ISOLATION AND CHARACTERIZATION OF AN ARSENITE-OXIDIZING CULTURE

by

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ABSTRACT

An arsenite-oxidizing enrichment culture was isolated from the northern tip of the Halls Brook Storage Area in suburban Boston in mineral medium and arsenite. The growth of three generations of cultures was followed for 1-3 months by measurements of arsenite oxidation and of absorbance. The bacteria grew optimally at pH lower than 9 and the final culture generation was shown to incorporate radiolabelled bicarbonate. Over the same period, direct counts yielded increases in culture density and the concentration of arsenite in the cultures decreased. Thus, it was shown that the culture was chemoautotrophic: capable of harvesting the energy released by the oxidation reaction for use in carbon fixation. The cells were estimated to have a doubling time of 8 hours.

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CHAPTER 1
INTRODUCTION

1.0 Introduction

Arsenic has gained infamy for its use as a murder weapon in arenas that vary from "Arsenic and Old Lace" to chemical weapons of mass destruction. However, the element and its compounds also have had many arguably beneficial uses. For instance, the very qualities of arsenic that render it such an effective murder weapon also render it an extremely effective pesticide. Arsenic also has important industrial applications. Potently-toxic arsine (AsH_3) is used as a doping agent in electronics. Arsenic is also used in leather tanning. Many arsenic compounds are also produced as a by-product of many industrial operations. For example, because arsenic is a widespread component of gold and sulfur ores, arsenic is often produced as a by-product of gold and sulfur mining operations, and from the processing of the metal ores. For example, smelting produces arsenic, as does the production of sulfuric acid. With the production and use of these hazardous chemicals comes their release into the environment. The Aberjona watershed in suburban Boston illustrates some effects of long-term aqueous arsenic release.

1.1 Brief History of the Aberjona Watershed

From the early-to-mid 1900s, the northern Aberjona watershed (Fig. 1) was home to several chemical manufacturers and leather processing companies. Unfortunately, environmental protection was not yet a popular goal, and the chemical manufacturers, notably those who produced sulfuric acid, released a great deal of arsenic into the area surrounding their factories. The area, including land now known as the Industri-Plex Superfund Site, is one of the largest hazardous waste sites in the country. The arsenic-laden wastes slowly leached their poisons into the Aberjona River. (Durant et al.) Various studies have followed arsenic as it traveled down the Aberjona River, ultimately arriving at the Mystic Lakes. (Knox; Solo)

Not surprisingly, this increased arsenic load has fostered some interesting microbial ecology. One previous study in the watershed drew sediment samples from the northern-most tip of the Halls Brook Storage Area (HBSA), an artificial pond located directly downstream from the Industri-Plex Site and directly upstream of the Aberjona River. (Fig. 2) The researchers isolated from these samples a bacterium able to harvest the energy released by using arsenate (As(V)) as a terminal electron acceptor (Ahmann et al.) A separate study demonstrated the existence of arsenate-reducing microorganisms in the waters of the Upper Mystic Lake. (Spliethoff) A third study shows the existence of an orpiment-producing bacterium isolated from the sediments of the Upper Mystic Lake. (Newmann) One further possible ecological niche that had not been explored in the Aberjona watershed is that of the arsenite-oxidizing bacterium.

Figure 1. Map of the Aberjona Watershed (Aurilio et al.)

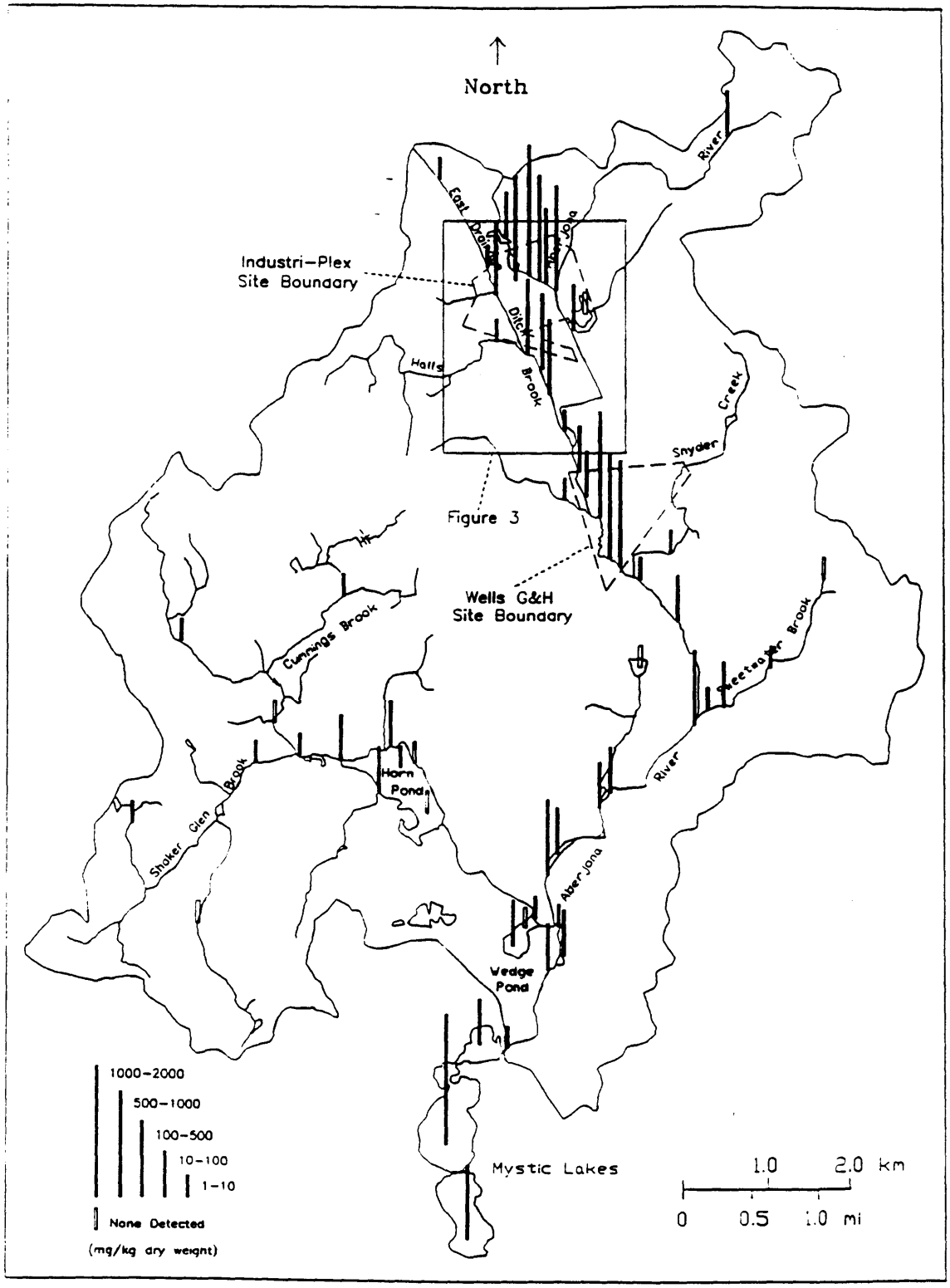
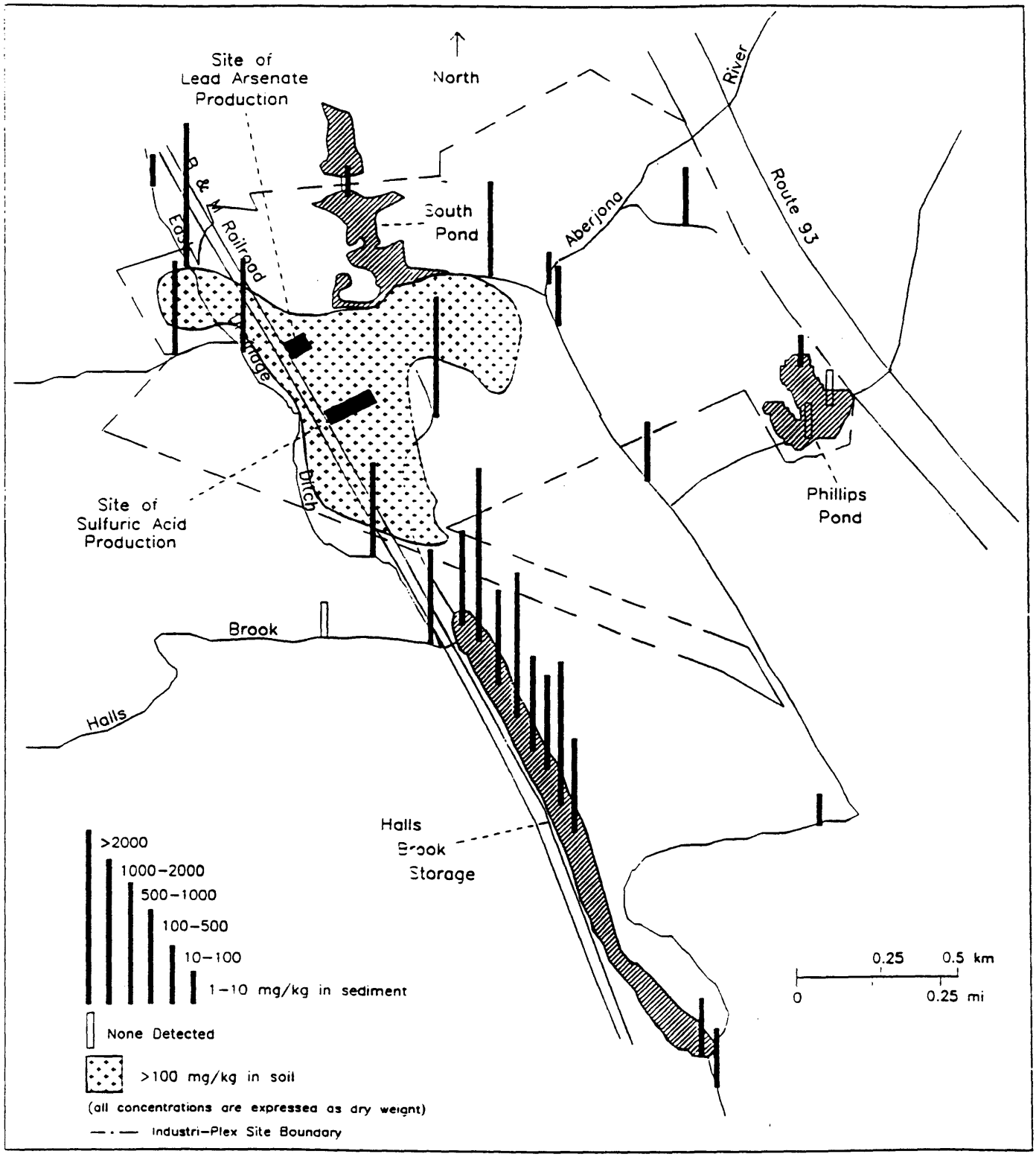


Figure 2. Map of the Halls Brook Storage Area (Aurilio et al.)



One particularly interesting study site is the northern edge of the HBSA. This location has several important characteristics. First, with sediment arsenic concentrations reaching 9800 mg/kg dry weight (Aurilio et al.), the area is highly enriched with arsenic. Second, this corner of the pond is shallow and actively fed by groundwater, and thus is a confluence of anoxic waters with oxic waters. In an oxic environment, arsenate is the thermodynamically favorable form of arsenic, chiefly taking the form of H_3AsO_4 (pKa_a=3.6, pKa_2=7.26, pKa_3=12.47) (Spliethoff). In anoxic environments, aqueous arsenic favors its arsenite form, present as H_3AsO_3. Thus, the unique environment of the northern tip of the HBSA provides both the oxic environment which favors arsenite oxidation, along with high concentrations of arsenite available for oxidation. These conditions together create an ecological niche for a microorganism which is not only resistant to arsenite but also actually capable of oxidizing the ion for energy consumption.

1.2 Microbial Energetics

It has been known for some time that the oxidation of arsenite yields enough energy that a chemoautotrophic bacterium could theoretically exist. Energetics calculations show that the reaction is more thermodynamically favorable with increasing pH.

PO_2 = 0.21 atm; $[HAsO_2] = [H_2AsO_4^-]$; $[HAsO_2]=[HAsO_4^{2-}]$; pH= 7; Temperature=298K

Reaction 1: $1/4\ O_2(g) + H^+ + e^- = 1/2\ H_2O$ $\quad pe^\circ = \log K = 20.75$
(Morel and Hering)

For pH<<7:
Reaction 2a: $1/2\ H_2AsO_4^- + 3/2\ H^+ + e^- = 1/2\ HAsO_2 + H_2O$ $pe^\circ = \log K = 5.63$
(Tomilov and Chomutov)

For pH>>7:
Reaction 2b: $1/2\ HAsO_4^{2-} + 2\ H^+ + e^- = 1/2\ HAsO_2 + H_2O$ $\quad pe^\circ = \log K = 7.44$
(Tomilov and Chomutov)

Reaction 1:
$$\frac{[H_2O]^{1/2}}{PO_2^{1/4}[H^+][e^-]} = 10^{20.75}$$

$$-1/4 \log PO_2 + pH + pe_{1w}^\circ = pe^\circ = 20.75$$

$$\mathbf{pe_{1w}^\circ = 20.75 - pH + 1/4\ logPO_2 \quad = 13.6}$$

Reaction 2a:

$$\frac{[HAsO_2]^{1/2}[H_2O]}{[H_2AsO_4^-]^{1/2}[H^+]^{3/2}[e^-]} = 10^{5.63}$$

$$1/2 \log([HASO_2]/[H_2AsO_4^-]) + 3/2\ pH + pe_{2a} = pe_{2a}^\circ = 5.63$$

$$\mathbf{pe_{2aw}^\circ = 5.63 - 3/2\ pH - 1/2\ log([HAsO_2]/[H_2AsO_4^-]) = -4.87}$$

Reaction 2b:

$$\frac{[HAsO_2]^{1/2}[H_2O]}{[HAsO_4^{2-}]^{1/2}[H^+]^2[e^-]} = 10^{7.44}$$

$$1/2 \log([HAsO_2]/[HAsO_4^{2-}]) + 2\ pH + pe_{2bw}^\circ = pe_{2b}^\circ = 7.44$$

$$\mathbf{pe_{2b} = 7.44 - 2\ pH - 1/2\ log([HAsO_2]/[HAsO_4^{2-}]) \quad = -6.56}$$

$\Delta G_w^\circ \sim= 2.3\ R\ T\ (pe_{2w}^\circ - pe_{1w}^\circ) = 5.7\ (pe_{2w}^\circ - pe_{1w}^\circ)$
(Morel and Hering)

ΔG_{aw}° ~= -105 kJ/mol for species favored under pH7[1]

ΔG_{bw}° ~= -115 kJ/mol for species favored over pH7[1]

These values compared favorably to biogeochemical reactions known to fuel other chemoautotrophic organisms.

Thermodynamic Coupling, pH7, T=298K	ΔG_w° [kJ/mol]	
$1/6\ NH_4^+ + 1/4\ O_2(g) = 1/6\ NO_2^- + 1/2\ H^+ + 1/6\ H_2O$	-45	*
$1/2\ NO_2^- + 1/4\ O_2(g) = 1/2\ NO_3^-$	-38	*
$1/8\ H_2S(g) + 1/4\ O_2(g) = 1/8\ SO_4^{2-} + 1/4\ H^+$	-98	*
$Fe^{2+} + 1/4\ O_2(g) + 5/2\ H_2O = Fe(OH)_3(s) + 2\ H^+$	-107	*
$1/2\ HAsO_2 + 1/4\ O_2(g) + 1/2\ H_2O = 1/2\ H^+ + 1/2\ H_2AsO_4^-$	-105	
$1/2 HAsO_2 + 1/4\ O_2(g) + 1/2\ H_2O = H^+ + 1/2\ HAsO_4^{2-}$	-115	

Table 1. Thermodynamic Couplings: Maintenance Reactions of Chemoautotrophic Bacteria (*Morel and Hering)

[1] Note that these are approximate calculations. In order to precisely determine the DG values must also include adjustments for the acid-base reaction separating the arsenate species $H_2AsO_4^-$ and $HAsO_4^{2-}$. Furthermore, no data could be found describing the electrochemistry of the pair $HAsO_2$ and H_3AsO_3; again, the data presented here should only be taken as *approximate* values of the natural reaction.

