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Microbial Transformation of Arsenic and the Characterization of Clostridium sp. strain OhILAs

Edward Fisher

Microbial Transformation of Arsenic and the Characterization of *Clostridium* sp. strain OhILAs

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by

Edward Fisher

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Thesis Advisor: Dr. John Stolz, Ph.D.

Thesis Committee:
Dr. Partha Basu, Ph.D.
Dr. Kyle Selcer, Ph.D.

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Abstract

The impact of electron donor (acetate, pyruvate, lactate, formate, and hydrogen) and arsenate concentration (1, 5, 10, and 20 mM) on microbial arsenate reduction in freshwater sediments from the Ohio River, Pittsburgh PA was investigated. Hydrogen (with acetate as the carbon source) appeared to be the best electron donor and 5 mM sodium arsenate the most effective for both growth and orpiment (As_2S_3) production. A mesophilic, spore-forming, motile, low-mole %GC gram-positive bacterium was isolated from Ohio River sediments on medium with 20 mM lactate and 10 mM arsenate. Although pH 8 was optimal for growth, 16S rRNA gene sequence analysis indicated that this species is most closely related to *Alkaliphilus* species, (A. *crotonoxidans* 95%, *A. auruminator* 95%, *A. metaliredigenes*, 94%). A strict anaerobic this species can ferment fructose and lactate and respire arsenate and thiosulfate. This bacterium is unique in that it can tolerate high arsenate concentrations (>40 mM) and readily degrades the organoarsenical roxarsone.

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I. Introduction

A. Background

Arsenic (As), a trace element naturally present in soil, water and air, poses a threat to wildlife and human populations, due to its toxic properties. Although the earth's crust contains about 0.0001% arsenic, making it the 20th most abundant element [1], there are terrestrial and aquatic environments that contain ppm (microgram/kilogram) levels of arsenic. Since its discovery, arsenic has impacted human life, as it has been used in medicine, agriculture, pigments and paints and even electronics; however, its most notorious use is as a poison.

The most common forms of arsenic are arsine (-3) elemental As (0), arsenate (+5), and arsenite (+3) [2]. Arsenate and arsenite are the more common species in soil and water and are associated with oxic and anoxic conditions, respectively [3]. The chemical structures and oxidation states of these two compounds give rise to the mechanisms for toxicity. Arsenate ($HAsO_4^{2-}$ and $H_2AsO_4^{1-}$) is an analog of phosphate and disrupts the oxidative phosphorylation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase . Arsenite ($HAsO_3^{2-}$) binds with thiol groups, thus acting in the destabilization of proteins [4]. The disruptive properties of these compounds can have a carcinogenic effect on the skin, liver, kidneys, bladder, lungs and prostate and can cause diabetes and heart disease [5].

B. Arsenic in Groundwater

Oral exposure to arsenic has gained great attention over the last decade as contamination of drinking water has been demonstrated in many countries around the world. In Vietnam, studies of rural ground water samples indicated levels ranging from 1 to 3050 μg/L with an average of 159 μg/L [6]. In comparison, the U.S. standard, as determined by the United States Environmental Protection Agency, is 50 μg/L with legislation to lower standards to 10 μg/L in progress [7]. Considering the surging agricultural production, growth of rural populations, and the necessity to shift to groundwater tubewells as the source of drinking water, the number of people exposed to As may increase significantly[6]. This has lead researchers to draw a parallel between the situation in Vietnam to the devastating events in Bangladesh, where between 35 million and 77 million people are consuming As contaminated drinking water [8, 9]. The United States is not exempt from arsenic contamination concerns. Sites of elevated concentrations can be found in the Southwest, Northwest, and the Northeast, particularly in California, Idaho, and Massachusetts (Fig. 1). For example, the recent realization of an "arsenic crescent" along the coast of New England indicates that many subsurface wells in Maine, New Hampshire, Massachusetts and Connecticut may contain significant levels (high ppb) of arsenic [10].

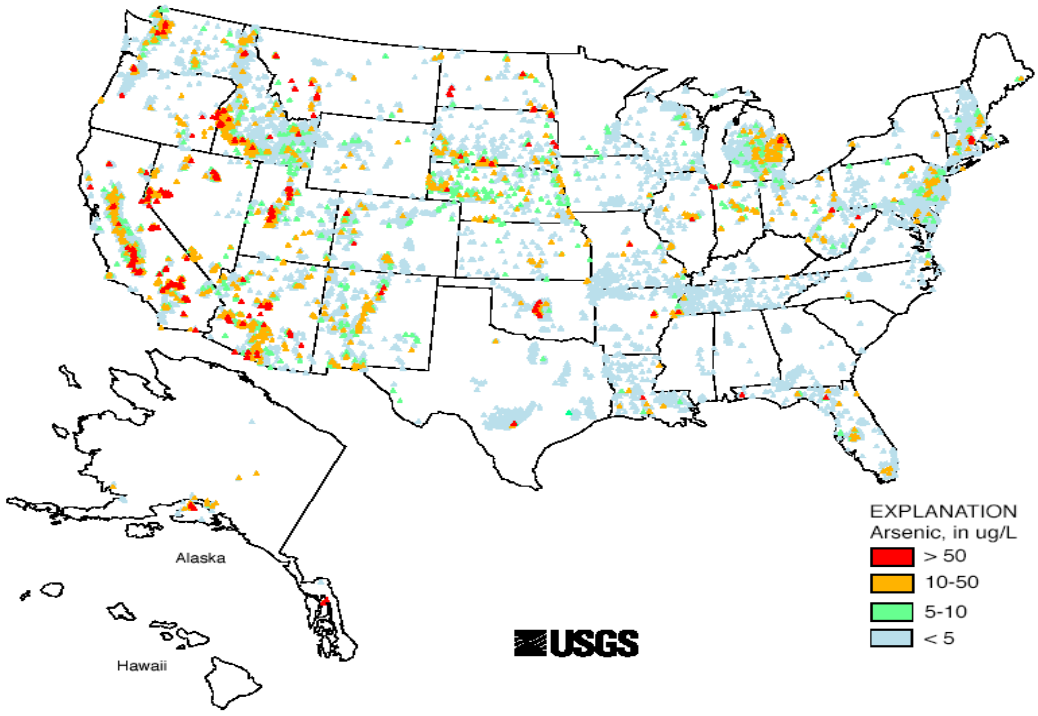

Figure 1. Occurrence of arsenic in groundwater in the United States from (USGS NAWQA http://water.usgs.gov/nawqa/).

C. Arsenic in food

The US Food and Drug Administration Total Diet Study estimates the average dietary intake of total arsenic as 0.03-0.04 mg per day. These fall below the safe level of arsenic ingestion of 130 µg inorganic arsenic set forth by the FDA. The leading dietary intake occurs with the consumption of seafood. The predominate forms of arsenic in fish, shellfish and bivalve mollusks are organic compounds such as arsenobetaine, arsenocholine, dimethyl arsenic, monomethyl arsenic, and the tetramethylarsonium ion. Organic species of arsenic are considered to be non-toxic as they are excreted unchanged, however there are limited studies on the toxicity of these organic species. Inorganic arsenic accounts for less than 1% of the total arsenic in seafood.

Table 1. Common arsenic compounds found in food.

Name	**Abbreviation**	**Chemical Formula**
Arsenite	As(III)	H_3AsO_3
Arsenate	As(V)	H_3AsO_4
Oxythioarsenic acid		H_3AsO_3S
Monomethylarsonic acid	MMA	$CH_3AsO(OH)_2$
Methylarsonous acid	MMA(III)	$CH_3As(OH)_2[CH_3AsO]_n$
Dimethylarsinic acid	DMA	$(CH_3)_2AsO(OH)$
Dimethylarsinous acid	DMA(III)	$(CH_3)_2AsO(OH)$
Trimethylarsine	TMA	$(CH_3)_3As$
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$
Arsenocholine	AsC	$(CH_3)_3As^+CH_2CH_2OH$
Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COO$-
Arsenic-containing ribosides	Arsenosugar X-XV, Arsenolipid	

The consumption of fish accounts for the greatest percentage of dietary intake of arsenic of Europe and North America, with rice following as a close second. However, in areas such as Bangladesh, rice accounts for 73% of the caloric intake. Arsenic in groundwater not only effects human life as a toxicant in drinking water, but also has deleterious effects on agricultural production and may lead to elevated concentration in the food products. The use of arsenic-contaminated groundwater for irrigation of crops has lead to elevated concentrations of arsenic in soils in Bangladesh, West Bengal, and other arsenic hotspots.

A study on the effects on the growth of rice as well as the uptake and speciation of arsenic in plants was conducted in a greenhouse, using two phosphate doses and seven different arsenic concentrations ranging from 0 to 8 mg As/L [11]. The increased arsenic concentrations were shown to decrease plant height, grain yield, the number of filled grains, grain weight and root biomass. The concentrations in rice grain increased but did not exceed the food hygiene concentration limit of 1.0 mg As/kg/L dry weight but were shown to increase in the root, straw, and husk of the rice plant. HPLC-ICP-MS analysis revealed that the inorganic arsenic species, arsenate and arsenite, were the predominant forms of arsenic found in the straw. It is important to consider that rice straw is used as cattle feed in many countries. Arsenic concentrations in rice straw is not covered by food hygiene regulations, thus increasing the potential for adverse health effects through the plant-animal-human pathway [11].

The use of organic compounds in chicken and pig feed is a current concern for both arsenic consumption and environmental impact. Roxarsone (3-nitro-4-hydroxybenzene arsonic acid) is fed to poultry to control intestinal parasites, thereby

improving feed efficiency and promoting rapid growth. Early studies indicated that little roxarsone was retained in the meat and most was excreted [12]. However, several recent studies [13, 14] have found higher levels (i.e., 0.5 – 1 ppm). Nevertheless, one would need to consume a significant amount of chicken on a regular basis for roxarsone to pose a risk [13]. The arsenic in chicken litter, however, is another story. From 1999 to 2000 seventy percent of the broiler industry employed roxarsone in concentrations of 22.7 to 45.4 g/ton [15]. Garbarino et al. [10] estimated that approximately $9x10^5$ kg of roxarsone was excreted unchanged in the manure, when considering that 8.3 billion broilers were grown in the United States during 2000.

Chicken manure is used as fertilizer or stockpiled in windrows [16]. The litter is applied at levels of 5 metric tons per hectare per application, introducing approximately 60-250g of arsenic per hectare. Studies have shown that rain and field irrigation can mobilize the roxarsone, while promoting growth of endemic microbial populations which may effect the stability of roxarsone. This may have severe implications on arsenic contamination of surface water and groundwater [15].

Garbarino et al. [10] examined poultry litter samples from various sites to identify the species of arsenic in the litter and the effects of composting on speciation and mobilization. Water leachates of litter samples (2g of litter per 20 ml of water) were used to determine extractability and speciation. One sample set showed that 90% of the total arsenic present was extractable with water and 91% of the extracted arsenic was in the form of roxarsone. This sample set differed from other samples collected at a different locale in which As(V) was the predominant arsenic species. This discrepancy suggests that poultry–litter management is important, as sunlight, elevated air temperatures and

precipitation promote the transformation of roxarsone to As(V) via photodegradation and biodegradation.

Composting experiments were performed on the samples comprised primarily of roxarsone. The roxarsone was degraded to As(V) in 3 to 4 weeks. The total concentration of arsenic extracted from the compost decreased from 90% in the initial reading to less than 40% by day 23. The use of sodium azide as a broad-spectrum microbial inhibitor suggested that biotic processes were important in the degradation of roxarsone. The roxarsone in the azide treated sample set was degraded below detection limits approximately 14 days longer than the untreated set suggesting microbial transformation of roxarsone for the untreated set.

More recently, Cortinas et al, [17] conducted a series of experiments on roxarsone degradation using sewage sludge slurries. They found that under anoxic conditions, roxarsone was rapidly (10 to 12 days for 1 mM roxarsone) transformed to 3-amino-4-hydroxybenzene arsonic acid (3A4HBAA). This degradation occurred under "methanogenenic" (no amendments) and "sulfidogenic" conditions (the slurries were amended with sulfate) but was inhibited under "denitrifying" conditions (the slurries were amended with nitrate). The further degradation of 3A4HBAA required prolonged incubations (25 - 200 days) and only then did they detect any arsenate or arsenite [17]. Although the microbial composition of the slurries was not determined, the conclusion of the study was that the degradation of roxarsone was primarily a biological process.

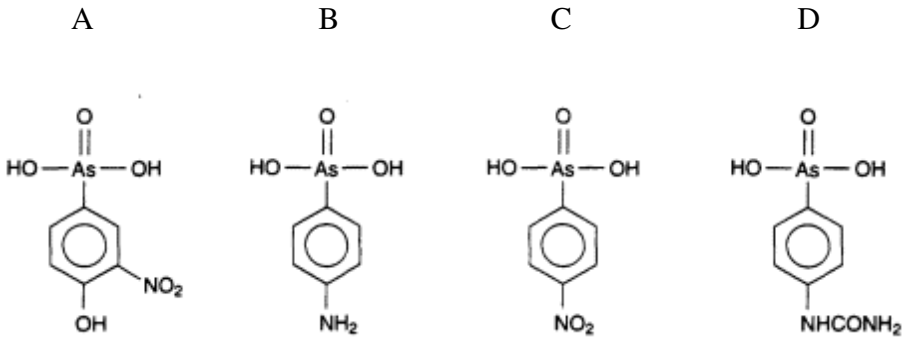

Figure 2. Feed additives containing arsenic (A) 3-nitro-4-hydroxybenzene arsonic acid, (B) p-arsanilic acid, (C) 4-nitrophenylarsonic acid, (D) p-ureidophenylarsonic acid.

D. Toxicity of Arsenic through Oral Exposure

Arsenic (As) contamination of drinking water is not the only means of arsenic ingestion. Oral exposures to arsenic occur through other means, such as accidental ingestion, suicide and homicide attempts, medicinal purposes, as well as food contents. Obviously human studies are rare, except for occupational, environmental, and acute accidental exposures. The nature of the human data lends itself to ambiguity concerning the chemical forms and concentrations of arsenic. Animal tests for specific target organs are more extensive, better characterized, and quantified. Inorganic arsenic is the most common, thus most studied form of arsenic. Collectively, human and animal studies provide insight to the toxic mechanisms and effects concerning lethality, specific systemic effects, immunological effects, neurological effects, reproductive effects, developmental effects, genotoxic effects and cancer.

Lethal doses in humans, occurring in a single ingestion event, ranged from 33-121 mg/kg for four individuals where the dose amounts were known. Immediate effects in cases resulting in death include vomiting, diarrhea, gastrointestinal hemorrhage, fluid loss and circulatory collapse [18]. Two individuals died from drinking water containing 110 ppm arsenic for a week, equivalent to approximately 2mg/kg/day [19]. Five children, ranging in age from 2-7 years, died from lifetime exposure to 0.05-.10 mg As/kg/day. The animal data is consistent with the human data. The LD_{50} for oral exposure of both As(III) and As(V) in rats and mice ranged from 15-175 mg As/kg [20].

Arsenic invokes a variety of systemic toxicities, including respiratory, cardiovascular, gastrointestinal, hematological, hepatic, renal, endocrine, dermal and ocular effects. Oral routes of arsenic exposure can lead to respiratory difficulties.

Respiratory distress, hemorrhagic bronchitis, and pulmonary edema were reported for doses of 8 mg As/kg. Cough, sputum, rhinorrhea, and sore throat coincide with repeated oral exposure of 0.03 to 0.05 mg As/kg/day [21]. Animal data for respiratory effects via the oral route is scarce.

Cardiovascular effects include myocardial depolarization, cardiac arrhythmias, and hypertrophy of the ventricular wall [22]. The vascular system is susceptible to long-term exposure evident in the expression of "Blackfoot disease". Necrosis and gangrene are the ultimate results of "Blackfoot disease", which is the loss of circulation in the hands and feet [23]. Alterations in vascular components have been demonstrated in rat studies subjected to oral doses of arsenic trioxide of 11mg As/kg/day for several weeks [24].

Gastrointestinal effects including nausea, vomiting, diarrhea, and abdominal pain coincide with acute high-dose exposure to inorganic arsenic. Acute exposure of 8mg/kg has prompted such adverse reactions. These symptoms are similar for long-term low-dose exposures (0.03-0.05 mg As/kg/day). The no observable adverse effect level for oral exposure and its effect on the GI tract is 0.01mg/kg/day [21].

Arsenic may have cytotoxic and hemolytic effects on the blood. Cases of anemia and leukopenia have been reported in acute, intermediate, and chronic exposures for doses of 0.05 mg As/kg/day or more [25]. Suppression of erythropoiesis has also been reported in some cases. However, hematological effects were not observed in all cases of exposure. Animal studies have shown a decrease in polychromatic erythrocytes in bone marrow of mice [22].

Hepatic effects, renal effects, musculoskeletal effects, and endocrine effects appear to be less severe or simply not as extensively studied. Hepatic effects include a swollen and tender liver for repeated exposures ranging from 0.01-0.1 mg/kg/day. Extensive exposure can lead to portal tract fibrosis. Renal studies in animals support the human data that the kidneys are not a major target organ. Musculoskeletal effects have not been studied. Studies pertaining to the endocrine system show an increase in incidence of diabetes mellitus [7].

One of the most common characteristics of arsenic ingestion is the visible dermal effect. Hyperkeratosis and warts or corns on the palms and soles are common. Chronic doses in humans of 0.01-0.1 mg/kg/day produced these skin abnormalities. A large study showed no effects to the skin at average doses of 0.00008 mg/kg/day. This number was used to calculate chronic oral MRL for inorganic arsenic at 0.0003 mg/kg/day. Skin cancer is also of great concern and will be considered below [23].

