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The Effects of Arsenic on *Thiobacillus ferrooxidans*

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EXECUTIVE SUMMARY

Arsenic is a heavy metal that occurs both naturally in the earth's crust and is caused by anthropologic means such as mining and combustion of fossil fuels. It is also an acute poison. Arsenic contamination of drinking water is an issue that has been receiving national and global attention. On a national level, the United States Environmental Protection Agency recently reduced the allowable limits of arsenic in drinking water from 50 parts per billion to 10 parts per billion. Internationally, the mass poisoning of the Bangladeshi people through groundwater drinking sources has received worldwide awareness.

Bioremediation is a technology that has begun to be perceived as a feasible option for water and soil clarification and remediation. However to make bioremediation a viable alternative to more traditional treatment technologies, more research needs to be conducted. Because the valance state of arsenic species determines its toxicity, bioavailability, and solubility, understanding the way in which various types of microbes and plants change arsenic speciation is imperative in the design of bioremediation techniques.

Laboratory experiments were conducted to determine the effects of As(III) and As(V) on *Thiobacillus ferrooxidans*. Even though *Thiobacillus ferrooxidans* is a widely studied bacterium due to its use in bioleaching of mining ores, new characteristics have been discovered through this study.

Three main conclusions can be drawn from the results of this research. First, As(III) and As(V) modify the physiology and the surface charge of *Thiobacillus ferrooxidans* in different ways. Secondly, this research suggests that As(V) is more toxic to *Thiobacillus ferrooxidans* than As(III) in the presence of iron. A finding of this research that has not been previously documented is fact that the *Thiobacillus ferrooxidans* in this study, whether adapted or unadapted to As(III), grow better in the presence of As(III) than without any arsenic at all. These findings have many implications and have spawned topics for further investigations.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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I. INTRODUCTION

Arsenic contamination is a worldwide environmental issue. Currently, there is a focus on the problem of arsenic in drinking water both nationally and globally. The arsenic issue in Bangladesh has recently been gaining international awareness.

In the 1960's, the people of Bangladesh were encouraged by the government and internal relief organizations to use groundwater from wells for their source of drinking water due to the bacterial contamination of the surface waters in the area. It has now been discovered that most of the ground water wells have very high levels of naturally occurring arsenic which has slowly been killing the people who have heeded the suggestions from the government and internal relief organizations.

Nationally, the Environmental Protection Agency (EPA) has recently reduced the arsenic limits in drinking water from 50 parts per billion (ppb) to 10 ppb due to its proven toxicity and detrimental health effects. This legislation became effective in February of 2002, while the water utilities must be in compliance by the beginning of 2006.

While several methods have been employed such as adsorption/coagulations, precipitation, oxidation, and ion exchange, at the present, there is no efficient, cost-effective method for the removal of arsenic from drinking water. Arsenic is one of the most difficult metals to remove from water, especially at low concentrations. It is a costly process that also requires the addition of many other chemicals to treat the water. Innovative removal methods need to be designed for this purpose.

Columbia University has been awarded a grant to research arsenic contamination in both Bangladesh and at sites within the United States. This research project is a

collaborative effort between various disciplines of the University; the School of Public Health, the Lamont Doherty Earth Observatory, the College of Physicians and Surgeons, and the Earth and Environmental Engineering Department. The Engineering department has been brought into the joint project to research ways in which to remediate arsenic contaminated sites.

This thesis research investigates ways to reduce the toxicity of arsenic using bioremediation. It involves the use of "biomimicry" which is a newly coined phrase that describes the concept of designing technology to imitate biological processes occurring in nature. It has been documented that some microbial and plant species have the capability to metabolize arsenic, although the mechanisms of this metabolism are not yet clearly understood (14). In addition, certain bacteria have been used to remove metals from the soil, a process called "bioleaching". These two concepts spawned the idea for my thesis research.

II. BACKGROUND INFORMATION

A. Arsenic

Arsenic is a heavy metal that occurs both naturally in the earth's crust and is caused by anthropologic means such as mining and combustion of fossil fuels. It is also an acute poison. Due to the fact that arsenic is odorless and tasteless, it is widely known for its criminal use in homicides.

1. Environmental Chemistry

Arsenic exists naturally in four valence states: $^{+}5$, $^{+}3$, 0, and $^{-}3$. The most common forms of arsenic are $^{+}5$, also denoted as As(V) or arsenate, and $^{+}3$, As(III) or arsenite. Arsenite is much more toxic than arsenate. The current literature states that arsenite is anywhere from 10 to 100 times more toxic than arsenate. (1,7,11).

Not only does toxicity vary with arsenic's valence state but also the solubility, bioavailability, and mobility (11). Although both As(III) and As(V) are soluble, As(III) has a higher solubility than As(V) and therefore has increased bioavailability and mobility as compared to As(V) (7).

2. Health Effects

Long term exposure to elevated levels of arsenic through ingestion of contaminated water or food causes many types of cancer such as skin, lung, liver, bladder, and kidney (3,6). Additionally, respiratory illnesses, cardiovascular disease, birth defects, and death have also been attributed to arsenic contamination (3). Skin lesions are a sign of advanced stages of arsenic poisoning (4,11). These lesions are

apparent in the population of Bangladeshis. A study conducted in 2001 found that of the 5,000 well owners interviewed in Bangladesh, twenty-one percent had skin lesions (6).

As previously mentioned, the EPA recently reduced its allowable levels of arsenic in drinking water from 50 ppb to 10 ppb. In Bangladesh the drinking wells have levels of arsenic contamination as high as 1000-2000 ppb, with a few isolated wells testing between 3000-4000 ppb (4). It is reported that over the half of the hand pumped wells in the country have arsenic levels higher than 50 ppb (5) and as many as 200,000 to 270,000 cancer deaths related to arsenic exposure have occurred. The source of contamination is naturally occurring, caused by arsenic rich river sediments leaching into the groundwater.

Although the issue in Bangladesh has been quoted as being the "largest mass-poisoning case in the world right now" (4), arsenic contamination of drinking water is not limited to Bangladesh. Other incidents have occurred in Taiwan, Mexico, India, Thailand, Poland, Peru, Chile, Argentina, Canada, Hungary, Japan, New Zealand, Spain, and even the United States (1,3,11). The contamination of these sites is due to both naturally occurring and anthropogenic sources.

3. Microbial Interactions

Oxidation and reduction reactions of the arsenic species occurs naturally, but at a very slow pace. Although, arsenic is toxic to most microorganisms, thus inhibiting their growth, certain microorganisms can facilitate these reactions through various processes such as As(V) respiration and As(III) oxidation (9). These reactions affect the mobility, solubility, and bioavailability of arsenic as a result of the valence state change. A more

detailed analysis of the interactions with specific types of bacteria and arsenic is discussed is the following section.

B. *Thiobacillus ferrooxidans*

Thiobacillus ferrooxidans (*T. ferrooxidans*) is a microorganism that was first discovered in acid mine drainage and isolated in 1947. It has been widely studied due to its bioleaching capabilities. The following sections will provide a background on bacteria's physiology and known interactions with arsenic. The last part of this section will discuss various species of bacteria that have been documented to oxidize or reduce arsenic.

1. Physiology

T. ferrooxidans is a gram-negative, non-spore forming bacterium. It is motile by use of its single polar flagellum. It is rod shaped with dimensions of approximately 0.5-0.6 μm wide and 1.0-2.0 μm long (15,17). It can be found single or in pairs, and also in short chains although this does not occur as often (15).

As a chemolithotrophic bacterium, *T. ferrooxidans* oxidizes iron or sulfur compounds as its source of energy. Iron is oxidized from ferrous (Fe^{+2}) to ferric (Fe^{+3}) by *T. ferrooxidans* according to the following equation (17):

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$

The growth of *T. ferrooxidans* is documented as being absolutely dependent on the oxidation of iron for survival (18).

T. ferrooxidans is autotrophic meaning that it able to survive with carbon dioxide as its sole carbon source. It is an aerobic organism, as well as mesophilic and acidophilic, with optimal growth at temperatures between 25 and 30°C and pH between 2.0 and 2.5 (17).

2. Arsenic Toxicity

Arsenic is toxic to *T. ferrooxidans*, causing a decrease in the growth rate and eventual death. The levels at which arsenic becomes toxic to *T. ferrooxidans* are not agreed upon. Even though some heavy metals can be toxic to *T. ferrooxidans,* the bacteria can be adapted to tolerate much higher concentrations than it naturally would allow. There have been a variety of arsenic toxicity levels documented with numbers ranging anywhere from 0.8 ppm (17) to 40,000 ppm (8). As(III) is documented as being three to eight times more toxic to *T. ferrooxidans* than As(V) (8,28).

Studies have also been conducted which argue that the toxicity of As(III) and As(V) on bacteria is dependent on the availability of an energy source (28). Furthermore, Breed *at al* stated that their results suggest that As(III) may not three times as toxic as As(V) as has been previously reported (28).

It has been documented that the addition of 10 ppm of As(III), Mn(III), Sn(II), Co(II), Cu(II), and Zn (II), Cl^-, and NO_3^- in the bacteria medium of *T. ferrooxidans* had no effect on the iron oxidation and therefore the growth of the bacteria (16). The study was conducted to ensure that the bioleaching abilities of *T. ferrooxidans* would not be compromised by other heavy metals that may be present in the ore.

3. Microbial Oxidation and Reduction of Arsenic

Due to the different characteristics of arsenic species, it is important to understand the way in which microbes oxidize and reduce arsenic. Microorganisms have been reported to oxidize As(III) and/or reduce As(V) (9,10,13,19,20). Some autotrophs have even been discovered to utilize As(III) as their sole energy source. Additionally, species of heterotrophs have the ability to convert As(III) to As(V) as a secondary source of energy or as a mechanism of detoxification (20).

The first identified arsenite-oxidizing bacterium, *Bacillus arsenoxidans*, was reported in 1918 in South Africa. Green discovered the bacterium in a cattle-dipping solution that used arsenite to protect against insect bites (20). *Bacillus arsenoxidans* was not tested to determine if it could grow with arsenite as its sole energy source and unfortunately will not be tested since the organism was lost.

Since that time, other arsenite-oxidizing bacteria have been documented. In 1949, 15 strains of bacteria were discovered again in cattle-dipping solution, but this time in Queens, Australia (20). Other arsenite-oxidizing bacteria have been discovered in arsenite enriched raw sewage, arsenic-contaminated sites, and geothermically active areas.

Most of the arsenite-oxidizing bacteria have been proven or assumed to be heterotrophic, which means they need organic material for their energy source and therefore cannot grow with arsenite as the only nutrient source. Another example of such a heterotroph is *Thermus aquaticus* and *Thermus thermophilus* that are found in areas of geothermal activities such as the hot springs of Yellowstone National Park (9).

A new isolate within the *Thermus* genus was discovered in 2001. This bacterium, designated, *Thermus* HR 13, was isolated from a geothermal environment in northern California (9) and has the ability to use arsenic as a secondary energy source.

Arsenite-oxidizing bacterium have been discovered that are chemolithotrophs and are able to utilize arsenite as their sole energy source, although to date only two such strains have been reported. The first isolated bacterium of this type was reported in 1981 in the former Soviet Union. It was a gram-negative, motile, rod-shaped bacterium isolated from an arsenopyrite gold mine, named *Pseudomonas arsenitoxidans*. The bacterium was found to have a slow growth rate with a doubling time of approximately 2 days (13, 20).

In 2000, a chemolithotrophic bacterium was reported from Australia, again from an arsenopyrite gold mine, which has a much more rapid growth rate than *Pseudomonas arsenitoxidans*, with a doubling time on the order of 7.6 hours. This bacterium was named NT-26 and has the ability to use arsenite as its sole energy source. It is gram-negative, motile, and rod-shaped, but possesses two subterminal flagella (13).

Some strains of *Alcaligenes faecalis* have been isolated that are not able to use arsenite as their sole energy source, but actually "survive better in the presence of arsenite than in its absence" (20). It is noted that this phenomena suggests that the bacteria is obtaining at least secondary energy from the oxidation of the arsenite. *Alcaligenes faecalis* are motile with a single peritrichous flagella, rod-shaped and approximately 1 μm.

It has been documented that *T. ferrooxidans* does not oxidize arsenite to arsenate (19), but *T. ferrooxidans* can oxidize arsenic-containing materials such as As_2S_3, FeAsS,

Cu_3AsS_4. It is thought that the newly formed arsenate in the cultures was either a result of oxidation from ferric iron or autoxidation in conjunction with the metabolite, but not the bacteria itself (20).

C. Bioremediation

Bioremediation is defined as the process by which living organisms act to degrade or transform hazardous organic contaminants (38) or stabilize, solubilize, or reduce the toxicity of inorganic contaminants. These living organisms are most commonly bacteria, fungi, algae, and/or plants. There are many applications of bioremediation from the treatment of mining drainage and other industrial sites to detoxifying sewer sludge and contaminated soil and water (33).

There is a major difference in the management of organic verses inorganic contaminates. Organic contaminants can be biologically broken down to environmentally safe compounds with carbon dioxide and water being the final products. This cannot be accomplished with inorganic contaminants because metals are already in their most fundamental form and cannot be destroyed (34).

1. Bioremediation Techniques

Several techniques are used in the field of bioremediation. Some of the more common ones will be presented here. The time frame, funds available, and concentration of contaminants are the deterministic factors involved in selecting a bioremediation technique (36).

Land farming is the process by which microorganisms and nutrients are mixed with contaminated soil, usually with a backhoe, to treat the contaminated land (36). This process requires a liner to contain the contaminants and the by-products of the reactions.

Another type of bioremediation uses a sequencing batch reactor (SBR). This process involves adding the contaminated soil or water in a reactor along with microbes and nutrients. This technique allows for optimization of removal parameters such as pH, contact time, and concentration since it is a controlled environment (36).

Composting uses microorganisms and aeration to convert organic matter into soil enrichment nutrients. This is a technique that has been used by farmers for many years (36), but it is not available to inorganic compounds.

2. Bioremediation Mechanisms for Inorganic Pollutants

This section will discuss the mechanisms by which organisms are able to perform bioremediation of inorganic pollutants. Biosorption is the uptake of metals by microbial cells in which no energy is required. This process enables the microbes to remove high amounts of metals from water and soil (37). Bioaccumulation is similar to biosorption, but in this case the uptake of the metal requires energy.

Bioremediation may also be accomplished by oxidation/reduction. These reactions can be catalyzed directly or indirectly. Some organisms have the capability to be the terminal electron acceptor. Other organisms facilitate the oxidation/reduction reaction by their excretions, also referred to as metabolites in the case of bacteria (37).

Precipitation is another mechanism by which inorganic contaminate can be remediated. This mechanism involves the reaction of a metallic ion with a product of

microbial metabolism (37). The reaction results in a precipitate that can then be filtered out of the waste stream and therefore rendered less toxic.

Finally, methylation is a process than can be used only if the product is nontoxic. A large number of metals can be methylated by microorganism, but many of the products are toxic. Therefore, this is a process that can be used only in very specific cases such as selenium (37).

3. Bioremediation and Arsenic

Many of the mechanisms mentioned above are possible in the bioremediation of arsenic. There is evidence of microbial oxidation/reduction as mentioned in previous sections. Also arsenic can be methylated, but as its products are toxic, this is not a technique that is viable for bioremediation. Arsenic is also subject to precipitation.

