THE INTERACTION OF SEDIMENT BACTERIA WITH ARSENIC COMPOUNDS

by

JAFARIAH JAAFAR

B.A. California State University, Chico,1981

M.Sc. University of Kentucky,1983

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

CHEMISTRY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February 1992

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at The University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

CHEMISTRY

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date: 26 FEBRUARY 1992

ABSTRACT

In general, bacteria are capable of biotransforming inorganic arsenic into methylarsenic acids and arsines. The microbial activity of lake sediments was examined with respect to the mobilization of mine tailings that have a high arsenic content. Aerobic and anaerobic mixed microbial populations were isolated from Kam Lake, Yellowknife, N.W.T.

An aerobic microbial population from 5 cm sediment depth, the layer immediately above the contaminated mine tailings, was capable of transforming arsenicals. Speciation of arsenicals in the culture medium, determined by using hydride generation - gas chromatography - atomic absorption spectrometry (HG-GC-AAS) shows that this bacterial population is able to methylate arsenicals and subsequently demethylate the product. However, only methylation was observed in media containing dimethylarsinic acid.

Anaerobic microbial populations, from all depths, produce a yellow precipitate upon incubation with arsenate for 10-14 days. The precipitate was identified as As_2S_3 by microanalysis and scanning electron microscope + energy dispersive x-ray (SEM + EDX). The anaerobic microbial population, which should not contain sulfate-reducing organisms, appears to be arsenic tolerant; there is no evidence of methylation of arsenic.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
AAS	Atomic absorption spectrometry
GC	Gas chromatography
ICP	Inductively coupled plasma
MMAA	Monomethylarsonic acid
DMAA	Dimethylarsinic acid
TMAO	Trimethylarsine oxide
MMA	Monomethylarsine
DMA	Dimethylarsine
PRAS	Pre-reduced anaerobically sterile
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
SEM	Scanning electron microscope
EDX	Energy dispersive x-ray
ORP	Oxidation reduction potential
pE	- log of electron activity

ACKNOWLEDGEMENT

I am grateful to my supervisors, Professors W.R. Cullen and K.J. Reimer, for their guidance throughout the course of this work.

I wish to give special thanks to Mr. Gary Hewitt for helping me in the bacterial isolation and introducing me to the microbial world, and to Ms. Deepthi Hettipathirana for helping me with the thesis stylesheet. Their time and patience spent during this work are very much appreciated.

I thank Ms. Eileen Cochien for helping me with the bacterial preservation during Summer 1991, and Mr. Hao Li for trouble shooting the HG-GC-AAS system. I also thank Ms. Mary Mager for teaching me the SEM+EDX. Thanks also to all my lab colleagues for their friendly and helpful endeavors.

Finally, I thank the Public Services Department, Malaysia for the financial support and Universiti Teknologi Malaysia for granting me the study leave.

DEDICATION

Affandi, Arina and Azman

CHAPTER I

INTRODUCTION

1.1 ARSENIC

Arsenic ranks as the twentieth most abundant element in the earth's crust.[1] It is widely dispersed at low levels, ranging from 0.1 to several hundred ppm (μg/g), depending on several factors such as geographical location and anthropogenic input.[2] The average arsenic concentration in the earth's crust is about 3 ppm,[1] but much higher concentrations, ranging from 0.4 to 6000 ppm, are found in sulfur containing ores that contain arsenopyrite (FeAsS), realgar (As_4S_4) and orpiment (As_2S_3).[3] Arsenic has been found to be a common contaminant of both freshwater and seawater in many parts of the world.

Arsenic compounds have different properties and uses.[4-5] Arsenic trioxide is well known as a poison. Both inorganic and organic arsenic compounds were widely used in medicine.[6] Arsenic, like mercury, has been used extensively in agriculture as herbicides and pesticides, and in industrial applications as wood preservatives. High arsenic concentrations in soils have been related to the mining industries, in particular, gold mining.

It is recognized that the toxic effects of arsenic depend on the concentrations of individual arsenic species, so the development of analytical techniques that enable the speciation of arsenicals has become an important objective for individuals and organizations interested in the biogeochemistry of the element.

1.2 BIOTRANSFORMATION OF ARSENIC

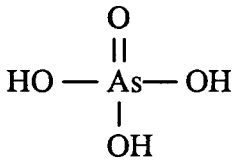
Three types of arsenic biotransformation have been found to occur in the environment: (1) oxidation and reduction between inorganic arsenite, As(III) and arsenate, As(V); (2) biomethylation of arsenic compounds to methylarsenic acids and the more volatile and toxic methylarsines; (3) biosynthesis of more complex organoarsenic compounds, such as arsenobetaine, arsenocholine and arsenosugars. This third class of transformation is carried out by organisms higher up along the food chain, and not, apparently, by microorganisms. However, Hanaoke et al.[7], reported the degradation of arsenobetaine to trimethylarsine oxide and dimethylarsinic acid, probably by the intestinal microorganisms in the chiton and only in an aerobic medium.

A list of arsenic compounds is shown in Figure 1.1.

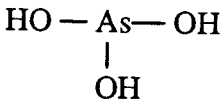
1.2.1 Arsenite and Arsenate Redox Transformation

In the aquatic environment, arsenic exists in two different oxidation states. In oxygenated water, arsenate is the dominant species, existing as $HAsO_4^{2-}$. Arsenite becomes the main species in anoxic basins and inlets, for example, Saanich Inlet, B.C.[8]

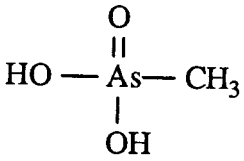
In air saturated waters the redox condition is set by the O_2/H_2O couple, with a pE of about 12.6.[9] Thermodynamic calculations for an oxygenated seawater at pH 8.1 predict the As(V)/As(III) ratio to be about 10^{26}.[10] However, significant amounts of arsenite are found in oxygenated surface waters, and conversely, arsenate is found in anoxic waters.