1.3 Previous Research on Arsenite- and Arsenate-Resistant Microorganisms

There is an eighty-year history of research on the resistance of bacteria to arsenic. Generally, the literature detailing these projects can be broken into a few categories: studies investigating the ability of a bacterium to oxidize arsenite (As(III)) to arsenate (As(V)), studies investigating the ability of a bacterium to reduce arsenate to arsenite, and studies describing a bacterium's particular mechanism for detoxification of arsenite and/or arsenate.

In 1918, Green isolated two novel arsenic-resistant bacteria from a cattle-dipping tank. One bacterium, which he called *Bacterium arsenoxydans* , was a Gram-negative, aerobic rod capable of oxidizing arsenite to arsenate. Presumably, this bacterium was at least partly responsible for the widespread oxidation of arsenite in arsenical livestock-dipping solutions. He called the other bacterium, also an aerobic Gram-negative rod, *Bacterium arsenreducens.* (Green) The bacteria lines were lost, preventing further research.

Several decades passed before researchers took an interest in Green's work. Finally, in 1949, Turner published a small report listing several arsenite-oxidizers which he'd isolated from a cattle dip. (Turner 1949) In 1953, Quastel demonstrated that organisms present in soil are also capable of oxidizing arsenite. However, the organisms responsible were not isolated: Quastel was chiefly interested in exploring the characteristics of soil rather than the characteristics of microorganisms within the soil. (Quastel)

Next, in 1954, Turner published Part I of a four part series: an extensive and detailed account of the organisms he mentioned in his 1949 work. All of the organisms were Gram-negative rods, non-sporeforming, aerobic, heterotrophic, and motile. These

fifteen strains fell into five distinct categories of novel organisms: *Pseudomonas arsenoxydans-unus, Pseudomonas arsenoxydans-duo, Achromobacter arsenoxydans-tres, Xanthomona arsenoxydans-quattor*, and *Pseudomonas arsenoxydans-quinque*. (Turner, 1954) In Part II of the series, Turner and Legge further analyzed *Pseudomonas arsenoxydans-quinque*, finding that it contained an induced enzyme system of arsenite oxidation and that it grew optimally at pH 6.4 and at 40°C. Furthermore, because the organism was capable of oxidizing arsenite anaerobically in the presence of a suitable electron acceptor (e.g. phenol blue), researchers deduced that the system included an arsenite dehydrogenase and cytochromes. Part III of the series expanded further upon the base *of P. arsenoxydans-quinque*. Researchers isolated and described a soluble, cell-free crude arsenite dehydrogenase. It was concluded that the dehydrogenase was not bound to the cell wall or to large organelles due to the ease with which the enzyme was separated from the crushed cells in low-speed centrifugation. Researchers also noted that there was "no good evidence" that sulfhydryl groups were associated with the activity of the enzyme. (Legge and Turner) Last, in Part IV, Legge investigated the properties of the bacterial cytochromes. The cytochromes were associated with the insoluble fraction of the ground and centrifuged cells. No evidence was found to support the existence of a carrier between the arsenite dehydrogenase and the oxidase, with the electron-transport chain consisting of arsenite(arsenite dehydrogenase) -> oxidase -> O_2. (Legge)

In 1976, noting the study by Quastel, Osborne and Ehrlich isolated an arsenite-oxidizing strain of *Alcaligenes* from soil. The bacterium most closely resembled *A. faecalis*, though the match was uncertain. The organism was peritrichously flagellated, non-sporeforming, Gram-negative, aerobic, heterotrophic, and rod-shaped. It acquired its arsenite-oxidizing enzyme system by growth-dependent induction. In contrast to *P. arsenoxydans-quinque*, the arsenite-oxidizing enzyme system of the *Alcaligenes* strain

appeared to use sulfhydryl groups and cytochrome C. The researchers proposed the following electron-transport chain: arsenite (oxioreductase) -> cytochrome C -> cytochrome oxidase -> O_2.

Only months later, in a similar though independent study, Phillips and Taylor reported isolation of *Alcaligenes faecalis* from raw sewage. The researchers found that when cultures were reared with no arsenite, no additional growth occurred with the addition and oxidation of arsenite. They also determined that Green's *B. arsenoxydans* and Turner's and Legge's *Achromobacter arsenoxydans-tres* were actually strains of *A. faecalis.* (Phillips and Taylor)

In 1978, Ehrlich published a review of the literature covering arsenic-related bacteria. He noted that the electron-transport chain of *A. faecalis* "suggests that the organism may be able to derive energy from the process." He described a study done by Welch, a student of Ehrlich, in which starved induced *A. faecalis* had higher survival rates with the addition of arsenite to the medium than without. This provided evidence, though certainly inconclusive, that *A. faecalis* may be able to derive maintenance energy from the oxidation of arsenite. (Ehrlich, 1978, Welch as cited by Ehrlich)

Next, in 1981, Abdrashitova, Mynbaeva, and Ilyaletdinov described two additional types of heterotrophic bacteria capable of oxidizing arsenite to arsenate. *Pseudomonas putida* and *Alcaligenes eutrophus* were isolated from gold-arsenic deposits. (Abdrashitova et al.) In the same year, Ilyaletdinov and Abdrashitova reported the exciting discovery of a novel bacterium which was not only capable of arsenite oxidation, but also capable of harvesting the energy released by the chemical reaction. The microorganism was also isolated from the mine waters of a gold-arsenic deposit. It was a Gram-negative, non-sporeforming rod with one flagellum; the bacterium lived fully autotrophically. The

researchers named the bacterium *Pseudomonas arsenitoxidans.* (Ilyaletdinov and Abdrashitova)

The next few years involved the fields of genetics and molecular biology more directly with the phenomena of arsenite- and arsenate-resistance among bacteria. Silver and Keach demonstrated the energy- and temperature-dependence of the arsenate efflux of both *E. coli* and *Staphylococcus aureus* which contained arsenic-resistance plasmids. (Silver and Keach) Next, Chen, Mobley, and Rosen investigated the arsenic-resistance plasmid R773. They showed that there were two separate regions for arsenite resistance and arsenate resistance.(Chen et al.) Then Rosen, Chen, SanFrancisco, and Gangola sequenced R773 and proposed that the plasmid encodes an arsenite pump and also encodes a modifier to allow arsenate as a pump substrate. (Rosen et al.) Dabbs and Sole then isolated a *Rhodococcus erythropolis* plasmid for arsenite- and arsenate-resistance. (Dabbs and Sole) Dey, Dou, and Rosen then showed that the R773 pump worked in vitro.(Dey et al.)

In 1986, Abdrashitova, Abdullina, and Ilyaletdinov showed that in *Pseudomonas putida* and *Alcaligenes eutrophus,* arsenite initiated cell lipid peroxidation, forming hydroperoxides of unsaturated fatty acids and the oxidation of arsenite to arsenate. Further, the researchers demonstrated that the bacteria actually over-synthesized unsaturated fatty acid lipids to accommodate the arsenite. (Abdrashitova et al., 1986) In 1990, Collinet and Morin demonstrated that *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* were both capable of oxidizing arsenopyrite. The researchers studied the tolerances of the bacteria to various concentrations of arsenite and arsenate. (Collinet and Morin) In 1994, Ahmann, Roberts, Krumholz, and Morel detailed a novel organism, tentatively dubbed "MIT13," which used arsenate as an electron acceptor and was able to

harvest the energy from the reaction. This arsenate reducing organism was vibrio-shaped, motile, and anaerobic.(Ahmann et al.)

1.4 Motivations for Research

This report details the isolation and characterization of an arsenite-oxidizing community of bacteria that was isolated from the northern edge of the HBSA. The role of this bacterium is interesting from a bioremediation standpoint. Arsenic(III) is chiefly present in waters as a neutral compound, whereas arsenic(V) is present as a charged compound. Charged species of arsenic will more preferentially adsorb to ferric oxyhydroxides than their neutral counterparts. This sorption renders them less environmentally mobile, allowing for their containment and perhaps treatment. Harnessing this biotechnology could be particularly important to people who enjoy the Mystic Lakes every summer through swimming on the public beaches, boating, and fishing. Were this bacterium cultivated at certain strategic points in the watershed, it might prevent much of the arsenic from reaching the Mystic Lakes, thus limiting human exposure to arsenic.

CHAPTER 2
METHODS

2.0 Description of the Site

The Halls Brook Storage Area is an anthropogenic pond located directly south of the Industri-Plex Superfund Site. It was dug to accommodate storm drainage from the Halls Brook. The northern edge of the pond is spring-fed with arsenite-laden groundwater. The sediment of "Arsenic Springs" is noteworthy due to its bright orange color, a result of the high concentrations of oxidized iron. The land surrounding the HBSA is marshy. The wildlife is varied. On separate field trips, a fox, a turtle, a woodpecker, and many types of waterfowl were seen on or around the pond.

2.1 Isolation of the Culture

2.1.1 Field Sampling

The field samples were drawn with acid washed polypropylene Nalgene bottles. The sample sites were selected from areas of the northern edge of the pond which were visibly spring-fed. Three times, the bottles were filled with spring water and shaken; then a sample was taken. The bottles were filled three-quarters with sample and taken directly to the laboratory where they were refrigerated tightly-lidded. Initially, the samples were often turbid orange due to suspended sediment. After a day, the refrigerated samples were clear with the orange sediment settled to the bottom.

2.1.2 Enrichment Culture

Because it was unclear whether the HBSA arsenite oxidizers would be chemoautotrophic or heterotrophic, the isolation techniques were modeled after previous work isolating the less unusual bacterial types: heterotrophic arsenite oxidizers. All cultures in this study were kept in the dark on a bench top shaker table set at 150 rpm. The temperature varied from 17°C to 25 °C within the laboratory. All cultures were maintained in triplicate with a killed control and an uninoculated control. Cultures were maintained in acid-washed, autoclaved 125 mL polypropylene Nalgene bottles, with caps loosely screwed on. The pipettes used in transferring and in analysis were acid washed and autoclaved.

A field sample was gathered August 22,1995. On August 24, 1995, an enrichment culture of WE medium was inoculated 1:9 with this HBSA sample. WE medium was an adaptation of that used to isolate the arsenite-oxidizing bacterium *Alcaligenes faecalis.* (Welch as quoted by Ehrlich, 1978) After five days, the culture had almost completely oxidized the arsenite. On September 6, #10-950824 was transferred, 1:9, to new medium. Six subsequent transfers into WE medium occurred: September 21, October 3, October 15, October 21, November 2, and last, November 12. On November 20, this final culture (#1-951112) was frozen in 30% autoclaved glycerol at -4° C.

At this point, an attempt was made to isolate a chemoautotrophic arsenite-oxidizing bacterium from this culture. A modified version of the chemically-defined medium was used by Abdrashitova, Mynbaeva, and Ilyaletdinov. (1981) On January 5, 1996, this medium was used to transfer culture #1-951112 1:9. The cultures failed to dramatically increase their turbidity.

On January 12, culture #7-960105 was transferred 1:9 into a second modification of The AMI medium. This was identical to the first except that it replaced the vitamin supplements with 0.4g/L yeast extract. The cultures thrived. On January 29, culture #32-960112 was transferred 1:500 into the same type of medium. After the culture became turbid and oxidized most of the arsenite, the culture (960129) was frozen in 30% autoclaved glycerol at -4°C.

Next came a third modification to the medium. On February 6, instead of using yeast extract, vitamin supplements 1 and 2 were again used along with two trace metals mixtures. Furthermore, the pH was adjusted to 7, rather than to 5.5, with sterile 2N NaOH. Culture 960129 was added to this medium, 1:100, giving cultures #27-#31-960206. The cultures became more turbid and also oxidized arsenite.

On March 10, #28-960206 was transferred 1:100 to a fourth modified version of the AMI medium· this time buffered with MOPS at pH=7. This generation was called #42-#46-960310. The final transfer was into a fifth and final modification of the medium; the only change was an increase in arsenite concentration to 10^{-2}M. Culture #44-960310 was used as an inoculum 1:100. This last generation, #1-#5-960323, was the fourteenth generation of cultures from the initial field sample of August 22, 1995, and the third generation in an entirely autotrophic medium. A summary of these transfers appears in table.

Culture Name	Medium Type	Inoculum: Medium	Source Inoculum
10-950824	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	HBSA sample 950822
22/23-950906	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	10-950824
4/5-950921	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	22/23-950906
30/31-951003	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	4/5-950921
2-951015	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	30/31-951003
28-951021	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	2-951015
27-951102	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	28-951021
1-951112	WE: 10^{-3}M As(III), yeast extract, ammonium citrate, pH5.5	1:9	27-951102
7-960105	PT: 10^{-3}M As(III), vits, pH5.5	1:9	1-951112
32-960112	PT: 10^{-3}M As(III), yeast extract, pH5.5	1:9	7-960105
960129	PT: 10^{-3}M As(III), yeast extract, pH5.5	1:500	32-960112
28-960206 (27-31)	PT: 10^{-3}M As(III), vits, pH7, metals	1:100	960129
44-960310 (42-46)	PT: 10^{-3}M As(III), vits, pH7 MOPS, metals	1:100	28-960206
3-960323 (1-5)	PT: 10^{-2}M As(III), vits, pH7 MOPS, metals	1:100	44-960310

Table 2: Transfers of Cultures

2.2 Analytical Methods

2.2.1 Measurement of Arsenite Concentration in Cultures

The concentration of arsenite was measured with a continuous-flow hydride generator system constructed by PSAnalytical, LTD. A peristaltic pump drew equal parts Tris buffer solution and sodium borohydride solution to a reaction vessel, each at approximately 3mL/min. The reaction mixture was purged with steadily-flowing argon (300mL/min) and hydrogen (0.1mL/min) gases.

During background measurements, distilled deionized water was pumped (approximately 8mL/min) into the reaction vessel in lieu of a sample. During arsenite measurements, diluted aliquots of the cultures were pumped (approximately 8mL/min) from acid washed sample cups into the reaction vessel. Due to the Tris buffer solution, the reaction mixture was maintained at a neutral pH, thus allowing the sodium borohydride to reduce only the arsenite to arsine gas, while maintaining the arsenate in solution. The newly-produced arsine was swept with the argon-hydrogen mixture to an air-hydrogen flame. The flame atomized the arsine gas, and the change in the flame's atomic fluorescence was directly proportional to the amount of arsenite in the initial sample.

Due to the high concentration of arsenite in the cultures, the samples required large dilutions prior to analysis. The analysis setup continued giving linear calibration curves from concentrations ranging from nm of arsenite to 20mM of arsenite. Thus, micromolar concentrations were chosen as the target dilution: the least dilution required to maintain a place on the linear portion of the calibration curve. The cultures with 10^{-3}M arsenite were diluted 1:1000 for analysis, whereas the cultures with 10^{-2}M arsenite were diluted 1:10,000. Every ten measurements, a calibration curve was taken. The six standards that constructed this curve ranged from 182nM arsenite to 2275nM arsenite. These standards were actually tertiary standards: further dilutions of a secondary standard that was a 1:1000 dilution of the 10^{-2}M sodium arsenite stock standard.

2.2.2 Measurement of Cell Density

2.2.2.a Turbidity

In order to measure how well the cells were growing, measurements of the density of the cultures were made. These measurements were taken on a Beckman Spectrophotometer DU 640 at a wavelength of 600nm. Aliquots of 1mL of culture were

compared to a baseline measurement of the absorbance of an aliquot of 1mL of deionized distilled water. Immediately prior to measurement, samples were slightly stirred; excessive mixing created bubbles which artificially raised the absorbance of the sample.