Phytoremediation is a type of bioremediation that has great potential to remediate arsenic contaminated sites. This topic will be discussed in detail in the following section.

4. Evaluation of Bioremediation

One of the major advantages of bioremediation is its cost benefits. Bioremediation techniques generally cost one-third to one-half of the price of more conventional methods (33). Because some of the bioremediation techniques can be applied on-site, bulk excavation is not required which reduces the cost and the potential for further contamination from the diesel trucks as well as spills or accidents (36). There is no additional environmental pollution caused by bioremediation which is a key advantage (33). Furthermore, large amounts of solid waste are not produced that then

need to be disposed of as in other treatment methods (35). Therefore, bioremediation is capable of remediating contaminated sites and not just transferring the pollutants to different mediums.

Although bioremediation is being used and has the potential to be implemented on a wider scale, there are still some issues that need to be reviewed in order for it to achieve its maximum capabilities. One of the disadvantages of bioremediation is the fact that it is more time consuming than the more conventional methods. Table 1 borrowed from Levin and Gealt (36) below summarizes some of the differences of various treatment methods.

Table 1 Comparison of Treatment Methods

Type of Treatment	Cost per cubic yard ($)*	Time required (months)	Additional factors/expense	Safety issues
Incineration	250-800	6-9	Energy	Air pollution
Fixation	90-125	6-9	Transport; long-term monitoring	Leaching
Landfill	150-250	6-9	Long-term monitoring	Leaching Air pollution**
Biotreatment	40-100	18-60	Time commitment of land	Intermediary metabolites and polymerization

*Note: This table was published in 1993, so the prices listed are subject to change.
** Item added by author.

Another disadvantage of bioremediation outlined in Table 1 is the intermediate products caused by microbial metabolism that may be more toxic than the original contaminants.

Currently, there are also problems with pollutants leaching from the treatment site if the bioremediation is conducted on-site. There is also no guarantee that the microorganisms on-site will stay there to remediate the contaminants.

Lastly, there is a possibility that not all the toxic or hazardous chemicals to be remediated will be treated. This is a troubling disadvantage of bioremediation because it

means that this treatment method gives no assurance that federal or state regulations will be met. Research is still needed to enhance bioremediation techniques so that they can be widely and accurately implemented.

D. Phytoremediation

There are some plants that can reduce the toxicity of arsenic or that can even thrive on arsenic as a nutrient, but the mechanisms by which these plants transform the arsenic are still not clearly understood. Phytoremediation is a form of bioremediation that uses plants to stabilize, transfer, or remove contaminants from soil or water. This is thought to be a promising "green" technology that is effective and inexpensive as compared to the other alternatives for pollutant remediation.

1. Types of Phytoremediation

There are four main types of phytoremediation: phytoextraction, phytofiltration, phytovolatilization, and phytostabilization. Phytoextraction is the process by which the plant removes contaminants from the soil and transports them to the above-ground shoots. Phytofiltration can be broken down further into two sub-groups: rhizofiltration, which uses the plant roots to remove toxic metals from contaminated effluents, and blastofiltration, in which seedlings are used to remove contaminants from effluent. Phytovolatilization involves using plants to extract volatile metals from contaminated soils. Lastly, phytostabilization is the process by which the plant will stabilize the soil

contaminants, not allowing them to leach from the site, or reduce the toxicity of the contaminants but not remove them from the soil (21, 22).

Two types of plant species are able to be used for phytoremediation. Pseudometallophytes are plants that are able to survive on both metal-contaminated and non-contaminated soils. Metallophytes are those that can only grow on metal-contaminated soil (24).

2. Mechanisms for Remediating Elemental Pollutants

There are three main mechanisms by which plants can remediate elemental pollutants such as arsenic, lead, and mercury. The first is adsorption onto the plant roots. The roots have a very large surface area, as well as the capability to bind to the elemental pollutants due to the fact that the roots are used to uptake elemental nutrients to the plant (23).

The second mechanism is hyperaccumulation which involves concentrating the toxic pollutants in the above-ground biomass of the plant. Hyperaccumulation is defined as the plant containing a concentration of a metal ion greater than 0.1-1% of the dry plant weight (23). Because elemental pollutants are toxic, it is thought that hyperaccumulation is used to compartmentalize the metal to keep it from interfering with the normal processes of the plant (14).

The last mechanism of elemental remediation in plants that will be discussed is transformation of the element to a less toxic species. As previously mentioned, characteristics of some elements are dependent upon their oxidation or valence states as

is the case with arsenic. In an effort to reduce the toxicity of these elements, the plant may transform the contaminant to a more harmless state (23).

3. Arsenic and Phytoremediation

Plants have been discovered that have the ability to remediate arsenic through the various mechanisms outline above. Some of these are naturally occurring, others have been grown on arsenic-contaminated lands, and others still have been genetically modified to have an affinity for arsenic (14,25,26).

The Brake fern, *Pteris vittata*, is one example of a plant that has been reported to naturally hyperaccumulate arsenic (14,25). It can contain levels of arsenic one hundred times that of the soil. It has been suggested that As(V) is converted to As(III) from the roots to the fronds (14). Other sources have indicated that it is much more efficient for plants to uptake As(V) as opposed to As(III) (12).

4. Evaluation of Phytoremediation

Phytoremediation is a promising technique that is still in its early stages of research. It has many benefits including that it is effective and economically viable. One project that used sunflowers and Indian mustard to clean up a contaminated lead site saved over $1 million by using phytoremediation as opposed to traditional methods (27).

In addition, the method is not very labor intensive as plants maintain themselves for the most part. Phytoremediation techniques are also solar energy driven therefore money is saved on power generation as well. Another benefit of phytoremediation is that it is capable of treating low concentrations of contaminants. Finally, phytoremediation is

a natural technique that allows plants to cleanse the environment without the addition of other chemicals and possibly more pollution. Because phytoremediation can be done in-situ, there is a reduction in the amount of large trucks that are needed to haul the contaminated soil to other locations and therefore air pollution caused by the diesel trucks is reduced.

There are some concerns with the use of phytoremediation for toxic contaminants. One of the limitations is the fact that the plants take time to grow and therefore the remediation is not instantaneous. It is even sited that some crops could take several months or even seasons to remediate the pollution to levels within the regulatory standards (27). Therefore, phytoremediation cannot be implemented on sites that are an immediate threat to human health.

Another limitation of phytoremediation is the process that is used by hyperaccumulators. Because the plants are drawing the pollutants up into their above-ground biomass, there is the risk that animals could consume the leaves and therefore bioaccumulate the pollutant, possibly resulting in the toxin making its way all the way up the food chain to humans. A possible solution for this is to keep the area fenced off. But then there is also the risk of pollinators carrying the toxins to other locations (26), which a fence would not prevent.

Additionally, inorganic metals cannot be rendered non-toxic or degraded as in organic contaminants. The metals will be recycled between the soil and the plant, as the plant uptakes the metal from the soil, storing it in its leaves or shoot. When the plant dies, the biomass will be degraded back into the soil, but the metals will still exist.

Therefore, the plants must be harvested and landfilled or incinerated in order to dispose of the toxic metal.

Furthermore, plants can only uptake contaminants that are within the reach of their roots. The majority of plants have a root mass that occupies only the top 30 cm of soil (39) which may not be where the pollutants are located.

Currently, there are also problems with pollutants leaching from the treatment site. The roots do not form an impervious barrier around the contaminated site. Also in reference to the soil ecology that works together with the plant roots, there is also no guarantee that microorganisms that aid in the uptake of pollutants will stay on-site.

Lastly, there is a possibility that not all the toxic or hazardous chemicals to be remediated will be treated. This is a troubling disadvantage of phytoremediation because it means that this treatment method gives no assurance that federal or state regulations will be met.

III. AIMS OF THE RESEARCH

The main purpose of this research is to investigate ways to remove or reduce the toxicity of arsenic in water. Because the behavior of arsenic is dependent on its valence state, it is important to understand the effects that each species, namely, arsenite and arsenate, have on microorganisms that are present in the environment.

This thesis research provides information on the effects of As(III) and As(V) on *T. ferrooxidans*. This study compares the zeta potential which is an indicator of surface electrochemical properties of unadapted bacteria, As(III)-adapted bacteria, and As(V)-adapted bacteria. It also investigates the effects of As(III) and As(V) on the growth of unadapted bacteria, As(III)-adapted bacteria, and As(V)-adapted bacteria through development of growth rate curves. Additionally, the morphology of unadapted and adapted bacteria is compared by use of the AFM.

IV. MATERIALS AND METHODOLOGY

A. *Thiobacillus ferrooxidans* Adaptation

T. ferrooxidans is a widely studied bacterium due to its bioleaching capabilities. Therefore, *T. ferrooxidans* was chosen for these experiments because of the availability information through the scientific literature. The growth medium used for the *T. ferrooxidans* was the 9K medium developed by Silverman and Ludgren (15) with 44.8 g/L of $FeSO_4 * 7 H_2O$ (Aldrich Chemical Company, 99% purity) as the energy source. The 9K medium consists of 3 g/L $(NH_4)_2SO_4$ (Fisher Scientific, 99.99% purity), 0.1g/L KCl (Aldrich Chemical Company, 99% purity), 0.5g/L K_2HPO_4 (Fisher Scientific, 100.1% purity), 0.5g/L $MgSO_4 * 7 H_2O$ (Aldrich Chemical Company, 99.99% purity), and 0.01 g/L $Co(NO_3)_2$ (Mallinckrodt, Inc., 98.5% purity) added to 80 mL of triple distilled water. The pH of the medium was adjusted to approximately 2 using H_2SO_4 (Amend Drug and Chemical Company, Inc., 95.8% purity) and then sterilized in an autoclave and subsequently cooled to room temperature. The $FeSO_4 * 7 H_2O$ was mixed with 20 mL of triple distilled water, the pH adjusted to approximately 2, then sterilized using a vacuum filter then added to the medium. It was inoculated with 10% bacteria by volume. The medium was contained in a 250-mL Erlenmeyer flask and kept on an orbital shaker at 200 rpm at room temperature.

The bacterial medium was adapted to As(III) and As(V) by repeated subculturing. This entailed incrementally increasing the amount of arsenic for each subsequent medium. Initially, 1mL of an As_2O_5 solution As(V) was added to the 9K medium inoculated with 10% unadapted *T. ferrooxidans*. After the bacteria reached full growth, approximately 6 days from inoculation, that bacterium was used to inoculate the next

flask in addition to 2 mL of the As_2O_5 solution. This process continued until 5mL of the As(V) solution was added which correlates to a concentration of 14.36 ppm.

The bacteria was adapted to As(III) in the same way, starting with unadapted bacteria, except that a $AsCl_3$ (Aldrich Chemical Company, 99.99% purity) solution was used. This solution was prepared by diluting the $AsCl_3$ with triple distilled water and adding 2.65 mL of HCl in order to dissolve the powder in solution. The solution was then filtered through 150 mm diameter filtered paper to remove any impurities. The final concentration of the highest level of As(III) solution added was 5 mL which correlates 12.38 ppm.

The triple distilled water used in these experiments was prepared by distilling single distilled water by boiling the water with KMnO4, a strong oxidant, to remove all organic contaminants. The resulting double water was then collected in a second chamber which was distilled again to remove all impurities. The product of the second distillation was then collected in a third chamber which resulted in triple distilled water which has a pH of approximately 6.5.

B. Zeta Potential

There are two stages involved in the zeta potential measurements. First, the bacteria cells were harvested so that the iron precipitate in the flasks would not affect the zeta potential readings. In order to accomplish this, the metabolite of the fully grown bacteria was filtered through 150 mm diameter filter paper. The filtered metabolite was then centrifuged at 10,000 rpm for 25 minutes. After the supernatant was poured off, the cell pellet was dispersed in a pH 2 solution prepared by adding H_2SO_4 to triple distilled

water . These cells were then centrifuged for 10 minutes at 1000 rpm and dispersed in pH 2 solution three more times. The washed cells were then stored in a refrigerator for future use. For the purpose of this research, it is assumed that washing does not have an effect on the zeta potential results. This can be concluded because the results were verified using two sample sets.

In order to prepare the samples for the zeta potential measurement, the following procedure was used. Ten drops of the washed cells were added to 200 mL of a 0.0005 M KNO_3 solution. The solution was mixed and divided into 10 vials of 20 mL each and the pH was adjusted with HNO_3 (Ruger Chemical Company, Inc., 69.9% purity) and NaOH (Fisher Scientific) so that a representative range of pHs were available. The ten samples were then put on an orbital shaker for at least an hour, after which the pH was measured again and readjusted if necessary. The zeta potential was measured for each of the samples using a Zeta-Meter System 3.0 by Zeta-Meter Incorporated. The average zeta potential and standard deviation were recorded for the various pHs of each sample.

C. Growth Rate

The growth rate curves of unadapted bacteria, various concentrations of both As(III) and As(V)-adapted bacteria, and two experimental mediums were developed. The medium for which the growth rate curve was to be developed was inoculated at time zero and kept on an orbital shaker at 200 rpm at room temperature following the procedure outlined in section A. *Thiobacillus ferrooxidans* Adaptation.

The medium was taken off the shaker at least 10 minutes prior to the growth measurements in order to let the precipitated iron settle. One drop of the bacterial

metabolite was extracted on a daily basis in order to record the number of viable bacteria cells using a Petroff-Hausser counting chamber. The cells were viewed at a magnification of forty through a monitor connected to the Nikon microscope.

D. Atomic Force Microscopy (AFM)

The samples of unadapted, As(III)-adapted, and As(V)-adapted bacteria were prepared by air drying one drop of each bacterial metabolite on glass for one hour. The glass was cut afterwards to fit the individual disks used for the AFM. Silicon-nitrite tips were used to scan the samples in contact mode. The AFM images were recorded using a MultiMode™ Scanning Probe Microscope and Nanoscope IIIa Scanning Probe Controller by Digital Instruments.

V. RESULTS

A. *Thiobacillus ferrooxidans* Adaptation

T. ferrooxidans was adapted to both As(III) and As(V). Table 2 outlines the concentrations of the adapted bacteria for this research.

Table 2 Arsenic Concentrations of Adapted Bacteria

Volume of Arsenic Solution added to 100mL medium	Concentration (ppm)
As(III)	
1 mL	2.59
2 mL	5.09
3 mL	7.56
4 mL	9.90
5 mL	12.38
As (V)	
1 mL	2.98
2 mL	5.90
3 mL	8.77
4 mL	11.59
5 mL	14.36

B. Zeta Potential

The zeta potential of the unadapted and adapted bacteria was measured in order to monitor surface electrochemical changes. It has been documented that unadapted *T. ferrooxidans* have an isoelectric point (IEP) at pH 2.4 (29). My research has verified these findings.

The results for the zeta potential readings are grouped by the concentration to which the bacterial cells were adapted. Two sets of measurements were taken for each.