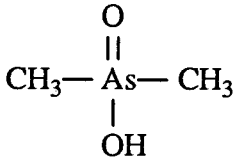
Arsenate



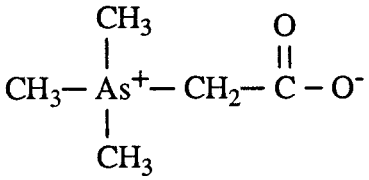
Arsenite



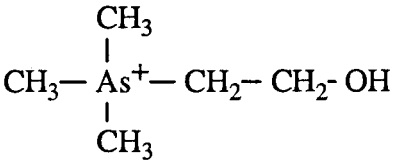
Monomethylarsonic acid



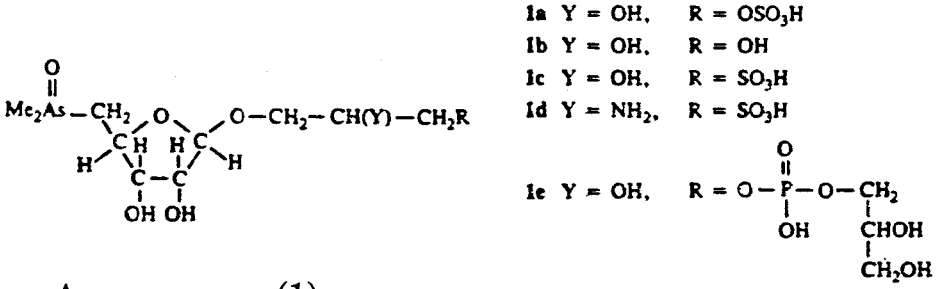
Dimethylarsinic acid



Arsenobetaine



Arsenocholine



Arsenosugars (1)

Figure 1.1 Structure of Selected Arsenic Compounds

Studies[11,12] found the As(V)/As(III) ratio to be in the range of 0.1 to 100, suggesting that the redox pair is not in thermodynamic equilibrium. In oxygenated seawater, bacteria and phytoplankton have been shown to be responsible for the presence of arsenite, by reduction of arsenate.[12-14] Johnson[12] showed that a mixed population of marine bacteria carries out this reduction in seawater medium.

Arsenite is slowly oxidized to arsenate in seawater, even under sterile conditions.[15] Some bacteria are also responsible for this oxidation, and Turner et al.[16] in 1954 demonstrated that bacteria from arsenical cattle-dipping fluids could oxidize arsenite to arsenate. The processes that control the oxidation of arsenite to arsenate in natural waters still need further study.

1.2.2 Biomethylation of Arsenic

The biomethylation of arsenic compounds has been known for many years; however, interest in this phenomenon has increased, in association with the discovery that microorganisms in natural lake sediments are able to methylate mercury to form a highly toxic methylmercury species.[17] The discovery that mercury is readily cycled from the sediments and concentrated by organisms along the food chain[18], has led to speculation that similar cycles may occur with arsenic.

Certain fungi, yeasts and bacteria are known to methylate arsenic to volatile arsines. Poisoning incidents resulting from fumes emitted from wall paper coverings were first investigated by Gosio in 1892.[19] He identified one

mould species, *Penicillium brevicaule* (current name: *Scopulariopsis brevicaulis*) as the producer of the gas which has a characteristic garlic odor, and incorrectly identified the gas as diethylarsine, $(C_2H_5)_2AsH$.

Challenger in 1932 correctly identified the gas as trimethylarsine, which was emitted by the mould *S.brevicaulis* growing on bread in the presence of arsenic compounds.[19] Challenger and coworkers further suggested that methylarsonic acid and dimethylarsinic acid were intermediates in the production of trimethylarsine. He favored the hypothesis that the methylation of arsenic involved the transfer of a methyl group from some already methylated compound such as betaine, choline or methionine. Challenger proposed a mechanism for the conversion of arsenate to trimethylarsine, shown in Figure 1.2. The arsenic (III) intermediates in braces are unknown. His proposed mechanism involved alternating oxidation and reduction steps.

du Vigneaud and coworkers' study on transmethylation[20] led Challenger to further study trimethylarsine production by *S.brevicaulis* in the presence of labelled precursors.[21] Only $^{14}CH_3$-labelled methionine was found to transfer its label to arsenite to a significant extent. The result indicated that an "active methionine", S-adenosylmethionine (SAM), Figure 1.3, is the methyl donor.

Work by Cullen and co-workers.[22-25] supported the idea that SAM is the source of CH_3^+. They demonstrated that the CD_3 group in L-methionine-methyl-d3, $CD_3SCH_2CH_2CH(NH_2)COOH$, is transferred intact by cultures of *S.brevicaulis*.