2.2.2.b Direct Counts

Later, when it became clear that turbidity was no longer an accurate measure of cell density, DAPI staining and fluorescence microscopy were used to achieve direct counts of the cells. Depending upon the cell density of the culture, 0.01mL-0.95mL aliquots of the culture were killed and preserved with 50mL formalin. These aliquots were maintained in the refrigerator for approximately 1 week prior to counting. Immediately prior to counting, 10mL of DAPI mixture was added in total darkness to the fixed culture. After the labeling had continued for ten minutes, the culture was filtered onto a black 0.2mm polycarbonate filter and counted under a 100W mercury arc lamp on a Zeiss microscope. The number of grid-fields counted depended upon the number of cells within the average grid. For each fixed sample, approximately 800 total cells were counted, though for some samples significantly more cells were counted.

2.2.3 Inorganic Carbon Uptake

The ^{14}C experiment was conducted as follows: A culture was spiked with radiolabeled bicarbonate and incubated for some fixed length of time. After incubation, the pH of the culture was lowered to encourage the degassing of all unincorporated $^{14}CO_2$. Any remaining radioactivity was associated with inorganic carbon that had been "fixed" into organic carbon: biomass. The amount of radioactivity was therefore directly proportional to the amount of increase in biomass. After the degassing, Fisher Scientific ScintiSafe Plus 50% liquid scintillation cocktail was added to allow counting of disintegrations per minute (dpm). Scintillation cocktail luminesces when in contact with

^{14}C. Thus, the amount of incorporated radioactivity was measured by a scintillation counter, which measured the luminescence of the scintillation cocktail/culture mixture.

Three separate trials were run to determine the correct amount of radioactivity to add to each sample, the appropriate incubation time, and also the correct length of degassing time. First, on May 9, 1mL of culture #3-960323 was incubated with .01mCi and 1mL was incubated with 0.1mCi. After twelve hours dark incubation on a shaker table at 150rpm, 100mL 2NHCl were added to each scintillation vial, along with 8mL Fisher Scientific Scinti-Safe Plus 50% . This mixture was incubated, loosely capped, in the dark at 150rpm, for two hours. After this incubation, there was no significant difference in measured counts or in dpm measurements between the two vials.

Next, 1mL of culture #1-960323 (a sister culture to #3-960323) was incubated with 0.1 mCi, and 1mL of uninoculated medium was incubated with 0.1 mCi. However, during this test, the cultures were allowed to degas for two hours prior to the addition of scintillation fluid. Again, there was little difference between the two scintillation measurements.

The third test used 1mL of culture #3-960323 incubated with 1mCi, and 1mL of uninoculated medium incubated with 1 mCi. Again, the cultures were allowed to degas for two hours prior to the addition of scintillation fluid. This time, there was a significant difference in incorporated radioactivity between the culture and the control. The two vials were then allowed to degas for four additional hours, after which an additional scintillation count was taken. It was found that this additional degassing time did indeed reduce the background count, though admittedly only by a few percent. Thus, it was settled upon that 1mCi would be added to 1mL of culture, incubation would last for twelve hours, and degassing time would be four hours.

On May 11 through May 12, four live 1mL aliquots of culture #3-960323, two autoclaved 1mL aliquots of culture #3-960323, and two uninoculated 1mL aliquots of sterile medium were each spiked with 1 mCi of ^{14}C-labeled sodium bicarbonate. Each scintillation vial was tightly capped and placed in the dark on a shaker table at 150 rpm. They were incubated for twelve hours. In addition were three non-radioactive vials: The original live culture #3-960323, one autoclaved 1mL aliquot of #3-960323 and one uninoculated aliquot of #3-960323 were also incubated in the dark on a separate 150-rpm shaker table. From these non-radioactive aliquots, samples were drawn several times during the incubation period for DAPI staining and cell counts, as well as for arsenite concentration analysis. After the twelve-hour incubation, 100mL of 2N HCl was distributed to each of the radioactive samples to promote the degassing of radiolabeled CO_2. The samples were loosely capped and again incubated on the shaker in the dark. After four hours of degassing, 8mL of scintillation fluid, Fisher Scientific ScintiSafe 50%, were added to each radiolabeled vial. The vials were capped tightly, shaken thoroughly, and placed in a Beckman LS 6500 Multi-Purpose Scintillation Counter for five-minute counts.

CHAPTER 3
RESULTS

3.0 pH of the Medium

As arsenite is oxidized to arsenate H^+ ions are released, decreasing the pH of a system. Not surprisingly, energetics calculations showed that arsenite oxidation is favored at high pH values. However, the pH of the northern tip of the HBSA was approximately 6. Yet, several different studies isolated arsenite-oxidizing bacteria in media that ranged from 6.8 to 8.5, though the most common pH was 7. (Abdrashitova, Mynbaeva, and Ilyaletdinov; Welch as quoted by Ehrlich, 1978; Phillips and Taylor; Osborne and Ehrlich).

Thus, 10^{-3}M As(III), MOPS-buffered media were prepared that varied in pH from 4 to 10, in an effort to find the optimum pH: the medium in which the bacteria oxidized the arsenite the most quickly. Following the arsenite concentrations for several days showed that the arsenite was oxidized equally quickly for the media ranging from pH4 to pH8, all cultures oxidizing the arsenite to completion after four days. However, the bacteria in the pH9 medium took five days to oxidize the arsenite to completion. The pH10 culture achieved no significant oxidation, having oxidized approximately ten percent of the arsenite after an entire week of incubation. (Fig. 3-10) These tests showed the unsuitability of the highest pH media. Presumably, better temporal resolution in the data would have helped distinguish the most favorable medium among the pH4 to pH8 media. However, according to these data, the lower pH media appeared.