Figure 1

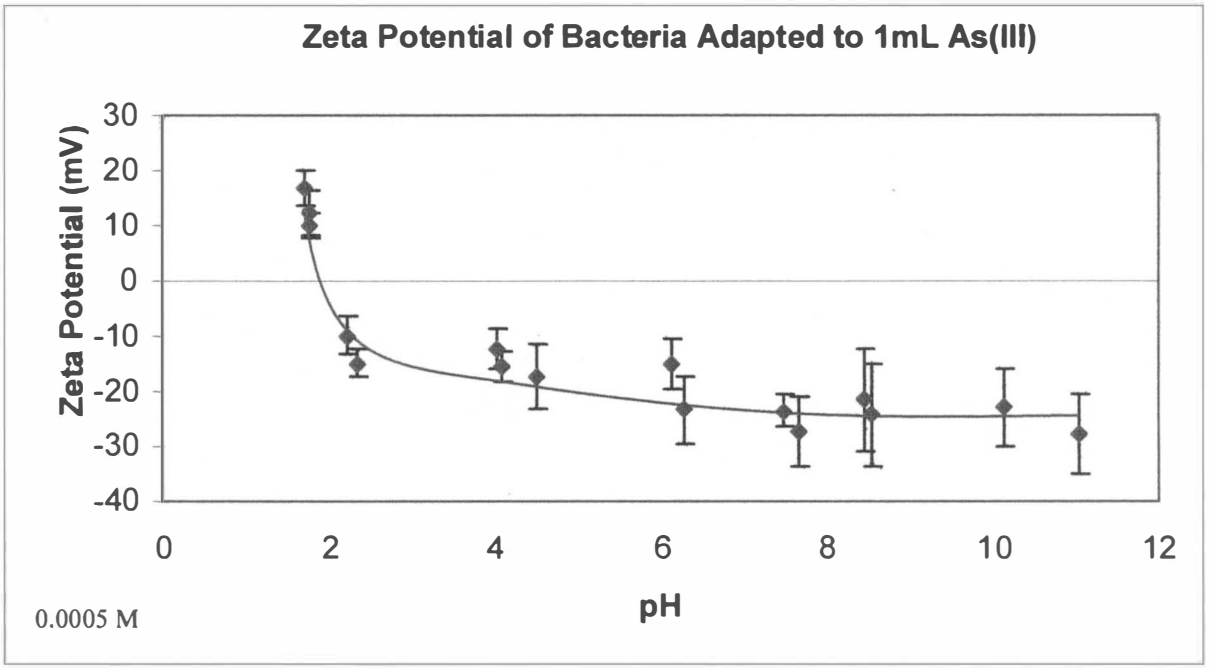


Figure 2

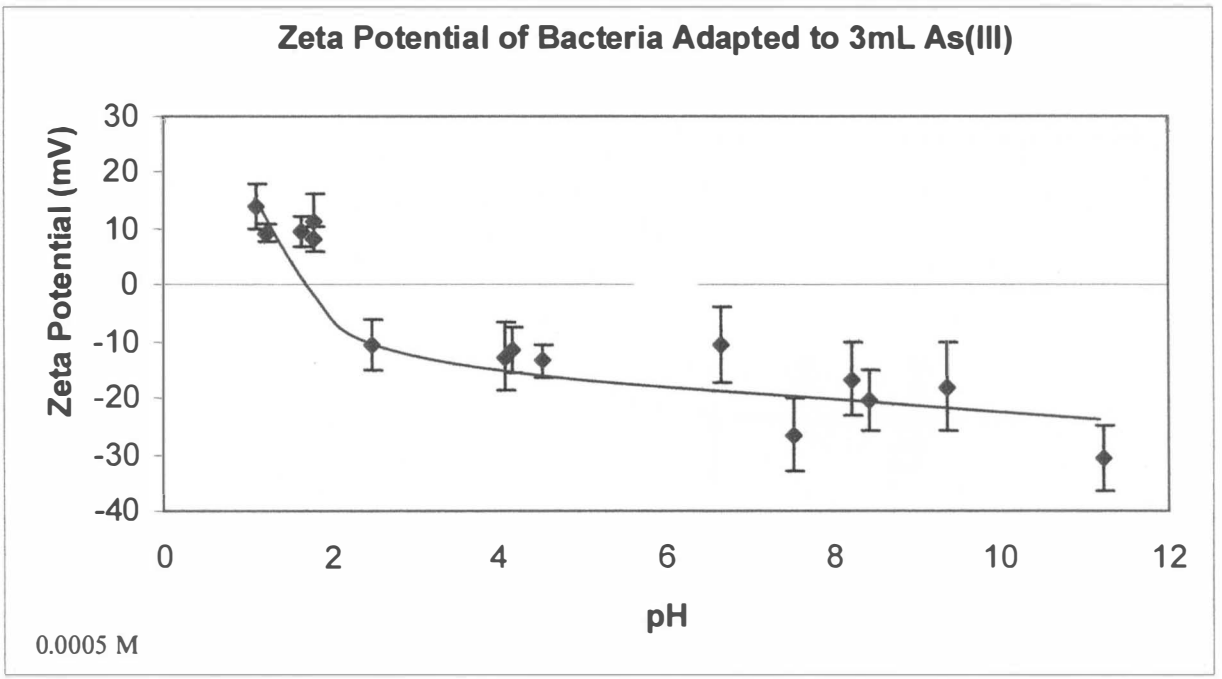


Figure 3

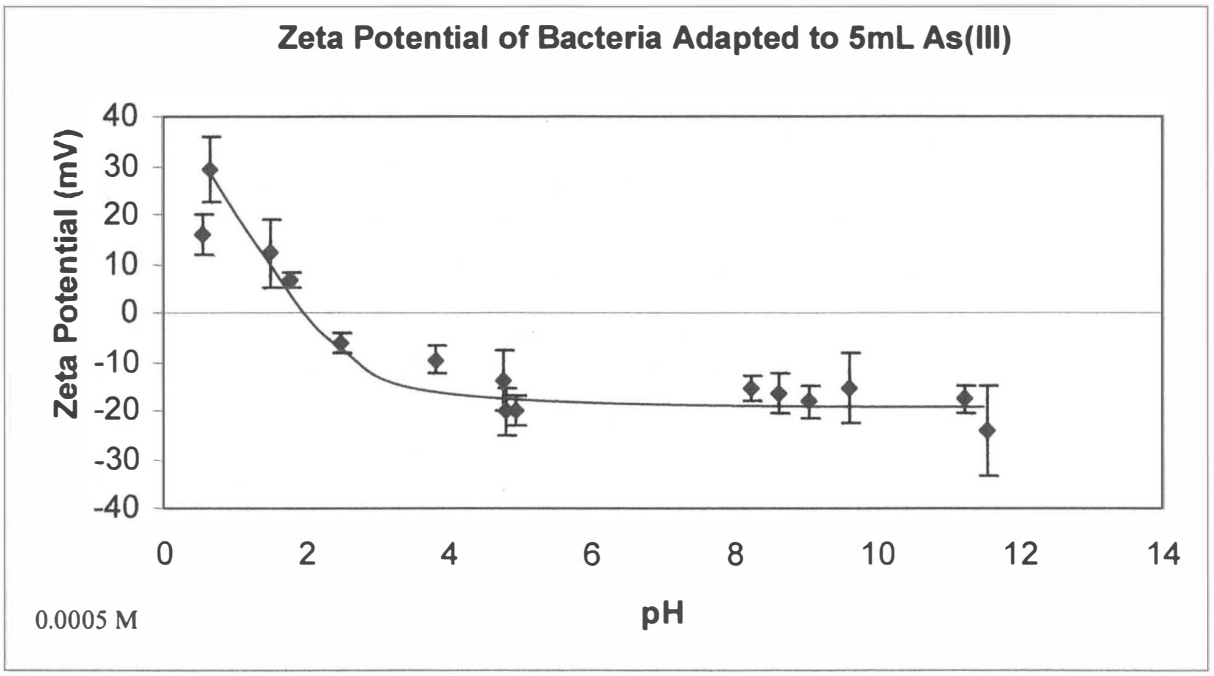


Figure 4

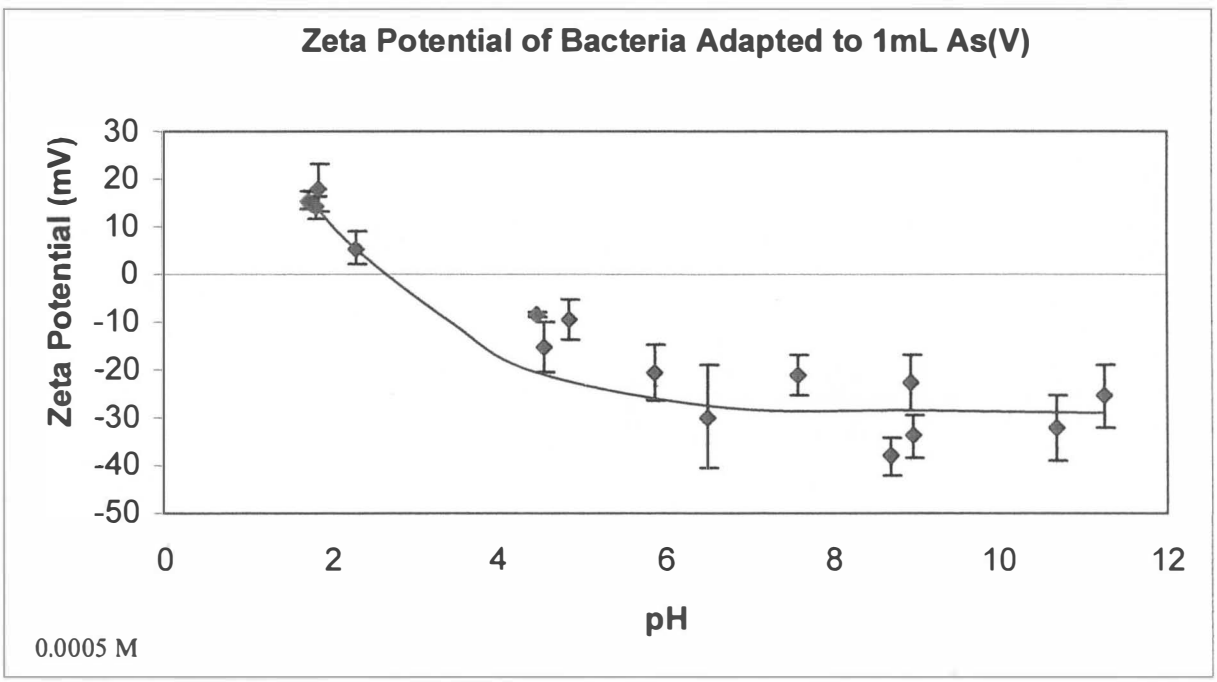


Figure 5

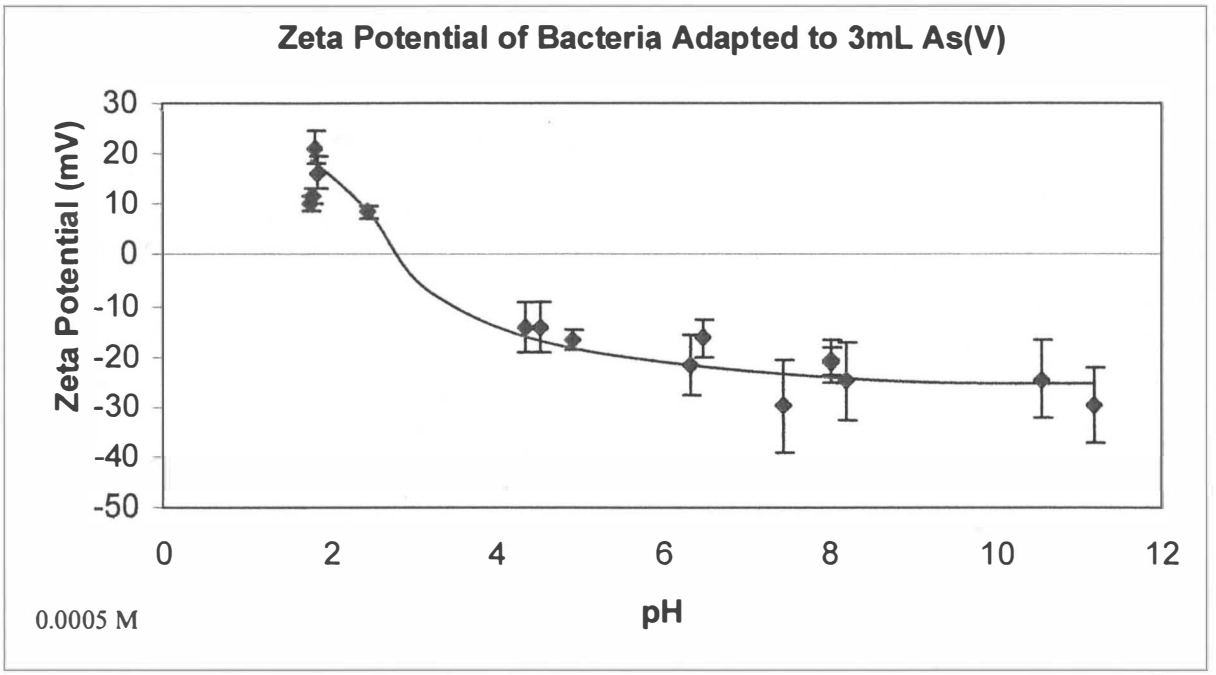
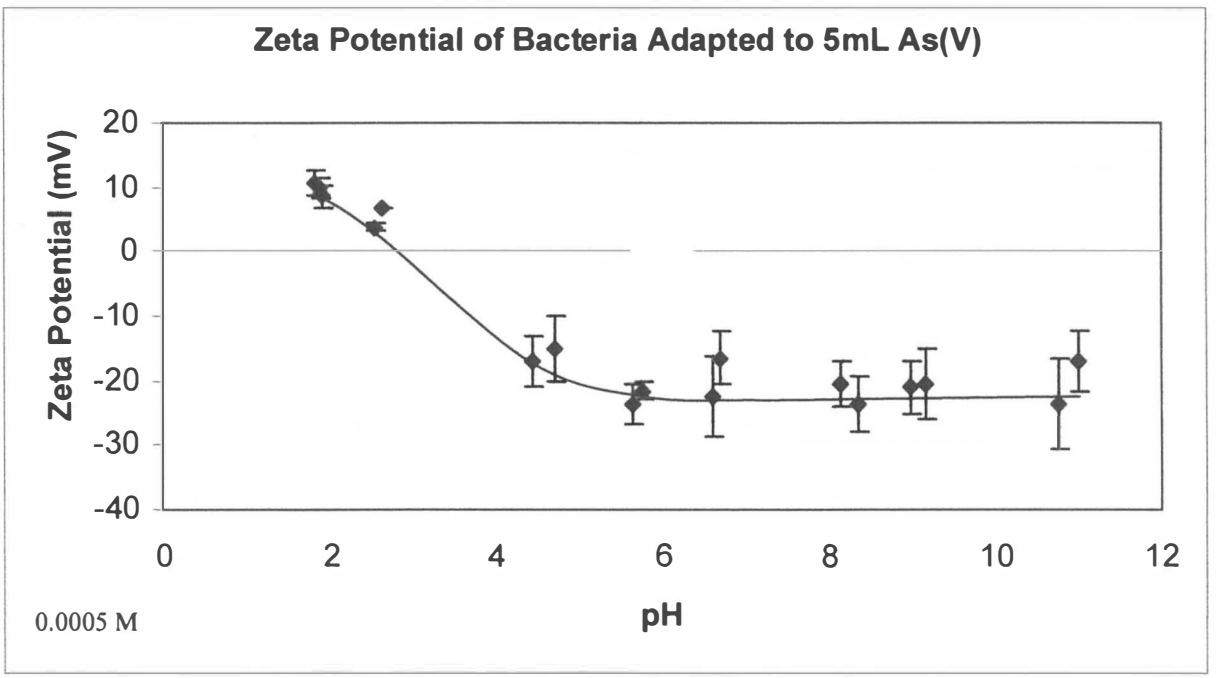