Neurological effects include encephalitis, characterized by headache, lethargy, mental confusion, hallucination, seizures and coma. This is common for acute high dose exposures of 2mg/kg/day [19]. Lower level doses of 0.03-0.1 mg/kg/day can cause numbness in feet and hands, followed by a pins and needles sensation. Sensory and motor neurons are affected by the toxicant. Histological examinations reveal a demyelination and dying of axons. Recovery is possible after exposure has ceased, but it is often slow and incomplete. Effects are not evident in populations exposed to 0.006 mg/kg/day [26].

The data for reproductive effects in humans is limited to one study. A 17-year-old mother consumed 0.39 mg/kg As III and suffered from acute renal failure within 24

hours. She delivered an infant weighing two-pound 7 ounces that died 11 hours after birth [27].

The studies on developmental effects in humans are limited to the case previously discussed. The studies are inconclusive in humans; however, animal studies are more detailed. Rat studies implicate high doses of inorganic arsenic in adverse developmental effects. Post-implantation loss and decrease in viable fitness per litter are indicative of high dose studies. Reduced body weight and skeletal effects were also associated to treatment with arsenic trioxide. The most sensitive test animal was the rabbit, which had shown developmental problems at 1.5 mg/kg/day. An interesting event with arsenic ingestion is that the death of the mother was found at the same or lower doses as the developmental effects of the fetus, suggest that the fetus is not more susceptible than the mother [28].

Genotoxic effects are characterized by chromosomal aberrations and p53 mutations in arsenic related skin cancer. Evidence from studies is limited. However, cancer studies are more complete and will be the focus of this section [29].

There is significant evidence from epidemiological studies implicating arsenic ingestion as a cause of skin cancer. Skin lesions, which are multiple squamous cell carcinoma, develop from the hyperkeratotic warts and corns previously discussed. Basal cell carcinoma is also a result of exposure.

The EPA has reviewed the current body of research on arsenic ingestion and based risk assessments on a 1968 study by Tseng et al, [30]. The study population and control groups were large (40,000 people and 7,500 respectively). The groups, including males and females were grouped into high medium and low exposure groups. Based on

this study the EPA was able to calculate the average daily doses of $1x10^{-4}$ to $1x10^{-7}$ mg/kg/day, which correspond to an excess cancer risk. This study has been met with great skepticism in regards to its relevancy to the U.S. population. Studies in the U.S. have not shown an increase in frequency of skin cancers at levels around 0.1-0.2 ppm [31].

Internal evidence of cancer is evident in many studies. Liver, bladder, kidney, lung and prostate tumors have been seen in epidemiological studies. A dose-response relationship was established between levels of arsenic and mortality. The elevated mortality for each cancer was 202 liver cancers, 304 lung cancers, 202 bladder cancers and 64 kidney cancers [7].

Studies of cancers in populations exposed to arsenic in drinking water in the U.S. are inconsistent with the extensive, large population studies in Taiwan, Mexico, and Chile. This may be due to a smaller population size of study, lower overall doses, and health and nutritional aspects among other variables. Animal studies using arsenate and arsenite produced little evidence of any type of cancers. In fact, some studies in mice have shown a decrease in the incidence of some tumor types with arsenic exposure. These difference from humans and animals maybe a species specific-phenomenon [32].

E. Arsenic Use

The implications of arsenic as a health hazard necessitated the research to examine the methods of its accumulation and mobilization. Anthropogenic sources such as Cu-smelting, oil and coal combustion, runoff from mine tailings, glassware production (decolorizing agent), electronics industry, (semiconductor production), ore production, ammunition factories (hardening of projectiles), dyes, wood preservatives, herbicides,

pesticides, pyrotechnics, pharmaceuticals, and agricultural feed additives impact the bioavailability of As [5]. The United States has taken great steps in finding alternatives to arsenic containing products.

The former Soviet Union, France, Mexico, Germany, Peru, and the United states are the highest arsenic producing countries, accounting for about 90% of the world production [33]. Until the 1970's, pesticides accounted for 80% of the As production in the form of inorganic salts. Inorganic arsenic species have been applied to crops such as citrus, cotton and potatoes. The long-term use of As in agriculture in the form of lead and calcium arsenate has led to soil concentrations exceeding 100mg kg^{-1}. Today only 50% of the arsenic used is attributed to pesticides, of which 90% are in organic forms. Another 30% was used in wood preservatives [5]. Wolmanizing with copper-chromated arsenic was a standard wood treatment. However, after studies indicated that the arsenic could leach from the wood, an agreement was reached between the EPA and the wood industry to voluntarily eliminate the use of CCA in wood for home applications and children's play areas by December 31, 2003 (http://www.epa.gov/oppad001/reregistration/cca/).

F. Natural Sources of Arsenic

Most of the arsenic in the environment (more than 99%) is found in rock, due to its ability to substitute for Si, Al, or Fe in silicate minerals. Table 2 gives the ratio of As concentrations in natural reservoirs with respect to soil [34].

Table 2. The ratio of As concentrations in natural reservoirs with respect to soil.

Reservoir	**Approximate ratio with respect to soil**
Rocks	25,000
Oceans	4
Soil	1
Biota (plants, man, microbes)	.0005
Atmosphere	.000001

Sedimentary rocks contain higher concentrations (5-10mg kg^{-1}) of As than igneous or metamorphic rocks. Sands and sandstones have low As concentrations. Sulfide minerals, oxides, organic matter and clays are large constituents of argillaceous deposits and account for higher As concentrations. Shales and coals often contain high concentrations of As and some of the highest concentrations have been found in Fe-rich rocks. Mining and fossil fuel utilization mobilize the arsenic from these sediment types.

The concentration of arsenic in igneous rocks is generally low, with volcanic glasses containing of about 6.0mg kg^{-1} [34]. Overall, there is little difference in As contents between igneous rock types. Volcanic rock, despite low concentrations of As, are often implicated in the production of high As waters. Metamorphic rocks reflect the concentrations of their igneous and sedimentary precursors.

The mineralogy of the sediments directly effects the concentration of As(V) and As(III) as both forms are adsorbed onto and desorbed from mineral substrates. Arsenic is a major constituent of more than 200 minerals, most of which are ore minerals (Table 3). Some of the most abundant arsenic containing minerals are arsenopyrite, arsenian pyrite, realgar and orpiment, yet a significant flux of soluble arsenic appears to stem from the elevated concentrations in hydrous metal oxides [35]. Arsenate has a high affinity for

insoluble compounds like ferryhydrite and other inorganic oxides. Arsenite is less reactive with such substrates and, therefore, exhibits greater solubility [3, 26]. Arsenite; however, displays a high affinity for Goethite (α-FeOOH) [36].

Iron-containing sediments are significant in that the biogeochemical cycling of iron and arsenic is coupled [37]. In reduced environments, the dissolution of iron oxyhydroxides will ultimately release arsenic. This occurs through biotic (dissimilatory reduction of iron) and chemical means. Under anoxic conditions, Fe(III) (ferrous) oxides can be reduced to Fe^{2+}. In oxic environments, low pH enhances the adsorption of arsenic to iron oxides, but as pH increases arsenic, desorbs from these substrates [35].

Table 3. Common arsenic minerals.

Mineral	**Composition**	**Occurrence**
Elemental Arsenic	As	Hydrothermal veins
Niccolite	NiAs	Vein deposits
Realgar	AsS	Vein deposits, deposits from hotsprings
Orpiment	As_2S_3	Hydrothermal veins, hot springs, volcanic sublimation product
Cobalite	CoAsS	Metamorphic rocks
Arsenopyrite	FeAsS	Mineral veins
Tennanite	$(Cu,Fe)_{12}As_4S_{13}$	Hydrothermal veins
Enargite	$CuAs_3S_4$	Hydrothermal veins
Arsenolite	As_2O_3	Formed by the oxidation of As minerals
Scorodite	$FeAsO_4.2H_2O$	Secondary mineral
Annabergite	$(Ni,Co)_3(AsO_4)_2.8H_2O$	Secondary mineral
Hoernesite	$Mg_3(AsO_4)_2.8H_2O$	Secondary mineral
Haematolite	$(Mn,Mg)_4Al(AsO_4)(OH)_8$	Secondary mineral
Conichalcite	$CaCu(AsO_4)(OH)$	Secondary mineral
Pharmacosiderite	$Fe_3(AsO_4)_2(OH)_3.5H_2O$	Oxidation product

G. Arsenic in the Environment

Elemental arsenic, atomic number 33, is a gray, crystalline material. Its atomic weight is 74.92, its density is 5.727 and its melting point is 817ºC [38]. Its chemical properties are similar to phosphorous.

Chemical factors, such as dissolved oxygen concentration, sediment type, pH, reducing conditions, and the presence of sulfide and carbonate, play an integral role in determining the species and mobility of arsenic. In stratified lakes, such as Mono Lake California, oxygen levels influence arsenic speciation as arsenate predominates in oxic environments while arsenite is more common in anoxic conditions as is thermodynamically favorable in each scenario [39, 40]. Anomalies do occur where

arsenate is found in greater concentrations in anoxic regions of lakes as was demonstrated in the meromictic Lake Pavin in France [26]. This conflicting report emphasizes the complexity of the chemical and biotic factors that drives elemental speciation.

Most trace-metal toxicants occur as cations in solution (Pb^{2+}, Cu^{2+} , Ni^{2+} , Cd^{2+}, Co^{2+}, Zn^{2+}) and become insoluble as pH increases. Near-neutral pH typical of most groundwaters severely limits the solubility of these cations. In contrast, most oxyanions such as arsenate become more mobile as pH increases. But As is set apart from other oxyanions, such as selenate, chromate, molybdate and vandate, because of its increased mobility under reducing conditions. The two most important triggers for the speciation of arsenic are high pH (>8.5) and strong reducing conditions at near neutral pH. $H_2AsO_4^-$ is dominant at low pH (<6.9) and $HAsO_4^{2-}$ at higher pH under oxidizing conditions. Under reducing conditions at pH less than 9.2, the uncharged arsenite species is most prevalent [34] (fig 3).

Speciation is also affected by reductants such as sulfide and oxidants such as Fe(III) and Mn(IV). Under acidic conditions sulfide will reduce arsenate into arsenite which in turn can be precipitated into orpiment [4]. Orpiment and other arsenic sulfides may leach arsenic upon carbonation [41]. Conversely, arsenite can be reoxidized to arsenate by Fe(III) and Mn(IV). Polyvalent anions such as phosphate may increase mobile levels of As, as the element is displaced from humic acids and hydrous oxides [42].

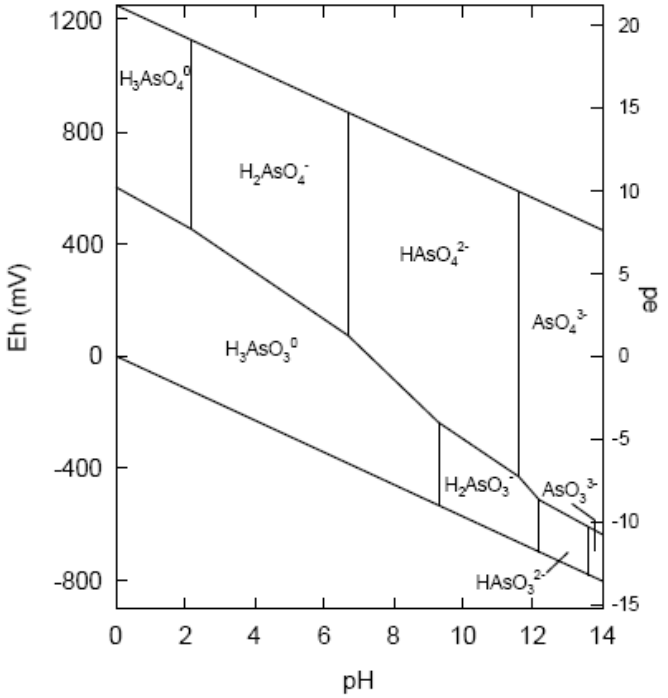

Figure 3. The effect of pH and Eh on the speciation of arsenic taken from [34] Smedley and Kinniburgh.

H. Global Scale Cycling of Arsenic

The microbial influenced speciation and mobilization of arsenic is only a component of the total biogeochemical cycle of the trace element. Estimates have been made for the As cycle on the global scale. The earth's crust is estimated to contain 4.01 x 10^{16} kg of arsenic. Of this, 1.715 x 10^{7} kg/year is liberated from the lithosphere by natural events, which are dominated by volcanic eruptions. The total input into the lithosphere from sedimentation and subduction is estimated to be 8.46-14.22 x 10^{7} kg/year [5].

The atmosphere stores an average of 1.74 x 10^{6} kg of arsenic [43]. This unevenly distributed among hemispheres due to the differences in land mass and highly industrialized centers. The arsenic remains in the atmosphere for an average of 7-10 days. Inputs for arsenic into the atmosphere is calculated to be 2.8 x 10^{7} and 8.36 x 10^{7} kg/yr for the northern and southern hemispheres respectively, each being dominated by anthropogenic sources [43]. Of these anthropogenic sources, coal combustion and copper smelting account for 60%. Approximately 20,000 t/yr of As is being deposited from the troposphere in the northern hemisphere and 7,600 t/yr in the southern. Deposition in oceans is an estimated 2,000-5,000 t/yr as dissolved input and 1,300-3000 t/yr as particualte As matter [43].

Soil concentrations of As are estimated to average 7.5 mg/kg [5]. This varies tremendously and soils rich in organic carbon contain the highest concentrations. When considering arsenic soil concentrations, it is important to note that the soil acts as a biogeochemical barrier and accumulates the element. In soils, As mainly exists as arsenate and readily binds to clay minerals, Fe and Mn oxyhydroxides/hydroxides, and

organic material. (As) is retained in the soil for an average of 1,000-3,000 years in moderate climates [44]. Retention time is much shorter in tropical environments. The inputs of As in soil are a result of pesticides, agricultural feed additives, as well as mining and smelting activities.

Arsenic is an important chemical component of water. The As constituent of freshwater is estimated to be 0.1µg/l. The As concentration in groundwater is higher with an average of 0.5-0.9µg/l (17). A normal range of 0.01-800µg/l takes into account the highly mineralized areas. The pH of aquatic environments plays an integral in the concentrations of arsenic in water. Acidic conditions lead to higher concentrations of arsenic mainly from dissolution from sediments (25). Dissolved oxygen concentrations also influence As concentrations as suboxic and anoxic waters contain more As than oxic waters. This is also is related to sediments as reduction of As containing minerals is significant. Marine waters contain an average of 1.1-1.9 µg/l at a salinity of 36% (17). Phytoplankton influence As concentrations and speciation by taking up arsenate and phosphate and releasing arsenite, methylarsenate and dimethylarsenate. A blackbox representative of the global As cycle as shown in Figure 4.

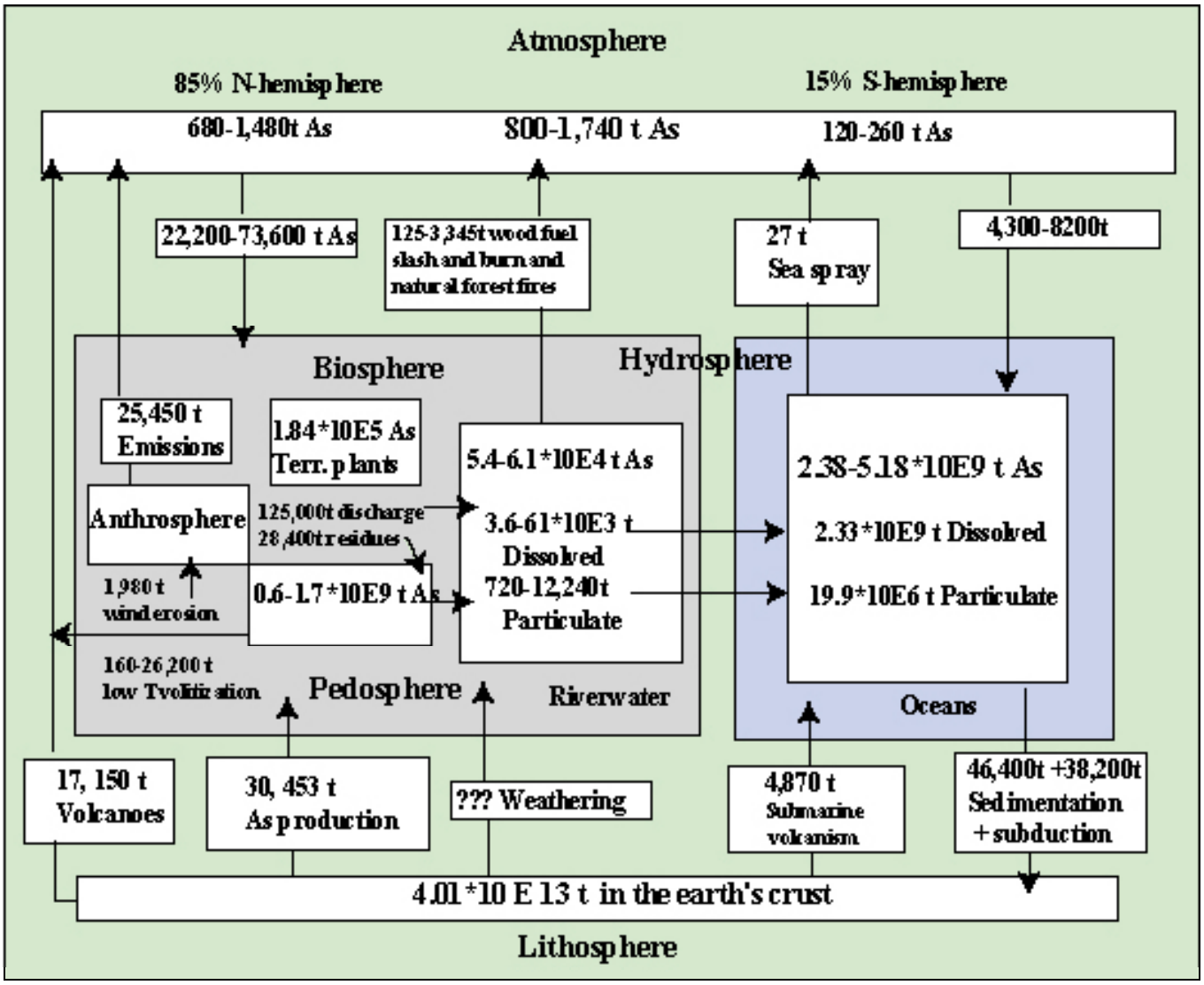

Figure 4. Global scale cycling of arsenic from [5] Matshullat, J.