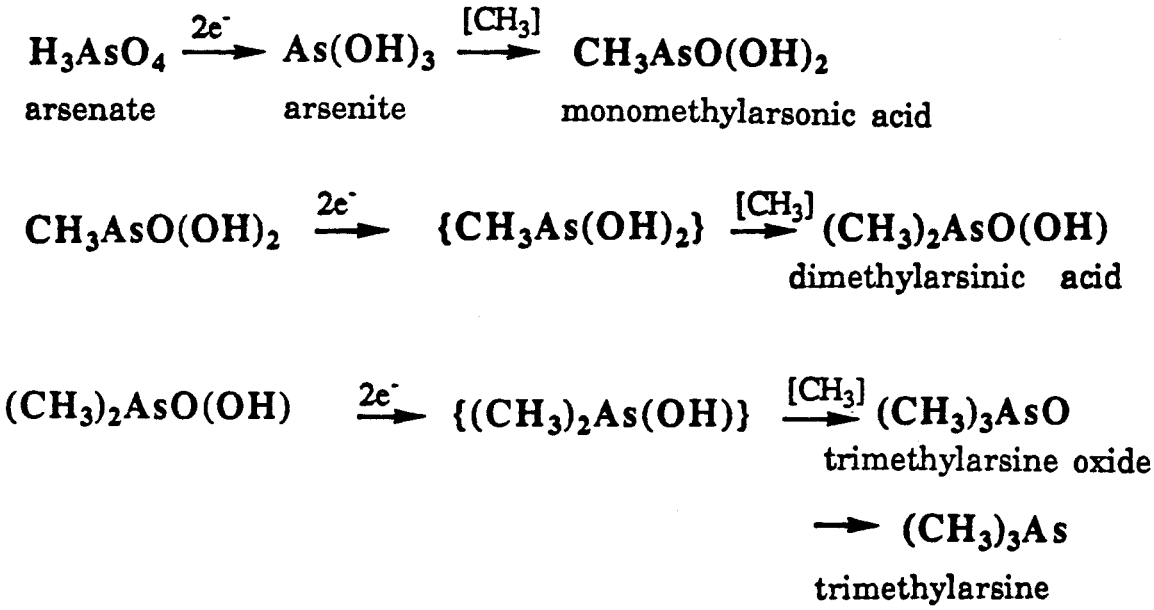


Figure 1.2 Challenger's mechanism for the biological methylation of arsenic

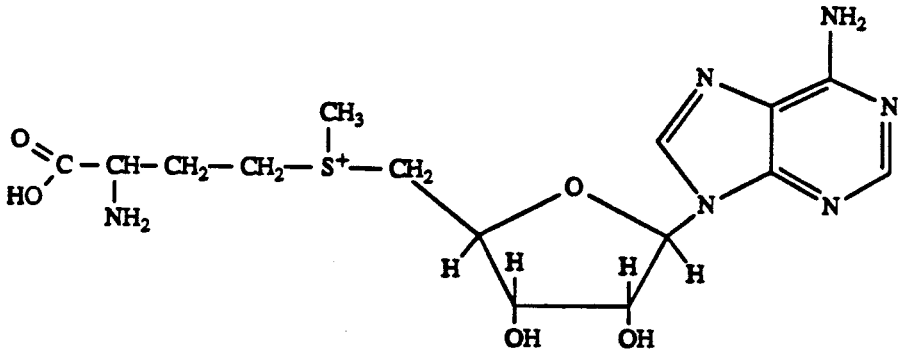


Figure 1.3 S-adenosylmethionine (SAM)

Cox and Alexander[26] isolated microorganisms from soil and sewage in cultures containing various arsenic compounds. The organisms were able to produce trimethylarsine from arsenite, arsenate, methylarsonic acid and dimethylarsinic acid when grown at pH 5. They suggested that the acidic condition in sewage might promote trimethylarsine production.

Shariatpanahi and coworkers[27] studied bacteria that were isolated from the environment and grown in the presence of arsenate (≤ 100ug/mL). Under aerobic conditions, they found the transformation of arsenate to dimethyl and trimethyl species.

In experiments with lake and river sediments using pure cultures of the bacteria *Aeromonas* and *Flavobacterium sp.* and *Escherichia coli*, Wong et al.[28] observed the production of monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide. They also observed the production of dimethyl- and trimethylarsines. The sediments were incubated with or without high levels of arsenic (at least three orders of magnitude higher than a typical non-polluted environment). These results were questioned by Andreae,[5] who suggested that this biomethylation is a response to extreme arsenic stress, and not a general process carried out by aerobic bacteria in the environment.

Additional work is required to understand the biomethylation process. The mechanism proposed by Challenger seems likely to occur in the aquatic environment with the reduction of arsenate and methylation to monomethylarsonic acid and dimethylarsinic acid. Trimethylarsine is not always the end product.

The biological function of methylated arsenic compounds is unknown, however, it is possible that the methylation serves as a detoxification process. Ferguson and Gavis[29] suggested that in an anaerobic environment, it may be energetically favorable for the organisms to transmethylate arsenic (and other metals) rather than to synthesize methane. This methylation might take place in the upper layer of the sediments. This process might be responsible for mobilizing methylarsines from sediments to solutions and to the food chain.

1.3 ARSENIC-SULFUR COMPOUNDS

In anoxic water systems, bacterial action would create a reducing environment and the pE will be set by the reduction of SO_4^{2-} to HS^-.[9] Under this reducing condition (pE value approximately -4) arsenite is the dominant species. Stable arsenic sulfides, realgar (As_4S_4) and orpiment (As_2S_3), will precipitate in the presence of sulfur under acidic anoxic conditions.[30]

Microorganisms play an important role in geochemical processes, and in the cycling of sulfur. Ehrlich[31,32] investigated the degradation of sulfide minerals, orpiment and arsenopyrite, by microorganisms. The rate of arsenic released by bacterial oxidation was twice as great as by spontaneous oxidation. The bacterial action released arsenate and arsenite. The biodegradation process results in the solubilization of metal compounds and can be applied practically for metal recovery.[33] Another role of microorganisms is in the partial generation of sulfide minerals. However, limited study has been done in this area.

1.4 METHODS FOR THE SPECIATION OF ARSENIC COMPOUNDS

Total arsenic has been monitored in environmental samples by using many analytical techniques: atomic absorption spectrometry,[34-38] atomic emission spectrometry,[39] voltammetry,[40] and neutron activation analysis.[41] Currently, there is an increased interest in determining the concentration of individual arsenic species, largely because of their different toxicities. The toxicity decreases in the order: arsines > arsenite > arsenate > alkylarsenic acids > arsonium compounds > metallic arsenic.[1]

A variety of techniques for the speciation of arsenicals has been reported. Generally, hyphenated techniques such as HPLC-AAS,[42] HPLC-ICP-MS,[43] GC-AAS,[44-46] and GC-MS[47] combined with selective hydride generation and cold-trapping,[48,49] are used. However, the problems associated with these techniques are long analysis time and their non-routine nature.

A number of arsenic compounds are volatile , such as arsine, or can be converted into volatile arsines by reduction with sodium borohydride. The reduction of arsenic compounds with sodium borohydride is pH-dependent,[49] and is related to the pKa of the individual arsenic acids (Table 1.1). Braman et al.[38] investigated the effect of pH on the fraction of undissociated arsenic acids present at equilibrium. A number of workers[44,46] have used the pKa differences of arsenate and arsenite to speciate them. However, Hinners[50] found that MMAA and DMAA interfered with the determination of As(III) and As(V).