Figure 6



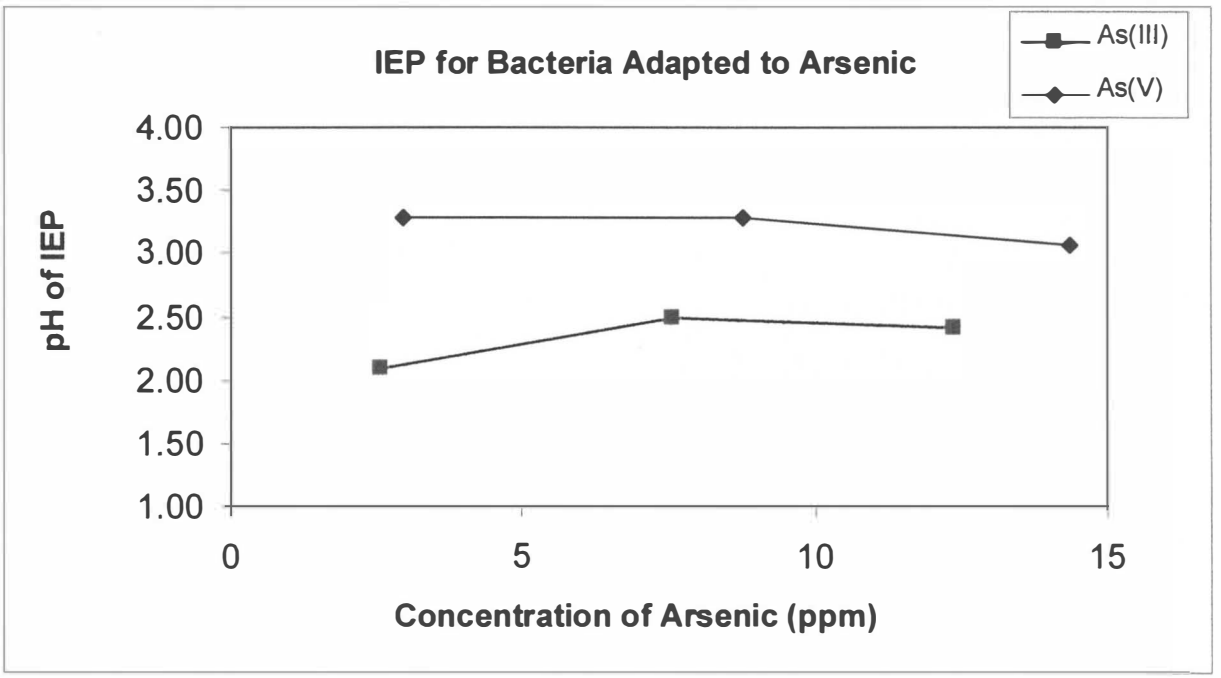
This research shows that there is a marked difference in surface electrochemical properties between As(III)-adapted and As(V)-adapted *T. ferrooxidans*. The following table contains the average IEP for each set of samples.

Table 3 **IEP Results**

Arsenic Concentration in Growth Medium (ppm)	Set 1 IEP (pH)	Set 2 IEP (pH)
As(III)		
2.59	2.0	2.0
7.56	2.2	2.9
12.38	2.2	2.8
As(V)		
2.98	3.2	3.3
8.77	3.4	3.2
14.36	3.2	3.0

The average IEP for the cells adapted to As(V) is 3.2 ± 0.1. The average IEP for As(III) is calculated to be 2.3 ± 0.3. The Figure 7 presents these results graphically.

Figure 7



In terms of minimum zeta potential results, the measurements are fairly consistent for the concentrations of arsenic to which the bacterial cells were adapted. The table below summarizes the results.

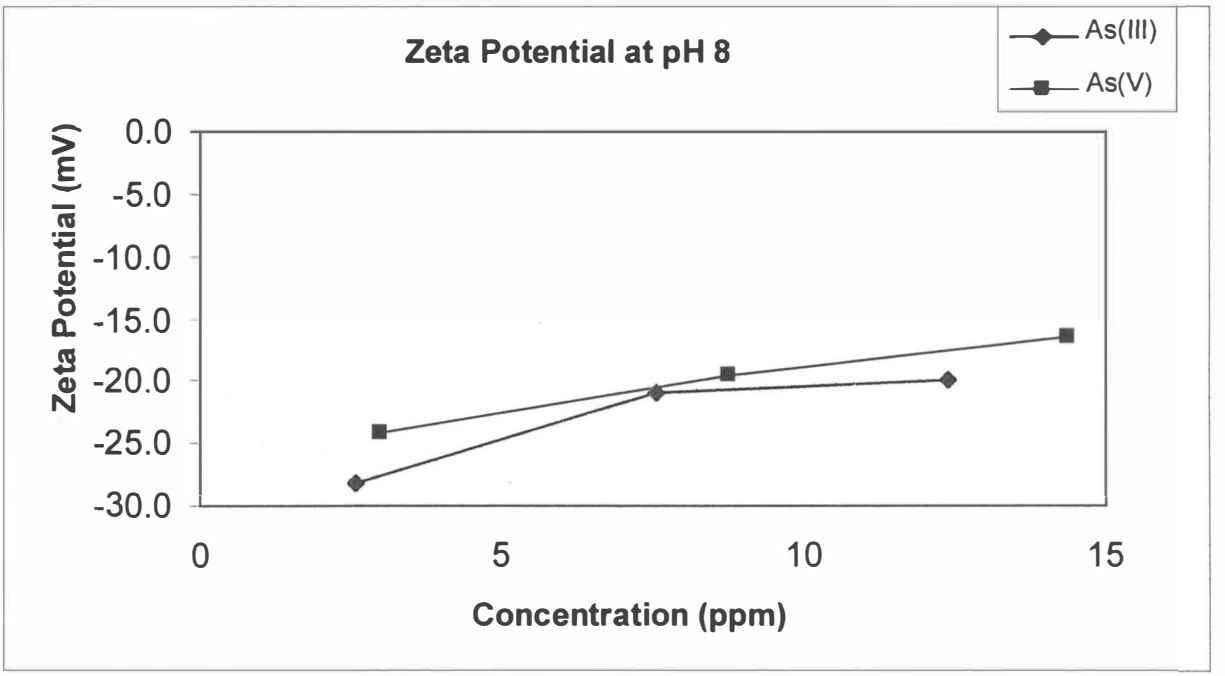
Table 4 **Minimum Zeta Potential Results**

Arsenic Concentration in Growth Medium (ppm)	Minimum Zeta Potential (mV)
As(III)	
2.59	-26.0 ± 1.7
7.56	-28.6 ± 2.1
12.38	-23.2 ± 2.0
As(V)	
2.98	-34.8 ± 6.3
8.77	-29.8 ± 0.1
14.36	-23.7 ± 0.1

As can be seen from the table, the average standard deviation for the minimum zeta potential for each concentration is approximately 2 millivolts.

The zeta potential of bacteria adapted to various concentrations of arsenic at a pH of 8 is presented in Figure 8 to demonstrate the way the zeta potential varies with bacteria adapted to the different arsenic species.

Figure 8



C. Growth Rate Curves

The following thirteen 9K mediums were prepared for the development of growth rate curves: unadapted bacteria ("control" in the figures below), 1mL As(III) solution + unadapted bacteria, 1mL As(III) solution + 1mL As(III)-adapted bacteria (Figure 9), 3mL As(III) solution + unadapted bacteria, 3mL As(III) solution + 3mL As(III)-adapted bacteria (Figure 10), 5mL As(III) solution + unadapted bacteria, 5mL As(III) solution + 5mL As(III)-adapted bacteria (Figure 11), 1mL As(V) solution + unadapted bacteria, 1mL As(V) solution + 1mL As(V)-adapted bacteria (Figure 12), 3mL As(V) solution + unadapted bacteria, 3mL As(V) solution + 3mL As(V)-adapted bacteria (Figure 13), 5mL

As(V) solution + unadapted bacteria, and 5mL As(V) solution + 5mL As(V)-adapted bacteria (Figure 14).

In addition, two experimental mediums were prepared. Twenty-five mL of As(III) solution was added which was inoculated with 10 mL of 5mL As(III)-adapted bacteria (Figure 15). The second experimental medium was used to investigate whether or not As(III)-adapted bacteria could use As(III) as its sole energy source. For this investigation, the 9K growth medium was prepared without the iron. Five mL of As(III)-solution was added and inoculated with washed 5mL As(III)-adapted bacteria cells (Figure 16).