Figure 3. Concentration of Arsenite in pH4 Cultures

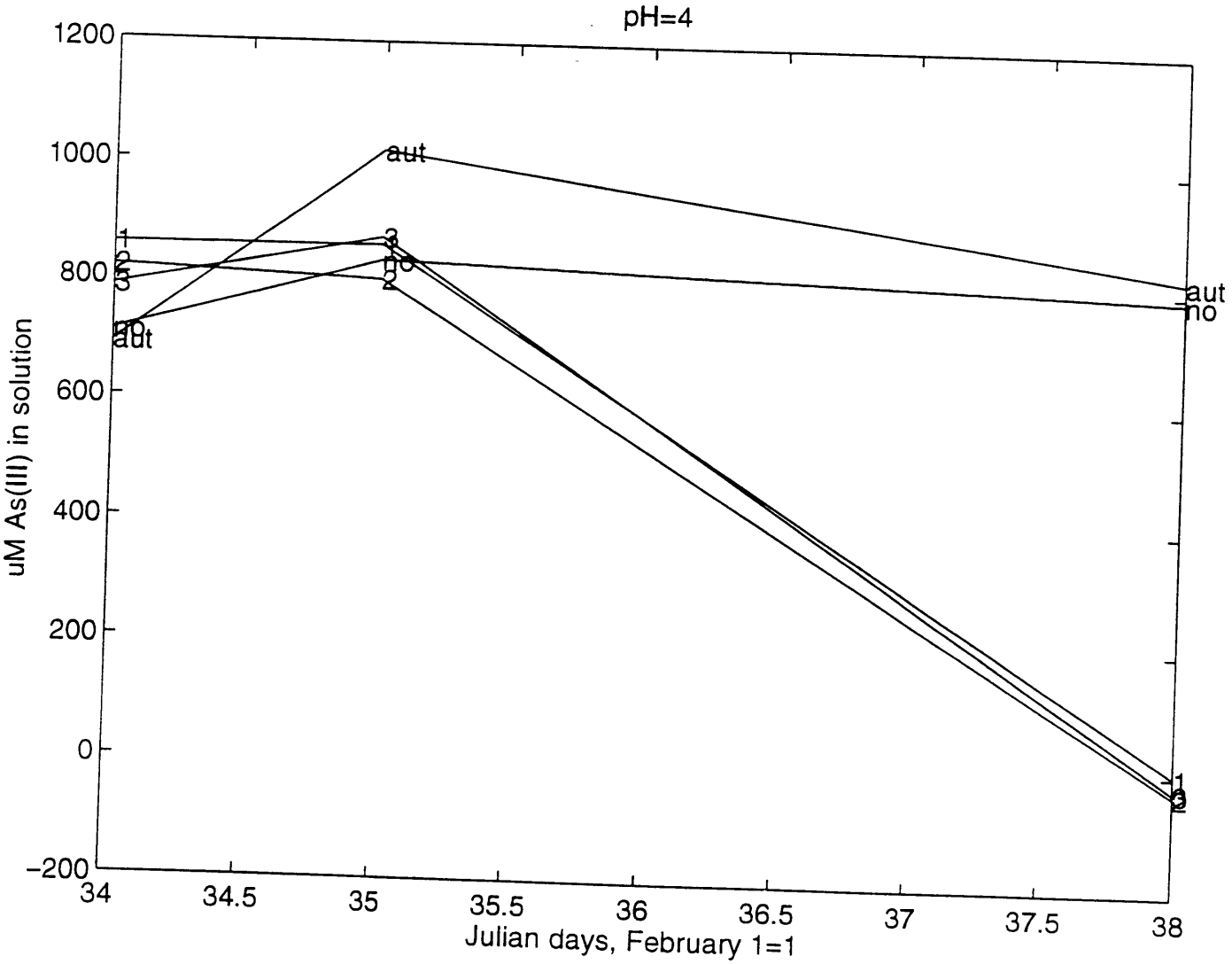


Figure 4. Concentration of Arsenite in pH5 Cultures

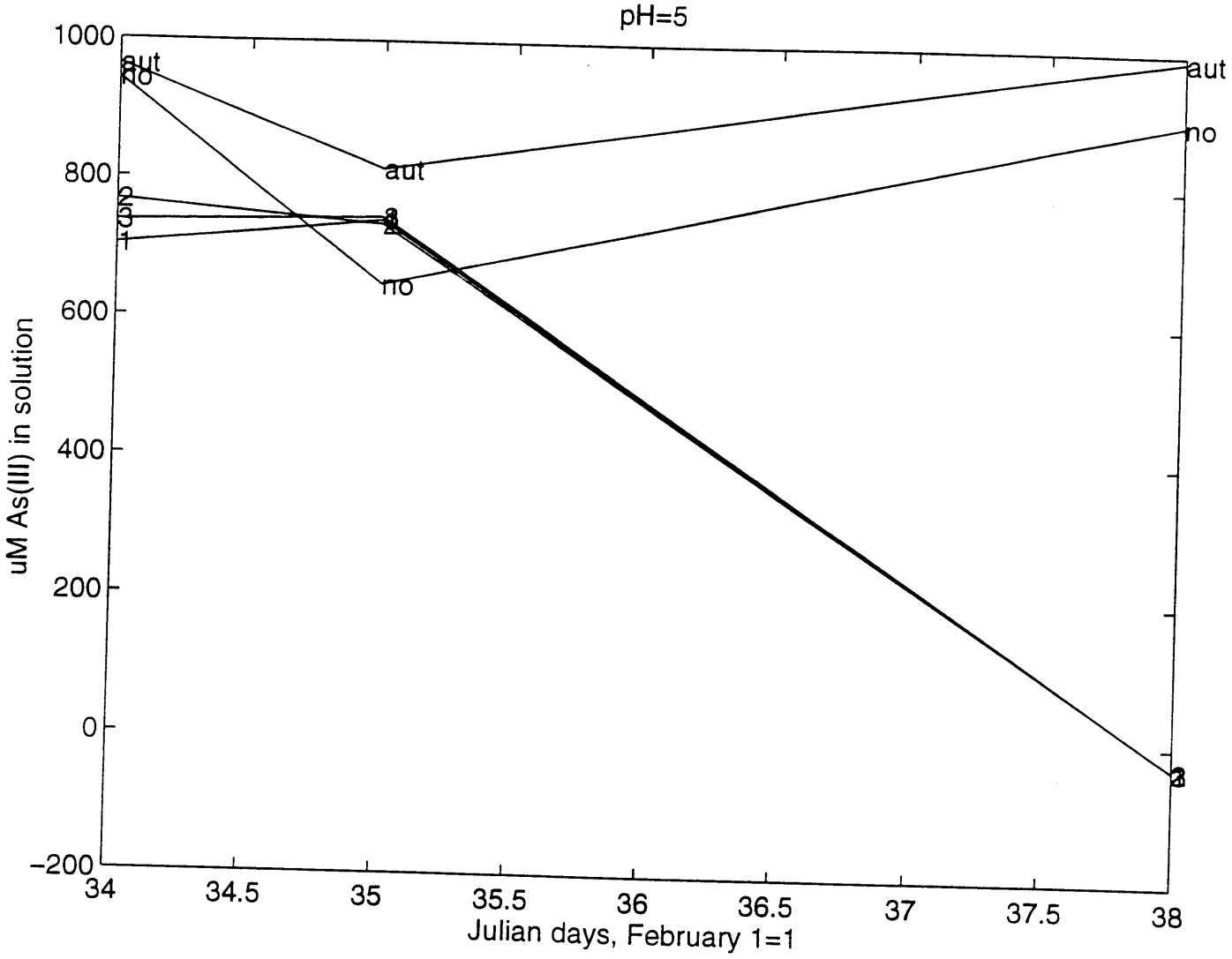


Figure 5. Concentration of Arsenite in pH6 Cultures

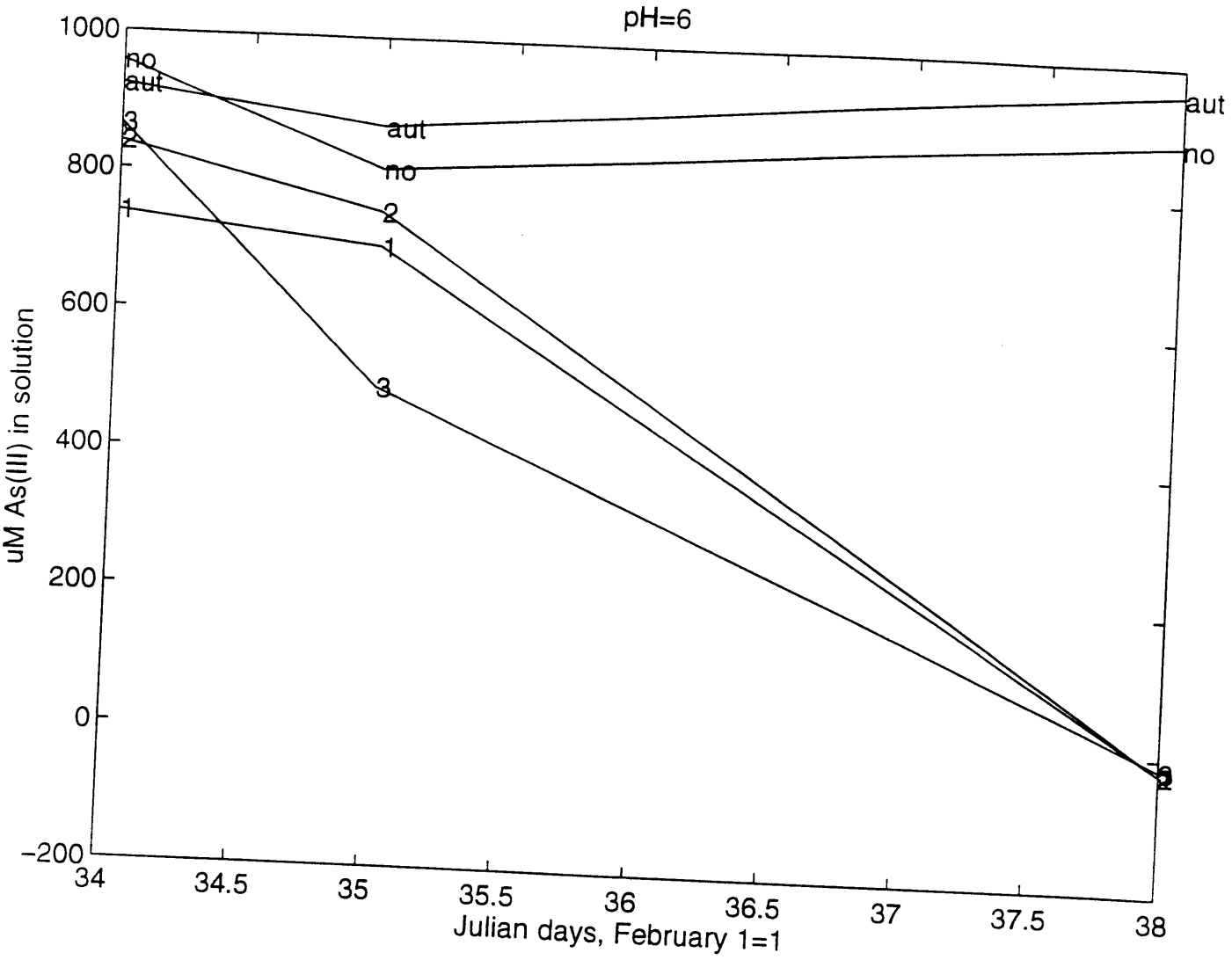


Figure 6. Concentration of Arsenite in pH7 Cultures

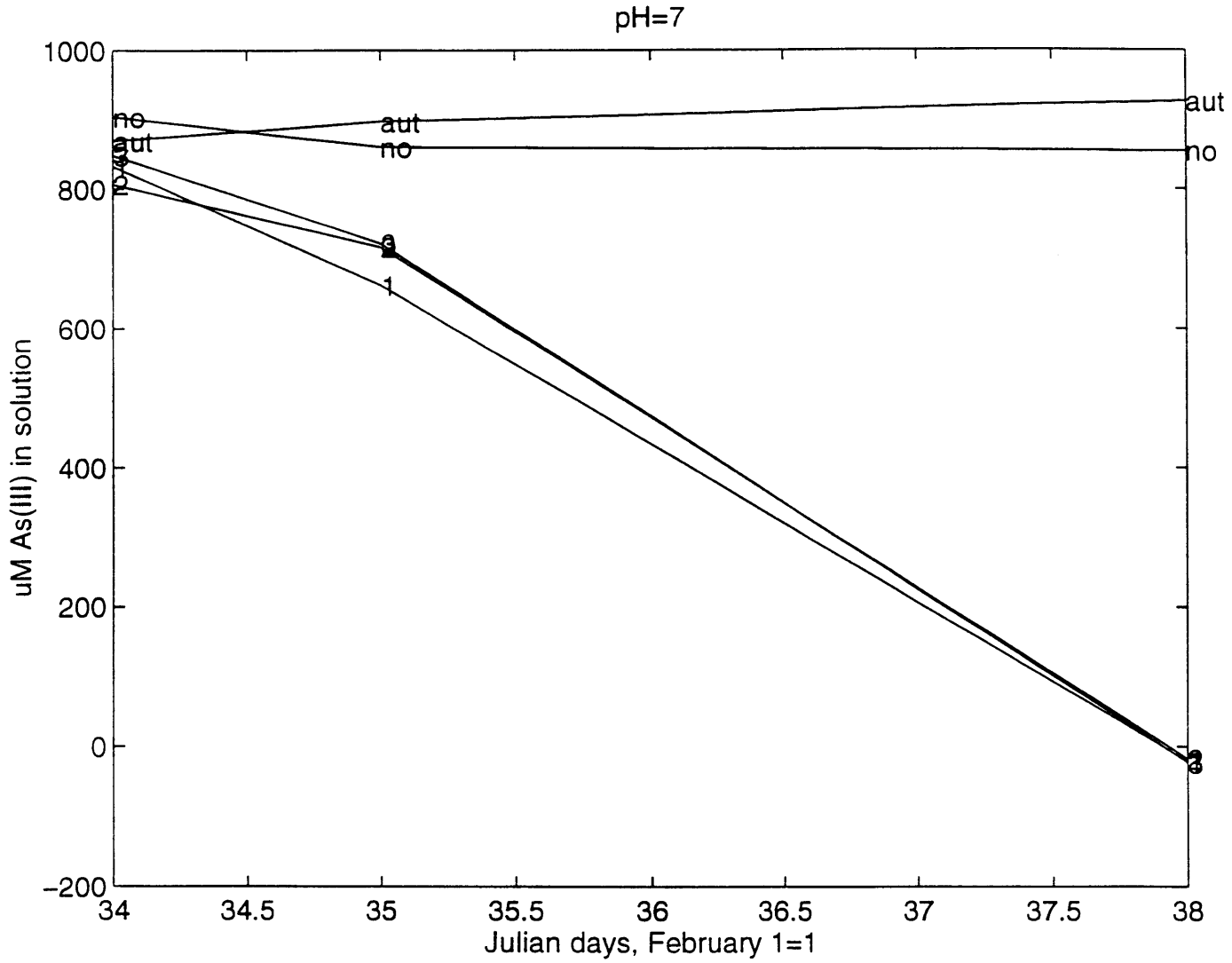


Figure 7. Concentration of Arsenite in pH8 Cultures

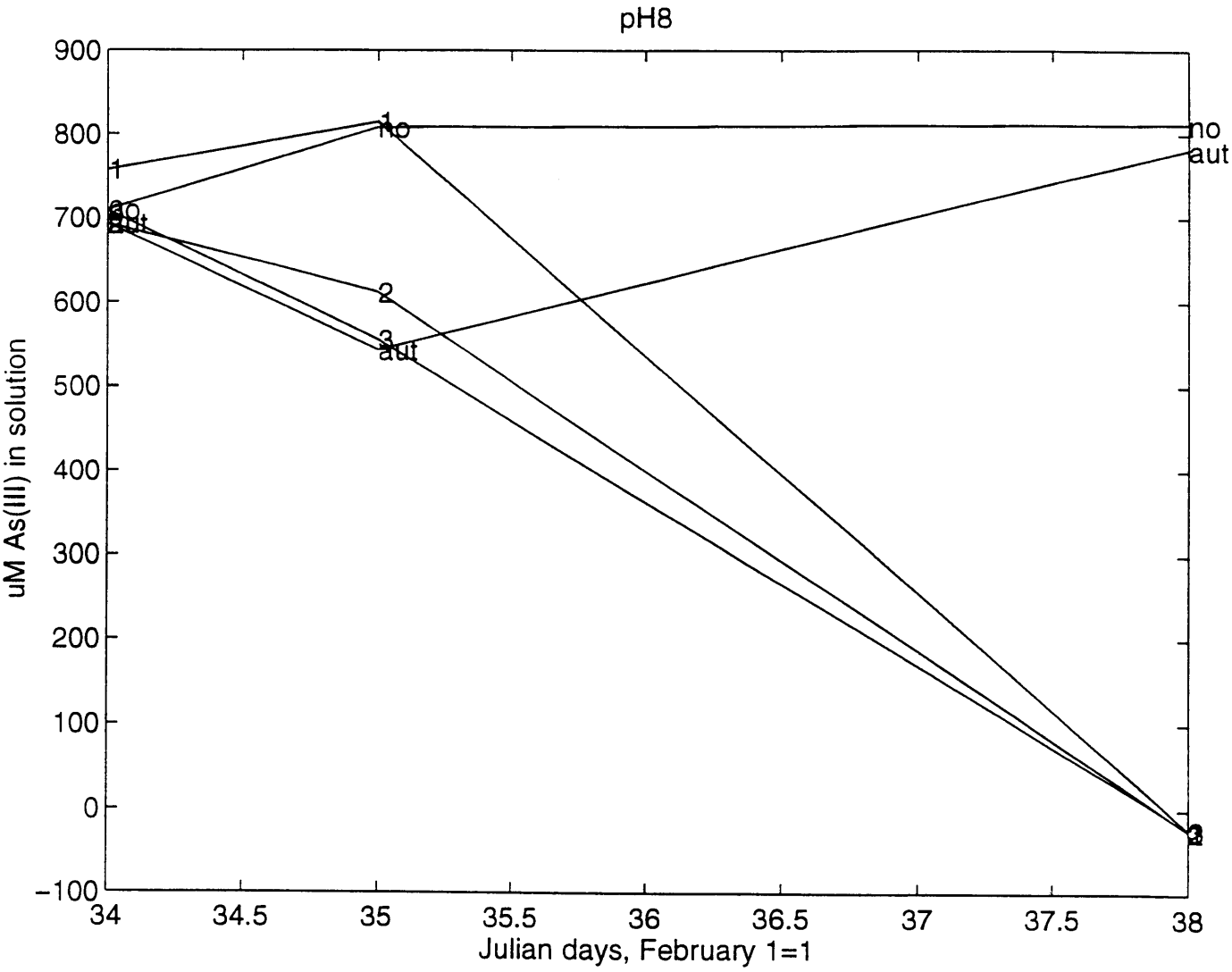


Figure 8. Concentration of Arsenite in pH9 Cultures

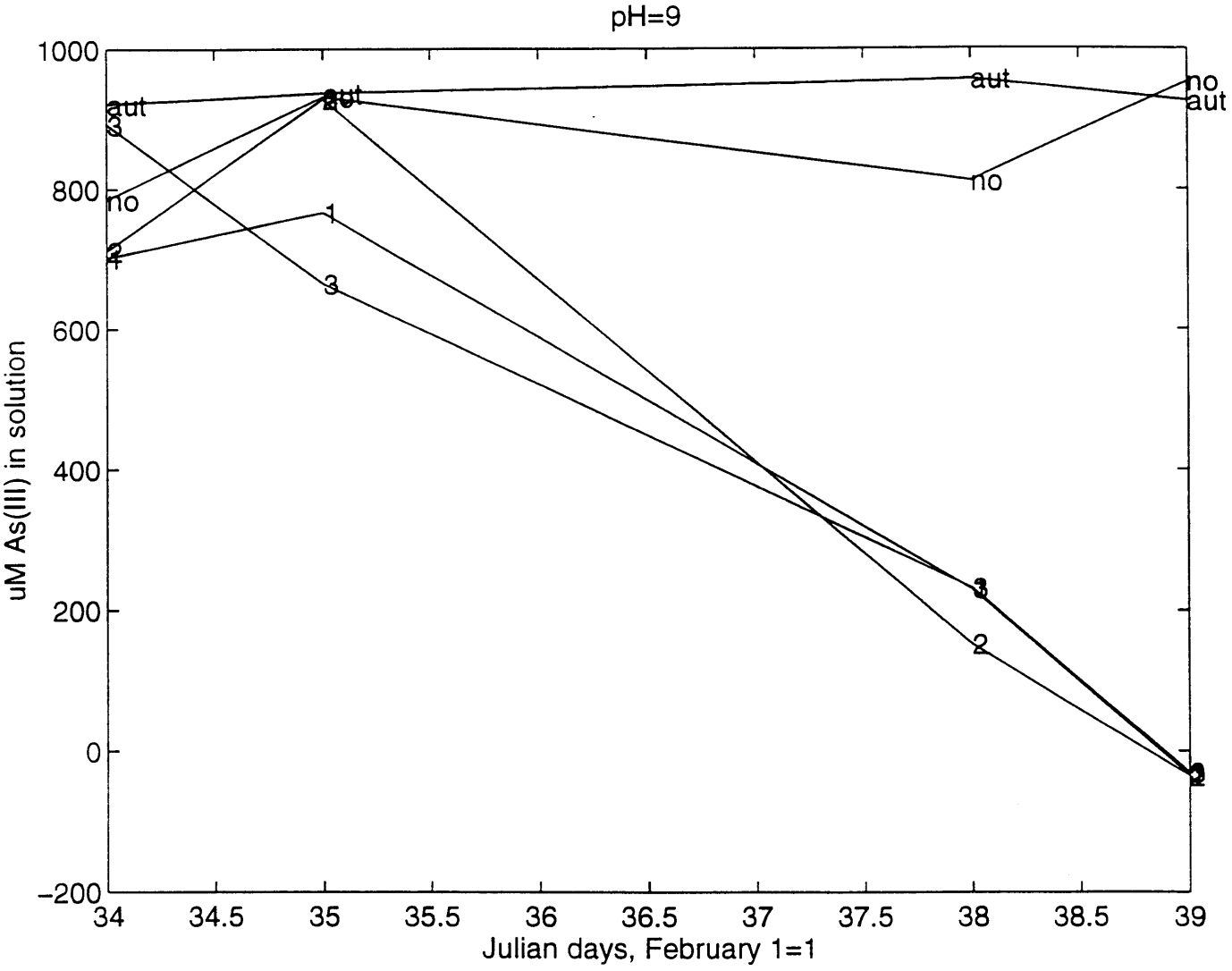


Figure 9. Concentration of Arsenite in pH10 Cultures

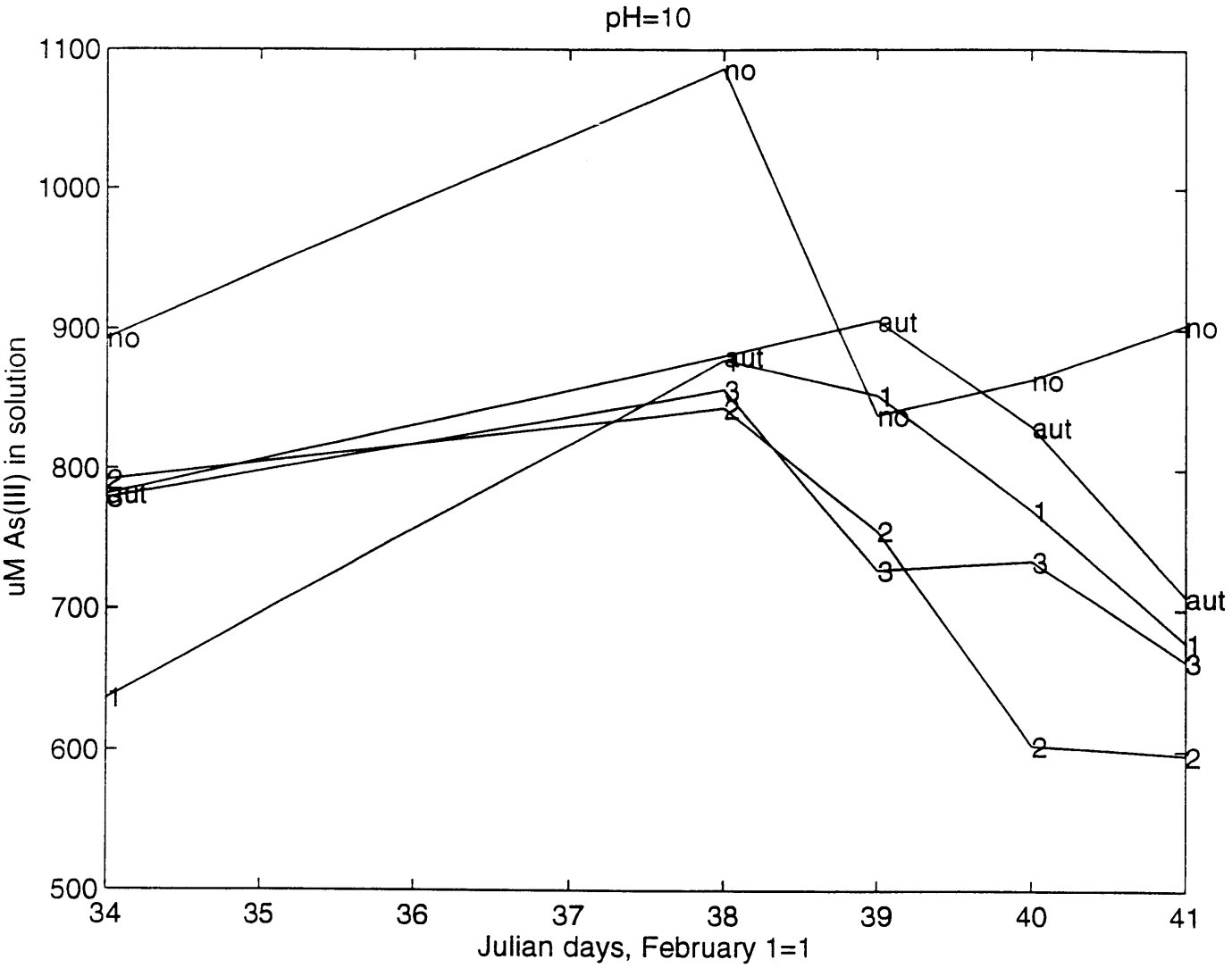
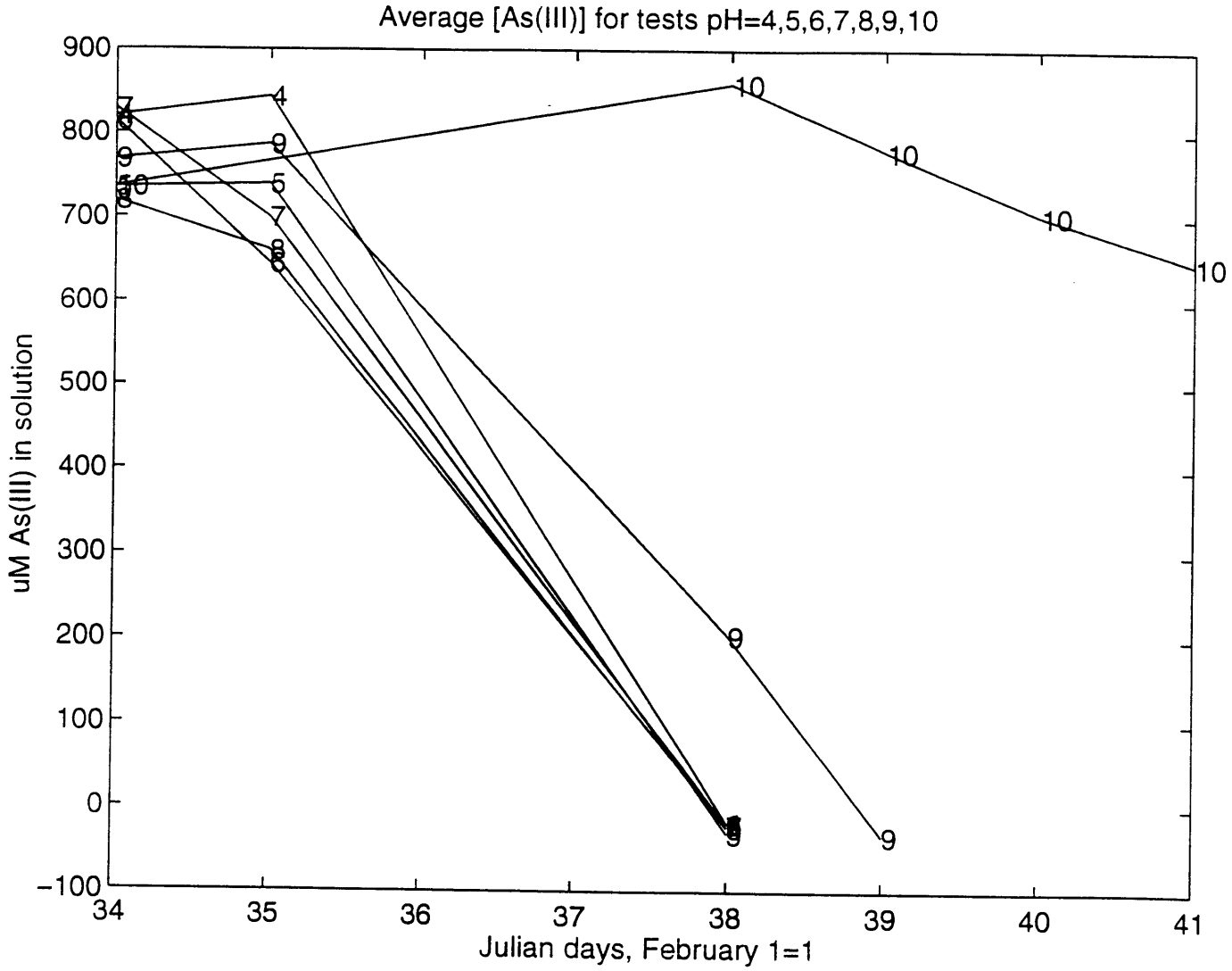


Figure 10. Average Concentration of Arsenite in Live Cultures in Media of pH 4, 5, 6, 7, 8, 9, 10



equally favorable. Ilyaletdinov and Abdrashitova isolated the chemoautotrophic *Pseudomonas arsenitoxidans* in a medium of pH7.5-pH8. Furthermore, energetics calculations did favor higher pH media for arsenite oxidation. Balancing these considerations with that of the natural pH of 6, a medium pH of 7 was settled upon.

3.1 Growth Patterns of the Bacteria

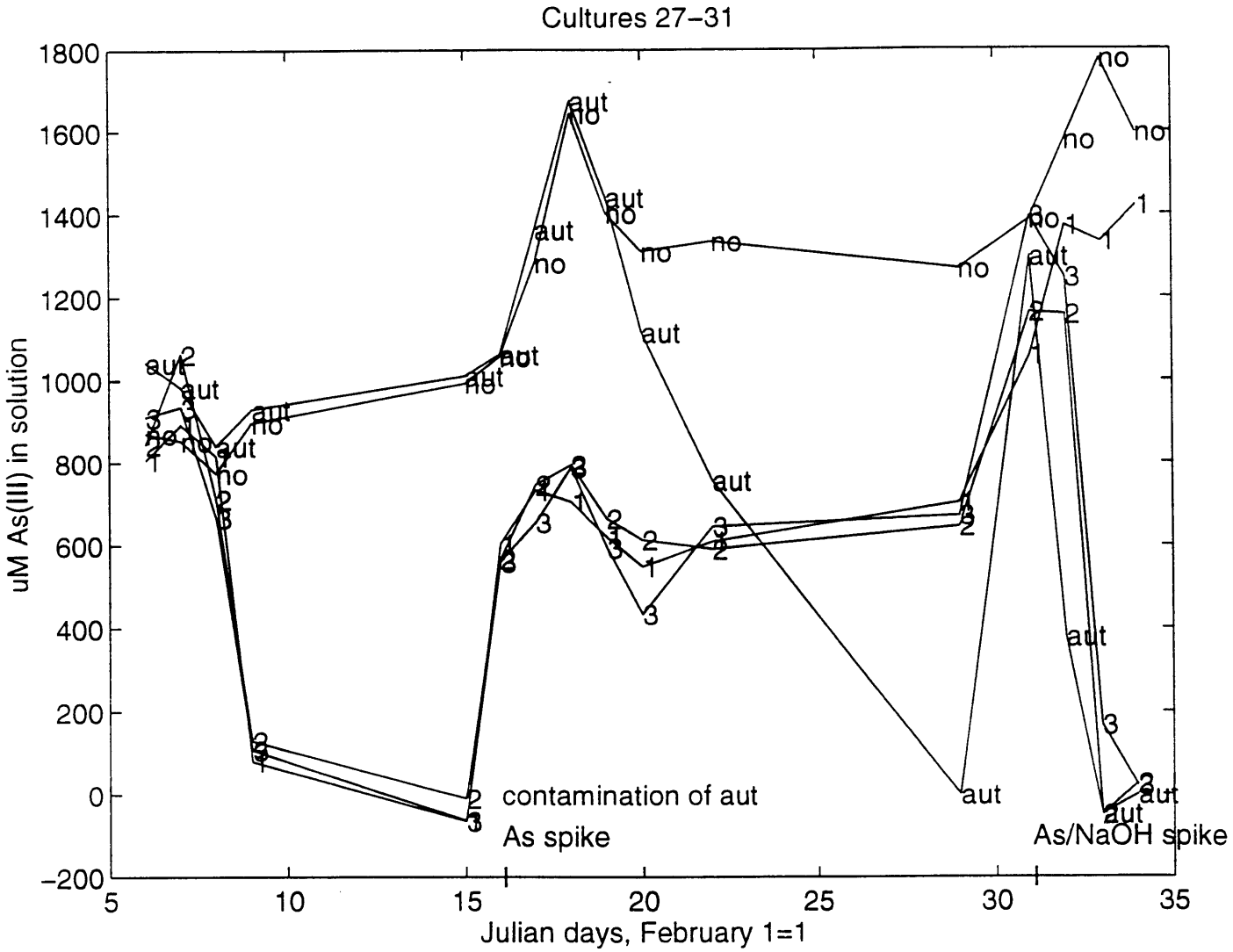
Next, the growth of the bacteria were analyzed. In particular, the three final generations of cultures were scrutinized closely. Turbidity measurements and arsenite concentration measurements were taken approximately once every two days.

3.1.1 Cultures #27-#31

3.1.1.a Arsenite Measurements

First, cultures #27-#31-960206 were followed for a period of approximately one month. #27, #28, and #29 were all live cultures, whereas #30 was an autoclaved control and #31 was an uninoculated control. After 9 days of incubation, each of the three live cultures had oxidized 