Anderson et al.[49] investigated several factors that influence the arsenic species determination. They used a continuous hydride generation with sodium borohydride and AAS for detection. Several combinations have shown promising selectivity towards the reduction of As(III), As(V), MMAA and DMAA: (a) 5M HCl for the determination of total inorganic arsenic; (b) citric acid-sodium citrate buffer at pH 6 for the determination of As(III); (c) 0.16M citric acid for the determination of DMAA.

The volatile property of the arsines enabled them to be removed from solution by sweeping them with an inert gas, collected on a liquid nitrogen cold trap, and separated by using gas chromatography. The separated arsines are then detected by a quartz-cuvette atomic absorption spectrophotometer.

Table 1.1: pKa values of arsenic species and reduction products

Species	pKa's	Reduction pH	Reaction Product	b.p. (°C)
Arsenate	2.2	1	AsH_3	-55
Arsenite	9.2	1-6	AsH_3	-55
MMAA	4.1	1	CH_3AsH_2	2
DMAA	6.2	1-2	$(CH_3)_2AsH$	35.6
TMAO	-	1-4	$(CH_3)_3As$	70

1.5 MICROBIAL SAMPLING

Microorganisms, either mixed communities or single species, for laboratory study are isolated by growing them under well-defined conditions as cultures. In nature, bacteria invariably occur as mixed communities containing more than one kind of microorganism. In the present work, enriched cultures of a mixed microbial population were isolated using a selective liquid media containing arsenate. The enrichment technique tends to select for the microorganism with the highest growth rate, by changing conditions in favor of the desired organism. This is because of a direct competition for nutrients among the mixed developing population.

Two techniques that are frequently used for the cultivation of strict anaerobes are the roll tube technique, developed by Hungate,[56] and the anaerobic glove box technique. In the roll tube method, agar medium is distributed as a thin layer over the internal surface of test tubes charged with an anaerobic atmosphere. Specimens are streaked on the surface of the agar layer on the wall of the tube. This technique provides a low oxidation-reduction potential of -150 mV and an oxygen-free atmosphere. Use of the glove box is more practical since all manipulations are performed in the reducing atmosphere of the box.

1.6 SCOPE OF WORK

Arsenic has frequently been concentrated in certain areas as a result of anthropogenic activities. For example, gold mining operations for years dumped arsenic as a waste by-product in their tailings. Mine sites, such as

those along the B.C. coast or those adjacent to lakes, frequently dumped tailings directly into these waters, and over time sediments rich in arsenic built up around the effluent outflow. Recent improvements in gold separation methods have reduced arsenic concentrations in effluents and cleaner tailings now lie on top of arsenic-laden layers.

Bacteria appear to be capable of mobilizing deposited arsenic into water soluble arsenic acids and arsines. MMAA, DMAA, TMAO and the highly toxic and volatile arsine and methylarsines are known products of bacterial metabolism.[19, 26-28]

To initiate an investigation of microbial mobilization of historically deposited arsenic, we decided to see if mixed microbial cultures obtained from sediments in a lake associated with the NercoCon Mine in Yellowknife, N.W.T. could metabolize arsenic. Kam Lake was chosen because it has a well defined sub-stratum with high arsenic concentration:[51] the layer resulted from a large spill of process effluent that would normally have been treated prior to disposal but was instead diverted to Kam Lake.

In a typical sediment, there is a predictable progression of nutritional microbial types that result from the lower oxygen concentration and increasing reducing condition as depth increases. Aerobes may predominate at the surface, whereas a variety of anaerobic types will predominate beneath the surface. In the present study, two microbial populations were initially sought that would represent aerobic and anaerobic microbial activity. Each mixed population was assayed for its ability to metabolize arsenate and DMAA. Hydride generation - gas chromatography - atomic absorption

spectrometry (HG-GC-AAS) was the analytical method used for arsenic speciation and characterization.

CHAPTER II

EXPERIMENTAL

2.1 REAGENTS AND CHEMICALS

2.1.1 Chemicals

Unless otherwise stated, all chemicals were obtained from commercial sources and were of analytical grade. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were obtained from Difco Laboratories. As_2S_3 and As_4S_4 were prepared by using literature methods.[52,53]

2.1.2 Chromatographic Supplies

Teflon tubing (1 mm i.d. by 600 mm length) was purchased commercially. Porapak PS (80/100 mesh) was obtained from Waters Chromatography Division/Millipore Corporation. Silyl 8, a GC column conditioner, was obtained from Pierce Chemical Co.

2.1.3 Prepared Solutions

2.1.3.1 Arsenate, arsenite, monomethylarsonic acid and dimethylarsinic acid standard solutions.

Stock solutions of 1000 μg/mL As were prepared by weighing the appropriate amount of $Na_2HAsO_4.7H_2O$, $NaAsO_2$, $CH_3AsO(ONa)_2.6H_2O$ and $(CH_3)_2AsO(OH)$ into separate 100.0 mL volumetric flasks, and diluting

with deionized water (Aquanetics Aqua Media System). The arsenate, arsenite and MMAA stock solutions were prepared as needed. The DMAA stock solution was prepared every two months.

Intermediate stock solutions of 10 μg/mL were prepared by serial dilution. The DMAA solution was prepared daily.

A working solution of 10 ng/mL each of As(V), As(III) and MMAA, and 20 ng/mL of DMAA was prepared daily by serial dilution.

2.1.3.2 Potassium borohydride solution.

A 4% solution was prepared by weighing 2.00g of KBH_4 in a polyethylene bottle. One pellet of sodium hydroxide was added. The solids were dissolved in deionized water and the solution was diluted to 50.0 mL.