I. Indirect Biotic Transformation of Arsenic

Arsenate's affinity to hydrous ferric oxides, increases the significance of dissimilatory reduction of iron as a means of As mobilization [45]. *Shewanella Alga* strain BrY and *Desulfosporus auripigmentum* strain OREX-4 mobilize arsenate from mineral scorodite ($FeAsO_4$ $2H_2O$) through respiration of Fe [46]. *Sulfurospirillum barnesi* strain SES-3 displays dissimilatory reducing capabilities for both Fe(III) and As(V) [47]. The reductions of iron III oxides hinder the adsorption of arsenate to these reduced sediments, allowing for the further reduction of arsenate to arsenite. *Desulfitobacterium sp*. Strain GBFH is capable of reducing As(V), Fe(III), Se(VI), Mn(IV) and oxidized sulfur species [48]. In culture, the sequential reduction of As(V) and oxidized sulfur forms orpiment [39]. These indirect biotic mechanisms for the mobilization of arsenic are complemented by more direct mechanisms.

J. Arsenic Reduction for detoxification

The early earth atmosphere, lacking in oxygen, would have contained little arsenate, therefore arsenite efflux pumps would have been sufficient for arsenic detoxification. However, introduction of oxygen into the atmosphere by cyanobacteria 3.8 billion years ago provided a selection pressure for the convergent evolution of at least three different arsenate reductases [49]. Arsenate oxyanions are transported into the cell through phosphate transport membrane systems such as the Pit and more phosphate specific Pst systems of *E. coli*. Arsenite is transported into the cells by aquaglyceroporins such as GlpF in E. coli and Fps in yeast [50, 51]. Once inside the cell the arsenate must be reduced to arsenite for efflux from the cell. At least three different resistance systems

have evolved convergently for arsenic detoxification. Bacteria such as *Escherichia coli, Klebsiella pneumoniae, Pseudomoas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis* utilize an ArsC to reduce As(V) to As(III) for efflux from the cell [52, 53]. The proteins which provide the mechanism of arsenic resistance are encoded by *ars* operons. These operons may be plasmid or chromosomal determined systems.

E. coli, a gram-negative bacterium, benefits from a chromosomal encoded *ars* RBC operon and may also have a plasmid (R733 plasmid) *ars* RDABC operon [54]. ArsC is a 14.8 kDa arsenate reductase complemented by ArsR and ArsD, the DNA binding proteins involved in regulation of the *ars* operon. ArsA is a soluble ATPase subunit activated by the presence of heavy metals. Ars A associates with the integral membrane protein ArsB. ArsB functions as an arsenite specific transporter expelling the toxic compound from the cell. Cys 12, a redox active cysteine residue of the arsenate reductase, binds to arsenate, which is reduced to arsenite by reduced glutathione [55]. There are three glutaredoxins, which act in the catalytic cycle; however, it is believed that Grx2 is the preferred electron donor, as it has the highest affinity for Ars C [54].

Plasmid pI258 of *Staphylococcus aureus* is representative of gram positive plasmid encoded systems. This *ars* operon shares low homology (10%) with the plasmid R773 encoded system for *E. coli* but utilizes similar Cys residues [56-60]. The operon is comprised of a contiguous *arsB* and *arsC* but no *arsA*. The source of electrons for arsenate reduction in pI258 system is reduced thioredoxin. The pI258 proteins are similar to the family of low molecular mass protein phosphotyrosine phosphatases found in microbes animals and humans [60, 61].

A third type of *ars* operon is represented by the yeast *Saccharomyces cerivisiae*. *ARR1*, *ARR2*, and *ARR3* are three contiguous genes conferring arsenic resistence [62]. *ACR1* is similar to arsR of bacteria and produces a transcription regulator. *ACR2* is the bacterial equivalent to the *arsC* gene and encodes for an arsenate reductase while *ACR3* encodes the membrane efflux protein[62].

K. Respiratory Arsenate Reduction

The ability for prokaryotes to use arsenate as a terminal electron acceptor in the oxidation of organic compounds or hydrogen for purposes of energy production was first demonstrated by Amann et al, [63] over a decade ago. Since then, over two dozen species have been described (Table 4). The diversity in metabolic capabilities is as broad as the variation in habitats in which arsenic respiring organisms have been found (Table 4). Electron donors include hydrogen, acetate, lactate, pyruvate, butyrate, malate, ethanol, and glycerol. Many arsenate-respiring organisms are capable of employing various electron acceptors in addition to As(V). Thus, with the possible exception of MLMS-1, the haloalkaliphilic microbe isolated from Mono Lake, these organisms are not obligate arsenate respirers.

Table 4. Arsenate Respiring Prokaryotes

Prokaryote	Isolated from	Phylogeny	Acceptors	Donors[a]
Bacillus macyae JMM-4	gold-mine	low G+C Gram+	NO_3^-	A,L, P, M, G
B. selenitireducens	Mono Lake, CA	low G+C Gram+	Se(IV), NO_3^-, Fe(III)	L,P
B. arsenicoselenatis	Mono Lake, CA	low G+C Gram+	Se(VI), NO_3^-, Fu	L,M,C
Desulfitobacterium sp. GBFH	lake sediments	low G+C Gram+	Se(VI), NO_3^- ,Fe(III), S	F, L, P, Fu
Desulfosporosinus auripigmenti	lake surface sediments	low G+C Gram+	Fumarate, $SO_4^=$, S, Thio	HA, L,P,M
Desulfosporosinus Y5	lake sediments	low G+C Gram+	$SO_4^=$, thio, Fe(III), NO_3^-	Bz, Ph,,Tol,, Sy, Fe
Bacillus sp. HT-1	hamster stool	low G+C Gram+	ND	HA
Clostridium sp. OhilAs	river sediment	low G+C Gram+	$SO_4^=$, NO_3^-	A,L,P
SLAS-1	Searles Lake, CA	Halanaerobacteria	As(V)	L
Chrysiogenes arsenatis	gold-mine	Chrysiogenetes	NO_3^-	A, L, P, Fu, M, S
HGM-K1	mine	Aquificales	Se(VI)	L
Deferribacter desulfuricans	hydrothermal vent	Deferribacteres	NO_3^-, $SO_4^=$	L
Citrobacter sp. str. TSA-1	termite hindgut	γ-Proteobacteria	Se(IV)	L, HA
Shewanella sp. str. ANA-3	As-treated wood	γ-Proteobacteria	O_2, Fu, NO_3^-, Fe(III), Mn(IV)	L,P
Wolinella sp. str. BRA-1	bovine rumen fluid	ε-Proteobacteria	ND	HA
Sulfurospirillum barnesii	Se-contaminated water	ε-Proteobacteria	Se(VI), NO_3^-, Fe(III), Fu, TMAO, DMSO	HA, L, P, Fu, C, S
S. arsenophilum	Aberjon sediments	ε-Proteobacteria	NO_2^-, NO_3^-, Thio	L,P,HA,Fu
S. deleyianum	freshwater mud	ε-Proteobacteria	NO_2^-, NO_3^-, Thio, TMAO, DMSO	P,F,Fu
S. multivorans		ε-Proteobacteria	NO_2^-,Se(VI)	L,P,F,Fu
S. halorespirans		ε-Proteobacteria	NO_2^-, NO_3^-	L,P,F,Fu
S. carboxydovorans		ε-Proteobacteria	NO_2^-, NO_3^- Thio, Se(VI), DMSO	L,P,F,Fu
Desulfomicrobium sp. Ben-RB	gold mine	δ-Proteobacteria	$SO_4^=$	L,P,F,Fu
MLMS-1	Mono Lake, CA	δ-Proteobacteria	Obligate As(V)	H_2S
Pyrobaculum aerophilum	hot spring	Crenarchaeota	Se(VI/IV), O_2NO3-, Thio	B, H_2
Pyrobaculum arsenaticum	hot spring	Crenarchaeota	Se(VI), Thio	B, H_2

[a]Donors: A: acetate, HA: H_2 + acetate, F: formate, L: lactate, P: pyruvate, M: malate, S: succinate,
B, butyrate, C: citrate, G: glutamate, Fu: fumarate, Bz: benzoate, Ph:phenol, Tol: toluene, Sy: syringic acid, Fe: ferulate
ND: not determined

Anaerobic respiration with alternative electron acceptors such as nitrate, sulfate, Mn(VI) and Fe(III), is well established, dissimilatory arsenate reduction; however, was only discovered within the last decade [63]. The process is restricted to prokaryotes in which arsenate is used as the terminal electron acceptor under anaerobic or microaerobic conditions [39]. The As(V)/As(III) oxidation/reduction potential is +135 mV, which is thermodynamically favorable for energy production when coupled to the oxidation of organic substrates or hydrogen. Table 5 is a comparison of the free energies for various electron acceptors coupled to H_2 oxidation. It is important to note that the free energy for arsenate reduction is significantly greater that sulfate reduction.

Table 5. Comparison of free energies for various electron acceptors coupled to hydrogen oxidation [64].

Thermodynamic Reaction	ΔG kcal/mol e^-
$1/4\ O_2(g) + 1/2H_2 \rightarrow 1/2\ H_2O$	-23.55
$1/2\ MnO_2(s) + H^+ + 1/2\ H_2 \rightarrow 1/2\ Mn^+ + H_2O$	-22.48
$1/5\ NO_3 + 1/5\ H^+ + 1/2\ H_2 \rightarrow 1/10\ N_2(g) + 3/5\ H_2O$	-20.66
$1/2\ SeO_4^{2-} + 1/2H^+ + 1/2H_2 \rightarrow 1/2\ HSeO_4^{2-} + 1/2\ H_2O$	-15.53
$1/8\ NO_3 + 1/4\ H^+ + 1/2H_2 \rightarrow 1/2\ H_2O$	-13.42
$1/3\ CrO_4^{2-} + 5/3\ H^+ + 1/2\ H_2 \rightarrow 1/3\ Cr^{+3} + 3H_2O$	-10.76
$Fe(OH)_3(am) + 2H^+ + 1/2\ H_2 \rightarrow Fe^{+2} + 3H_2O$	-10.4
$1/4\ HSeO_3^- + 2H^+ + 1/2\ H_2 \rightarrow 1/4\ Se + 3/4\ H_2O$	-8.93
$1/2\ H2AsO4- + 1/2\ H_2 \rightarrow 1/3\ As + 1/2\ H_2O$	-5.51
$1/3\ H_3AsO_3 + 1/2\ H_2 \rightarrow 1/3\ As + H_2O$	-2.58
$1/8\ SO_4^{2-} + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ HS^- + 1/2\ H_2O$	-0.10

Dissimilatory arsenate reducing prokaryotes, or DARPs, are phylogenetically diverse, with representatives of the gamma, delta, and epsilon Proteobacteria, low G+C gram positive bacteria, thermophilic Eubacteria and Crenarchaea [65].

Sulfurospirillum arsenophilus and *S. barnesii*, members of the sulfurospirillum clade of the epsilon proteobacteria, were the first microbes found to reduce arsenate for dissimilatory purposes. *S. arsenophilum* strain MIT-13 was isolated from arsenic contaminated sediments of a Superfund site in Woburn, MA [63]. This bacterium couples the reduction of arsenic to the complete oxidation of lactate to CO_2 [47]. MIT-13 may also use H_2 +acetate, pyruvate, and fumarate as electron donors. Electron acceptors also include nitrate and fumarate. *S. barnesii* strain SES3 was isolated from a selenate respiring enrichment of a marsh in the Stillwater Wildlife Management Area of western Nevada [66]. SES3 may grow on acetate, pyruvate, lactate as well as H_2 as electron donors and a variety of electron acceptors including selenate, nitrate, nitrite, fumarate, Fe(III), thiosulfate, elemental sulfur, DMSO, and trimethylamineoxide [67].

Desulfosporosinus auripigmenti strain OREX-4, isolated from the freshwater sediments of Upper Mystic Lake in Winchester, MA is a gram positive which metabolizes compounds used by sulfate reducers. H_2, H_2+acetate, butyrate, pyruvate, lactate, malate, glycerol and ethanol are electron donors metabolized by OREX-4, while electron acceptors include sulfate, sulfite, thiosulfate, As(V), and fumarate. Orex-4 was the first sulfate reducing bacterium shown to grow with arsenate as the terminal electron acceptor [64]. This fact gave evidence to the physiological diversity of dissimilatory arsenic reducing bacteria.

Two bacillus species further supported the diversity of arsenic reducers as they were isolated from Mono Lake, California, an alkaline, hypersaline, arsenic rich water body [68]. *Bacillus arsenicoselenatis* strain E1H, a spore forming rod which can reduce Se(VI) to Se(IV) and *B. selenitireducens* strain MLS-10, a nonspore forming rod that reduces Se(IV) to Se(0) are members of the low G+C gram positive group of bacteria. Strain E1H is able to use lactate, starch, malate and citrate as donors coupled to the reduction of arsenate, fumarate, nitrate, and Fe(III). Strain MLS-10 utilizes lactate, pyruvate, galactose, as electron donors and O_2 (10% or less), arsenate, fumarate, trimethylamine oxide, nitrate and nitrite as terminal electron acceptors.

Chrysiogenes arsenatis is the first bacterium shown to couple arsenate reduction to the oxidation of acetate [69]. The arsenate reductase from this species has been purified and characterized and differs from the detoxifying arsenate reductases, ArsC, of *E. coli* and *S. aureus*. The arsenate reductase is a soluble enzyme located in the periplasm and is comprised of two subunits of different molecular masses, ArrA (87kDa) and ArrB (29kDa) that form a heterodimer. Cofactors of the enzyme include molybdenum, iron-sulfur centers, and zinc. The genes are induced by the presence of arsenate, which is the only electron acceptor used by the enzyme [70]. In contrast the ArsC proteins are comprised of a single subunit, which is smaller than any of the subunits of the arsenate reductase of *C. arsenatis*. Also, the presence of cofactors and location of the enzymes also differ as the dissimalatory arsenate reductase of *C. arsenatis* contains cofactors and is located in the periplasm wheras the detoxifying arsenate reductases are localized in the cytosol and lack cofactors [70].

A similar enzyme was recently identified from the haloalkaliphilic bacterium, *Bacillus selenitireducens* strain MLS10. The arsenate reductase of MLS10 is a membrane bound heterodimer consisting of a 110kDa ArrA and a 34kDa ArrB. Metal analysis of the holoenzyme and sequence analysis of *arrA* (the gene that encodes the catalytic subunit) indicate it is a member of the DMSO reductase family of molybdoproteins [71].

L. Clostridium sp. strain OhILAs

Clostridium sp. strain OhILAs is a low mole% GC Gram +, spore forming rod isolated from the sediments of the Ohio River using medium containing 10 mM As(V) and 20 mM Lactate (A. Dawson unpublished). It was purified through tindalization and serial dilution. It may form short chains of several cells and has small terminal spores (Fig. 5). Phylogenetic analysis using the 16S rRNA gene indicated that it was most closely related to alcaliphilic species of clostridia (Fig. 6), namely *Alkaliphilus auruminator* (95%), as well as mesophilic species like *Clostridium aceticum*, *C. formicoaceticum* and *C. felsineum*. This specie's preliminary characterization was initiated by A. Dawson, who found it to produce arsenic trisulfide in medium containing sulfate and arsenate.

Members of the genus Clostridium, which means small spindle, are low G+C, Gram positive rods, which form endospores. Clostridia may be motile or nonmotile. Most are chemoorganotrophic, while some are chemoautotrophic or chemolithotrophic. Clostridia may be saccharolytic, proteolytic, neither or both. Metabolism of carbohydrates, alcohols, amino acids, purines, steroids, and other organic compounds are

common to the genus. Most Clostridia are obligately anaerobic, with optimum growth occurring at a pH between 6.5 and 7 and temperatures between 30ºC and 37ºC. The mol G+C of the type species *Clostridium butyricum* is 27-28 (tm). Others range from 22-55 (tm). Clostridia are commonly found in soil, sewage, marine sediments, animal and plant products, as well as the intestinal tract of insects and vertebrates [72].