10^{-3}M arsenite to completion, and the controls had not oxidized any of their arsenite. After a second arsenite spike on day 16, however, the live cultures were no longer able to oxidize their arsenite. However, by day 20, autoclaved control culture #30 had oxidized a significant portion of the total arsenite in its medium. By day 29, the live cultures had oxidized none of their second spike. In contrast, the autoclaved control had oxidized to completion not only the initial arsenite in its medium, but also the second spike. The uninoculated control had oxidized none of the arsenite in its medium. (Fig.11)

Figure 11. Concentration of Arsenite in Cultures 27-31

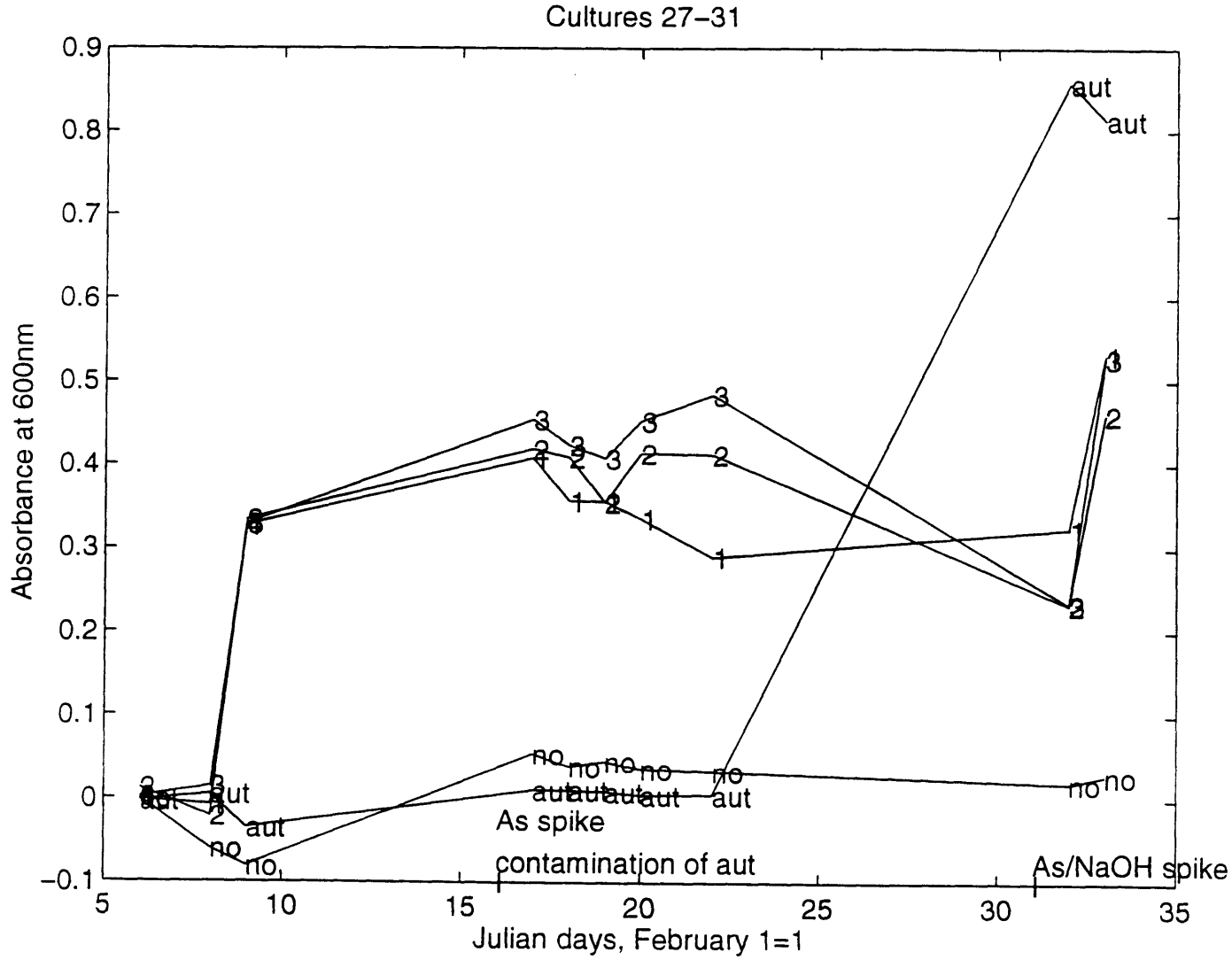


This implied a few things. First, culture #30 had been accidentally inoculated. Most likely, the source culture was #29, which was always pipetted immediately before #30. Furthermore, the inoculation probably occurred on day 16, the date of the second spike, because the same pipette was used to spike each culture. After this experiment, new sterile pipettes were used for each spiking. The second thing these results suggested was that the failure of cultures #27, #28, and #29 to oxidize the second spike was likely due to pH factors, rather than to cell death. Culture #30 demonstrated that it was possible for the cell strains to oxidize $2*10^{-3}$M As(III) to completion given favorable circumstances. Therefore, on day 31, the cultures were spiked with arsenite a third time. However, this time they were also spiked with NaOH to pH7. After only three days, cultures #28, #29, and #30 had oxidized to completion. Culture #27 failed to oxidize the arsenite, possibly due to cell death; uninoculated control culture #31 failed to oxidize its arsenite as well.