2.2 INSTRUMENTATION

2.2.1 Chambers for Incubation of Anaerobic Cultures.

Incubation of anaerobes requires pre-reduced media and elimination of oxygen from the growth environment. Oxygen can be scavenged within a sealed jar by catalytic hydrogenation over a palladium catalyst.

BBL anaerobic chambers (Becton Dickinson Microbiology) and chambers built by the Mechanical Engineering Service, Department of Chemistry, U.B.C. based on the design of Balch et al.[54] were used in conjunction with BBL GasPak H_2 plus CO_2 generator envelopes for all anaerobic incubations. GasPak envelopes contain a tablet of sodium

borohydride and a tablet of sodium bicarbonate plus citric acid. To generate H_2 and CO_2, a top corner is cut to allow addition of 10 mL of water. The water is wicked through a channel - the time required is sufficient to allow chambers to be sealed before gas generation begins - to the two tablets, and upon contact the gases are evolved. The hydrogen reacts with oxygen over Pd coated alumina pellets (1/8"; Aldrich Chemical Co.) held in a wire basket secured to the underside of the chamber headplate to produce water. The appearance of condensate on the chamber walls is an indication of reaction progress. Anaerobic indicator strips (GasPak) soaked with methylene blue indicator change from blue to colorless to confirm the removal of oxygen. Carbon dioxide does not participate in the development of anaerobiosis but is a required nutrient for some anaerobes (e.g. methanogens).

All anaerobic sample and culture manipulations were performed under conditions designed to minimize oxygen exposure. Initial sediment core samples were acquired in sealed gravity core sampler tubes[55] which were later opened in a glove bag (Instruments for Research and Industry) under an atmosphere of oxygen-free nitrogen. Subsequent culture manipulations were performed in a modified glove box (KSE, Adrian, Michigan) charged with a gas mixture of CO_2 (5%), H_2 (10%) and N_2 (85%) and supplied with Pd-coated alumina pellets. Anaerobic indicator strips were used to confirm anaerobic conditions, and catalyst was replaced routinely.

2.2.2 Gas Chromatograph

Two types of gas chromatograph were used: a Hewlett Packard 5830A model with a 18850A GC terminal, and a Hewlett Packard Series 5890. A home-packed column of Porapak PS was prepared.

2.2.3 Atomic Absorption Spectrophotometer (AAS)

The AAS was used as the detector for the GC effluent. A Jarrell Ash Model 810 AAS (Fisher Scientific Co.) was used for arsenic speciation and was equipped with Waters QA-1 Data System.

Total arsenic was determined on Varian model AA 1275 series. The AA hollow cathode lamp was operating at 8 mA and the monochromator was set at 193.7 nm, one of the resonance lines of arsenic. The spectrophotometer was equipped with a deuterium background corrector and a Hewlett Packard 82905A printer.

2.2.4 Autoclave

Amsco, Eagle Series-3041 Gravity autoclave was used to sterilize all culture vessels and heat-stable media.

2.2.5 Microscope

The microbial growth was observed by using an Olympus BH-2 microscope.

2.2.6 Scanning Electron Microscope + Energy Dispersive X-ray (SEM + EDX)

A Hitachi S-570 Scanning Electron Microscope (SEM) was used. It was equipped with a Kevex Super8000 Energy-Dispersive X-ray Analyser (EDX). This instrument is located in the Metals and Materials Engineering Department, UBC.

2.3 CULTURE PROCEDURES

2.3.1 Media Preparation

All aerobic media were prepared on the open bench, and sterile media were handled in a Class II (laminar flow) biohazard cabinet (Mechanical Engineering Services, Department of Chemistry, UBC). Pre-reduced anaerobically sterile (PRAS)[56,57] media were prepared by flushing filter-sterilized (glass wool packed tube) nitrogen gas over the headspace of the boiled media in an air-tight Pyrex bottle.

All transfers were done in an anaerobic glove box containing palladium catalysts and under an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 (from a commercial cylinder). A methylene blue indicator strip was used to check for anaerobic condition. All glassware was flushed with nitrogen gas before being sterilized. All anaerobic incubations were done in the anaerobic chamber.

Arsenate augmented Tryptic Soy Broth at quarter strength (Medium A) was prepared by adding 10.0 mL of 100.0 μg/mL As(V) solution to 7.50 g of

the commercially available powder (Table 2.1) and dissolving this in 1000 mL of distilled water. Ten mL aliquots of this broth were pipetted into screw cap test tubes (16 mm diameter by 120 mm) and autoclaved at 121°C for 15 min. The final pH of this medium was 7.0.

Table 2.1 Tryptic Soy Broth (Medium A)

Bacto Tryptone (Pancreatic Digest of Casein)	15 g
Bacto Soytone (Pancreatic Digest of Soybean Meal)	5 g
Sodium chloride	5 g

"Arsenate-reducer" medium (Medium B) was prepared from the ingredients shown in Table 2.2. All these (except sodium bicarbonate) were dissolved in water and diluted to 960 mL. The medium was prepared PRAS, and autoclaved by using a gravity cycle at 121°C for 15 min., with no drying time. The sodium bicarbonate was filter-sterilized by using a 0.45 μm Millipore membrane filter (HV type). The autoclaved solution was cooled, placed in the anaerobic glove box, and 40 mL of the sterile sodium bicarbonate was added aseptically. The final pH of the medium was adjusted to 7.4 with 4M HCl or NaOH. This medium was pipetted (10.0 mL portions) into screw cap test tubes and allowed to stand overnight in the glove box. The tubes were then placed in the anaerobic chamber which was charged with CO_2 and H_2 from a GasPak envelope, and removed from the glove box.

Table 2.2 "Arsenate-reducer" (Medium B)

Ingredients	g/L
$NaHCO_3$	2.5
$CaCl_2.2H_2O$	0.1
KCl	0.1
NH_4Cl	1.5
$NaH_2PO_4.H_2O$	0.6
$NaCH_3COO$	2.7
Difco yeast extract	0.05
NaCl	0.1
$MgCl_2.6H_2O$	0.1
$MgSO_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.005
$Na_2MoO_4.2H_2O$	0.001
$FeSO_4.7H_2O$	0.001
$Na_2HAsO_4.7H_2O$	3.12

2.3.2 Bacterial Isolation

The microbiological sampling was done in the field by Mr. Gary Hewitt of Department of Chemistry, UBC and Dr. Doug Bright of RRMC.