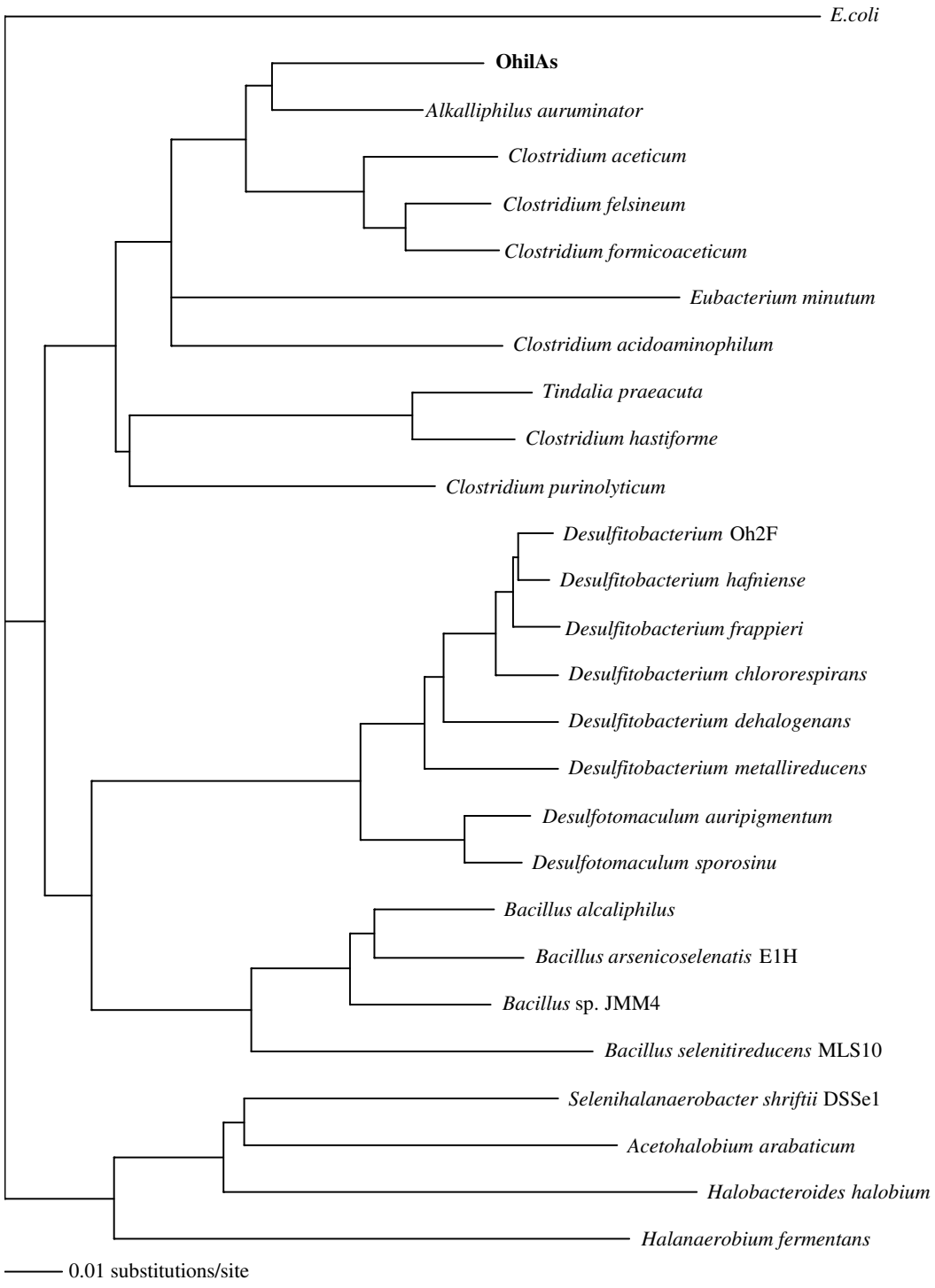

Figure 5. Phylogenetic tree (Neighbor Joining) showing the relatedness of *Clostridium sp.* strain OhILAs to *Alkaliphillus* species and other *Clostridium* species.

II. Specific Aims and Experimental Approach

A. Characterization of *Clostridium* sp. strain OhILAs

The apparent ability for *Clostridium* sp. strain OhILAs to reduce As(V) to As(III) warranted its more complete characterization. It is possible that the production of arsenic trisulfide in culture might be linked not with respiration, but with arsenate reduction for resistance. Our hypothesis was that *Clostridium* sp. strain OhILAs was capable of dissimilatory arsenate reduction.

Hypothesis I: *Clostridium* sp. strain OhILAs oxidizes lactate to acetate and CO_2 coupled to the dissimilatory reduction of As(V) to As(III).

Specific Aim I: Demonstrate dissimilatory arsenate reduction by and complete the physiological characterization of *Clostridium* sp. strain OhILAs.

Experimental Approach: Determine the ability for *Clostridium* sp. strain OhILAs to utilize electron donors and electron acceptors, as well as the optimal salinity, temperature, and pH for growth. Determine the stoichiometry for the coupling of lactate oxidation to arsenate reduction.

Preliminary studies also indicated that this strain might also be capable of transforming the organoarsenical 3-nitro-4-hydroxyphenylarsonic acid (roxarsone). A second hypothesis for *Clostridium* sp. strain OhILAs was proposed.

Hypothesis II: OhILAs transforms the organoarsenical 3-nitro-4-hydroxybenzene arsonic acid to 3-amino-4-hydroxybenzene arsonic acid.

Specific Aim II: Demonstrate and determine the rates and products of 3-nitro-4-hydroxybenzene arsonic acid transformation by *Clostridium* sp. strain OhILAs.

Experimental Approach: Provide *Clostridium* sp. strain OhILAs with 3-nitro-4-hydroxybenzene arsonic acid as a electron donor, electron acceptor, carbon source, or fermentable substrate. Test the growth of *Clostridium* sp. strain OhILAs with different concentrations of 3-nitro-4-hydroxybenzene arsonic acid to determine the minimal inhibitory concentration (MIC).

B. Ohio River Sediment Enrichments

Efforts to enrich and isolate arsenate respiring bacteria have resulted in a diverse group of organisms that include low mole% GC Gram positive bacteria from soda lakes [73] and river and aquifer sediments, ε Proteobacteria from aquifer sediments [63], and γ Proteobacteria from marine waters [74]. Whether this is the result of the different environments sampled or the formulation of the enrichment medium used was not known. For example, at least three different concentrations of arsenate (e.g., 1, 5, and 10 mM) have been used by different investigators. It is possible that the concentration of arsenic can have a selective affect. Electron donor and carbon source may also potentially have a selective affect.

Hypothesis III: Electron donor and the concentration of arsenate can impact the selection for specific arsenate-respiring bacteria.

Specific Aim III: Determine whether different electron donors and concentrations of arsenate influence the enrichment of arsenate respiring bacteria.

Experimental Approach: Use a matrix with different electron donors (none, formate, acetate, hydrogen + acetate, pyruvate, and lactate) and different concentrations (0, 1, 5, 10, and 20 mM) of sodium arsenate in a minimal medium. Monitor the enrichments for arsenic trisulfide production and observe the microbial populations by light microscopy. Quantitate As(V) reduction to As(III) using HPLC analysis.

III. Materials and Methods

A. Stock Media and Culture Conditions

Clostridium sp. strain OhILAs was grown on fresh-water acetate or lactate (electron donor) media with sodium arsenate as the terminal electron acceptor, based on the medium for *Sulfurospirillum barnessii* strain SES 3 [75]. Ammonium chloride and magnesium chloride were used in place of the sulfate ($(NH_4)_2SO_4$ and $MgSO_4$) compounds and the pH raised to 7.5 (Appendix I). Wheaton bottles (125ml), containing 100ml of media, were degassed with 80:20 N_2:CO_2 for 5 minutes for the liquid and 2 minutes for the headspace to produce anoxic conditions. Bottles were sealed with butyl rubber stoppers and capped with aluminum crimp tops before autoclaving. Cells (2-3ml

innoculum per 100ml of fresh medium) were transferred once or twice per week. The transfer syringes were degassed with 80:20 N_2:$C0_2$. Cultures were incubated at room temperature.

B. Growth Experiments

Clostridium sp. strain OhILAs was grown on 20 mM As(V) and 20 mM lactate medium (Trial 1), 20 mM As(V) and 20 mM acetate (Trials 2 and 3), for 48 hours at room temperature. A 48-hour and 72-hour growth experiment was also conducted using 5 mM selenite and 20 mM acetate (Trials 4 and 5) at 37°C. Three bottles (125ml Wheaton) containing 100ml of media were inoculated with 3ml of innoculum.

Three samples (1ml each) for each bottle were collected every six hours starting after the inoculation through 48 hours. One milliliter from each bottle was used to measure optical density using the Perkin Elmer Lambda 2 spectrometer. A second milliliter of sample was treated with 1 drop of filtered (0.2 µm Acrodiscs, GelmanScience, Ann Arbor MI) 0.1% acridine orange. Cells were filtered onto a Poretics membrane filter (Livermore, CA) backed with a 0.45 µm grid filter. The membrane filter was mounted on the slide, treated with a drop of immersion oil, and examined by fluorescence microscopy under oil immersion. Cells were counted (10 fields of view per slide) and cell concentration calculated by:

Cells/ml = (mean cells/field)x(filter area/field area)x(1/ml filtered)

A third milliliter sample was filtered (0.2 µM polycarbonate), acidified (using 10µl of 9M H_2SO_4), and placed in 0°C freezer in preparation for HPLC analysis.

The growth rate (μ) was determined by least-squares linear fit to the formula $\mu = \ln (N/N_0)/t$, where N_0 is the initial number of cells [76].

i. Optimum Arsenate Concentration

Clostridium sp. strain OhILAs was grown on 20 mM acetate and concentrations of 1, 5, 10, 20, 30, and 40 mM As(V) using the SES3 medium. Absorbance at 600 nm was measured every six hours starting with the inoculation and concluding at demonstrated cell growth decline.

ii. Electron Donor Determination

Clostridium sp. strain OhILAs was grown on 10 mM As(V) and either 20 mM acetate, pyruvate, formate, lactate, fumarate, glycerol, or no donor (control). The media was prepared in 20 ml volumes in 50ml Wheaton bottles. Each media type was inoculated and transferred six times before evaluation. Absorbance at 600 nm was measured after inoculation and at 24 hours.

iii. Electron Acceptor Determination

Clostridium sp. strain OhILAs was grown on 20 mM acetate and 20 mM sodium arsenate, sodium arsenite, sodium nitrate, sodium sulfate, sodium thiosulfate, sodium selenate, sodium selenite, or dimethylsulfoxide. The media was prepared in 20 ml volumes in 50 ml Wheaton bottles. Each type of medium was inoculated with *Clostridium* sp. strain OhILAs initially grown on lactate and arsenate, and transferred six

times before evaluation. Absorbance at 600 nm was measured after inoculation and at 24 hours.

iv. Optimum pH Determination

Clostridium sp. strain OhILAs was grown on medium containing 10 mM arsenate and 20 mM acetate at different pH (4, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9). The media was prepared in 20 ml volumes in 50 ml Wheaton bottles. Three bottles were used to ascertain growth by absorbance at 600 nm for a 24 hour growth period. The pH was measured before and after autoclaving.

v. Optimum Salinity Determination

Clostridium sp. strain OhILAs was grown on medium containing 10 mM arsenate and 20 mM acetate with different concentrations of NaCl (0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, and 20 mM). The media was prepared in 20 ml volumes in 50 ml bottles. Absorbance at 600 nm was measured to determine growth after 24 hours. All experiments were done in triplicate.

vi. Optimum Temperature Determination

Clostridium sp. strain OhILAs was grown on 5 mM arsenate and 10 mM formate and grown at4, 10, 22, 32, 37, 44, or 50°C. The media was prepared in 20 ml volumes in 50 ml bottles. Growth was determined after 24 hr by determining the absorbance at 600 nm.

vii. Arsenite Inhibition Determination

Clostridium sp. strain OhILAs was grown on 10 mM thiosulfate and 10 mM acetate and concentrations of arsenite measuring 0, 0.1, 0.5, 1, 5, and 7, 8, 9, 10 mM. The media was prepared in 20 ml volumes in 50 ml bottles. Absorbance at 600nm was measured at 0, 24, and 48hr.

viii. Minimal Inhibitory Concentration of Roxarsone

Clostridium sp. strain OhILAs was grown on 20 mM lactate and 20 mM thiosulfate with different concentrations of roxarsone (0, 0.1, 0.5, 1.0, 2.5, and 5.0 mM). *Sulfurospirillum barnesii* strain SES3 was grown on 20mM lactate and 20 mM fumarate and and different concentrations of roxarsone (0, 0.1, 0.5, 1.0, 2.5, and 5.0 mM). The media was prepared in 20 ml volumes in 50 ml bottles. Three of the bottles served as the experimental bottles, while the fourth bottle was used as a control and blank for spectroscopy. Absorbance at 600nm was measured after 48 hours of growth.

ix. Roxarsone Growth Determination

Clostridium sp. strain OhILAs was grown in 100ml of base medium amended with 10 mM lactate and 1 mM roxarsone, 10 mM thiosulfate, 10 mM lactate and 10 mM thiosulfate or 10 mM thiosulfate and 1 mM roxarsone. Each medium was tested in triplicate and monitored every 6 hours for 48 hours. Absorbance at 600nm was determined to monitor growth and the calculated optical densities were compared to a calibrated growth curve that was calibrated by direct cell counts. The disappearance of roxarsone was measured using a UV-Vis scan from 600 nm to 200 nm and the

measurements were compared to a standard curve calibrated with different concentrations of roxarsone.

C. Analytical

As(V) and As(III) were determined using chromatographic analysis in collaboration with Ganna Polshyna [77]. A Waters HPLC with 717 autosampler, 510 pump, and 484 Absorbance detector were used. Integration curves were obtained on an HP 3396A integrator. The separations were performed on an Aminex HPX-87H ion exchange column with a 0.0075M H_2SO_4 solution sparged with helium as the mobile phase. The column temperature was 30°C and absorbance measured at 195nm. A flow rate of 1ml/min measuring 1500 psi was used for injection of 20µl of sample. As(V) and As(III) were identified by comparison of the retention times of standard solutions and quantified with calibration curves [77].

D. River Enrichments

Sediment samples of the Ohio River were obtained with a ponar dredge sampler aboard the The Pittsburgh Voyager April 17, 2002. The pH of the water was 7.9 and the temperature 11°C. A slurry was made by injecting approximately 5 grams of sediment into 100 ml of anoxic base media (in a 125 ml Wheaton bottle) that did not contain electron donor or acceptor. Additional sediment samples were collected in 50 ml polypropylene tubes (Fisher Scientific, Pittsburgh PA). All samples (including the slurry) were then placed in ice and kept in the dark for transport back to the laboratory.

The bottles of medium with the different electron donors and arsenate concentrations (the matrix) were inoculated immediately upon arrival back at the laboratory (within four hours). One ml of the slurry was injected into each bottle. The matrix consisted of 50 ml bottles containing 40 ml of base medium (Appendix I) amended with different electron donors and different concentrations of sodium arsenate (Table 6).

Table 6. Electron donors and concentrations of sodium arsenic used in the enrichment of arsenic transforming microbes.

Electron Donor (20 mM)	**Sodium Arsenate Concentration (mM)**				
Formate	0	1	5	10	20
Acetate	0	1	5	10	20
Acetate+ H_2	0	1	5	10	20
Lactate	0	1	5	10	20
Pyruvate	0	1	5	10	20
No donor	0	1	5	10	20

The bottles were monitored for arsenic trisulfide (orpiment) production on a weekly basis and documented with a digital camera. For arsenic species, 1ml samples of the media were filter sterilized and acidified (as described above) and collected in 1.5 ml microfuge tubes. As(V) and As(III) were measured using HPLC as described above.

IV. Results

A. Characterization of Clostridium sp. strain OhILAs

i. Optimal As Growth Conditions of *Clostridium sp*. strain OhILAs

The affect on growth of As(V) concentration for *Clostridium sp*. strain OhILAs was determined using different concentrations of As(V) (1, 5, 10, 20, 40 mM) with 10 mM acetate. Exponential growth occurred in all concentrations of As(V) tested except for 1 mM (Figure 6). The highest cell yields were obtained at concentrations of 5 mM and 10 mM arsenate while growth in 30 mM and 40 mM arsenate was less. Growth on 1 mM arsenate was significantly less than all other concentrations suggesting that the concentration was not sufficient to sustain respiratory growth.. The growth curves for all other concentrations tested were similar as exponential growth occurred from hour 6 to hour 24. Maximum cell yield and the death phase were achieved earliest (18 hr vs. 24 hr) with 5 mM As(V), suggesting that this was the optimal concentration for growth.

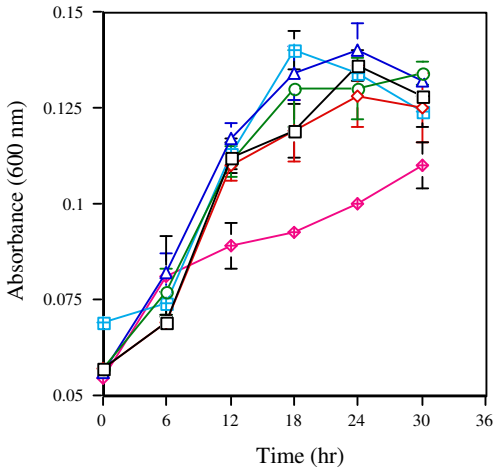

Figure 6. Optimum arsenic concentration for OhILAs growth measured every 6 hours at 600 nm. Growth medium tested contained 20 mM acetate and arsenic concentrations of 1 mM (u), 5 mM (┼) 10 mM (△), 20 mM (□), 30 mM (◇), and 40 mM (○)**.** Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

ii. Arsenite Inhibition

The affect on growth of As(III) concentration for *Clostridium sp*. strain OhILAs was determined using different concentrations of As(III) (1, 5, 10, 20, 40 mM) with 10 mM acetate and 10 mM sodium thiosulfate (Figure 7). Growth of OhILAs was completely inhibited by 10 mM As(III) and the experimental sets for the 9 mM concentrations demonstrated little growth. interestingly, growth seemed to be enhanced at low concentrations of As(III) including 0.1, 0.5, and 1 mM. The addition of HCl caused the formation of orpiment in sample sets containing concentrations of 0.5 mM As(III) and up giving evidence to the reduction of thiosulfate (data not shown).