Figure 9

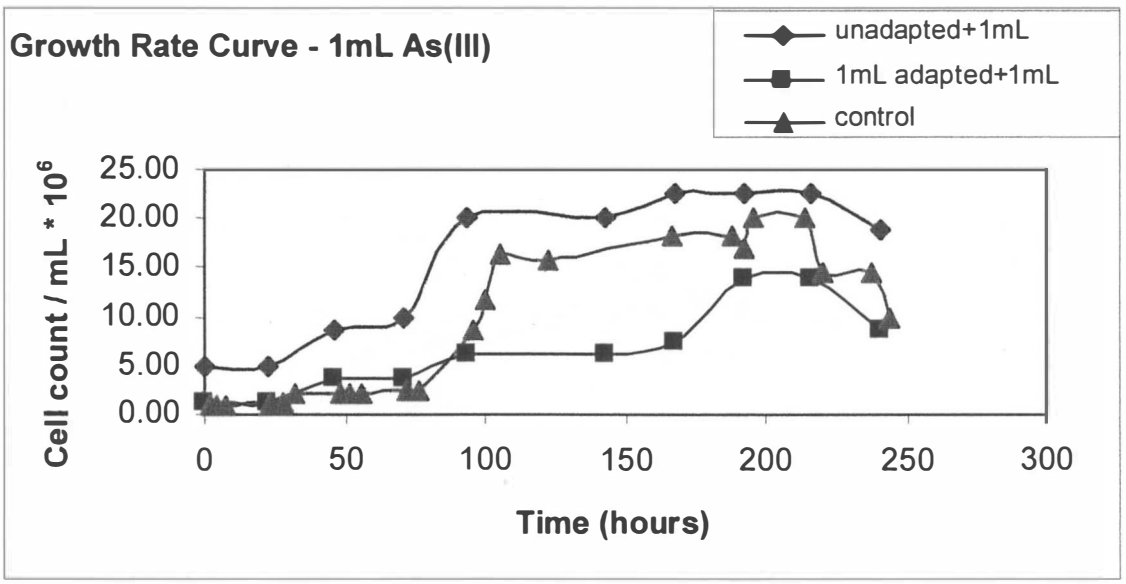


Figure 10

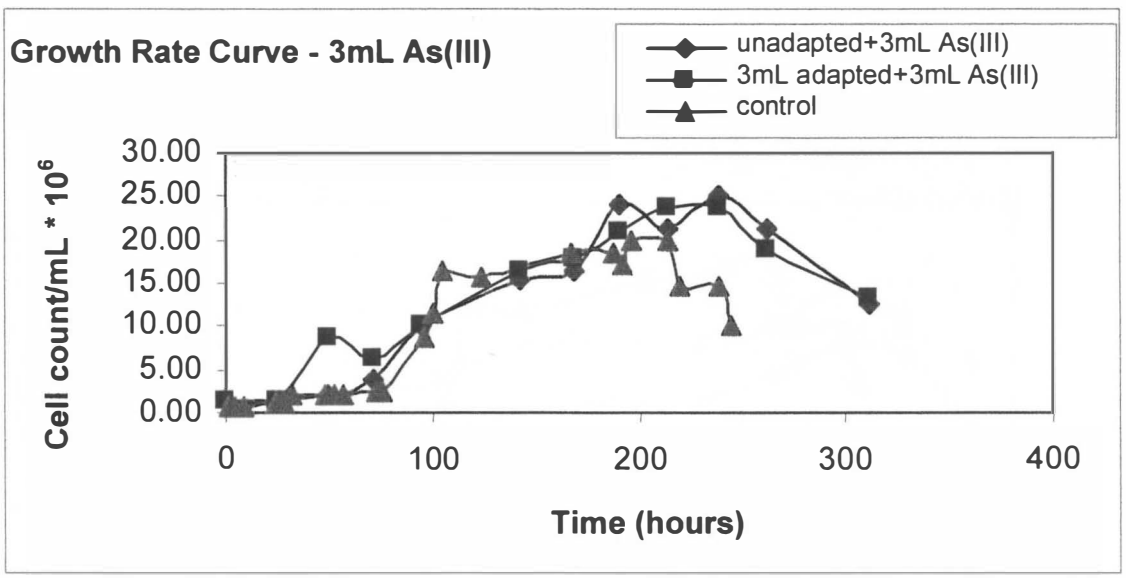


Figure 11

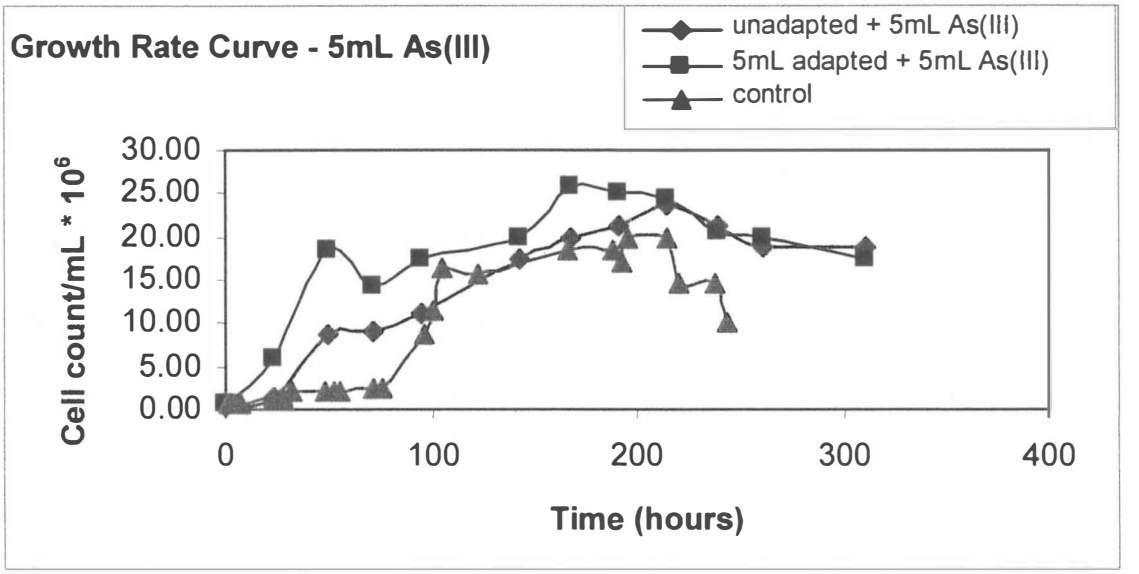


Figure 12

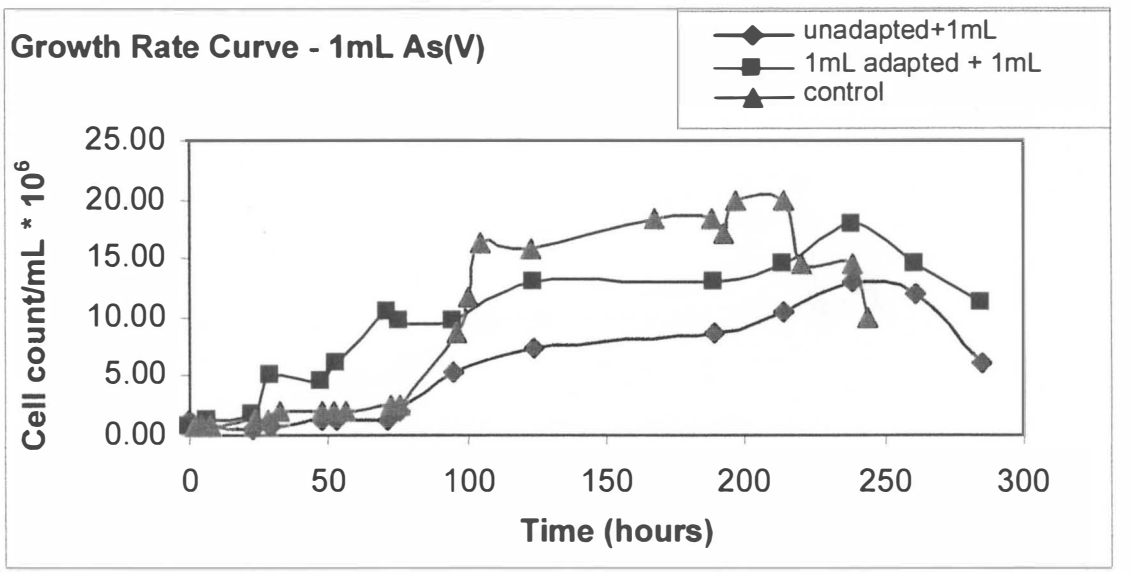


Figure 13

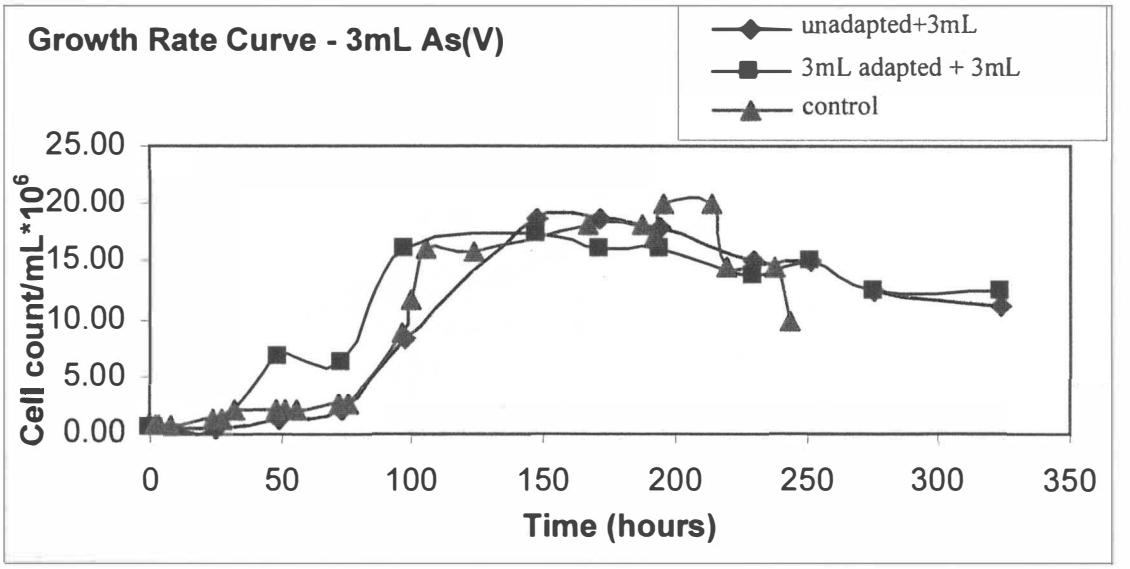


Figure 14

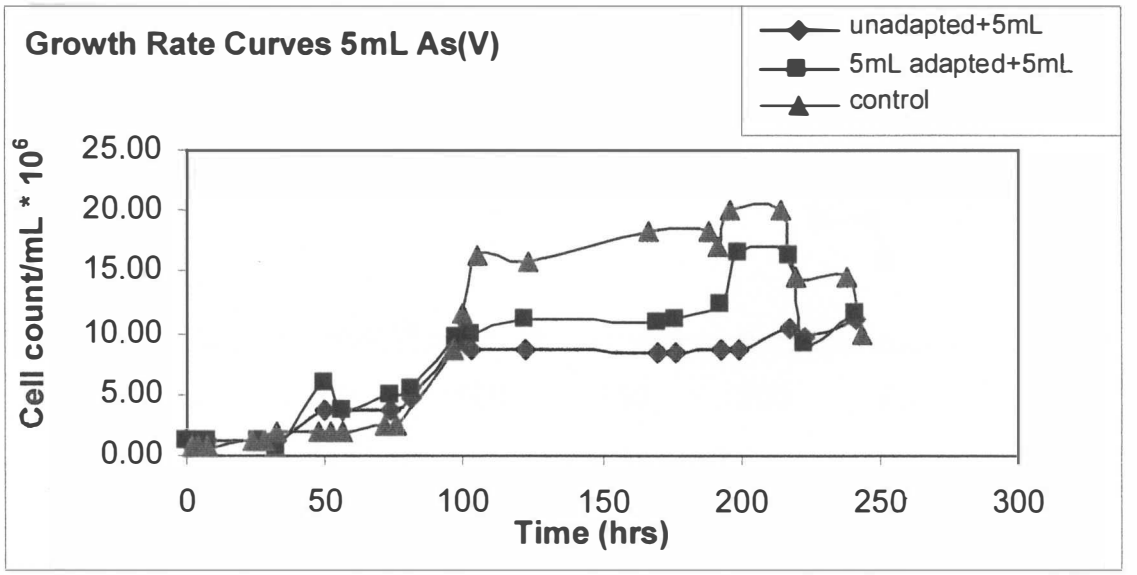


Figure 15

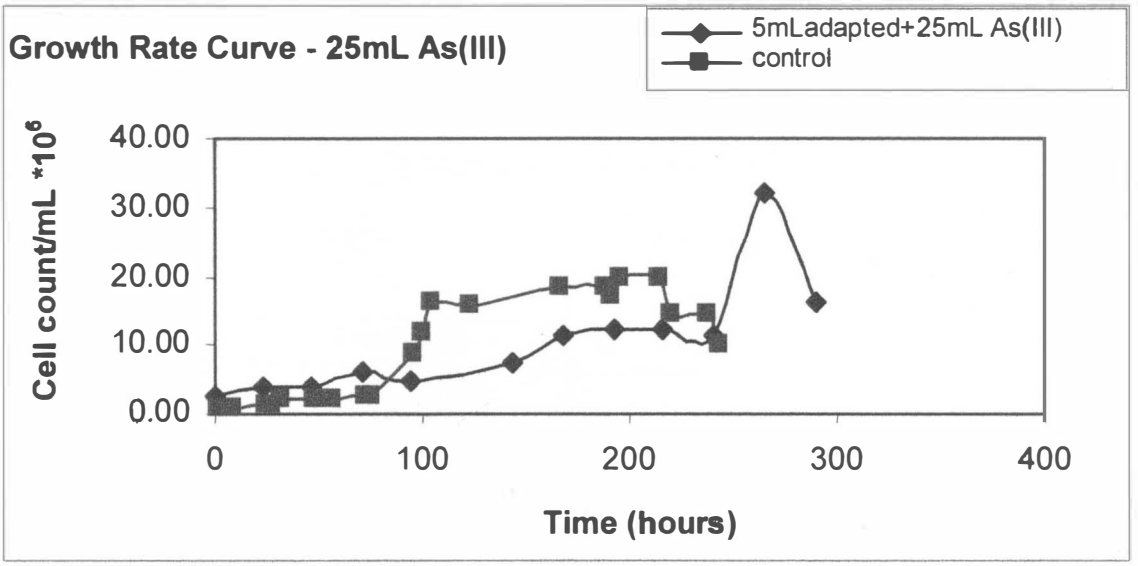
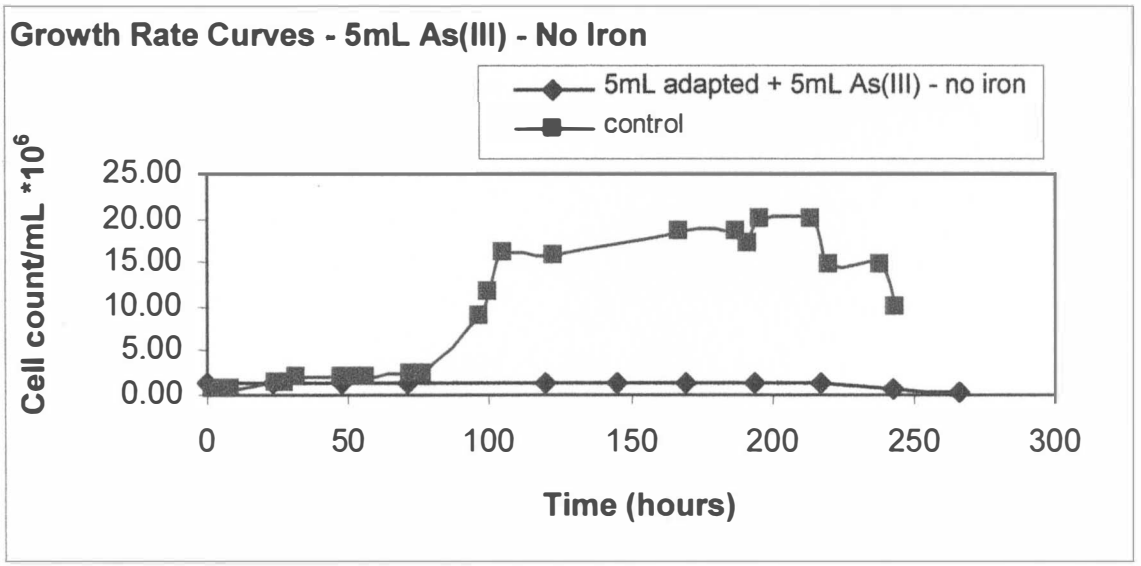


Figure 16



The table below summarizes the duration of the lag and exponential growth phase of the sample mediums.

Table 5 Duration of Growth Phases

Sample		Lag (hours)	Exponential Growth (hours)
	Unadapted	76	29
As(III)			
1mL	Unadapted	23	71
	Adapted	23	71
3mL	Unadapted	49	142
	Adapted	24	190
5mL	Unadapted	24	190
	Adapted	<24	168
25 mL		95	73
No Iron		>250*	0
As(V)			
1mL	Unadapted	75	50
	Adapted	23	102
3mL	Unadapted	73	74
	Adapted	25	72
5mL	Unadapted	74	23
	Adapted	32	90

* Note: There was no recorded growth for this sample

The data in Table 5 is represented graphically in Figures 17 through 20 below.