Sediment core samples taken from Station 1, Kam Lake, Yellowknife, Northwest Territories (Figure 2.1) were obtained by hand lowering a gravity core sampler from an aluminum power boat. Plastic core tubes for assay of pH, temperature and oxidation-reduction potential (ORP) and for microbial sampling had 1 cm holes drilled each 2 cm of their length. The holes were covered with duct tape for sample acquisition and for sealed transport back to the laboratory. Standard core tubes were of 3.75 cm diameter and 60 to 80 cm long. Cores for microbial sampling were transported from Kam Lake to the Department of Indian and Northern Affairs' water testing laboratory in Yellowknife, and opened in a glove bag (Instruments for Research and Industry) under an atmosphere of oxygen-free nitrogen. The holes were uncovered and sediment samples from different depths were aseptically removed with a sterile spatula after the layer closest to the wall was removed. Roughly 1 mL of sediment was then inoculated into each test medium.

Cultures of aerobic heterotrophs, Medium A, were removed from the glove bag and incubated with loose caps. Cultures in "arsenate-reducer" medium (Medium B) were placed in an anaerobic chamber before removal from the glove bag. All cultures were transported to the Department of Chemistry, UBC for further work.

Subsequent culture manipulations were performed in a glove box as described in section 2.2.1. Room temperature (approximately 21°C) was used for all incubations.

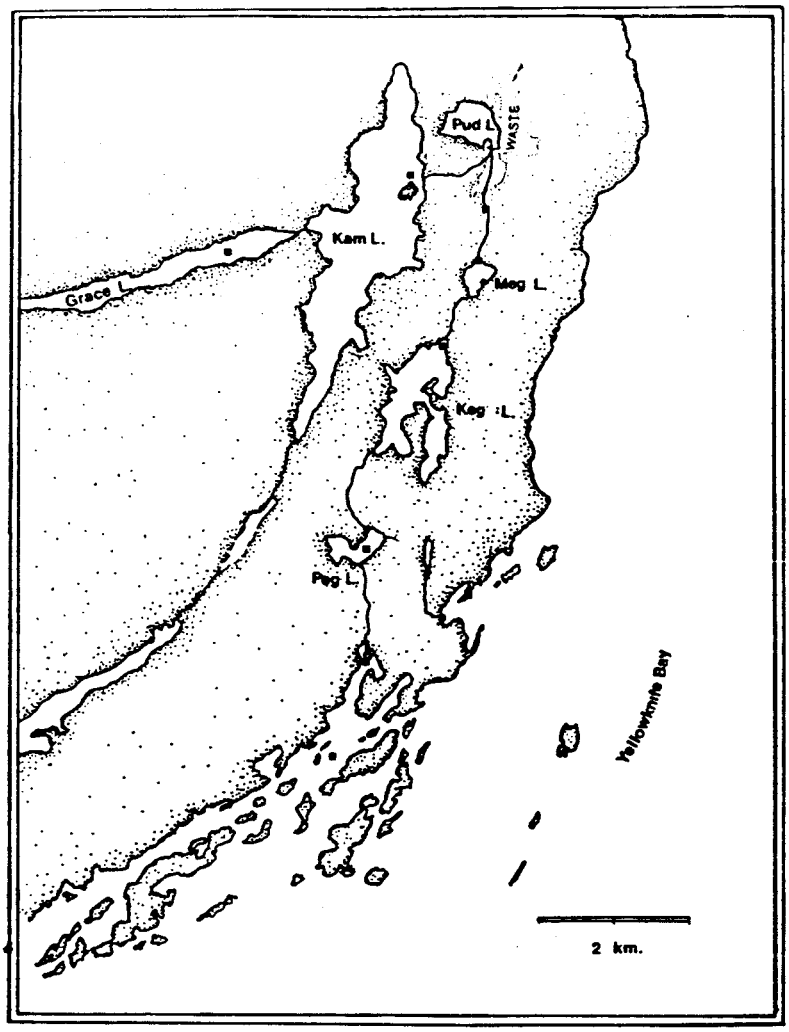


Figure 2.1 Map of Yellowknife Lakes System, Northwest Territories. ■ indicates sampling site.

2.3.3 Bacterial Enrichment

Enrichments were made by serial subculture. The mixed aerobic culture in Medium A was enriched once by transferring 1.0 mL of the culture, to a sterile, 10.0 mL of Medium A. Medium A was incubated for two days.

One mL of the mixed anaerobic culture was transferred, in each enrichment step, into a sterile 10.0 mL of Medium B. Medium B was incubated in the anaerobic chamber for 10-14 days between each of the three enrichment steps.

2.3.4 Bacterial Preservation

The two-day old aerobic subculture was centrifuged for 30 min at a speed setting of 80 in a Dynac Centrifuge (Becton Dickinson Co.) and the supernatant was discarded. One mL each of TSB (double strength) and 20% glycerol were added to the bacterial cell to achieve a final concentration of 10% (v/v) glycerol and single strength TSB. The cells were resuspended and 1.0 mL aliquots were dispensed into cryovials (1.2 mL capacity, NUNC), pre-cooled on dry ice, and finally stored in a cryogenic refrigerator (Taylor Warton, model 34 HC) charged with liquid nitrogen.

Enriched anaerobic cultures were centrifuged by the same procedure and returned to the glove box. The supernatant was discarded and 1.0 mL each of Medium B and 20% glycerol (PRAS) was added to resuspend the cells. One mL of the cell suspension was dispensed into cryovials and sealed. The tubes were removed from the glove box, pre-cooled on dry ice and immediately preserved in the cryogenic refrigerator.