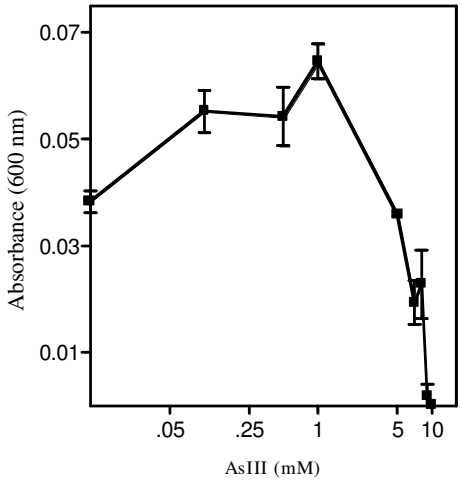

Figure 7. Arsenite inhibition on the growth of OhILAs. Cells were grown on 10 mM thiosulfate and 10 mM acetate with concentrations of 0.1, 0.25, 0.5, 1.0, 5.0, 7.0, 8.0, 9.0, 10.0 mM As(III). Cell growth was measured as absorbance at 600 nm for samples performed in triplicate and plotted along a log (2) X-axis. Error bars represent the standard deviation of the average growth of the triplicate experimental sample set.

iii. Optimal Temperature, Salinity, and pH

Optimal temperature, salinity, and pH for *Clostridium sp*. strain OhILAs was determined using growth medium with 10 mM sodium arsenate and 10 mM acetate. The optimal temperature for the growth was 37ºC (Figure 8A). Growth occurred at 32ºC and 44ºC but was inhibited at temperatures below 22ºC and above 50ºC.

The optimal concentration of NaCl was in the range between 0.1 g/l and 2.5 g/l with significant growth occurring up to 5 g/l (Figure 8B). Growth was inhibited at NaCl concentrations above 10 g NaCl/l.

The optimum pH was approximately 8.5 with growth occurring in between 8.0 and 8.75 (Figure 9). Little growth occurred below pH 7.5.

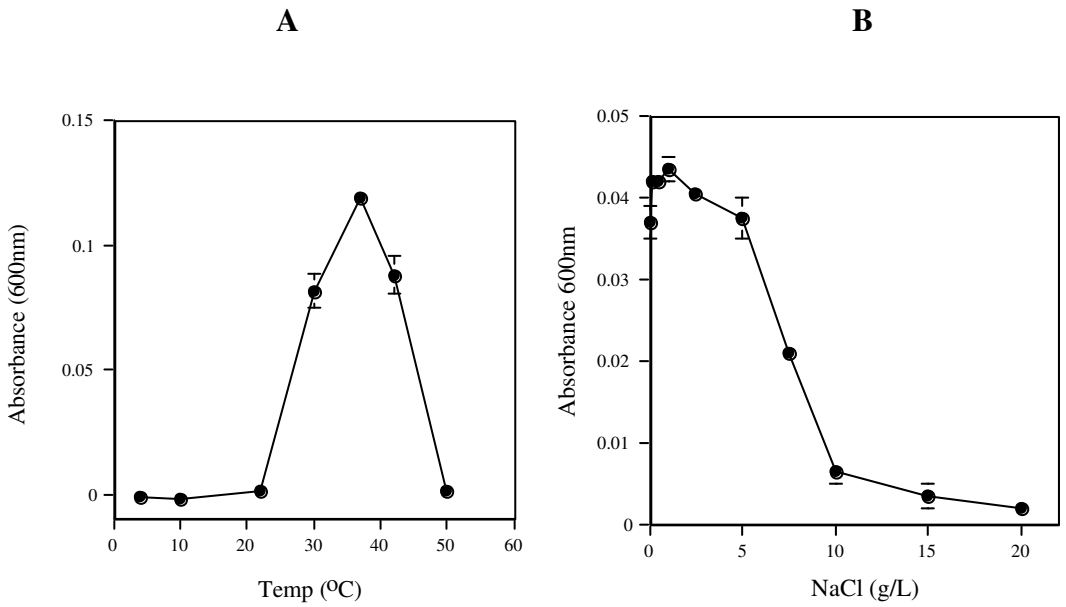

Figure 8. (A) Optimum temperature and (B) optimum salinity for the growth of OhILAs. The growth medium contained 10 mM arsenate and 20 mM acetate with varying temperatures (A) of 4, 10, 22, 32, 44 and 50ºC and NaCl concentrations (B) of 0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15 qnd 20 mM. Cell growth was measured as absorbance at 600nm. Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

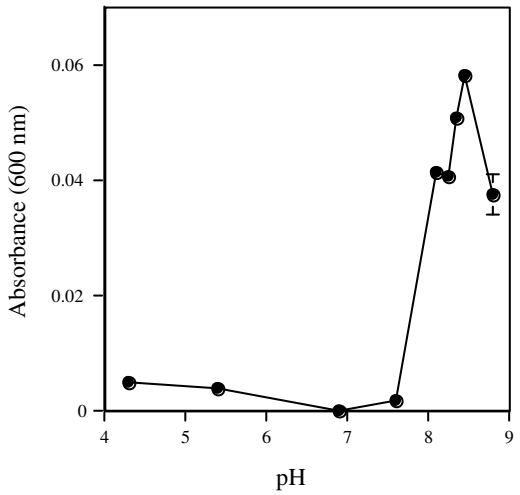

Figure 9. Optimum pH determination for the growth of OhILAs. The growth medium contained 10 mM arsenate and 20 mM acetate with varying pH levels of 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9. Cell growth was measured as absorbance at 600nm. Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

iv. Growth Kinetics

The growth kinetics of *Clostridium* sp. strain OhILAs was determined using medium containing 20 mM sodium arsenate and 10 mM sodium acetate. Cell growth was concomittant with the reduction of arsenate (Figure 10). Equimolar amounts of As(V) were reduced to As(III) (Figure 10B, Polshyna, 2003).

Exponential growth occurred between the 6^{th} and 18^{th} hour, which coincides with the exponential reduction of As(V) to As(III) The death phase occurred at the 42^{nd} hour, which coincides with the loss of As(III) production. The calculated growth rate $\mu = \ln (N/N_0)/t)$.

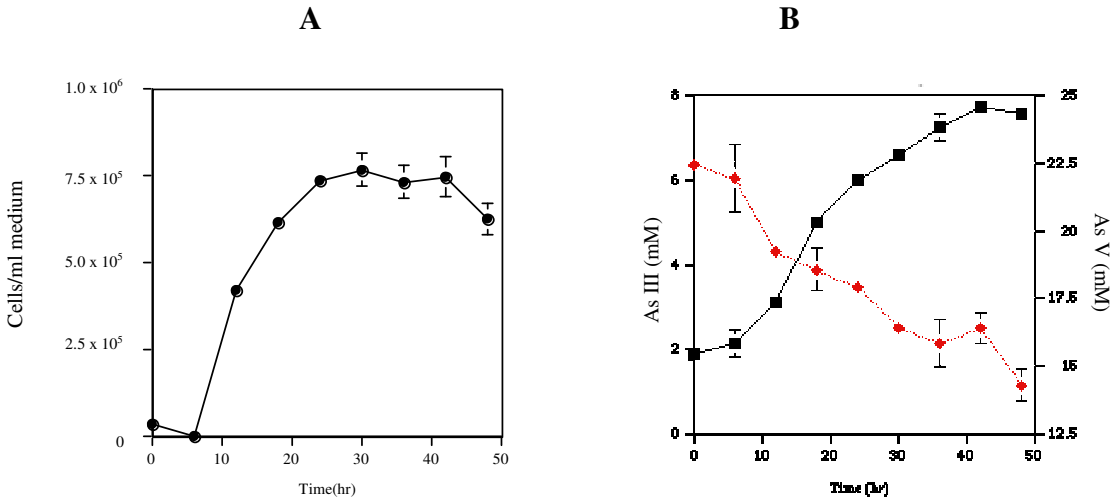

Figure 10. Growth kinetics of OhILAs grown in medium containing 20 mM sodium arsenate and 10 mM sodium acetate. (A) Growth (l) of OhILAs over a 48 hour period given in cells/ml medium. (B) Reduction of As(V) (♦) and production of As(III) (n)given in mM As(V) and As(III) (from [77] Polshyna). Error bars represent the standard deviation of the average growth of a triplicate sample set.

v. Electron Donor Determination

Clostridium sp. strain OhILAs exhibited metabolic versatility as it could couple the reduction of As(V) to the oxidation of a variety of electron donors (Figure 11). It was also capable of fermentative growth. Growth on acetate, formate, fumarate, and lactate was enhanced with the presence of As(V) when compared to the bottles containing only the donor (Figure 11). Growth on pyruvate and glycerol occurred fermentatively. The bottles containing pyruvate but no As(V) exhibited greater growth than the bottles containing pyruvate and As(V) (Figure 11). Growth on glycerol and As(V) was greater than glycerol alone, however, a subsequent transfer from the glycerol/As(V) medium to medium containing glycerol alone exhibited growth for 5 successive transfers at 1/100ml dilutions.

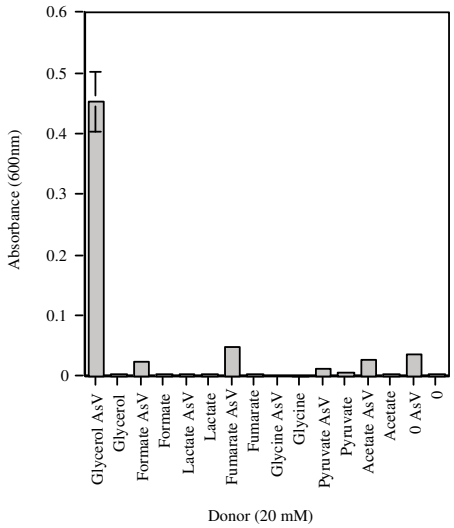

Figure 11. Electron donor determination for OhILAs. OhILAs grown on donors (20mM) acetate, formate fumarate, glycerol, glycine, lactate, and pyruvate and no donor with and without 10 mM As(V). Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

vi. Electron Acceptor Determination

Clostridium sp. strain OhILAs appears to couple the oxidation of lactate to the reduction of arsenate, and thiosulfate, as growth was significantly greater than with lactate alone (Figure 12). Growth was not enhanced over control (lactate only) in the presence of nitrate, sulfate, or fumarate (Figure 12), or selenate or selenite (data not shown). It was also apparent that 10 mM arsenite was inhibitory (Figure 12).

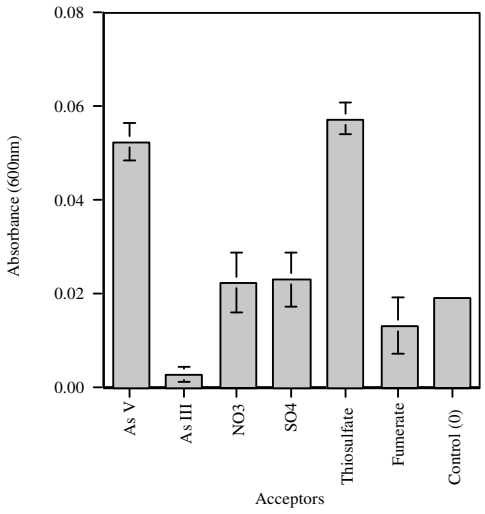

Figure 12. Terminal electron acceptor determination for OhILAs. OhILAs grown in liquid medium containing 20 mM lactate and 10 mM arsenate, nitrate, sulfate, thiosulfate, fumarate, 5 mM arsenite and no electron acceptor. Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

vii. Selenite Growth Kinetics

Although *Clostridium* sp. strain OhILAs did not respire selenite, it did reduce it elemental selenium (Figure 13). The medium changed in color from a yellow to a dark red, evident of production of elemental selenium (data not shown The growth kinetics did not correspond with the steady reduction of selenite as determined by cell counts and HPLC (Figure 13).

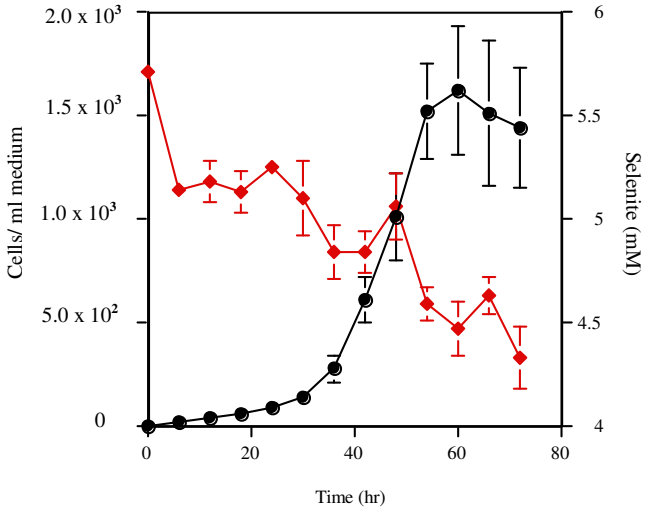

Figure 13. Growth kinetics in liquid medium containing selenite (5 mM) and acetate (20 mM). Growth (l) of Clostridium sp. stain OhILAs over a 48 hour period determined by direct cell count and reported in cells/ml. Loss of selenite (♦) in mM (courtesy of G. Polshnya, unpublished data). Error bars represent the standard deviation of the average growth of experimental samples performed in triplicate.

B. River Enrichment

i. Observations of River Enrichment

The initial experiment of the matrix was conducted to determine whether orpiment formation would occur and how long it would take to observe. Orpiment formation occurred within 7 days. The bottles of 0 mM and 1 mM As(V) for all electron donors had a strong odor of sulfide and the bottles turned from tan to black in color (Figure 14). Bottles of the contained either 0 mM or 1 mM As(V) with H_2 and acetate, generated negative pressure as affirmed by air contents of syringes were pulled into the bottle. This suggests the use of H_2 as an electron donor. At 5 mM concentrations, an abundance of orpiment production was evident as there was copious yellow precipitate (Figure 14). This suggests the biological reduction of arsenate to arsenite and the consequent co-precipitation of arsenite with sulfide as orpiment. Further supporting the biological nature of the transformation of arsenic, the bottle containing 5 mM arsenate, but no electron donor, did not show orpiment formation. At the higher concentrations of As(V), 10 mM and 20 mM, no orpiment was produced (Figure 14) suggesting either that either arsenate or sulfate reduction was inhibited.

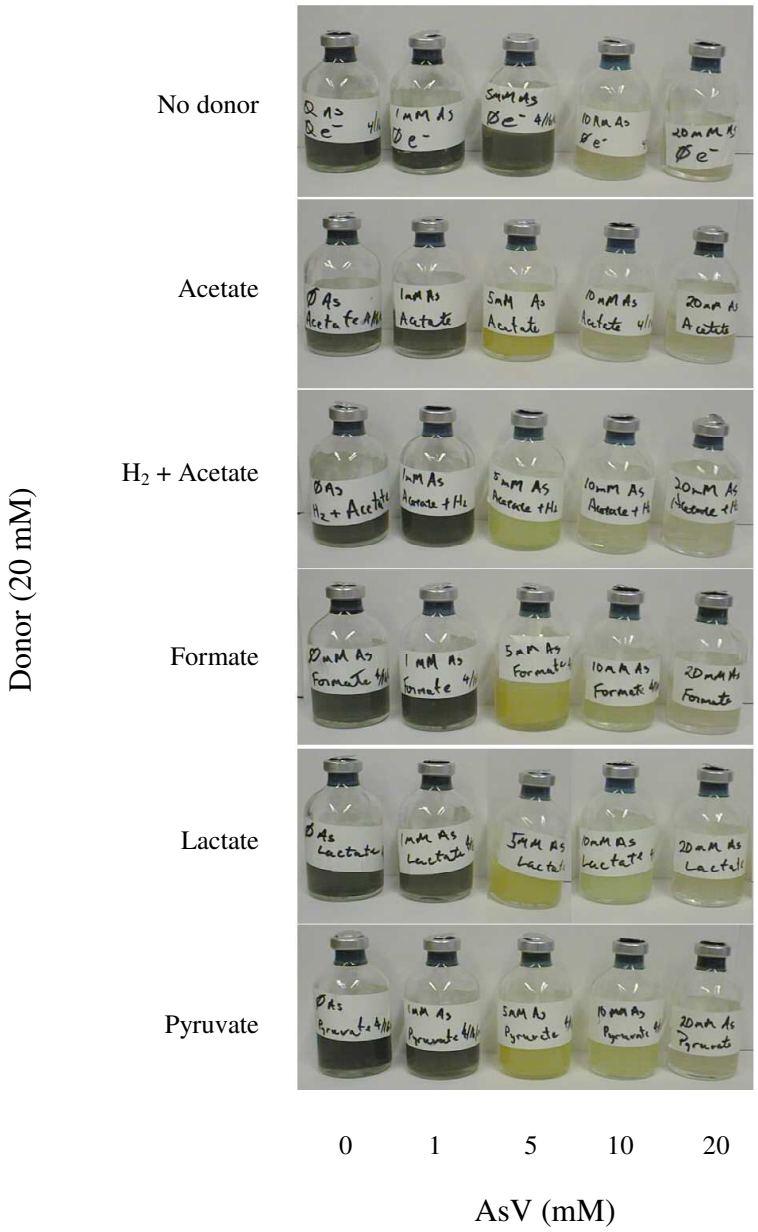

Figure 14. An enrichment for arsenic transforming bacteria from Ohio River sediments with media containing various electron donor sources (no donor, acatate, H2 + acetate, formate, lactate, and pyruvate) and various arsenic concentrations. Observations of changes in medium color and odor were made and noted daily. This picture was taken 7 days after inoculation. The yellow coloration at the 5 mM As levels is indicative of the formation of orpiment (arsenic trisulfide). Bottles with a darkening of the medium (the 0 and 1 mM As sets) had a strong odor sulfide.

ii. HPLC Analysis of River Matrix

The matrix was repeated to include heat killed controls and included HPLC analysis for As(V) and As(III). Visual observation again indicated the production of orpiment in bottles containing 5 mM As(V). It was also revealed that there was some color change in the bottles that were heat killed indicating some abiotic chemical reactions (i.e., arsenate reduction) between the medium and sediment. However, there was no further transformations over the time course of the experiment (data not shown).

a) Comparisons of Day 0 and Day 7 for 10 mM A(V)

Bottles containing 10 mM As(V) were used as a control to measure changes from day 0 to day 7 for the inoculated, autoclaved-killed and uninoculated sets. All other concentrations were measured only for the 7^{th} day for the inoculated, autoclaved-killed and uninoculated sets and will be discussed below.