Figure 17

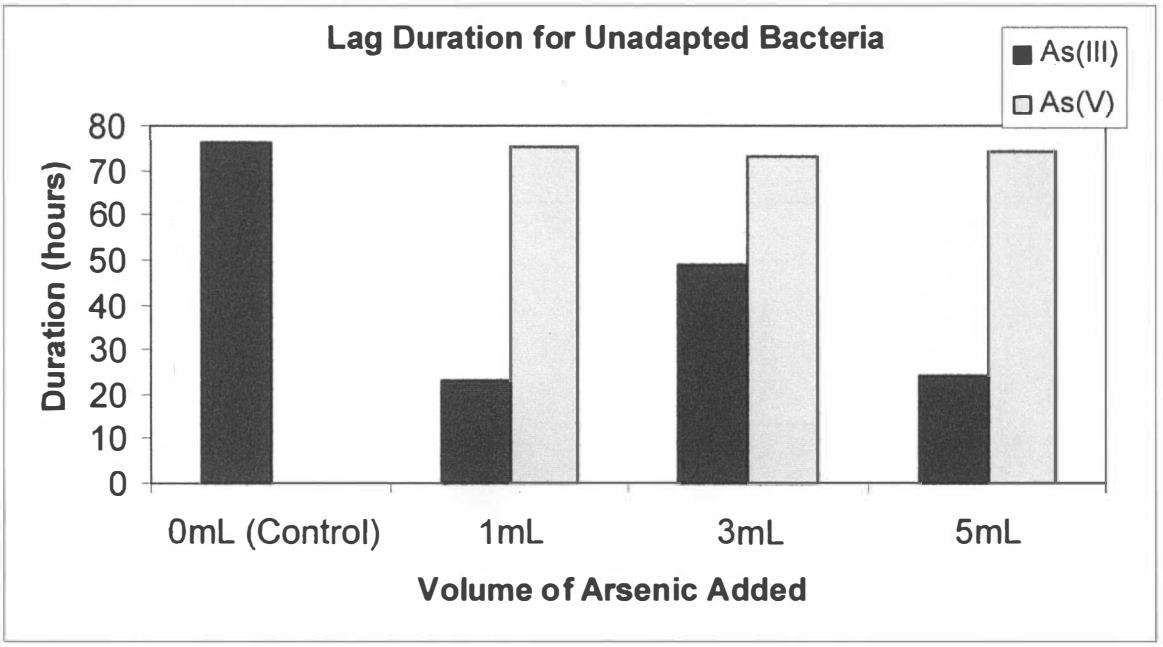


Figure 18

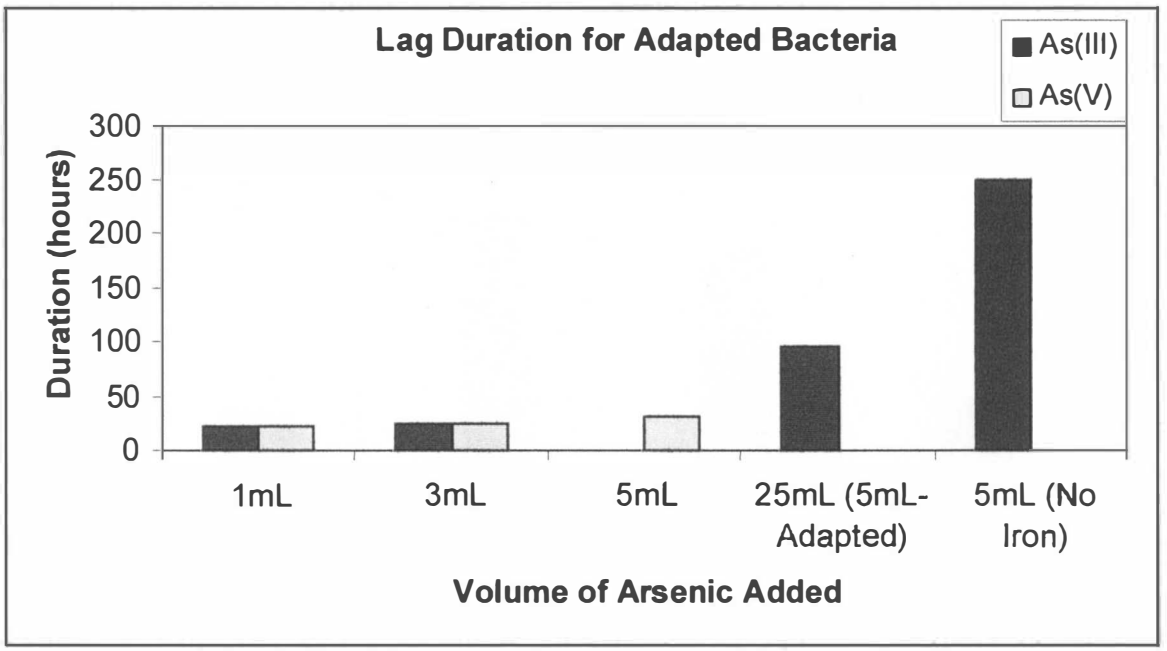


Figure 19

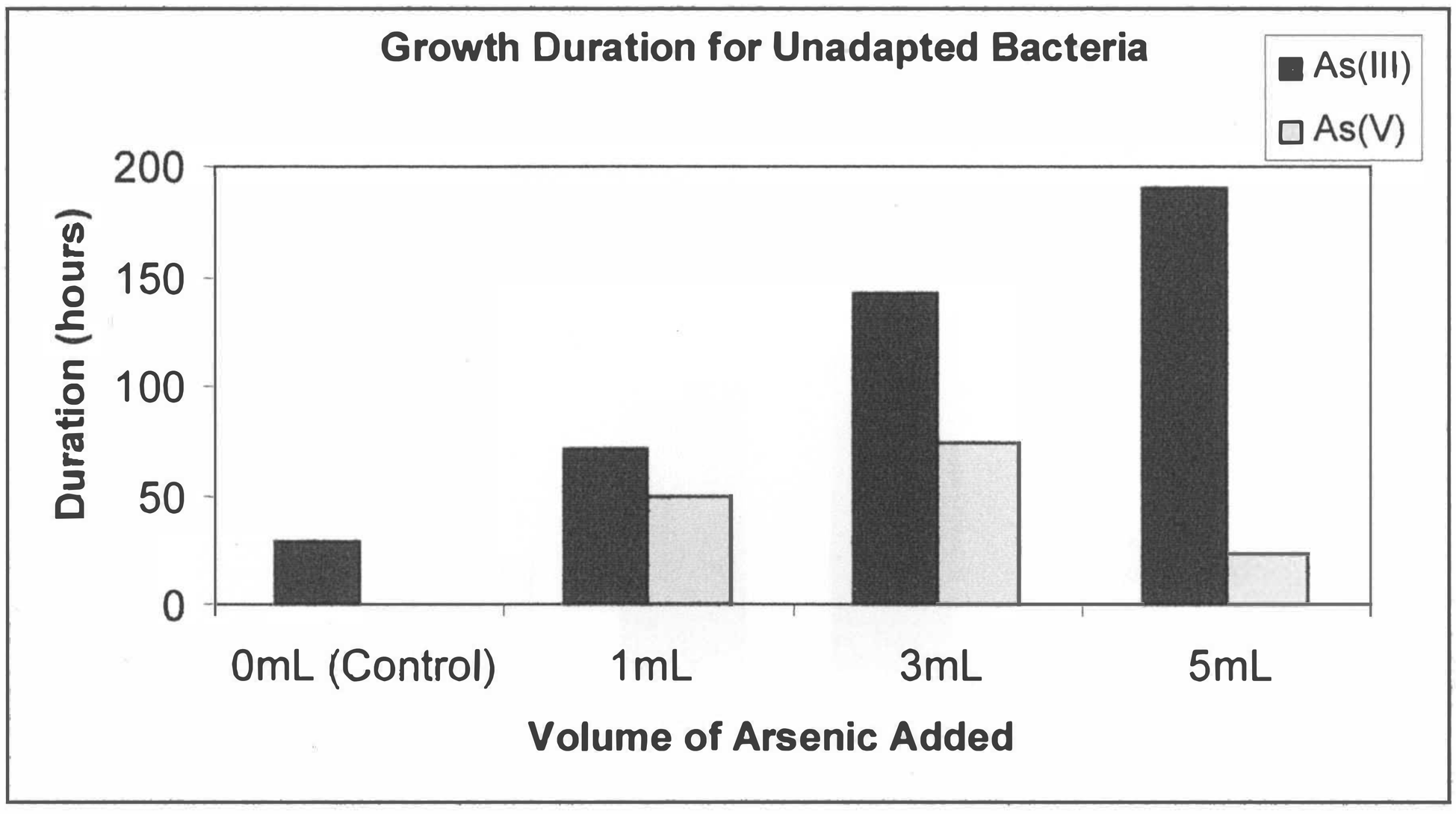
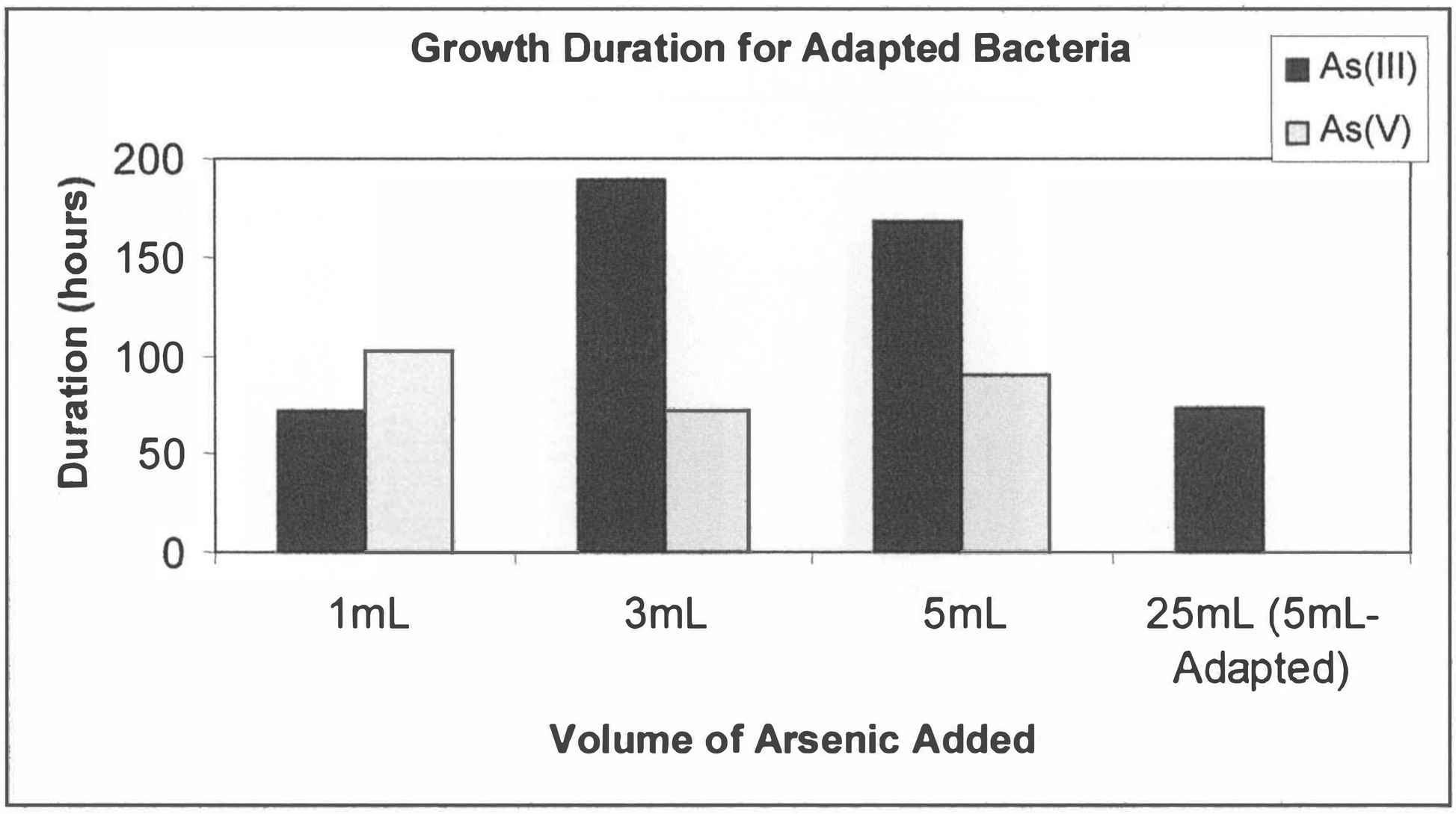


Figure 20



Figures 21 and 22 show the cell count for unadapted and adapted bacteria exposed to As(III) and As(V) for 150 hours.

Figure 21

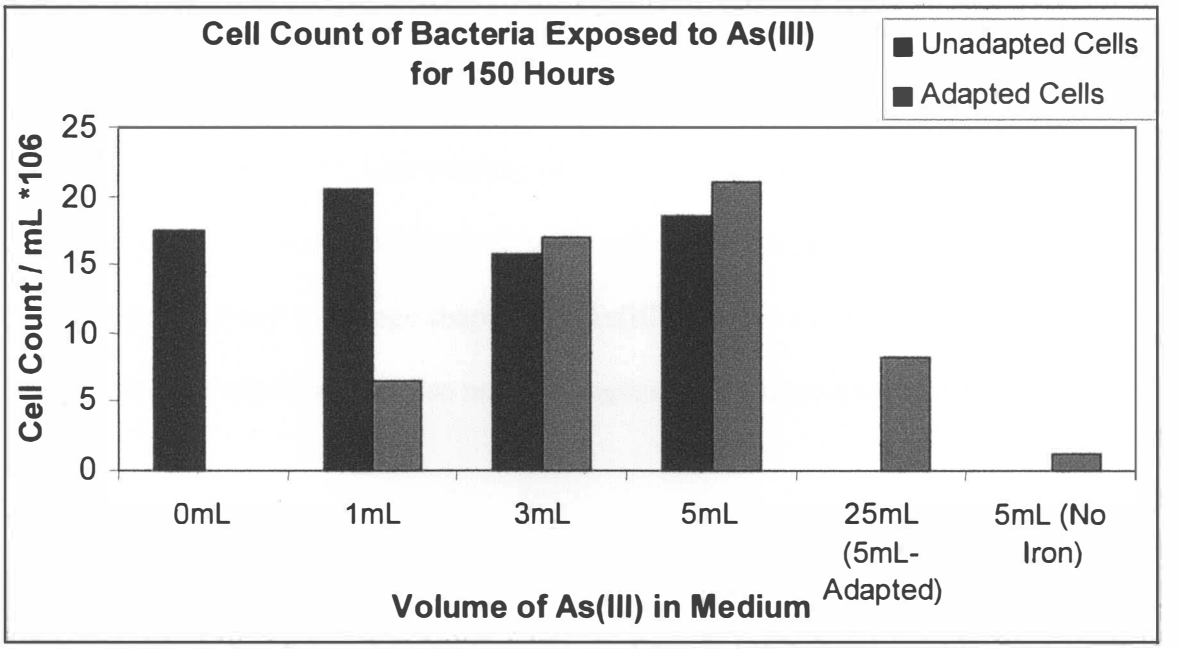
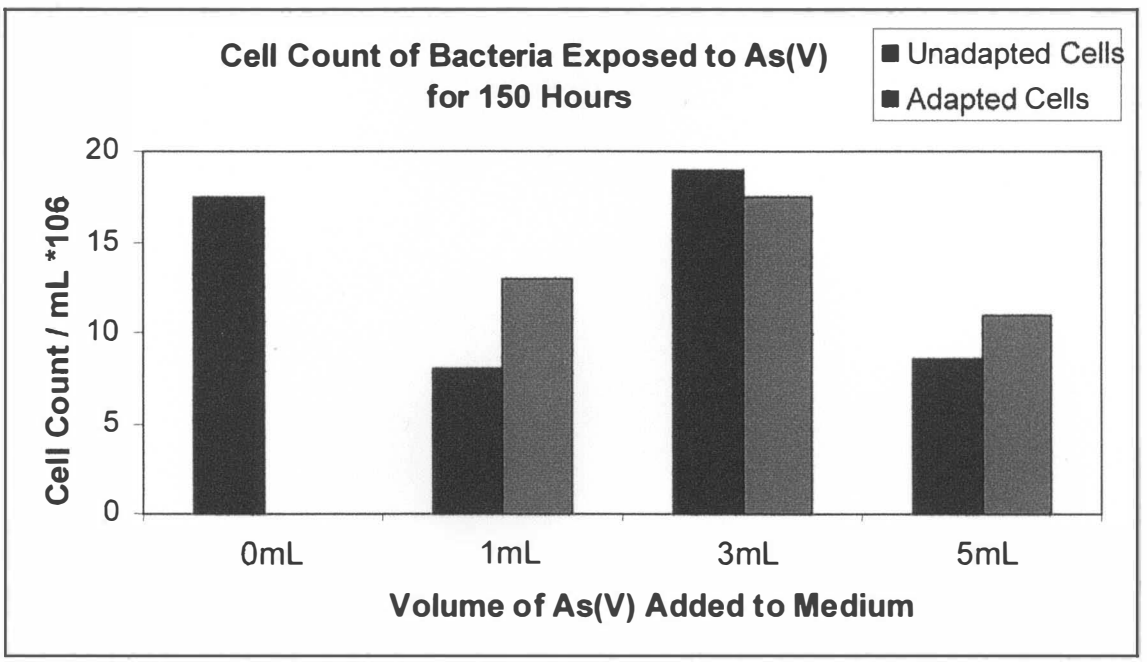


Figure 22

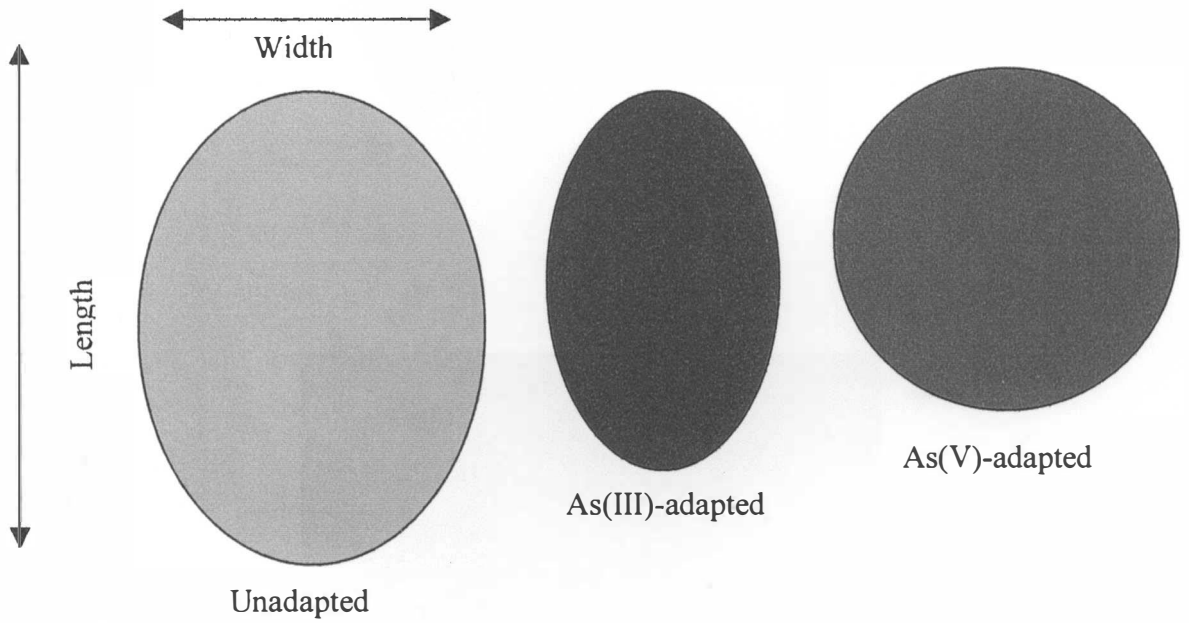


D. AFM

AFM was used to understand the changing in morphology due to arsenic exposure of the arsenic adapted bacteria as compared to the unadapted bacteria. The experiment yielded images presented in Figures 23, 24, and 25. Additionally, the size of the bacterial cells was measured and is listed in the table below. It can be seen that the unadapted *T. ferrooxidans* are rod-shaped which confirms the well established documentation (15, 17). The bacteria adapted to As(V) is spherical, which shows that the presence of a toxic substance causes the cell to change shape. The As(III)-adapted bacteria also has a rod-shape, which is not spherical, but also not as elongated as the unadapted cell.