2.4 ARSENIC METABOLISM

2.4.1 Aerobic Experiment

The medium used was TSB containing 50 ng/mL As(V) solution (Medium C). Twenty mL portions of the sterile medium was pipetted aseptically into a series of Evergreen tubes (50 mL capacity, polypropylene tube).

The preserved aerobic heterotrophs were thawed and grown on Tryptic Soy Agar (TSA) + 1.0 μg/mL As(V) plates. The plates were incubated at room temperature for 2 days to allow colonies to develop. Approximately 7 mL of Medium C was added to the plate and any growth was scraped to resuspend it. The cell suspension was pipetted to a test tube containing Medium C and the turbidity was measured spectrophotometrically at 420 nm with a Spectronic 20. Appropriate volumes of the cell suspension and Medium C were mixed to obtain an absorbance reading of 1.0. Two mL each of the standardized inoculum was added to 20.0 mL Medium D in 7 Evergreen tubes. The tubes were incubated aerobically at room temperature. All cultures were shaken daily to resuspend the growth. One tube was centrifuged each day, and the 'snift test' was carried out to detect the production of arsines, and then the supernatant was transferred to a sterile tube and frozen prior to analysis for arsenic compounds. The solid phase and bacterial cells were discarded. The process was repeated for 7 days.

Controls, containing Medium C, and blanks (TSB) were carried through the same incubation condition and frozen daily for arsenic analysis.

A second set of cultures was prepared. In addition to the 50 ppb As(v) in half strength TSB (Medium D), 50 ppb DMAA in half strength TSB (Medium E) were prepared. Standardized inoculum of the aerobic heterotrophs were added to Media D and E. The tubes were incubated as before. One each of eleven days supernatant samples were frozen for arsenic speciation. Controls (Media D and E) and TSB blanks were similarly prepared.

2.4.2 Anaerobic Experiment

One mL of the anaerobic culture solution from the third enrichment step was added to 10.0 mL Medium B. The test tubes were incubated in the anaerobic chamber for 10-14 days. A heavy yellow precipitate formed and was separated from the solution by centrifugation for 30 min at a speed setting of 80. The precipitate was washed three times with distilled water, freeze-dried and its composition was investigated by using mass spectrometry, elemental analysis, and SEM + EDX. The supernatant was analyzed for arsenic compounds by HG-GC-AAS.

2.5 ANALYTICAL METHODS

2.5.1 Hydride Generation-Gas Chromatography-Atomic Absorption Spectrometry (HG-GC-AAS)

Arsenic speciation studies involved a selective hydride generation of volatile arsines, which were then concentrated by cryogenic trapping. The

arsines were separated by gas chromatography and detected by atomic absorption spectrophotometer. The HG-GC-AAS system is shown in Figure 2.2.

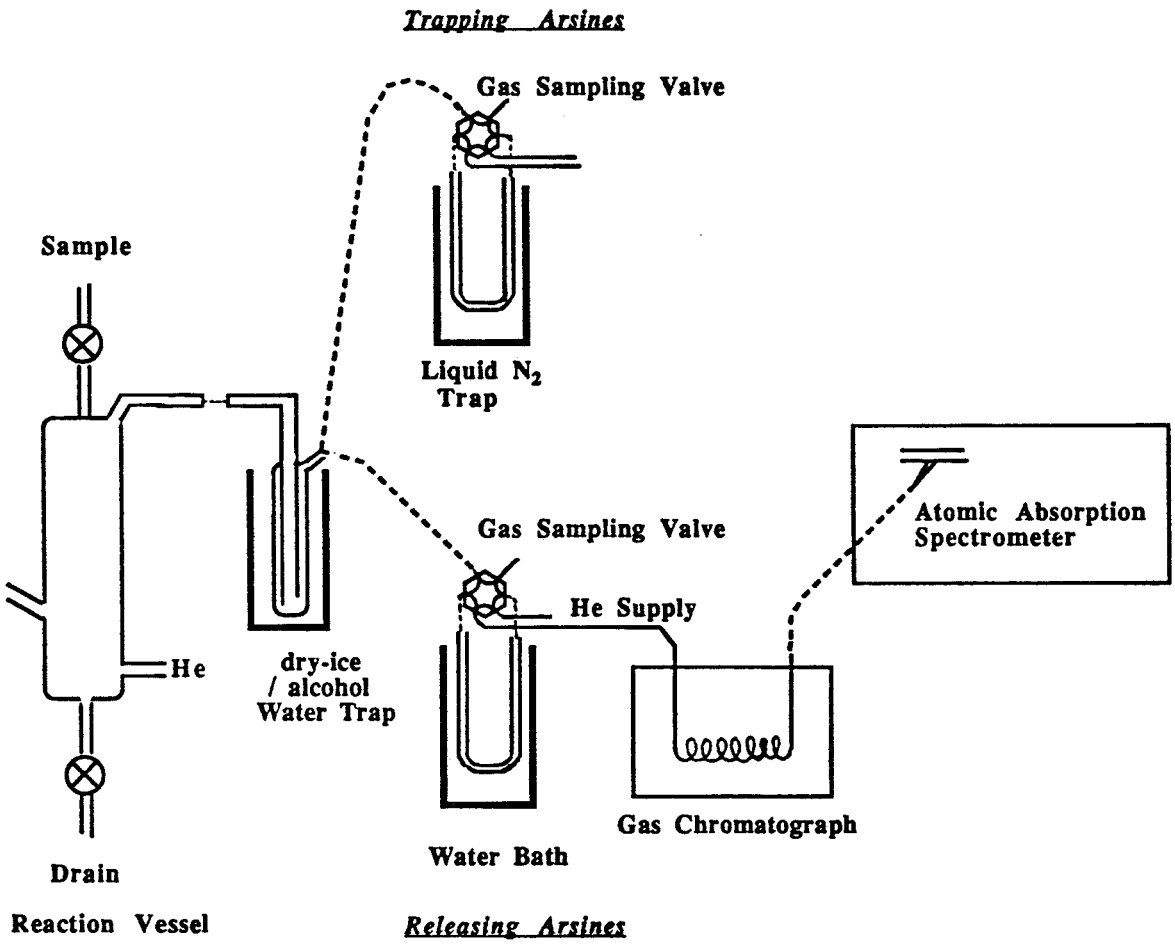


Figure 2.2 Schematic of HG-GC-AAS System

2.5.1.1 Hydride Generation

One to two mL of the sample solution was injected into the Teflon reaction vessel containing 50 mL deionized water and 4 mL, 4M HCl. The mixture was flushed with a helium flow for 5 min. Four mL of 4% KBH_4 was slowly injected into the vessel and the generated arsines were swept by a helium flow through an acetone-dry ice trap to remove water and were collected for 5 min in a trap made of Teflon tubing immersed in liquid nitrogen. The liquid nitrogen was removed and the tubing was warmed in a water bath and the arsines were flushed onto the GC column.