3.1.1.b Turbidity measurements

The turbidity measurements at 600nm show similar patterns. By day 9, the turbidity of the three live cultures had increased markedly. However, until the third spike, the turbidity of these three cultures remained approximately constant, with a small rise before the second spike of arsenite, and a slow decline after the second spike. Their turbidity increased markedly after the third arsenite spike, which was accompanied by a spike of NaOH to pH7. The turbidity of the no-inoculum control, #31, remained negligible for the duration of the experiment. However, after day 23, the turbidity of the autoclaved control, #30, shot up, becoming twice as turbid as the live cultures, #27, #28, and #29. (Fig. 12)

Figure 12. Turbidity of Cultures 27-31



3.1.2 Cultures #42-#46

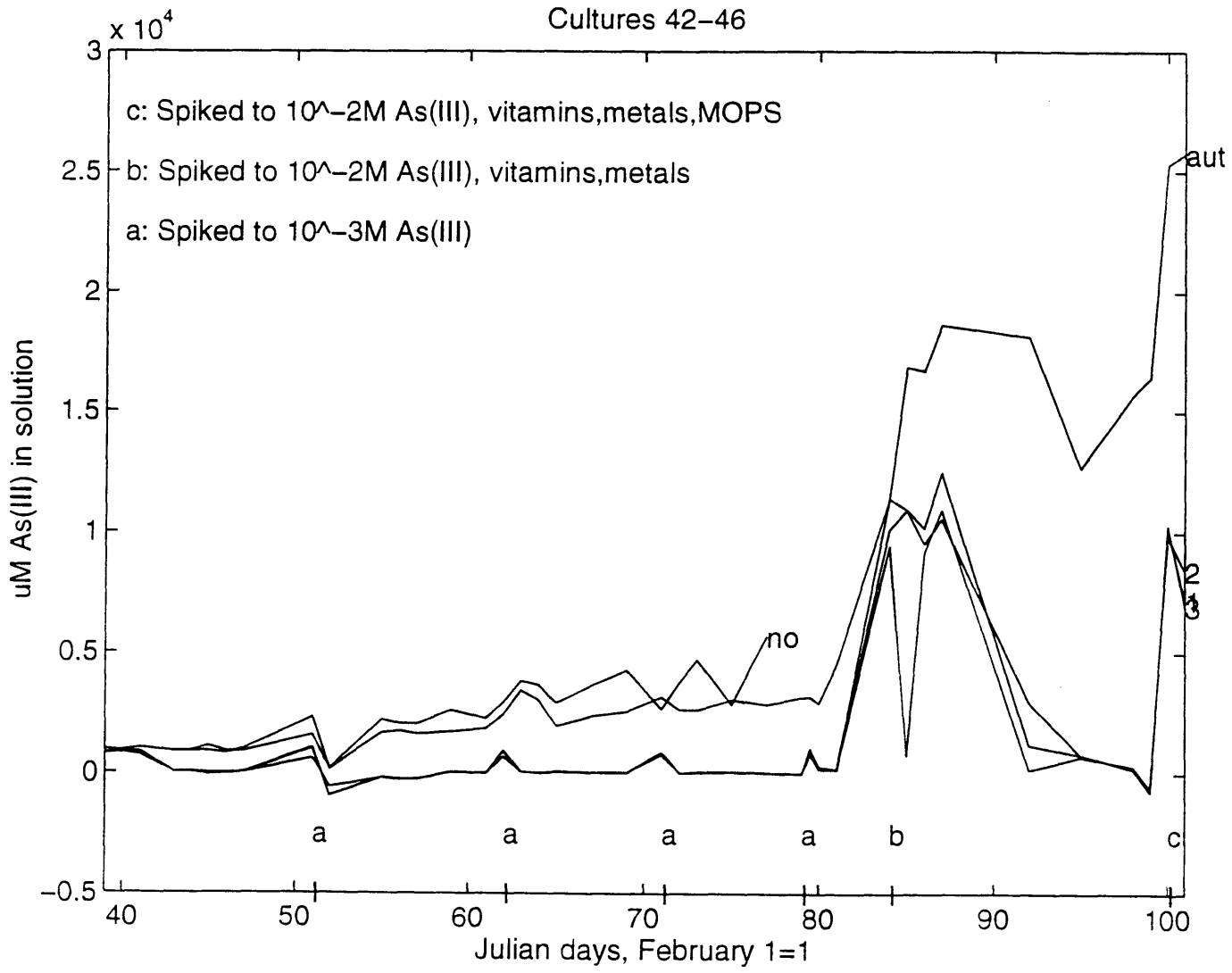
3.1.2.a Arsenite Measurements

The next generation of cultures (spiked by #28 of the previous generation) was buffered at pH7 to avoid the problem of a drop in pH due to arsenite oxidation. Live cultures #42-#44, autoclaved control culture #45, and uninoculated control culture #46 were inoculated and initially supplied with 10^{-3}M arsenite. Neither the autoclaved control, #45, nor theuninoculated control, #46, displayed any oxidation of the arsenite throughout the 60-day trial. On day 49, uninoculated control #46 was partially spilled; the culture was completely depleted due to sampling by day 77. The live cultures depleted their initial 10^{-3}M arsenite spike after approximately 10 days. They were subsequently spiked with 10^{-3}M As(III) on day 51, then days 62, 71, and 79. After each spike, the live cultures oxidized to completion after 1-3 days. Next, on day 84, the cultures were spiked with 10^{-2}M arsenite, along with a new supply of vitamins and trace metals. This new supply of arsenite was oxidized to completion eight days later. One last spike was administered on day 100, this time including 10^{-2}M arsenite, MOPS buffer at pH7, vitamins, and trace metals. (Fig. 13)

3.1.2.b Turbidity Measurements

The turbidity measurements of cultures #42-#46 told a different story from what might be expected given the arsenite concentration measurements. After the four 10^{-3}M arsenite spikes, the two control cultures remained at approximately zero. However, after the first 10^{-2} M arsenite spike, the turbidity of the killed control culture, #45, began to slowly rise, although remaining consistently less turbid than the live cultures, #42, #43, and #44. The live cultures behaved somewhat as expected, displaying small turbidity peaks, increasing turbidity by a factor of two, immediately after oxidizing the initial arsenite in the medium, and then again after oxidizing the arsenite in the spike on day 52.

Figure 13. Concentration of Arsenite in Cultures 42-46



However, after the 10^{-3}M arsenite spike on day 62, the live cultures' turbidity measurements began to be less predictable. By day 67, they had quickly shot up to three times their previous turbidity, and then by day 70, sank to previous baseline turbidity values. The live cultures exhibited similarly interesting behavior after the 10^{-3}M arsenite spike on day 71. Culture #42 shot up to four times its original turbidity by day 72, then sank again to its baseline by day 73. Culture #43 remained at its baseline turbidity until day 76. By day 77, culture #43 was up to four times its baseline turbidity; then by day 80, it returned to its baseline measurement. Culture #44 displayed the most dramatic behavior. On day 71, the turbidity of #44 was at its baseline value. By day 73, the turbidity had increased nine times. Then, falling just as quickly, by day 76, the turbidity of #44 fell back to its baseline value.

The arsenite spike on day 79, then the arsenite/vitamins/trace-metals spikes on day 84 supplied small peaks in the three live cultures, bringing their turbidities to approximately three times the baseline values. (Fig. 14)

3.1.3 Cultures #1-#5

3.1.3.a Arsenite Measurements

The arsenite measurements of cultures #1-#5 were relatively straightforward. The live cultures, #1, #2, and #3, oxidized their original 10^{-2}M arsenite in approximately 20 days. The next spike demanded 8 days for complete oxidation of the arsenite.. The third spike required 5 days to be oxidized to completion. (Fig. 15)

Figure 14. Turbidity of Cultures 42-46

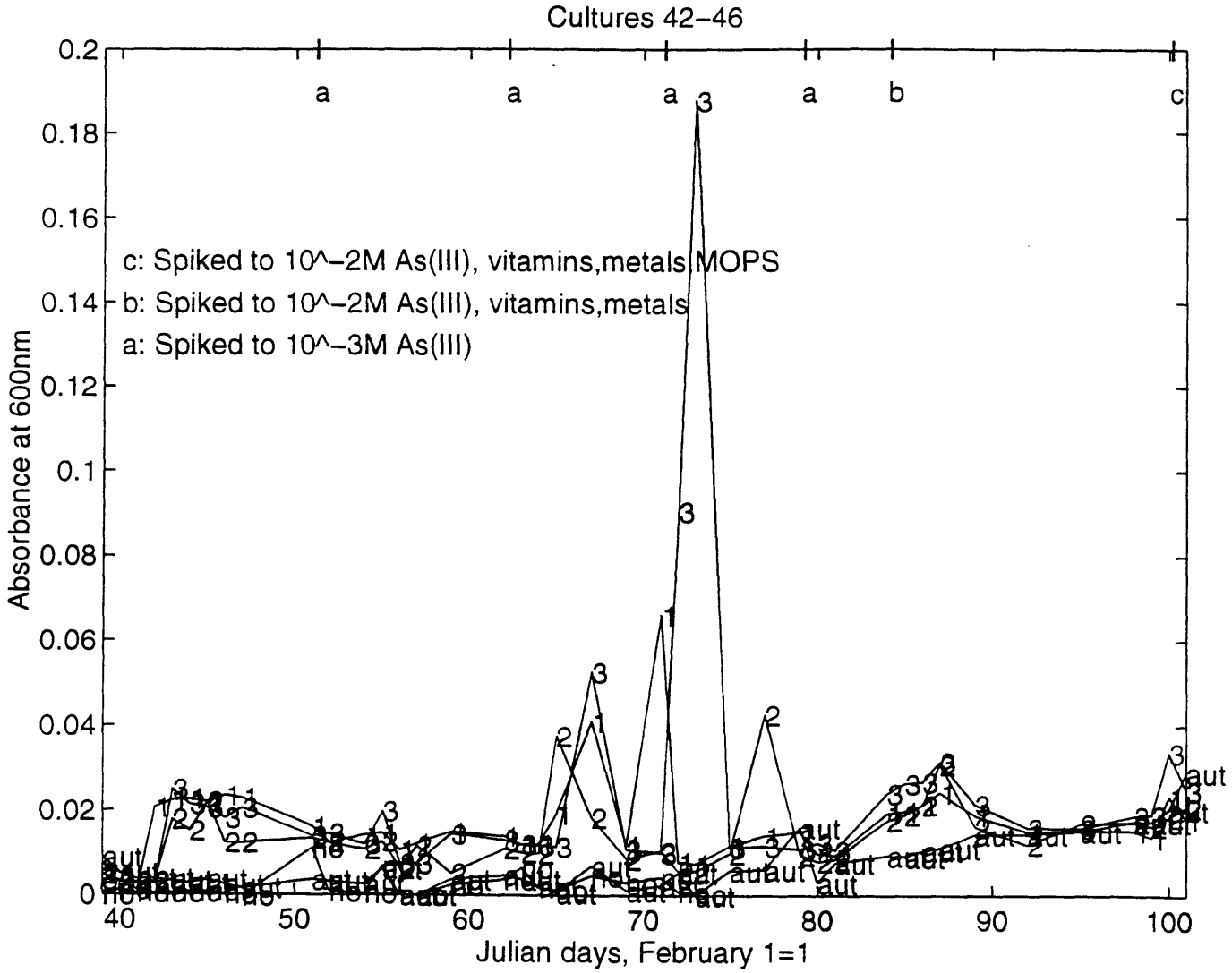
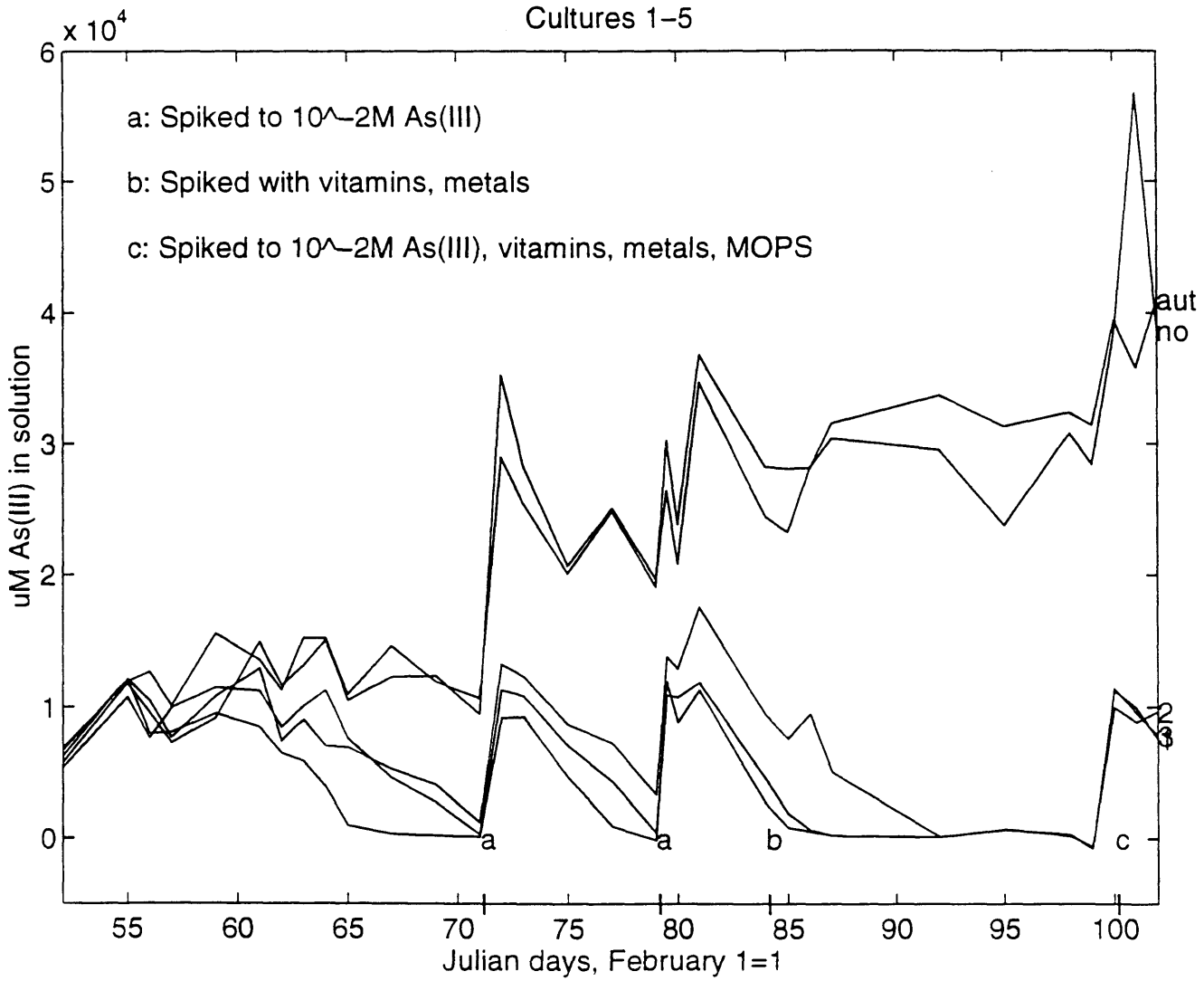


Figure 15. Concentration of Arsenite in Cultures 1-5



3.1.3.b Turbidity Measurements

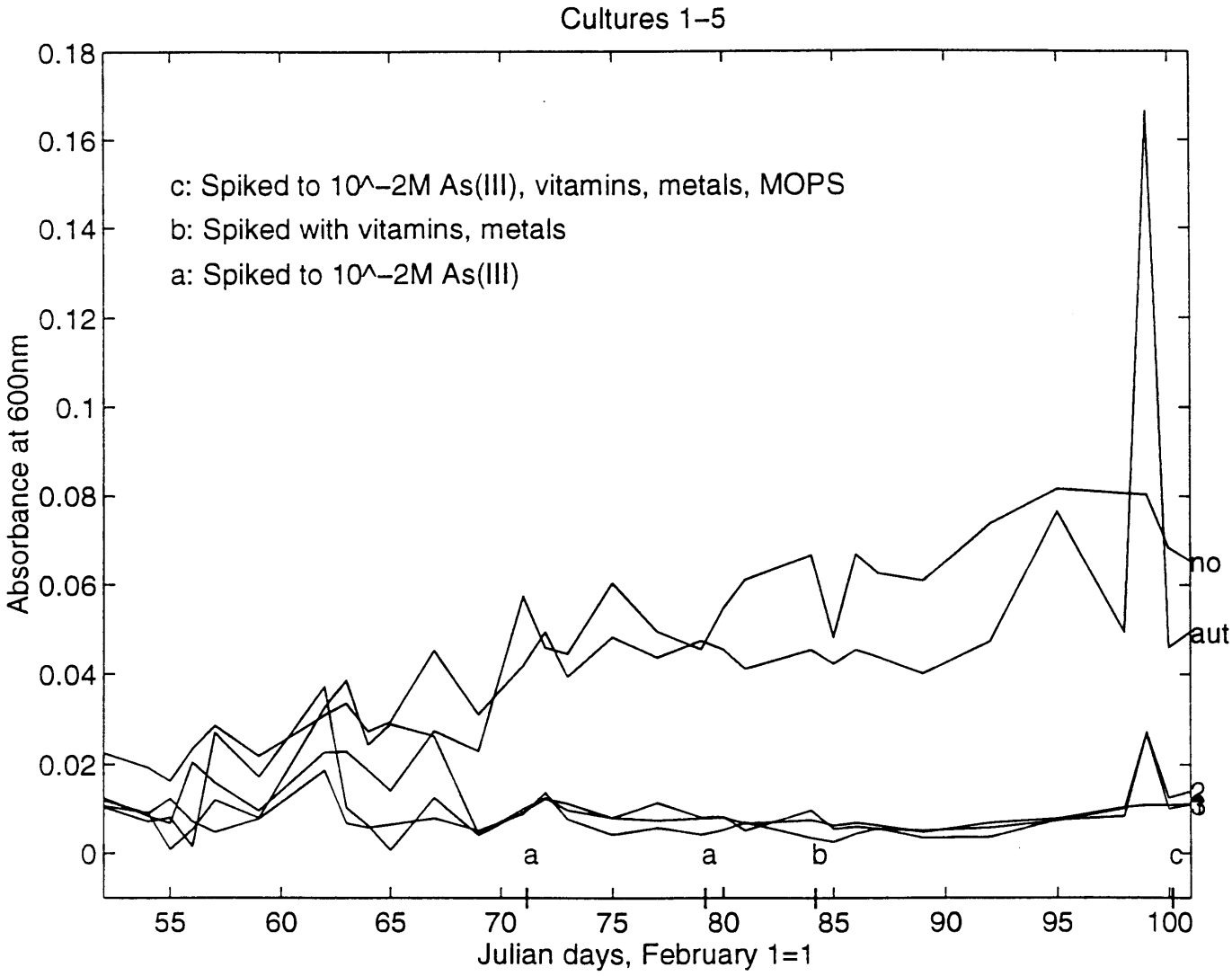
The turbidity measurements of cultures #1-#5 were as interesting as those for #42-#46, though for separate reasons. First, the live cultures displayed only very slight increases in turbidity following spikes and subsequent oxidation of arsenite. Second, the controls both displayed consistent increases in turbidity, with values several times the baseline values for the live cultures. Initially, it was thought that this signaled another accidental inoculation of controls. However, microscopy evidence contradicted this theory. Instead, it showed that while there were no cells in bottles #4 and #5, there was a great deal of inorganic precipitate. Apparently, the high concentrations of arsenite reacted with one of the other compounds in the medium, leading to the whitish precipitate. This precipitate was not detected in the live cultures by turbidity measurements and by microscopy. Thus, it was learned that turbidity was not an accurate measure of cell density when considering cultures with concentrations of arsenite higher than 30mM. (Fig. 16)

3.2 Confirmation of Chemoautotrophy

The previous experiments confirmed that there were indeed microorganisms in culture that were capable of oxidizing arsenite to arsenate. However, it was still unclear why the cells performed this oxidation. The cells could have been heterotrophs, subsisting on the dilute remnants of heterotrophic medium transferred from culture number 960129. They might have been oxidizing the arsenite simply as a detoxifying mechanism.