Table 6 Size Comparison of Unadapted and Adapted Bacterial Cells

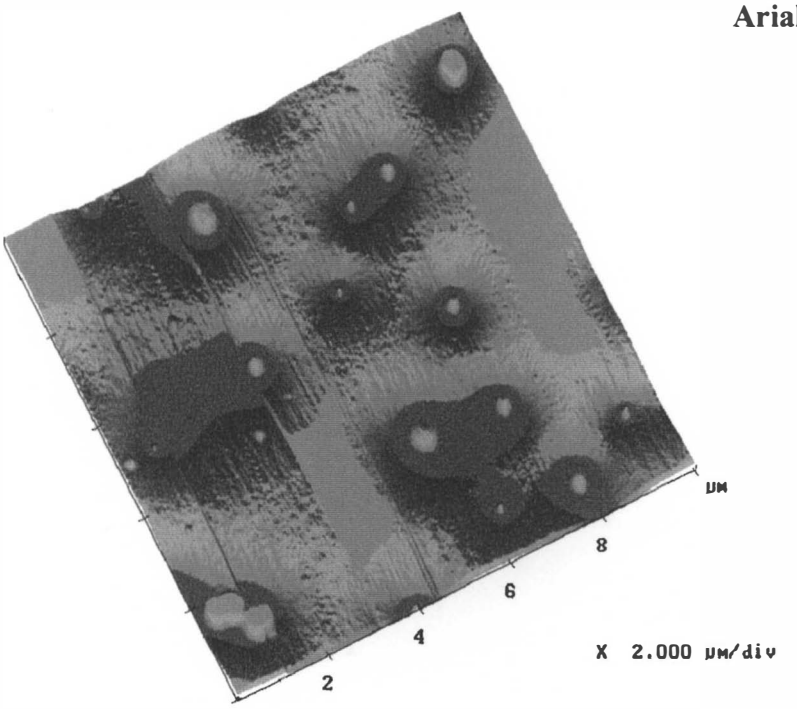
	Length (μm)	Width (μm)	Area** (μm^2)
Unadapted cells	2.46	1.80	3.48
As(III)-adapted cells	1.97	1.21	1.87
As(V)-adapted cells	1.78	1.78	2.49



* The cells shown above are to scale in dimensionless units. The diagram is for comparison purposed only.
** The area calculation is assuming that the cells are perfect ellipses or spheres.

Figure 23 Unadapted AFM Images

Arial View



2D View

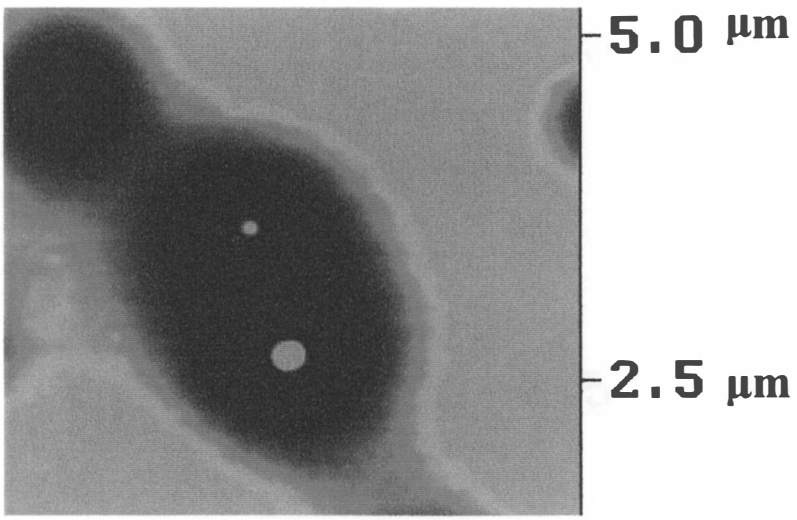
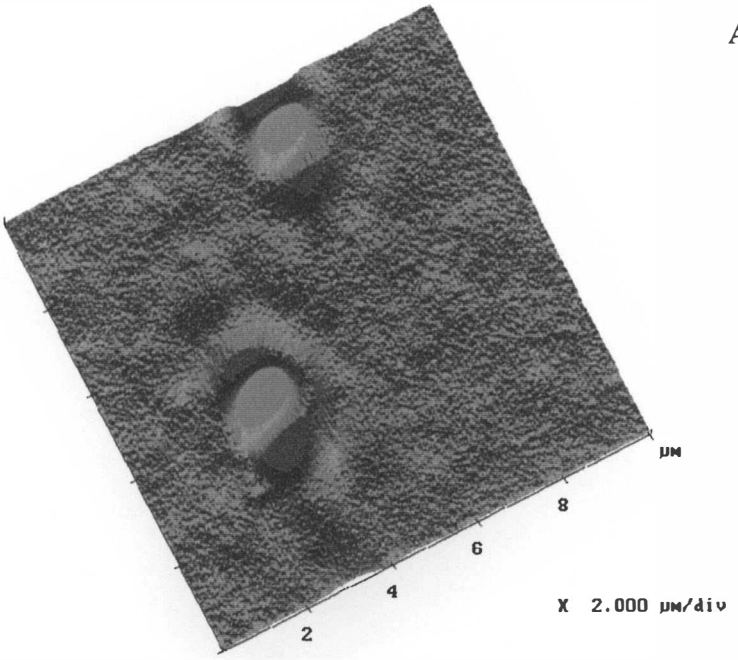


Figure 24 Bacteria Exposed to As(III)

Arial View



2D View

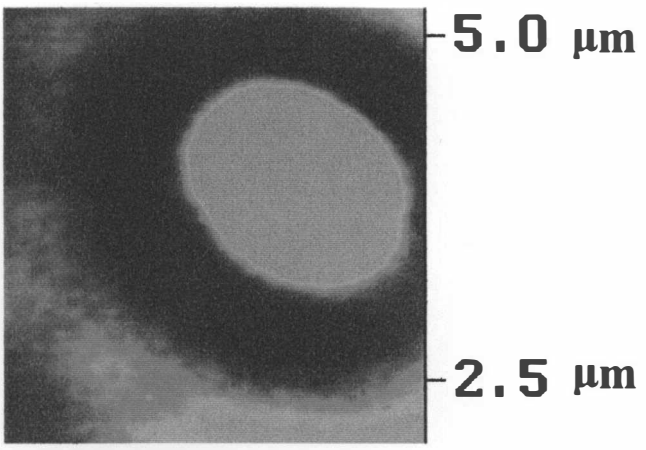
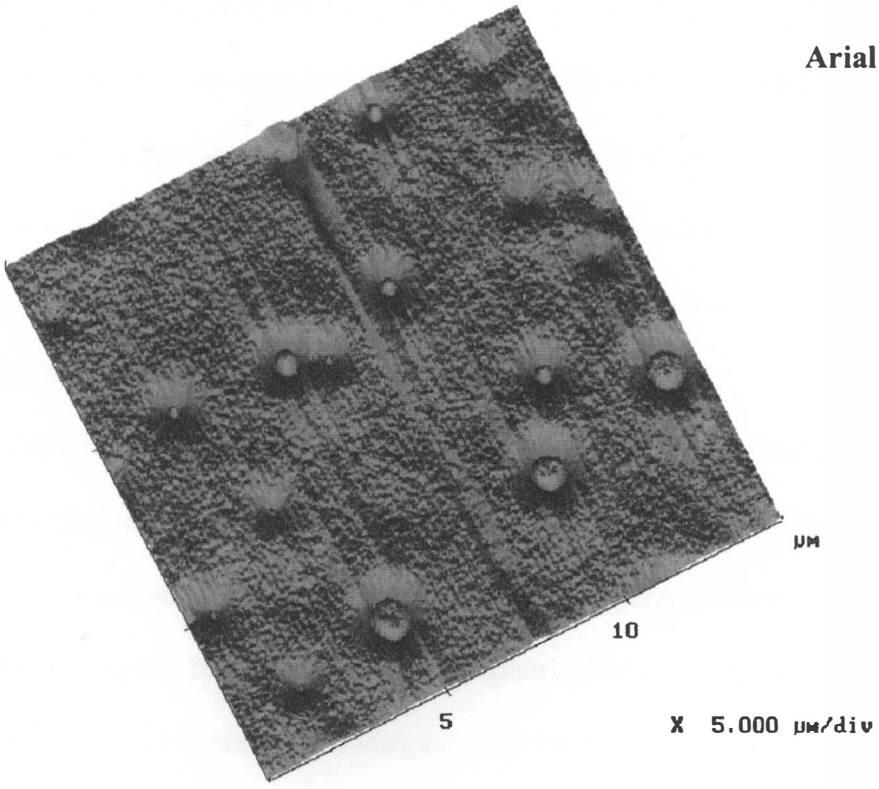
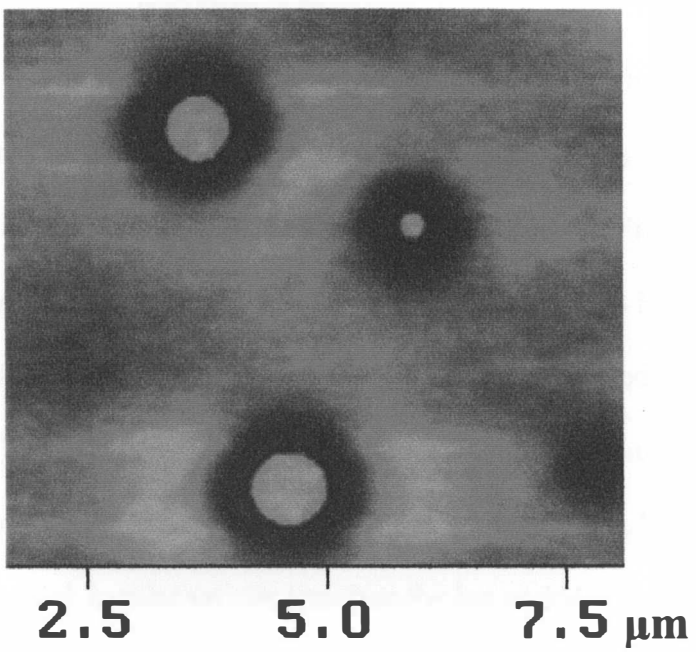


Figure 25 Bacteria Exposed to As(V)

Arial View



2D View



VI. DISCUSSION OF RESULTS

The results of the experiments have shown that not only does *T. ferrooxidans* have a response to arsenic exposure, but that it behaves differently to As(III) and As(V). The IEP determined by the zeta potential measurements of the unadapted cells as compared to the cells adapted to As(III) and As(V) at various concentrations shows that the different species of arsenic alter the surface charge of the cells in diverse ways possibly due to the secretion of different products on the cell surface.

The AFM results show the morphology of the arsenic adapted bacterial cells. When bacterial cells are exposed to toxic substances, they change their surface morphology. It is apparent that the As(V) is more toxic to *T. ferrooxidans* as the cell shape of the As(V)-adapted bacteria is completely spherical as compared to the As(III)-adapted bacterial cells which are still rod-shaped. It is interesting to note that the As(III)-adapted cells have the smallest idealized area. As(V)-adapted bacteria cells are 1.3 times larger and unadapted cells are 1.9 times larger than the As(III)-adapted cells.

The growth rates curves were very instrumental in determining the effects of As(III) and As(V) on the growth of the unadapted and adapted cells. The average lag period for all the adapted cells was fairly uniform with exception of the 5mL As(III)-adapted bacteria which had lag duration of less than 24 hours, refer to Figure 18 for a graphical representation. The average lag for the adapted cells is 25 ± 3 hours, if the duration of 5mL As(III)-adapted bacteria is not included. The unadapted cells with various levels of As(V) added have a lag duration of 74 ± 1 hours. The unadapted cells which were exposed to As(III) do not have such a consistent lag period, but the average value is 32 ± 11 hours which is substantially less than the lag period of the As(V). A

comparison of the lag durations can be seen in the bar graph in Figure 17. This suggests that As(V) is more toxic to *T. ferrooxidans* than is As(III) because the bacteria takes approximately twice as long to acclimate to the arsenic before growth can begin.

The exponential growth durations varied more than did the lag phases, however there are general trends that can be seen. The average duration for As(V)-adapted bacteria with As(V) added to the medium was 88 $\pm$ 11 hours and 49 $\pm$ 17 hours for unadapted bacteria with the addition of As(V). For the mediums with As(III) added, the adapted bacteria had an average of 143 $\pm$ 48 hours and 134 $\pm$ 42 hours for unadapted bacteria. In both arsenic species, the adapted bacteria exhibited a longer growth phase than did the unadapted bacteria. However, the growth phase was substantially longer with the addition of As(III) than with As(V) whether or not the bacteria was adapted as seen in Figures 19 and 20. This suggests that *T. ferrooxidans* grow better in the presence of As(III) or may be able to use As(III), directly or indirectly, as a nutrient source.

In Figures 9 through 11, it can be seen that the growth of the bacteria is not inhibited by the addition of 1 to 5 mL of As(III) solution to the medium. In fact with the exception of adapted bacteria in the 1mL As(III) growth curve, both the unadapted and adapted bacteria have higher cell counts with As(III) than without the addition of arsenic at all, see Figure 21. This again suggests that *T. ferrooxidans* prefers As(III) in its growth environment and may possibly be oxidizing As(III) to use as a direct or indirect nutrient source for growth.

Figures 12 through 14 as well as Figure 22 pertain to the bacterial mediums with various levels of As(V) added. It can be seen that the growth of the adapted and unadapted bacteria is inhibited by As(V) and the adapted cells have a higher growth rate

than the unadapted cells. This trend however does not hold true for bacteria with 3mL of As(V) added, neither the unadapted or adapted cells seem to be affected by the addition of As(V). The general trend suggested that As(V) may be more toxic to *T. ferrooxidans* than As(III).