Figure 15 A and B shows the concentrations of arsenate and arsenite detected for acetate and H_2 and acetate alone respectively. In each donor type, production of arsenite was evident in the uninoculated and sterilized subsets for both day 0 and day 7. This is due to the effects of autoclaving the media, as high temperatures reduce some of the arsenate to arsenite. In the experimental set, the biotic reduction of arsenate is evident as a production of approximately 7.5 mM As(III) is apparent in the acetate medium while the H_2 and acetate medium exhibits a complete reduction of As(V). The H_2 and acetate medium provides more flexibility in the use of an electron donor making the utilization of such a donor source possible for a wider range of genera of bacteria found in the sediments.

Formate and lactate (Figure 16A and B) and pyruvate (Figure 17 A) show similar results as As(V) was no longer detectable by day 7, indicating microbial transformation of the toxic element. Bottles that did not have added donor also showed significant As(V) reduction (Figure 17 B).

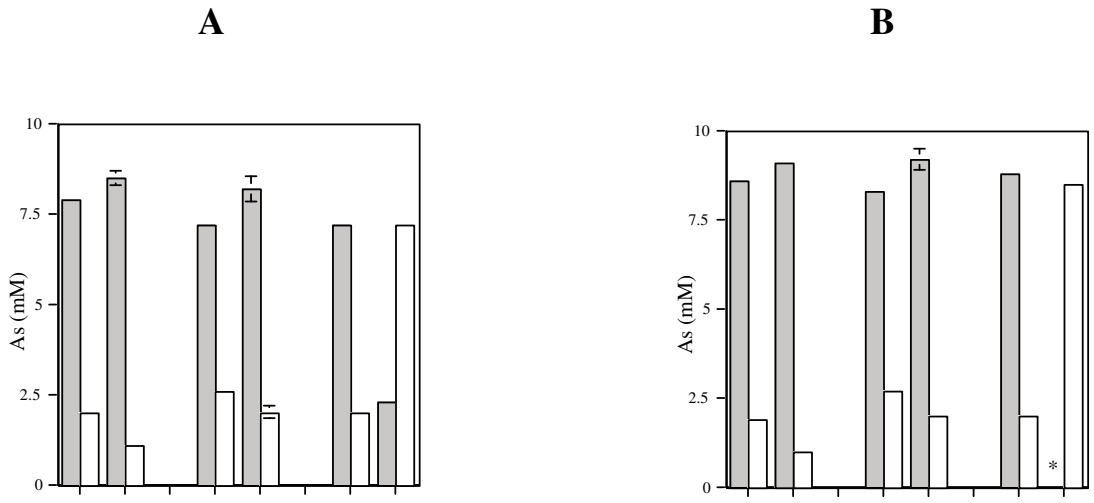

Figure 15. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 10 mM arsenate and (A) acetate and (B) acetate and H_2 for 0 and 7 day incubation periods. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

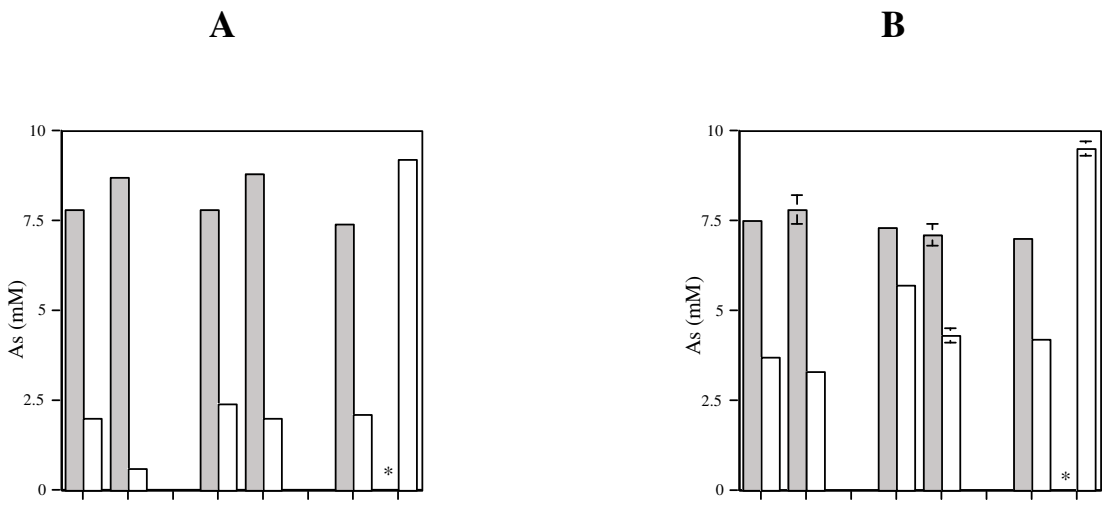

Figure 16. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 10 mM arsenate and (A) formate and (B) lactate for 0 and 7 day incubation periods. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

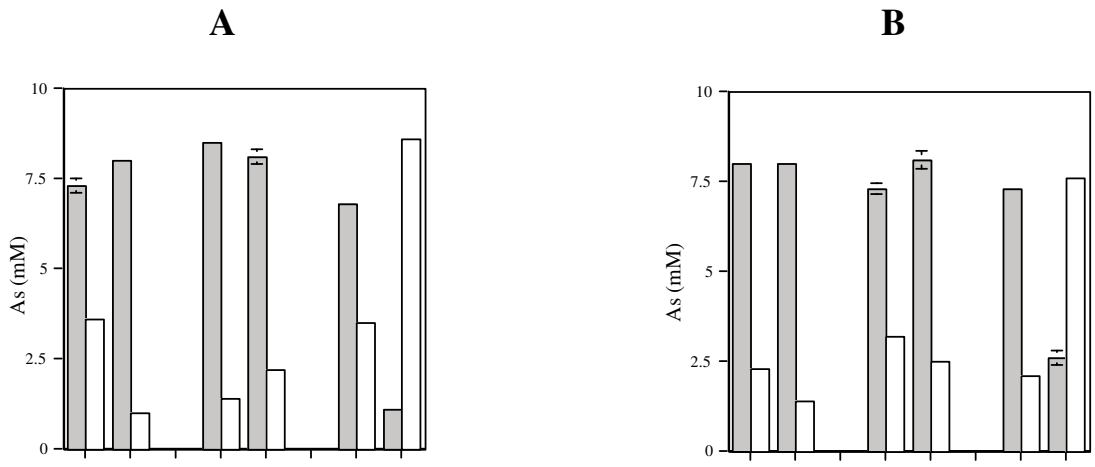

Figure 17. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 10 mM arsenate and (A) pyruvate and (B) no donor for 0 and 7 day incubation periods. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

b) Acetate and As(V)

The reduction of As(V) to As(III) with acetate as the electron donor was evident in bottles containing 5, 10 and 20 mM As(V) (Figure 18B,C and D). At 1 mM concentrations, no change occurred between the uninoculated, sterilized and experimental sets (Figure 18A). Each had measurements of 0.4 mM As(V) and 0.6 mM As(III). The production of As(III) can be attributed to the abiotic effects of the heating of the autoclave (Figure 18A). At 5 mM As(V) a slight reduction of As(V) was detected in both the uninoculated and sterilized sets, roughly 4.5 and 3.5 mM As(V) respectively (Figure 18B). Nevertheless, the bottles inoculated with sediment showed a complete reduction of As(V), to As(III). As(V) was not detected while the concentration of As(III) was approximately 4.5 mM. This difference can be attributed to the production of orpiment, which is in the solid phase and cannot be measured by HPLC.

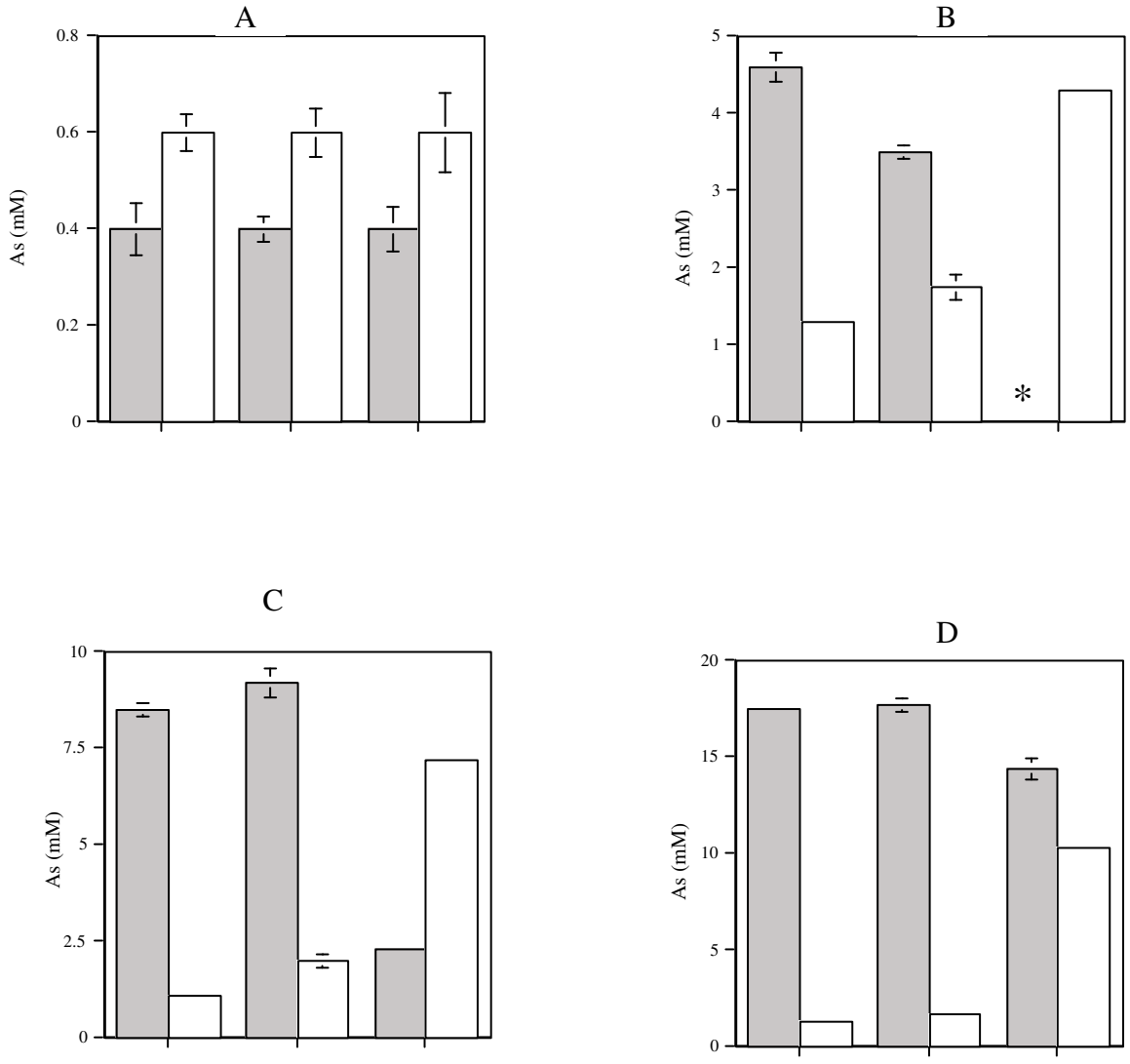

Figure 18. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 20 mM acetate and (A) 1 mM As(V), (B) 5 mM As(V), (C) 10 mM As(V), and (D) 20 mM As(V) after a 7 day incubation. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

c) Acetate and H_2

A complete reduction of As(V) to A(III) was demonstrated for the experiemtal set for the 5 and 10 mM concentrations of As(V) for Acetate and H_2 (Figure 19B and C). The uninoculated and sterilized bottles for the 5 mM As(V) showed moderate reduction to about 0.8 mM and 1.5 mM As(III) respectively (Figure 19B). The effect of the autoclaving is less significant as the concentrations of As(V) increase as is evident in the 10 mM set (Figure19 C). The uninoculated and heat killed controls showed only a slight reduction of As(V). The 20 mM As(V) sediment samples showed an approximately 18 mM conversion of As(V) to As(III), while the uninoculated and sterilized sets showed little reduction in As(V) (Figure19 D).

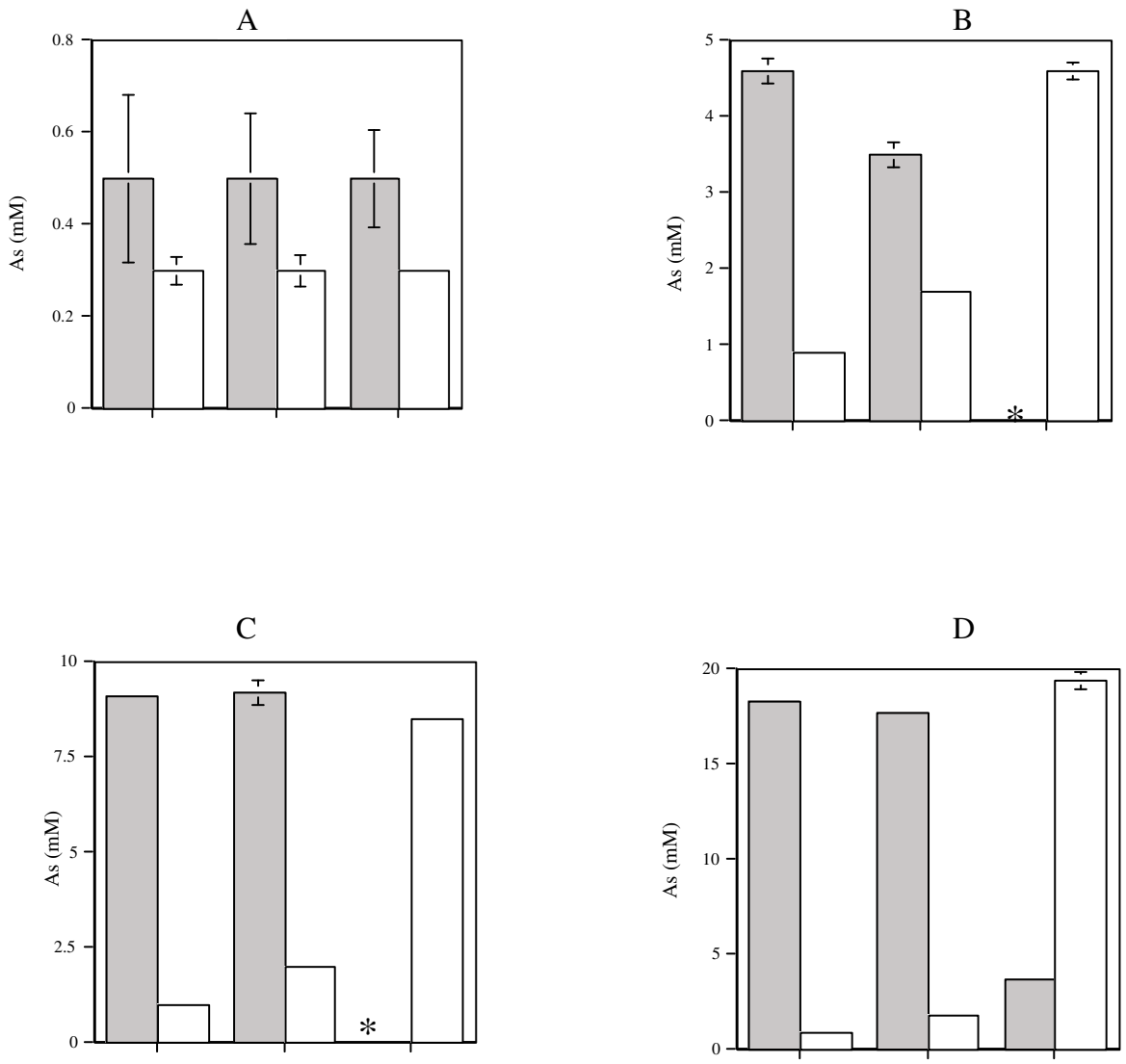

Figure 19. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 20mM acetate + H2 and (A) 1 mM As(V), (B) 5 mM As(V), (C) 10 mM As(V), and (D) 20 mM As(V) after a 7 day incubation. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

d) Formate

With formate as the electron donor, complete reduction of As(V) was apparent in the 5 and 10 mM sets (Figure 20 B and C) , while a 15 mM reduction of As(V) occurred in the 20 mM experimental set (Figure 20D). Reduction of As(V) was not evident in the 1 mM experimental set when compared to the uninoculated and sterilized samples (Figure 20A). However the reduction from autoclaving seems to be less pronounced with this particular donor as only a 0.2 mM As(III) was reported for all samples in the 1 mM set.