In order to establish that the cells in culture were chemoautotrophic, several steps were taken. First, photosynthetic organisms were prevented by keeping the cultures in darkness. Second, heterotrophic organisms were excluded by adding no organic carbon to the medium. Third, several transfers with small inocula were made, ensuring that the

Figure 16. Turbidity of Cultures 1-5



microorganisms that did survive were well-suited to these conditions. Fourth, the concentration of arsenite was measured over time. Last, a ^{14}C fixation experiment was conducted to confirm that despite these precautions, the bacteria were still growing and incorporating carbon.

There were three separate elements to the ^{14}C fixation experiment. The bacteria had to incorporate radiolabeled bicarbonate above background levels. Next, the bacteria had to multiply over the same time that they incorporate the radiolabeled bicarbonate. Last, the culture had to oxidize arsenite over this same time period.

3.2.1 ^{14}C-Fixation

The four live cultures spiked with radioactivity each displayed incorporation of radioactivity that was significantly higher than the incorporation of the controls.

Culture Name	^{14}C dpm
#3 Live Culture Sample 1	2405.05
#3 Live Culture Sample 2	1408.5
#3 Live Culture Sample 3	937.21
#3 Live Culture Sample 4	2518.46
Average Value for #3 Live Samples 1-4	**1817.3**
#3 Killed Sample 1	481.97
#3 Killed Sample 2	396.63
Average Value for #3 Killed Samples 1-2	**439.3**
Uninoculated Medium Sample 1	414.13
Uninoculated Medium Sample 2	404.77
Average Value for Uninoculated Medium Samples 1-2	**409.45**
Average Value for Both Control Types	**424.4**

Table 3. Incorporated 14-C in Spiked Cultures

These data showed two important facts. First, neither of the controls incorporated significantly more inorganic carbon than did the other. This was noteworthy because of the

concern that the inorganic carbon might chemically diffuse into the cells rather than be actively incorporated by the bacteria. However, the killed controls, which initially contained equal amounts of cellular matter as did the live controls, did not incorporate any more inorganic carbon than did the uninoculated controls. Because the radioactivity incorporated only through diffusion was negligible, this set aside the concerns that it would be impossible to track radioactivity that was incorporated simply through diffusion.

The second important result of these data showed that the live controls did indeed incorporate much more carbon than did their killed and uninoculated counterparts. The average dpm incorporated by live cultures was 4.3 times the average dpm incorporated by the controls. This ratio of dpm values would presumably have increased had the cultures had even more time to degas. This result confirmed that there was indeed carbon fixation in the cultures.

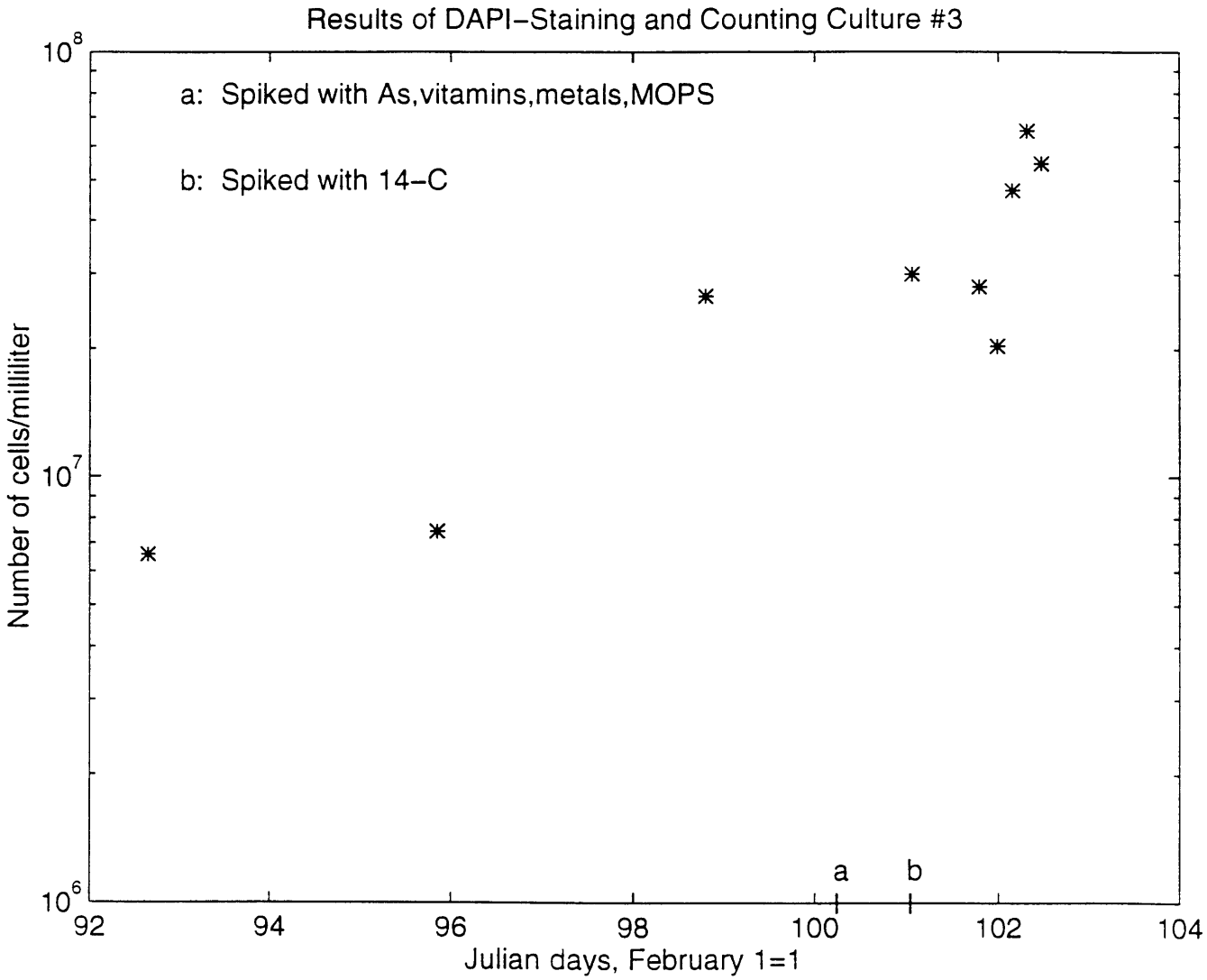
3.2.2 DAPI Staining and Cell Counting

Next, the cells had to be shown to multiply during the period of demonstrated carbon fixation. This would link the incorporated carbon directly to increased biomass. Thus, at several points before and during the carbon-fixing experiment, aliquots of culture #3 were taken and fixed. These samples were diluted and stained with DAPI. The results of the counting were significant. The number of cells clearly increased. Over the period of the carbon-fixing experiment, the number of cells increased by approximately 3 times. Thus, the fixed radioactivity was correlated to the increased biomass. A plot of these data follows. (Fig. 17)

3.2.3 Arsenite Concentration Measurements

The last requirement was that the live culture would oxidize arsenite over the course of the experiment. At the same times that aliquots of culture #3 were fixed for DAPI

Figure 17. Number of Cells/Milliliter in Culture 3 During 14-C Experiment



counting, samples were taken to measure the arsenite concentration of the culture, of a killed control of #3, and of an uninoculated control. The results for this test were perhaps the least definitive of the three tests. Although the concentration of arsenite remained constant for the uninoculated control, and the concentration of arsenite consistently fell for the live culture, the results for the killed control were less certain. In particular, the arsenite concentration of the autoclaved control appeared to decrease until the final measurement, when it shot back up. It is possible that this behavior was due to post-autoclaving arsenic chemistry. However, this remains a puzzling unresolved problem. (Fig. 18)

3.3 Estimations of Growth Rate and Cell Size

Thus, over twelve hours, the number of cells in culture increased from approximately $2*10^7$ cells/mL to $6*10^7$ cells/mL: The cells underwent 1.25 doublings. This yields a doubling time of approximately 8 hours.

Based on this number, the dpm added, and the dpm incorporated, a rough estimation of cell size was found:

Total amount of inorganic carbon in system prior to ^{14}-C spike:

$[H_2CO_3^*]=10^{-8}$ mols

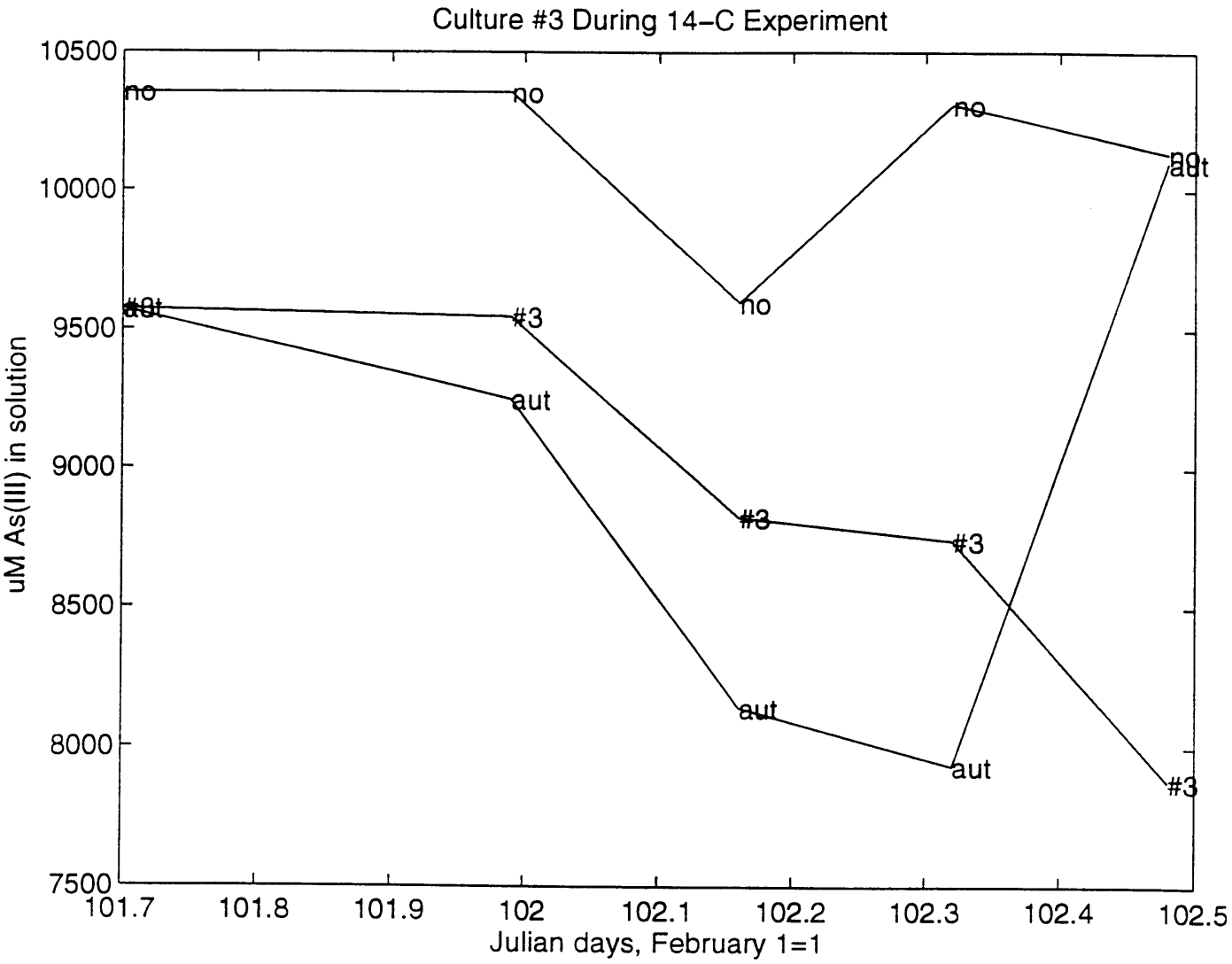
$[HCO^{3-}]=10^{-7.3}$ mols

$[CO_3^{2-}]=10^{-10.6}$ mols

CO_2 in headspace$=10^{-6.5}$ mols

Sum = **$10^{-6.4}$ mols inorganic C present in scintillation vial prior to ^{14}C spike**

Figure 18. Concentration of Arsenite inCulture 3 During 14-C Experiment



Total mols carbon added:

1mCi* (10 mg $NaH^{14}CO_3$/mCi)*(1g/10^6mg)*(1mol $NaHCO_3$/84g $NaHCO_3$)=

=$10^{-6.9}$ mols added in ^{14}C spike

Total mols inorganic carbon in system:

$10^{-6.9} + 10^{-6.4} = 10^{-6.3}$ **mols total**

Total mols incorporated carbon over course of experiment (12 hours):

$$\text{total mols incorporated} = \frac{(\text{total mols inorganic carbon in culture})*(\text{dpm incorporated})}{(\text{dpm added to culture})}$$

=$10^{-6.3}$mols*1400dpm/(2.2*10^6dpm)

=$10^{-9.5}$ mols inorganic carbon incorporated into biomass.

Total mols carbon/cell:

=($10^{-9.5}$mols carbon)/(4*10^7 cells) = 10^{-17}mols carbon/cell

=$10^{-15.9}$ g carbon/cell

=$10^{-15.6}$ g/cell

This result shows the cells to be impressively small. When the cells were examined at 100X, the cells, short rods, were noted to be approximately 1mm long and 0.4mm wide. The cells were therefore small, even by bacterial standards. According to Brock and Madigan, the dry mass of cells varies from 10^{-15}g to 10^{-11}g. Assuming that carbon comprises 50% of the dry mass of the cell, the size of these chemoautotrophs falls at the tail of the distribution of cell sizes.

CHAPTER 4
DISCUSSION

4.0 Implications of This Research

The isolation of this chemoautotrophic culture sheds some light on the biogeochemistry of the Aberjona watershed. It has now been demonstrated that not only are native microorganisms capable of and responsible for arsenate reduction (Ahmann, Spliethoff), but also of and for the reverse reaction: arsenite oxidation. This research explains one more geomicrobial transformation in arsenic's complicated aquatic chemistry.