Because the bacteria appeared to be proliferating on As(III), the growth rate curves for two experimental bacteria mediums were developed. The first one measured the growth of 5mL As(V)-adapted bacteria when 25 mL of As(III) were added to the medium, see Figure 15. There was very little growth at the beginning with a lag period of 95 hours. This duration is significantly longer than the 32 hours that it took unadapted bacteria to acclimate to even 5 mL of As(III) this comparison can be seen in Figure 18. The initial growth phase had a growth rate of $1.47*10^6$ cells/mL per day, which then jumped to a growth rate of $20*10^6$ cells/mL per day after the 241^{th} hour. This shows that although it took the bacteria longer to adjust to the higher level of As(III), it had the highest cell count of all the various bacterial samples. These results again suggest that As(III) directly or indirectly provides a source of nutrients for *T. ferrooxidans*.

The second experimental medium was inoculated with 5mL As(III)-adapted washed bacterial cells and 5mL of As(III), but contained no iron as a nutrient source, see Figure 16. The *T. ferrooxidans* did not grow with As(III) as the sole energy source. It also did not begin the death phase until after 218 hours which again suggests that As(III) is not as toxic to *T. ferrooxidans* as has been previously reported.

VII. CONCLUSION

Bioremediation is a field in which much more research is needed for it to become a viable treatment technology that can compete with the more traditional technologies for heavy metal remediation. In terms of heavy metals, such as arsenic, in which their characteristics are dependent upon their oxidation states, it is important to examine the effects that the various oxidation states have on bacteria. This thesis provides information on the effects of As(III) and As(V) on *T. ferrooxidans* which is vital if the bacterium is to be used effectively for bioremediation techniques. Three main conclusions can be drawn from this research.

First, As(III) and As(V) modify the morphology of *T. ferrooxidans* in different ways. This is evident in the results of the zeta potential readings as well as the AFM. The zeta potential shows that the surface charge of the bacterial cells is altered depending on which species of arsenic the bacteria is exposed. Additionally, the AFM has shown that the shape of the bacterial cell is varies depending on whether it is exposed to As(III) or As(V).

Secondly, this study suggests that As(V) is more toxic to *T. ferrooxidans* than As(III) in the presence of iron. This can be seen in the growth curves. This conclusion is supported by the results of Breed *et al* (28) that arsenic toxicity on a mixed culture, including *T. ferrooxidans,* is dependent on the availability of an energy source and that As(III) may not have the toxicity levels that have been previously reported.

A finding of this research that has not been previously documented is fact that the *T. ferrooxidans* in this study, whether adapted or unadapted to As(III), grows better in the presence of As(III) than without any arsenic at all. But as formerly recognized,

T. ferrooxidans cannot grow with As(III) as its sole source of energy. The bacteria could be using the As(III) as a secondary source of energy in conjunction with the iron by indirectly oxidizing the As(III) to As(V) by the bacterial metabolites. This reaction could in turn cause the iron to be reduced from Fe(III) to Fe(II), making the ferric iron available to be oxidized by the bacteria as a nutrient source. Another type of bacteria has been documented with this property to "survive better in the presence of arsenic", *Alcaligenes faecalis* (20). This bacteria is also motile, rod-shaped, and approximately the same size as *T. ferrooxidans*.

VIII. RECOMMENDATION

This research provides the foundation for which further research can expand upon. There are many directions that can be explored. As this research was conducted to investigate how *T. ferrooxidans* could be used to remove or detoxify arsenic in drinking water, the bacteria was adapted to arsenic levels within the vicinity of 10 ppb which is new allowable level of arsenic in drinking water for the United States and 4000 ppb which is the concentration of some of the highest arsenic contaminated sources in Bangladesh. The bacteria could be adapted to higher levels for research involving the remediation of arsenic contaminated industrial sites which reach concentrations in the tens of thousands ppb. Additionally, research could be conducted on the use of *T. ferrooxidans* for bioremediation of heavy metals other than arsenic.

Phytoremediation is also a plausible technique for arsenic remediation, although more research is needed. It is important to understand the microbial activity in conjunction with oxidization/reduction of arsenic compounds as it relates to the soil ecosystem. Because the arsenic species determines its toxicity, bioavailability, and solubility, understanding the way in which various types of microbes and plants change arsenic's speciation is imperative in the design of bioremediation techniques. It is documented (12) that most plants uptake As(V) much more readily than As(III). If this is the case, bacteria that oxidizes As(III) could be used as a pretreatment to convert As(III) to As(V).

On a genetic level, there are many opportunities for further investigation and exploration of the effects of arsenic on *T. ferrooxidans*. Sectional analysis of the bacterial cells of the arsenic adapted bacteria could be performed. This would give

insight into the mechanisms that are involved in cell's metabolism of As(III) and As(V). Additionally, an analysis could be conducted to determine the products that are secreted on the cell surface from the interactions with As(III) and As(V). Further research could also be conducted to find out why As(III) is less toxic than As(V) for *T. ferrooxidans* when for most species it is As(V) that is more detrimental.

Another very interesting question is why does *T. ferrooxidans* appear to survive better in the presence of As(III) than without any arsenic at all. This preliminary research suggests that the *T. ferrooxidans* is using As(III) as a secondary nutrient source through the oxidation/reduction reactions between the arsenic and iron. The species of arsenic in the medium before, during, and after growth could also be determined to see how much of the As(III) is being converted to As(V). Although the arsenic speciation determination alone would not be enough to conclude that the *T. ferrooxidans* is using As(III) as a nutrient source because it has been documented that the metabolites from *T. ferrooxidans* are capable of oxidizing As(III) as well. As stated before, it is possible that the metabolites are oxidizing the arsenic which in turn in reducing the iron, making it an available source of nutrients. Therefore, the species of iron in the medium before, during, and after growth should be determined as well to understand the oxidation/reduction reactions that are taking place in the bacterial medium.

There are also many experiments that could be conducted to more acutely focus this finding that As(III) is less toxic than As(V) for *T. ferrooxidans* when a nutrient source is present. The parameters involved with this phenomena need to be investigated such as the required ratio of the nutrient source to As(III). Another question to be explored could be at what point is the As(III) not only harmless to the bacteria, but the

bacterial metabolites are able to facilitate oxidation/reduction reactions with the arsenic. If it is proven that this type of reaction is indeed taking place in which the As(III) being converted to As(V), then more research should be conducted on the optimal levels of As(III) and As(V) the bacteria can tolerate in the growth medium, as this study has shown that As(V) is more toxic to *T. ferrooxidans* in the presence of iron.

There are many directions that this introductory study may lead from the small scale of genetics and microbiology to the larger scale implementation of remediation techniques. This study has opened the door for many intriguing queries to be investigated and answers discovered.

REFERENCE

1. Viraraghavan, T., Subramanian, K.S., Aruldoss, J.A. 1999. Arsenic in Drinking Water- Problems and Solutions. *Water Science Technology* 40(2):69-76

2. Somasundaran, P. 1999. Project Description: Study of Assessment and Remediation of Arsenic Enrichment in Groundwater. Department of Earth and Environmental Engineering, Columbia University

3. World Health Organization. 2002. Arsenic in Drinking Water Fact Sheet. www.who.int/inf-fs/en/fact210.html accessed October 23, 2002

4. Lepkowski, Wil. 1998. Arsenic Crisis in Bangladesh. *C&EN Washington.* November 16, 1998: 27-29

5. Hering, Janet, Dutta, Subijoy. 2000. In-Situ Remediation of Arsenic-Contaminated Groundwater: Proposal For A Pilot in West Bengal, India.

6. Turpeinen, Riina, 2002. Interactions between Metals, Microbes, and Plants – Bioremediation of Arsenic and Lead Contaminated Soils. Department of Ecological and Environmental Sciences, University of Helsinki

7. Parvez, M.F., Ahsan, H., Chen, Y., van Geen, A., Hussain, A.Z.M.I., Momotaj, H., Horneman, A., Dhar, R., Zheng, Y., Stute, M., Simpson, H.J., Slavkovich, V., Lolacono, N.J., Shahnewas, M., Ahmed, K.M., Graziano, J.H. 2001. The Arsenic Problem and Its Awareness in Bangladesh Population: Results of a Large Population-based Survey. Arsenic in Drinking Water an International Conference at Columbia University, New York, November 226-27, 2001

8. Harvey, P.I., Crundwell, F.K. 1996. The Effect of As(III) on the Growth of *Thiobacillus ferrooxidans* in an Electrolytic Cell under Controlled Redox Potentials. *Minerals Engineering* 9(10): 1059-1068

9. Gihring, Thomas M., Druschel, Gregory K., McClenskey, R. Blaine, Jamers, Robert J., Banfield, Jillian F. 2001. Rapid Arsenite Oxidation by *Thermus aquaticus* and *Thermus thermophilus*: Field and Laboratory Investigations. *Environmental Science Technology* 35: 3857-3862

10. Gihring, Thomas M., Banfield, Jillian F. 2001. Arsenite Oxidation and Arsenate Respiration by a New *Thermus* isolate. *FEMS Microbiology Letters* 204: 335-340

11. Bhawan, Jal Vigyan. 2000. Arenic: Occurrence, Toxicity and Speciation Techniques. *Water Resources* 34(17): 4304-4312

12. Ritchie, A.W., Harrington, C.F., Haris, P.I., Jenkins, R.O. 2002. Mobilisation of Arsenic from Contaminated Soil via Bacterial Arsenate Respiration. www.math.ohio-state.edu/~tanveer/icben02final/parvezhans.oct15.doc accessed March 6, 2003

13. Santini, Joanne M., Sly, Lindsay I., Schnagl, Roger D., Macy, Joan M. 2000. A New Chemolithoautotrophic Arsenite-Oxidizing Bacterium Isolated from a Gold Mine: Phylogenetic, Physiological, and Preliminary Biochemical Studies. *Applied and Environmental Microbiology*: 92-97

14. Meharg, Andrew A., Hartley-Whitaker, Jeanette. 2002. Arsenic Uptake and Metabolism in Arsenic Resistant and Nonresistant Plant Species. *New Phytologist* 154: 29-43

15. Jensen, Anders B., Webb, Colin. 1995. Ferrous Sulphate Oxidation Using *Thiobacillus ferrooxidans*: a Review. *Process Biochemistry* 30(3): 225-236

16. De, G.C., Oliver, D.J., Pesic, B.M. 1996. Effects of Heavy Metals on the Ferrous Iron Oxidizing Ability of *Thiobacillus ferrooxidans*. *Hydrometallurgy* 44:53-63

17. Natarajan, K.A. 1998. Microbes Minerals and Environment. Department of Metllurgy, Inida Institute of Science, Bangalore. Geological Survey of India. 43-56

18. Sugio, Tsuyoshi, Fuhioka, Akira, Tsuchiya, Mikio, Shibusawa, Naiki, Iwahori, Kenji, Kamimura, Kazuo. 1998. Isolation and Some Properties of a Strain of the Iron-Oxidizing Bacterium Thiobacillus ferrooxidans Resistant to 2,4-Dinitrophenol. *Journal of Fermentation and Bioengineering* 86(1): 134-137

19. Ehrlich, Henry Lutz, 2002. *Geomicrobiology,* Fourth Edition, Marcel Dekker, Inc. New York, NY. 303-326

20. Frankenberger, William T. Jr. 2002. Environmental Chemistry of Arsenic, Marcel Dekker, Inc. New York, NY. 313-361

21. Chaney, Rufus L., Malik, Minnie, Li, Yin M., Brown, Sally L., Brewer, Eric P., Angle, J. Scott, Baker, Alan JM. 1997. Phytoremediation of Soil Metals. *Current Opinion in Biotechnology* 8: 279-284

22. Rasjin, Ilya, Smith, Robert D., Salt, David E. 1997. Phytoremediation of Metals: Using Plants to Remove Pollutants from the Environment. *Current Opinion in Biotechnology* 8: 221-226

23. Meagher, Richard B., 2000. Phytoremediation of Toxic Elemental and Organic Pollutants. *Current Opinion in Plant Biology* 3: 153-162

24. Dahmani-Muller, H., van Oort, F., Gelie, B., Balabane, M. 1999. Strategies of Heavy Metal Uptake by Three Plant Species Growing Near a Metal Smelter. *Environmental Pollution* 109:231-238

25. Ma, Lena Q., Komar, Kenneth M., Tu, Cong, Zhang, Weihua, Cai, Yong, Kennelley, Elizabeth D. 2001. A Fern that Hyperaccumulates Arsenic. *Nature* 409, 579

26. Weise, Elizabeth. 2002. Poison-Craving Plant, Germ Designed to Suck Up Pollutants; 'Bio-remediation' Shows Promise. *USA Today*, October 8, 2002

27. Revkin, Andrew C. 2001. New Pollution Tool: Toxic Avengers With Leaves. *The New York Times*, March 6, 2001

28. Breed, A.W., Glatz, A., Hansford, G.S., Harrison, S.T.L. 1996. The Effect of As(III) and As(V) on the Batch Bioleaching of a Pyrite-Arsenopyrite Concentrate. *Minerals Engineering* 9 (12):1235-1252

29. West, R.J., Stephens, G.M., Cilliers, J.J. 1998. Zeta Potential of Silver Absorbing *Thiobacillus ferrooxidans*. *Mineral Engineering* 11(2): 189-194

30. Helfferich, Carla. 1994. Domesticating the Gold Bugs, and the Copper Bugs Too. Geophysical Institute, University of Alaska, Fairbanks, *Alaska Science Forum*, June 16, 1994

31. Tenny, Ron, Adams, Jack. 2001. Ferric Salts Reduce Arsenic in Mine Effluent by Combining Chemical and Biological Treatment. *Environmental Science and Engineering*. www.esemag.com/0101/ferric.html. accessed October 15, 2002

32. Oremland, Ronald. 2002. Microbial Redox Cycling of Arsenic Oxyanions in Anoxic Environments. *U.S. Geological Survey Artificial Recharge Workshop Proceedings*, Sacremento, California, April 2-4, 2002

33. Bosecker, K. 2001. Microbial Leaching in Environmental Clean-up Programmes. *Hydrometallurgy* 59: 245-248

34. Valls, Marc, de Lorenzo, Victor. 2002. Exploiting the Genetic and Biochemical Capacities of Bacteria for the Remediation of Heavy Metal Pollution. *FEMS Microbiology Review* 26: 327-338

35. Costly, S.C., Wallis, F.M. Bioremediation of Heavy Metals in a Synthetic

Wastewater Using a Rotating Biological Contactor. *Water Resources* 35 (15): 3715-3723

36. Levin, Morris A., Gealt, Micheal A. 1993. Overview of Biotreatment Practices and Promises. In: Biotreatment of Industrial and Hazardous Waste. McGraw-Hill, Inc. pp. 1-18

37. Alexander, Martin. 1999. Biodegradation and Bioremediation. Academic Press

38. Bioremediation and Phytoremediation Glossary. 1998. http://members.tripod.com/~bioremediation accessed April 13, 2003

39. Hinchee, Robert, Means, Jeffery L., Burris, David R., 1995. Bioremediation of Inorganics. Battelle Memorial Institute