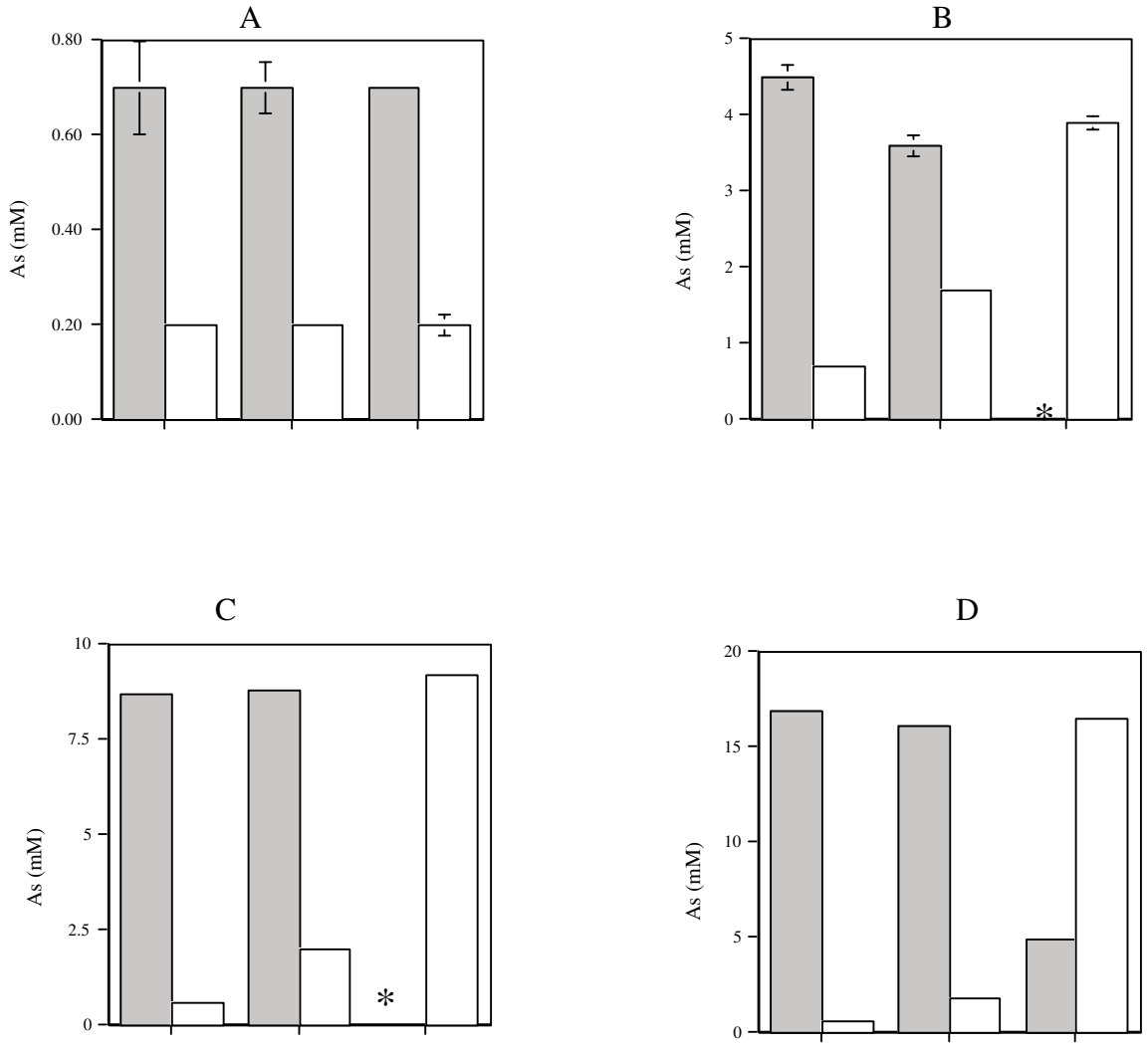

Figure 20. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 20mM formate and (A) 1 mM As(V), (B) 5 mM As(V), (C) 10 mM As(V), and (D) 20 mM As(V) after a 7 day incubation. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

e) *Lactate*

The medium containing lactate proposed some detection limitations for the 1mM set and thus the graph was not shown. Total reduction of As(V) was evident for the 5 and 10 mM experimental sets (Figure 21A and B). However, it must be noted that the effects of the autoclave were more prominent with this donor as approximately half (2.5 mM As(V)) was reduced in the uninoculated samples of the 5 mM set (Figure 21A). Likewise, the 10 mM set showed significant reduction in the uninoculated and heat killed controls (Figure 21B). At 20 mM As(V) the reduction in the experimental bottle was comparable to other donors tested (Figure 21C). Despite the limitations in the detection level of HPLC analysis for lactate, biotic reduction of As(V) was demonstrated.

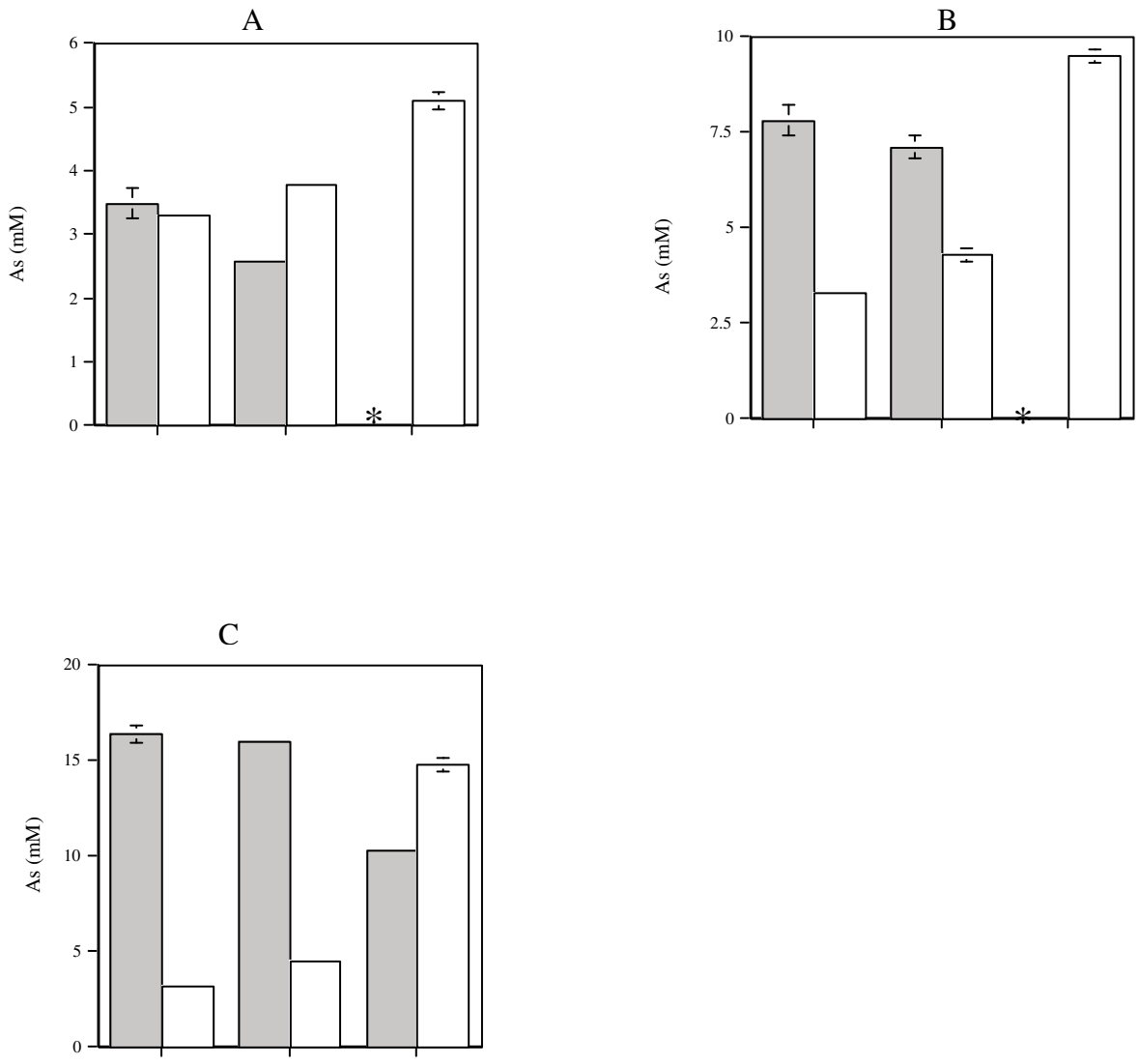

Figure 21. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 20mM lactate and (A) 5 mM As(V), (B) 10 mM As(V), and (C) 20 mM As(V) after a 7 day incubation. A measurement of 0 mM is denoted by a (*) symbol. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated. The 1 mM As(V) data was omitted due to its HPLC resolution limitations in lactate media.

f) Pyruvate

The medium containing pyruvate also proposed detection limitations at the 1 mM and 5 mM As(V) concentrations. Reduction of As(V) occurred in both the 10 mM and 20 mM As(V) (Figure 22A and B). At 10 mM As(V) a full transformation from As(V) to As(III) did not occur. A As(III) level of 7.5 mM was found, while As(V) measured approximately 2.5 mM (Figure 22A). These results are similar to those found for acetate. All other donor types demonstrated a full reduction of As(V) to As(III) in medium cotaining 10 mM. The results for 20 mM As(V) also demonstrated less As(V) transformation for pyruvate than all other donor types (Figure 22 B).

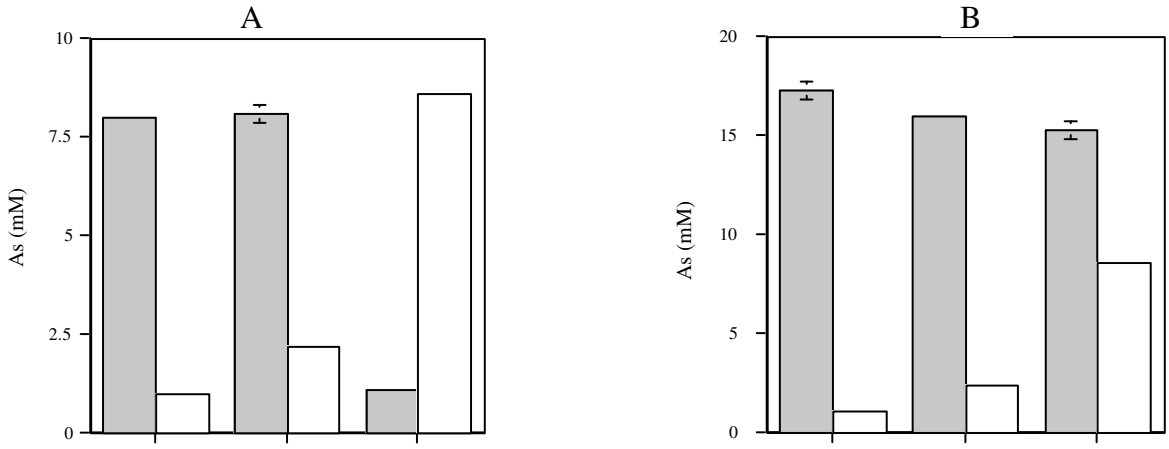

Figure 22. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media containing 20 mM pyruvate and (A) 10 mM As(V), and (B) 20 mM As(V) after a 7 day incubation. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated. The 1 mM and 5mM As(V) data were omitted due to its HPLC resolution limitations in lactate media.

g) *No Donor*

Samples that were not amended with an electron donor, nevertheless, showed complete reduction of As(V) in medium with 5 mM As(V) (Figure 23B). Reduction at the 10 mM concentration was comparable to pyruvate and acetate samples (Figure 23C). Interestingly at 20 mM As(V), no reduction was detected over the uninoculated or heat killed contols (Figure 23D). This demonstrates the importance of the type of electron donor of the given environment when considering dissimilatory arsenic respiring bacteria.

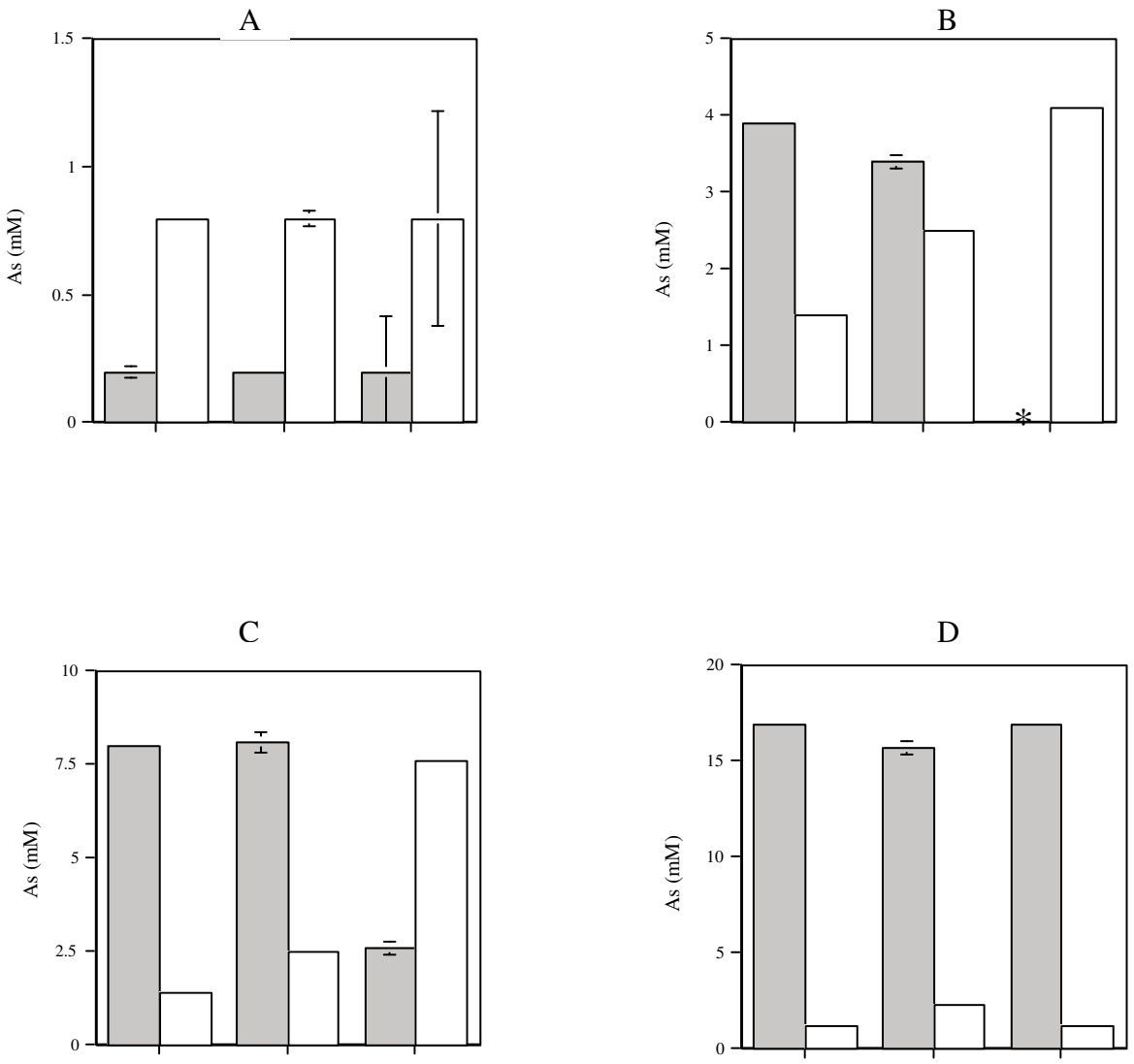

Figure 23. HPLC measurement of arsenate (grey column) and arsenite (white column) of uninoculated, sterilized, and experimental sets of the Ohio River enrichment media lacking an electron donor and (A) 1 mM As(V), (B) 5 mM As(V), (C) 10 mM As(V), and (D) 20 mM As(V) after a 7 day incubation. The uninoculated set was comprised of the sample medium without the addition of river sediments. The sterilized set included the addition of heat killed river sediment samples to the sample medium. The experimental set included the addition of fresh, biologically active river sediments to the sample medium. Each condition was performed in triplicate and average concentration and standard deviation (error bars) calculated.

C. Biotransformation of Roxarsone

i. Minimal Inhibitory Concentration

Roxarsone is a nitrophenol and has a distinctive yellow color. This color could be monitored by eye and by spectrophotometry. The higher the concentration of roxarsone the more pronounced the yellow color was (Figure 24) *Clostridium* sp. strain OhILAs grew in medium amended with roxarsone. Interestingly, the yellow color was no longer visible after 48 hr in medium containing up to 1 mM roxarsone (Figure 24). This suggested that *Clostridium* sp. strain OhILAs was not only resistant to roxarsone, but might be able to degrade it.

Figure 25 shows the growth curves for *Clostridium* sp. OhILAs and that of *Sulfurospirllium barnessi* strain SES3. *Clostridium* sp. OhILAs was able to grow in the presence of 2.5 mM roxarsone and growth was actually enhanced for the 0.5 and 1.0 mM roxarsone. The 5mM concentration of roxarsone proved to completely inhibit growth (Figure 25). *S. barnesii* strain SES3 did not exhibit significant growth in any of the concentrations tested, but did exhibit a greater resistance to roxarsone as compared to OhILAs. No loss of yellow color was noticed in the *S. barnesii* strain SES3 culture medium.

Figure 24. Degradation of roxarsone by *Clostridium sp.* strain OhILAs. OhILAs was grown in liquid medium consisting of 20mM lactate and 20mM thiosulfate amended with 5 mM, 2.5 mM, 1 mM, 0.5 mM, 0.1 mM, and 0 mM roxarsone. The top row of bottles is the experimental set, inoculated with OhILAs, and the bottom row is the corresponding uninoculated set. The disappearance of the yellow hue in the inoculated sets is indicative of the degradation of roxarsone.