4.1 Suggestions for Future Research

There are many logical extensions of this research. First and foremost, the chemoautotrophic bacterium responsible for arsenite oxidation should be isolated from the mixed culture. This may prove to be a difficult task, but it is an important one, and will allow even more interesting issues to be tackled. For instance, the isolated microbe may be identified by the host of stains and varying media that are used to phylogenetically peg bacteria. Next, the bacterium may be positively identified with 16SRNA sequencing. It would be interesting to compare the 16SRNA sequence of this bacterium to that of *Pseudomonas arsenitoxidans*, isolated by Ilyaletdinov and Abdrashitova. It is not unlikely that this microbe is a novel organism, and comparing it to the only isolated chemoautotrophic arsenite oxidizer ever isolated would confirm or deny this hypothesis. Next, the enzyme systems responsible for the bacterium's arsenite oxidation can be

identified and studied. Last, and most significantly from an environmental engineering standpoint, a field test of the bioremediation possibilities of this bacterium can be carried out. If the arsenite flowing through the groundwater into the HBSA can be successfully contained and harvested within the northern edge of the HBSA, this tiny bacterium may find its way into many bioremediation efforts across the country, and indeed, throughout the world.

APPENDIX A
INGREDIENTS OF MEDIA AND OTHER CHEMICAL SOLUTIONS

A.1 Ingredients of Media

WE medium contains 0.9 L deionized distilled water, 5g ammonium citrate, 5g asparagine, 0.4g yeast extract, 8g K_2HPO_4, 0.5g $MgSO_4$, 0.05g $MnCl_2$, a trace of $FeSO_4$, NaOH/HCl to pH=5.5; the mixture was autoclaved, then 100mL of sterile 10^{-2} M sodium arsenite standard was added.

Modified AMI medium contains 0.9L deionized distilled water, 1g$(NH_4)_2SO_4$, 0.5g KH_2PO_4, 0.05g KCl, 0.05g $MnCl_2$, 0.1g $Ca(NO_3)_2$, 0.5g $MgSO_4$, 10mL vitamin supplement 1, 2mL vitamin supplement 2, and NaOH to pH=5.5; mixture was autoclaved, then 100 mL 10-2 M sodium arsenite standard was added.

The fourth version of AMI medium contained: 900mL deionized distilled water, 0.5g KH_2PO_4, 0.05g KCl, 0.05g $MnCl_2$, 0.1g $Ca(NO_3)_2$, 0.5g $MgSO_4$; the mixture was autoclaved, then 10mL vitamin supplement 1, 2 mL vitamin supplement 2, 1mL of SL10, 1mL selenite-tungstate, 10.5mL MOPS standard, and 100mL 10^{-2}M sodium arsenite standard were added.

A.2 Ingredients of Media Additives

The arsenite standard solution was made as follows: A balance was tared to an acid washed, autoclaved 1L polypropylene bottle. The bottle was filled with distilled deionized water and autoclaved. The sterile water was then brought to 1000g with a sterile pipette. Next, approximately 200mL of the water were removed to a second sterile bottle. 0.01 mols of sodium arsenite were weighed out to four decimal places and dissolved in the 200mL. Last, this solution was filter-sterilized and added to the waiting 800mL of sterilized distilled deionized water.

Trace Metal Mixture 1, SL10, contains:1000mL distilled, deionized water, 8.5mL HCl, 2.1g $FeSO_4 \cdot 7H_2O$, 100mg $MnCl_2 \cdot 4H_2O$, 190mg $CoCl_2 \cdot 6H_2O$, 144mg $ZnSO_4 \cdot 7H2O$, 6mg H_3BO_3, 24mg $NiCl_2 \cdot 6H_2O$, 2mg $CuCl_2 \cdot 2H_2O$, and 36mg $Na_2MoO_4 \cdot 2H_2O$; mixture is autoclaved.

Trace Metal Mixture 2, selenite-tungstate mixture, contains: 1000mL distilled, deionized water, 0.5g NaOH, 6mg $Na_2SeO_3 \cdot 5H_2O$, 8mg $Na_2WO_4 \cdot 2H_2O$; mixture is autoclaved.

Vitamin Supplement 1 contained: 1000 mL distilled, deionized water, 0.025g pyridoxal hydrochloride, 0.1g thiamine, 0.1g calcium pantothenate, 0.1g riboflavin, 0.1g niacin, 0.05g p-aminobenzoate, 0.2g pyrodoxine hydrochloride, 200mg vitamin B_{12}. Solution was stored at -20°C.

Vitamin Supplement 2 contained: 200mL distilled, deionized water, 25mg folic acid, 500mg biotin. Solution was stored at -20°C.

MOPS is 3-[N-Morpholino] propane-sulfonic acid. A sterile standard was made: 20g MOPS/100ml deionized distilled water. This standard was adjusted to pH=7 with concentrated HCL. The solution was autoclaved and the standard was added directly to the autoclaved medium, 10.5 mL/L.

A.3 Ingredients of Solutions Used in Analysis

Tris buffer solution contains 58g Tris buffer, 36 mL HCl, and deionized distilled water to 1L.

Sodium borohydride solution contains 1000mL deionized distilled water, 9.6g sodium borohydride, and 2.4mL 2N NaOH.

Formalin contains 63% deionized distilled water, 37% formaldehyde.

APPENDIX B
ARSENITE
CONCETRATION DATA

B.1 Arsenite Concentrations for Cultures 27-31 for Dates 6-34.[μM] (February 1,1996=1)

0	6	7	8	9	15	16
27	805.7	892.4	816.2	79.9	-66.2	603.3
28	843.6	1063.5	712.2	129.3	-10.2	565.9
29	1043.1	935.4	667.4	107.2	-64.2	560.2
30	870.5	982.1	940.2	929.1	1009.8	1062.3
31	870.5	852.4	774.6	896.8	991.8	1056.6

17	18	19	20	22	29	31
733.3	705.1	619.3	548.6	608.7	703.1	1058.7
746.1	794.1	664.9	612.6	590.1	644.5	1161.1
655.3	787.6	594.3	433.9	645.1	671.1	1393.9
1363.4	1674.8	1441.2	1115.3	755.8	no datum	1296.4
1282.9	1644.8	1402.5	1310.2	1335	1267.0	1385.6

32	33	34
1371.2	1332.8	1420.1
1156	-49.4	27.2
1246	167.7	21
379.4	-48	0
1577.5	1776.4	1596

Table 4. Concentration of Arsenite in Cultures 27-31

B.2 Arsenite Concentrations for Cultures 42-46 for Dates 39-101[μM]

0	39	40	41	43	44	45
42	817	805	708	8	26	-28
43	755	831	771	2	34	-87
44	813	847	865	8	26	-74
45	943	900	1007	867	888	1091
46	959	893	1014	859	880	867

46	47	51	52	55	56	57
-23	-4	602	-588	-198	-275	-256
-25	-4	995	-960	-179	-260	-257
-20	16	1058	-960	-198	-275	-244
882	863	1577	127	1668	1733	1621
791	962	2325	160	2220	2052	2044

59	61	62	63	64	65	67
41	-20	890	36	-6	53	26
33	-12	914	45	6	71	21
47	-20	679	44	3	53	30
1716	1849	2440	3427	3053	1954	2380
2614	2271	2945	3820	3661	2925	3655

69	71	72	73	75	77	79
34	774	22	39	60	22	-15
22	826	-1	60	67	31	-6
34	867	25	49	60	26	-15
2574	3189	2638	2644	3073	2871	3175
4277	2679	3789	4711	2882	5690	no sample

80	80.5	81	84	85	86	87
885	288	158	11409	10934	9530	10564
1019	272	158	9426	753	9182	10931
796	178	171	10133	10934	10200	12484
3195	2940	4522	11409	16870	16711	18634
no sample	no sample	no sample	no sample	no sample	no sample	no sample

92	95	98	99	100	101
2976	774	197	-770	10159	7240
153	696	283	-628	985	8428
1184	767	197	-770	10313	6986
18144	12666	15699	16468	25323	25779
no sample	no sample	no sample	no sample	no sample	no sample

Table 5. Concentration of Arsenite in Cultures 42-46

B.3 Arsenite Concentrations for Cultures 1-5 for Dates 52-102 [μM]

0	52	55	56	57	59	61
1	6281	12144	10472	7698	10793	12909
2	5409	10749	7630	9937	11465	11210
3	6708	12144	8010	8075	9413	8460
4	6893	11959	12661	10067	15566	13556
5	5799	11808	9560	7294	9153	14932

62	63	64	65	67	79	71
7454	9064	7045	6935	5273	4091	1224
8476	10085	11269	7634	4597	2757	307
6481	5876	3909	0987	0317	0213	124
11276	15247	15209	10973	14642	11934	10658
11637	13164	15077	10507	12293	12378	9449

72	73	75	77	79	80	80.5
13254	12321	8692	7294	3311	13865	12920
11300	10845	7097	4341	428	10925	10750
9186	9283	4685	937	-154	11961	8887
28949	25395	20112	24822	19060	23870	23870
3569	28292	20690	25069	19681	26387	20830

81	84	85	86	87	92	95
17548	9437	7611	9530	5062	153	696
11858	4565	1906	655	232	146	739
11300	2686	840	544	239	153	696
36786	28229	28079	28138	31528	33700	31297
34669	24401	23233	28159	30360	29504	23753

98	99	100	101	102
283	-770	11266	9947	7605
197	-628	9940	8865	9666
291	-770	11413	9571	7877
32391	31400	39439	35780	41048
30781	28411	38318	56693	38682

Table 6. Concentration of Arsenite in Cultures 1-5

APPENDIX C
TURBIDITY DATA

C.1 Turbidity Data for Cultures 27-31 for Dates 6-33

0	6	8	9	17	18	19
27	-.0012	-.0074	.3267	.4076	.3557	.3553
28	.0120	-.0212	.3333	.4187	.4082	.3541
29	.0017	.0143	.3285	.4540	.4232	.4074
30	-.0029	.0050	-.0350	.0109	.0104	.0088
31	0	-.0605	-.0806	.0537	.0386	.0444

20	22	32	33
.3352	.2891	.3237	.5320
.4137	.4119	.2328	.4598
.4529	.4834	.2341	.5274
.0048	.0053	.8583	.8143
.0359	.0331	.0192	.0282

Table 7. Turbidity of Cultures 27-31

C.2 Turbidity Data for Cultures 42-46 for Dates 39-101

0	39	40	41	42	43	44
42	.0047	.0032	.0013	.0207	.0223	.0225
43	.0049	.0030	.0031	.0031	.0178	.0151
44	.0034	.0033	.0026	.0026	.0249	.0213
45	.0088	.0051	.0037	.0037	.0033	.0027
46	0	.0013	.0005	.0005	.0006	.0007

45	46	47	51	52	54	55
.0219	.0234	.0228	.0162	.0123	.0141	.0147
.0219	.0122	.0122	.0133	.0123	.0106	.0126
.0205	.0181	.0205	.0138	.0144	.0119	.0198
.0036	.0024	.0016	.0037	.0032	.0026	.0062
.0008	.0002	-.0003	.0110	.0009	.0005	.0069

56	57	59	62	63	64	65
.0105	.0121	.0149	.0138	.0119	.0119	.0192
.0062	.0115	.0051	.0108	.0099	.0097	.0376
.0073	.0074	.0144	.0129	.0119	.0117	.0113
0	.0001	.0037	.0046	.0038	.0022	.0006
0	0	.0024	.0037	.0064	.0018	.0013

67	69	71	72	73	75	77
.0410	.0116	.0661	.0077	.0073	.0115	.0140
.0183	.0085	.0103	.0057	.0057	.0097	.0426
.0525	.0107	.0099	.0905	.1879	.0112	.0115
.0069	.0012	.0023	.0053	0	.0057	.0062
.0046	.0030	.0042	.0009	.0004	no sample	no sample

79	80	81	84	85	86	87
.0149	.0097	.0093	.0193	.0198	.0213	.0242
.0095	.0079	.0084	.0176	.0190	.0213	.0309
.0108	.0123	.0106	.0240	.0264	.0268	.0315
.0159	.0031	.0076	.0093	.0091	.0105	.0111
no sample	no sample	no sample	no sample	no sample	no sample	no sample

79	80	81	84	85	86	87
.0149	.0097	.0093	.0193	.0198	.0213	.0242
.0095	.0079	.0084	.0176	.0190	.0213	.0309
.0108	.0123	.0106	.0240	.0264	.0268	.0315
.0159	.0031	.0076	.0093	.0091	.0105	.0111
no sample	no sample	no sample	no sample	no sample	no sample	no sample

89	92	95	98	99	100	101
.0188	.0150	.0153	.0152	.0136	.0235	.0180
.0162	.0119	.0169	.0173	.0163	.0186	.0209
.0215	.0160	.0158	.0188	.0190	.0338	.0249
.0143	.0144	.0151	.0175	.0174	.0204	.0292
no sample	no sample	no sample	no sample	no sample	no sample	no sample

Table 8. Turbidity of Cultures 42-46

C.3 Turbidity Data for Cultures 1-5 for Dates 52-101

0	52	54	55	56	57	59
1	.0119	.009	.0123	.0072	.0048	.0078
2	.0107	.0092	.0009	.0054	.0120	.008
3	.0104	.0072	.0081	.0015	.027	.0171
4	.0225	.0192	.0162	.0234	.0286	.0218
5	.0125	.0085	.0068	.0204	.0158	.0096

62	63	64	65	67	69	71
.0186	.0068	.0058	.0065	.0079	.0051	.0089
.0326	.0386	.0243	.0289	.0262	.0044	.01
.0372	.0103	.0063	.0007	.025	.0041	.0093
.0309	.0335	.0273	.0294	.0453	.0310	.0419
.0227	.0228	.0186	.014	.0274	.0229	.0574

72	73	75	77	79	80	81
.0136	.0077	.0042	.0058	.0042	.0053	.0069
.0125	.0097	.0080	.0114	.0081	.0082	.0051
.0122	.0112	.0080	.0074	.0079	.0081	.0067
.0494	.0394	.0483	.0437	.0475	.0455	.0412
.0459	.0445	.0604	.0495	.0455	.0547	.0611

84	85	86	87	89	92	95
.0035	.0026	.0045	.0056	.0035	.0037	.0076
.0097	.0055	.0060	.0055	.0051	.0058	.0074
.0075	.0063	.0069	.0063	.0048	.0069	.0079
.0454	.0423	.0454	.0438	.0401	.0473	.0764
.0667	.0482	.0669	.0626	.0609	.0736	.0816

98	99	100	101
.0084	.0269	.0101	.0111
.0102	.0272	.0126	.0140
.0104	.0109	.0109	.0112
.0494	.1665	.0460	.0495
.0806	.0803	.0684	.0655

Table 9. Turbidity of Cultures 1-5

APPENDIX D
CARBON-FIXATION DATA

D.1 DAPI-Stained Cell Counts

Culture number	Date	Average # Cells/Frame	mL on Filter	[15400 frames/filter]* [cells/frame]/ [mL on filter] =Cells/mL
#3-960323	92.65	41	.095	$6.6*10^6$
#3-960323	95.85	459	.95	$7.4*10^6$
#3-960323	98.81	164	.095	$2.7*10^7$
#3-960323	101.06	185	.095	$3.0*10^7$
#3-960323	101.79	173	.095	$2.8*10^7$
#3-960323	101.99	112	.1	$1.7*10^7$
#3-960323	102.16	306	.1	$4.7*10^7$
#3-960323	102.32	42	.01	$6.5*10^7$
#3-960323	102.48	35	.01	$5.4*10^7$

Table 10. DAPI Cell Count for Culture 3

D.2 Arsenite Concentrations During ^{14}C Experiment [μM]

	101.99	102.16	102.32	102.48
culture #3	9540	8823	8740	7877
autoclaved culture #3	9250	8140	7934	10102
uninoculated medium	10350	9595	10310	10134

Table 11. Concentration of Arsenite in Culture 3 During 14-C Experiment

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