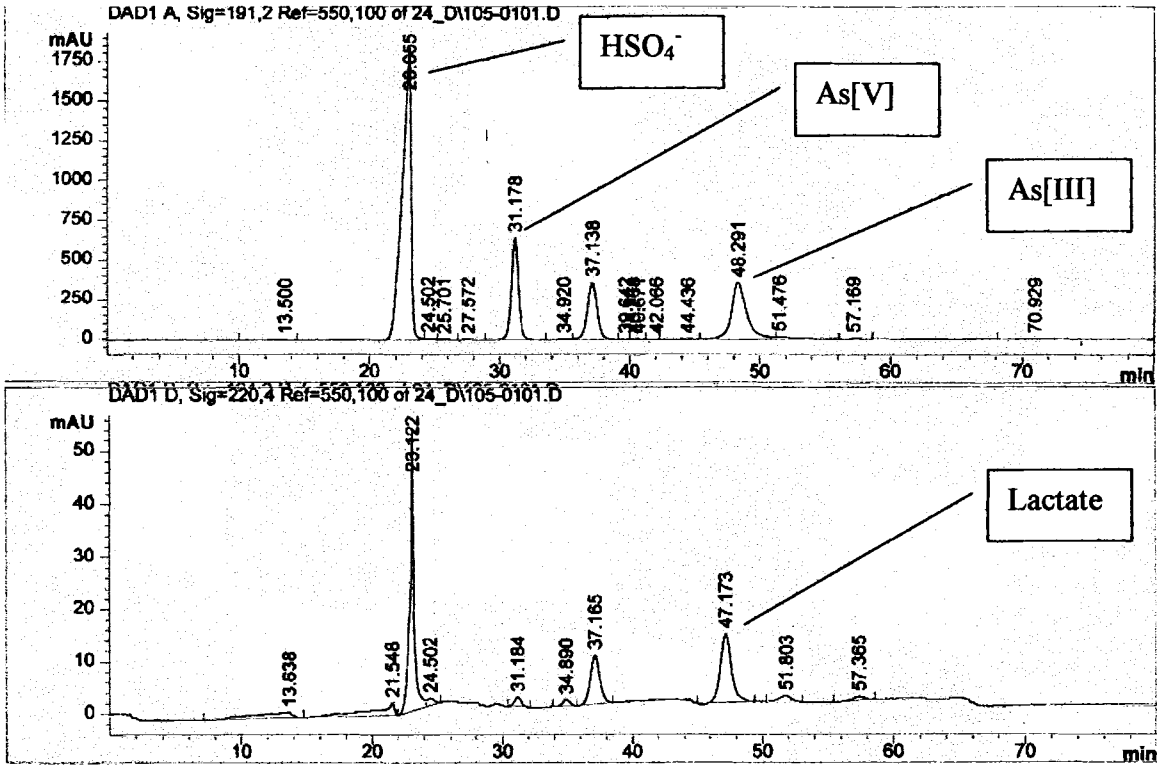


Figure E.2. Example chromatogram with 80-minute elution time.

NP4 culture media, time = 1 day.



BIOGRAPHY OF THE AUTHOR

Kevin A. McCaffery was born in Syracuse, New York on September 19, 1976. He was raised in Pompey, New York and graduated from Fabius-Pompey Central School in 1994. He attended Jefferson Community College in Watertown, New York and in 1997 received an Associate of Science degree with honors in Engineering Science. He then went on to study Environmental Science at the State University of New York at Plattsburgh, receiving his Bachelor of Science degree, *cum laude*, in 2000.

Kevin is a member of the American Society of Civil Engineers and completed the Fundamentals of Engineering examination in 2002. He was inducted into Chi Epsilon in 2001, Phi Kappa Phi in 1999, and the National Honor Society in 1993. Kevin is currently a practicing engineer with Kleinschmidt Associates of Pittsfield, Maine where he is a hydraulic engineer and hydrologist. Kevin is a candidate for the Master of Science degree in Civil Engineering from The University of Maine in August, 2002.