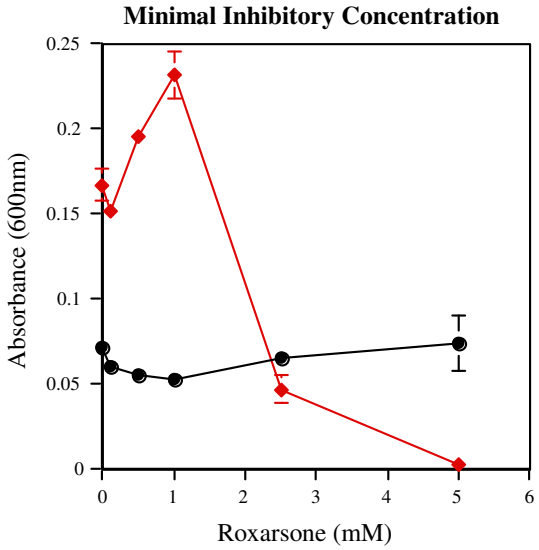

Figure 25. Minimal growth inhibitory concentration of roxarsone for *Clostridium sp*. OhILAs and *Sulfurospirillum barnesii.* OhILAs growth (♦) in liquid medium containing 20 mM lactate and 10 mM thiosulfate and *S. barnesii* growth (l) in liquid medium containing 20 mM lactate and 10 mM fumarate was determined by absorbance at 600nm. Both media types were amended with 0, 0.1, 0.5, 1, 2.5, and 5 mM roxarsone. Each experimental set was performed in triplicate and the average growth and standard deviation (error bars) was determined.

ii. Growth Kinetics on Roxarsone

a) Glycerol and Roxarsone

To begin the investigation of the possible physiological role of roxarsone, growth experiments were carried out. There was no apparent difference in cell yields between cultures of *Clostridium* sp. strain OhILAs grown on 10 mM glycerol alone and 10 mM glycerol amended with 1 mM roxarsone(Fig 29). This suggests that *Clostridium* sp. strain OhILAs is growing fermentatively on glycerol even in the presence of roxarsone.

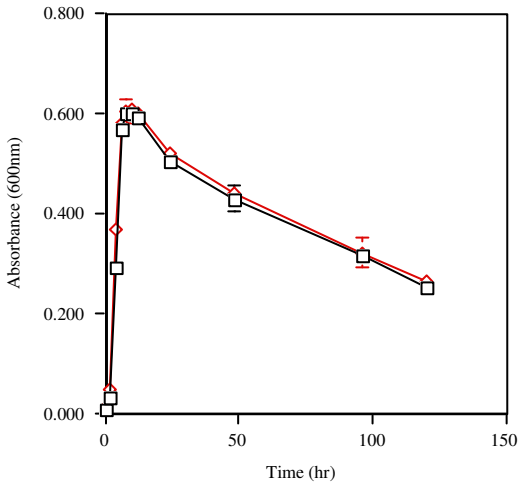

Figure 26. Growth of *Clostridium* sp. strain OhILAs in liquid medium contianing 10 mM glycerol (n) and 10 mM glycerol with 1 mM roxarsone (◇) measured every 3 hours for the first 18 hrs followed by measuerments performed every 12 hours. The experiment was carried out in triplicate and the average growth (measured at 600 nm) and standard deviation (error bars) determined.

b) Roxarsone as a Donor or Acceptor

Roxarsone was then tested as a donor or acceptors. *Clostridium* sp. strain OhILAs was grown in medium containing lactate alone, thiosulfate alone, lactate and thiosulfate, lactate and roxarsone (as acceptor), and thiosulfate and roxarsone (as donor). *Clostridium* sp. strain OhILAs appeared to grow best with lactate and roxarsone suggesting that roxarsone could be used as an electron acceptor. All other media types tested had similar growth, although cells grown on thiosulfate and roxarsone showed a slight increase in turbidity compared to the cultures grown in the absence of roxarsone. Roxarsone provides two side groups, the nitro and the arsenate group that could be used as electron acceptors. A third pathway has been demonstrated for a *Clostridium sp.* and involves a transfer of a phosphate group from an unknown donor to phenol, forming phenylphosphate which is then further metabolized through the benzoyl CoA pathway which involves ultimate ring fission [78] The lactate and roxarsone seemed to produce what may be secondary growth in the 48^{th} hour. One possibility is that the nitrate group is being attacked first as nitrate provides the most energetically favorable reaction for anaerobic respiration. A possible secondary growth stage may occur in the reduction of the arsenate group. Interestingly, preliminary in gel activity assays demonstrate that the arsenate reductase of *Clostridium* sp. strain OhILAs is induced in the presence of roxarsone (data not shown).

The purpose of using roxarsone in the presence of thiosulfate was to determine if roxarsone could be used as an energy source. In this process aromatic ring cleavge must occur. However, with the proteolytic properties of OhILAs it is difficult to determine if the roxarsone was being used as an energy substrate in this experimental set. It is

possible that OhILAs was coupling the oxidation of an energy source provided in the yeast extract to a functional group on the aromatic ring of roxarsone.

Figure 27 shows the growth curve for *Clostridium* sp. strain OhILAs on lactate and roxarsone as well as the degradation of roxarsone. Complete transformation of roxarsone from approximately 1 mM to 0 mM occurred over the 48 hour period. The exponential growth rate coincides with the exponential degradation of roxarsone from approximately the 6^{th} hour until the 30^{th} hour.

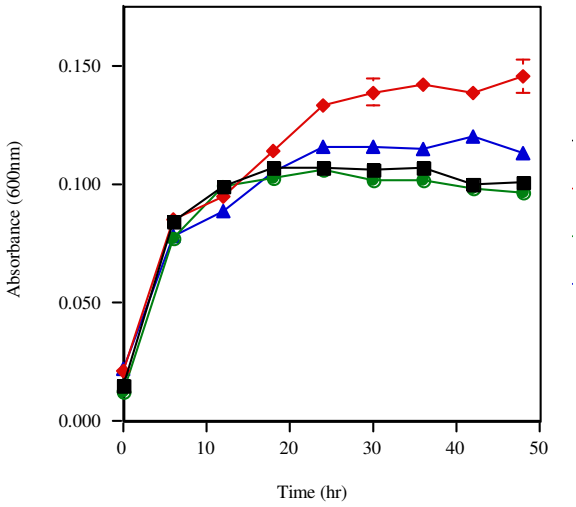

Figure 27. Growth of *Clostridium* sp. OhILAs with roxarsone as an electron donor and acceptor measured at 600nm over a 48 hour period. Average growth on thiosulfate with no electron source (n), lactate and roxarsone (♦), lactate and thiosulfate (l), and thiosulfate and roxarsone (▲). Each sample set performed in triplicate and the average growth and standard deviation (error bars) was determined.

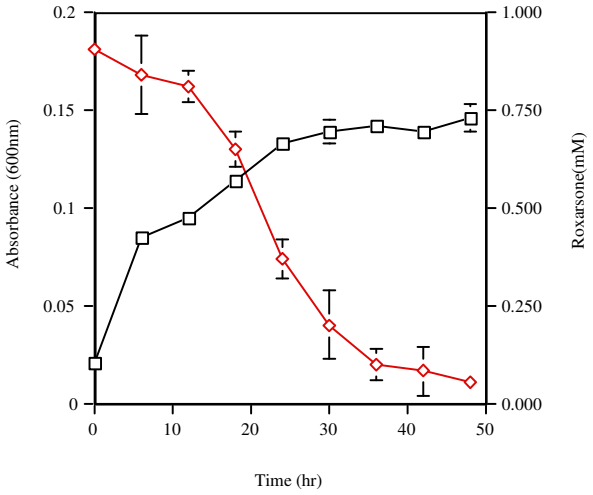

Figure 28. Growth kinetics for Clostridium sp. OhILAs in liquid medium medium containing 10 mM lacatate and 1.0 mM roxarsone. Average growth of samples performed in triplicate was determined by absorbance at 600nm (○) and disappearance of roxarsone (◇) was measured (at 400 nm) every 6 hours over a 48 hour period. The error bars represent the standard deviation of the average growth and roxarsone degradation.

V. Discussion

A. Characterization of *Clostridium* sp. strain OhILAs

Clostridium sp. strain OhILAs joins an ever-increasing list of documented dissimilatory arsenate respiring prokaryotes (DARPs). OhILAs is an important and unique for its ability to metabolize the organoarsenical roxarsone. Characterization has also provided valuable information on the capabilities and limitations of its arsenate reductase. Furthermore, its characterization has provided insight into arsenic metabolism in natural environments as it was isolated from Ohio River sediments.

Clostridia exhibit a wide range of metabolic capability despite being strictly anaerobic and fermentative. It is this diversity which presented problems in deciphering the metobolic physiology of *Clostridium* sp. strain OhILAs. Determination of suitable electron donors and terminal electron acceptors was hindered by the need to supplement the growth medium with yeast extract. In the 1930's, Stickland and peers described what is known as the Stickland reaction in numerous species of the genus *Clostridium*. In this process one amino acid (e.g., alanine, leucine) serves as an electron donor while another amino acid (e.g., glycine, proline) serves to take up the reducing equivalents [79]. Although attempts to grow *Clostridium* sp. strain OhILAs on Stickland pairs were unsuccessful, it may be the lack of added selenium to the medium. Both glycine reductase and proline reductase are selenoenzymes in *Clostridium* sp. strain OhILAs (as determined by analysis of the annotated genome).

Clostridium sp. strain OhILAs was able to couple the oxidation of acetate, formate, fumarate, lactate and glycerol with the reduction of As(V). Growth was supported in both lactate and acetate containing media strongly suggesting *Clostridium*

sp. strain OhILAs has the capability to completely oxidize lactate to CO_2**.** Many DARPs can couple As(V) reduction to lactate oxidation, however most exhibit an ability to only partially oxidize lactate to acetate. The coupling of formate with As(V) reduction is also a rare among the characterized DARPs. Among the low G+C Gram-positive bacteria, only the *Desulfitobacterium spp*. have been reported to have this capability. Fumarate oxidation is rare in the Gram-positive low G+C.

Optimal temperative, salinity, and pH were comparable to other Gram-positive low G+C bacteria. *Clostridium* sp. strain OhILAs was able to grow at temperatures ranging from approximately 25˚C and 45˚C with an optimum temperature of 37˚C. *Desulfitobacterium sp*. strain GBFH also has an optimal temperature of 37˚C but has the ability to grow at 15˚C. The salinity optimum was 0.1 g/L NaCl, and growth was significantly inhibited at concentrations greater than 10 g/L NaCl. The pH optimum was 8.4, and the organism did not grow well above pH 9. This is significant as the closest relatives as determined by 16S rRNA sequence are members of the genus *Alkaliphilus*. *A. metalliredigenes*, for example has a pH optimum of 9.6 and 20 g/L NaCl when grown under dissimilatory iron reducing conditions [80] and other species (e.g., *A. transvaalensis*) grow at pH above 12 [81]. Thus, it appears that strain OhILAs may be classified as a *Clostridium*.

The physiological response to arsenic was quite interesting in *Clostridium* sp. strain OhILAs. It was extremely resistant to As(V) as growth was not inhibited by 40 mM As(V) (the highest concentration tested). However, complete inhibition of growth occurred in the presence of 10 mM As(III) and growth was decreased by 50% from 1 mm As(III) to 5 mM As(III). From the As(III) inhibition it can be concluded that OhILAs can

only reduce up to 10 mM As(V) as the 1:1 conversion to the product, As(III), proves to be too toxic at that concentration in a closed system.

Gram-positive bacteria lack a "true" periplasmic compartment and the arsenate reductase in such bacteria is anchored to the membrane. Nevertheless, the gene encoding the catalytic subunit of the respiratory arsenate reductase, *arrA*, includes a twin arginine motif in the leader sequence at the 5' end. This suggests that as in a Gram-negative cells, the catalytic subunit of the respiratory arsenate reductase is oriented towards the peptidoglycan layer and not the cytoplasm.

Saltikov et al, [74] demonstrated that the Gram-negative *Shewenella sp*. strain ANA-3 exhibits increased growth, arsenate reduction and lactate (substrate) oxidation with a assistance of a functional *ars* detoxification operon. *Clostridium* sp. strain OhILAs also has an *ars* operon that also includes a homolog of the methylase ArsM. Thus is can deal with As(V) and As(III) that manages to enter the cell. As(V) is transported through phosphate transporters, in specific, Pit (inorganic phosphate transporter) and Pst (phosphate specific transporter). Pit functions under high phosphate conditions and is less discriminatory towards arsenic where as the Pst is more specific reducing arsenic uptake by 100 fold while scavenging for phosphate. It is possible that the transport of phosphate in high concentrations of As(V) is via the Pst system in *Clostridium* sp. strain OhILAs. As(III) in water is an inorganic equivalent if nonionized glycerol and enters the cell through glycoporin membrane channel proteins, which may explain why *Clostridium* sp. strain OhILAs is more sensitive to As(III) [82].

B. River Enrichments

The assessment of the river enrichment data has indicated that media formulation can be an important factor in enriching for arsenate respiring prokaryotes. At low concentrations of arsenic (<1 mM arsenic), respiration of As was not evident. Interestingly, sulfide reduction was not inhibited, as evidenced by a strong odor of sulfide emanated from the bottles that contained less than 5 mM arsenic. Although As(V) is favored over sulfate as an electron acceptor [64], concentration may supercede energetics (e.g., there was not enough As(III) to support respiratory growth). Another issue may concern sediment composition, as As is readily adsorbed to iron-sulfides, this would make the arsenic less readily available.

Medium containing 5 mM As(V) appeared to yield the best results, regardless of electron donor. The concomitant reduction of arsenate and sulfate resulted in copious orpiment formation. The 5 mM As set lacking electron donor did not development of orpiment, suggesting that the organics in the sediment could not support arsenate reduction.

Interestingly, orpiment production was inhibited a the higher concentrations of As (10 mM and 20 mM) even though there was significant arsenate reduction. One explanation is that higher concentrations of As inhibit the reduction of sulfate. It is possible that these As levels may be toxic to certain bacteria such as sulfate reducers. Also the precipitation of orpiment is dominant at high (20:1) S:As ratios [83]. This supports the positive detection of orpiment at the 5 mM level and the lack of production at higher levels of As.

The results also suggest that hydrogen is the best electron donor. While all donors tested gave comparable results with 5 mM As(V), the results at higher concentrations were

different. More importantly, these results suggest that a matrix approach, using a combination of different electron donors and different concentrations of As(V) will provide a more confident assessment of the natural community.

C. Transformation of Roxarsone

The spectrophotometric analysis of 3-nitro-4-hydroxy benzene arsonic acid (roxarsone) and its potential degradation products indicated that spectrophotometry could be a useful tool in monitoring the transformation of roxarsone. Roxarsone was found to have an absorbance maximum at 400 nm. 3-amino-4-hydroxy benzene arsonic acid was found to have a distinct absorbance peak at 280 nm, but was indistinguishable from para-nitrophenol. A calibration curve could be used to quantify the transformation of roxarsone. Acidification of the samples was found to alter the absorbance spectrum for both roxarsone and 3-amino-4-hydroxy benzene arsonic acid. Thus separate samples need to be taken for cell counts, spectrophotometric analysis, and HPLC/LC-MS analysis.

Clostridium sp. strain OhILAs was able to readily and rapidly convert 3-nitro-4-hydroxy benzene arsonic acid (roxarsone) to 3-amino-4-hydroxy benzene arsonic acid. The initial hypothesis was that *Clostridium* sp. strain OhILAs could use roxarsone either as an electron donor and carbon source, or as an electron acceptor. The results with thiosulfate indicate that while roxarsone was transformed, the thiosulfate was not. The results with glycerol were equivocal. Again, this may be due to the amount of yeast that was used in the experiment. When lactate was provided as a potential electron donor, roxarsone was also transformed. However, it is equally probable that the lactate was used as a fermentable substrate and the roxarsone served as a sink for the reducing equivalents. Clearly, only the nitro group was reduced under all growth conditions tested.

Clostridia are a common component of both the chicken cecum and chicken litter [84, 85]. *Clostridium* sp. strain OhILAs was not isolated from chicken litter but does have many characteristics in common with clostridial species that were isolated from chicken litter.

D. Summary

This work has demonstrated that *Clostridium* sp. strain OhILAs is capable of respiring As(V). It is also capable of thiosulfate reduction and the fermentation of lactate and glycerol. More significantly, it can transform the organoarsenic roxarsone. The recent completion of the annotation of its genome holds great promise, as many aspects of its physiology can now be explored.

The river matrix experiments also provided insight into the effects of arsenic concentration and electron donor. The results demonstrate that the concentration of As(V) can have a dramatic impact on enrichment cultures and incubations. A concentration of 5 mM As(V) is suggested as the most appropriate single concentration; however, a matrix using different electron donors and concentrations of As(V) is recommended.

More importantly, this study showed that roxarsone was readily transformed, at least to 3-amino-4-hydroxy benzene arsonic acid under anaerobic conditions, by a strain of *Clostridium*. As clostridia are commonly associated with the chicken digestive tract (e.g., cecum) and litter, roxarsone transformation should naturally occur during composting of the litter.

VI. Appendices

A. Appendix 1 : Basal Salt medium

Constituents	g/L
Potassium Phosphate Dibasic	0.225
Potassium Phosphate Monobasic	0.225
Sodium Chloride	0.46
Ammonium Sulfate	0.225
Magnesium Sulfate	0.117
Yeast Extract	0.5
Sodium Lactate* (10mM)	2.24
Sodium Bicarbonate	4.2
Cysteine	0.5
Additions	
Mineral Mix	10ml
Vitamin Mix	10ml
Terminal Electron Acceptor (10mM)	
Sodium Arsenate	3.0
Sodium Thiosulfate	2.48

Vitamin Mix	**mg/L**
Biotin	2
Folic Acid	2
Pyridoxine	10
Riboflavin	5
Thiamine	5
Nicotinic Acid	5
Pantothenic Acid	5
p-aminobenzoic Acid	5
Thioctic Acid	5
B_{12}	0.1

Mineral Mix	**g/L**
Nitrilotriacetic acid	1.5
Magnesium sulfate	3.0
Manganous Chloride	0.44
Sodium Chloride	1.0
Ferric Chloride	0.067
Calcium Chloride	0.1
Cobalt Chloride	0.1
Zinc Sulfate	0.274
Cupric Sulfate	0.01
Aluminum Potassium Sulfate	0.01

Boric acid (granular)	0.01
Sodium Molybdate	0.025
Nickelous Chloride 6-Hydrate	0.024
Sodium Tungstate Hydrate	0.025

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