2.5.1.2 Gas Chromatography

A Porapak PS column was prepared as follows: A 600 mm length of Teflon tubing was plugged at one end with a small piece of Teflon wool. Porapak PS (80/100 mesh) was packed into the tubing and the tube was hand-spun for 5 minutes to ensure tight packing. The open end of the column was then plugged with Teflon wool.

To condition the column, one end of the packed column was connected to the injection line, while the exit was left unconnected in the GC oven. The column was conditioned by flushing the column with helium gas as the temperature was raised from 60°C to 150°C, at a rate of 30°/min. The temperature was then held at 150°C for an hour and subsequently cooled to 60°C. One end of the column was injected with 50 mL of Silyl-8. The column was heated again at 150°C for 45 min. The column silanization was repeated for the other end of the column.

Temperature programming was used to enhance the separation of the arsines, by the column and the program used was as follows:

Temp 1	60°C
Time 1	6 sec
Rate	30°C/min
Temp 2	150°C
Time 2	3 min.

2.5.1.3 Atomic Absorption Spectrometry

The effluent from the GC was detected in a hydrogen-air flame in a quartz-cuvette of the AAS. Arsines produced from inorganic arsenic [As(V) + As(III)], MMAA, DMAA and TMAO were detected. Standards arsenicals were injected to establish the retention times and the standard calibration curves.

2.5.2 Wet Digestion of the Freeze-dried Yellow Precipitate

The dried precipitate (5 mg) was added to concentrated nitric acid (3 mL), concentrated sulfuric acid (1 mL) and 30% hydrogen peroxide (3 mL) in a 100 mL round bottom flask. The flask was fitted with Teflon stopper and air condenser. The flask was heated at reflux for 2 hours by using a heating mantle. The flask was then cooled to room temperature and the digested sample was diluted to 100 mL with deionized water in a volumetric flask. Total arsenic was determined by flame-AAS, after calibration with 0.5 to 50 ppm As(V) standards.

2.5.3 SEM + EDX

The solid sample was fixed with carbon Dag (colloidal carbon in mixed alcohols) onto a spectrographic-grade graphite stub and the surface was coated with carbon (200A) in a vacuum evaporator (Japan Electron Optic Laboratory).

The stub was examined with a SEM using an accelerating potential of 30 KeV and a working distance of 35 mm. The x-rays generated by the electron beam were detected, identified and quantified by using the computer associated software package (Kevex Quantex IV).

CHAPTER III

RESULTS AND DISCUSSION

3.1 MICROBIAL SAMPLING

3.1.1 Study Site

An investigation was initiated by Reimer and coworkers on the Yellowknife Lakes system to investigate the speciation, redistribution and bioavailability of arsenic compounds from contaminated mine tailings.[51] As part of this program, the microbial activity of lake sediments was examined with respect to the mobilization of mine tailings that contain a high arsenic content. Details of the geochemical cycling or arsenic and various metals in these lakes are presented in a report.[51] Kam Lake was chosen as one study site. It has a maximum depth of 12 meters. According to Wagemann et al.[59] the dissolved arsenic concentration ranges from approximately 2000 ppb to more than 5000 ppb in the water column of Kam Lake. Bright and coworkers[51] observed an arsenic concentration ranging from 2200 ppm in the upper layer of the sediment to about 50 ppm in deeper layers.

The sediment of Kam Lake sampled by Bright and coworkers[51] in 1990 showed a profile that runs from an oxygenated surface layer to a sub-oxic zone and finally an anoxic layer. This typical profile is likely to promote a sequence of microbially mediated redox processes in which the aerobes dominate at the surface and anaerobes below the surface.

3.1.2 Kam Lake Core Description

The sediment core (core #6) that was used for the microbiological sampling was obtained on 3 August 1991. Table 3.1 gives a detailed description of the core at various depths. The temperature and pH of the core were not recorded in order to maintain the sterility of the sample. However, a similar core (core #3) showed a decreasing pH with depth (pH 7.4 - 6.7) and temperature of 18°C to 20°C (Table 3.2).

3.2 CULTIVATION AND SUBCULTURE

Once collected the cores were kept cool (0°C) for shipment to the laboratory in Yellowknife. The core was then exposed and sampled in a glove bag under an atmosphere of oxygen-free nitrogen. Less than 8 hours elapsed between the time of sample acquisition and inoculation into test media in Yellowknife. All cultures were transported back to UBC, Vancouver for subsequent studies.

The microbial growth in liquid media was monitored by visual observation of physical changes. The aerobes took about 48 hours to produce a turbid suspension. The anaerobes did not produce a turbid solution, however, the formation of a heavy yellow precipitate was used as the growth indicator. It took about 10-14 days for the anaerobes to produce the precipitate. The amount of precipitate formed decreased slightly with each subculture.

Table 3.1 Oxidation-reduction potential (ORP) measurement for core #6 taken at Kam Lake on 3 August 1991.

Hole#	Sediment Depth[a] (cm)	ORP (mV)
1	1	-
2	3	-225
3	5	-
4	7	-425
5	9	-
6	11	-358
7	13	-
8	15	-328
9	17	-
10	19	-
11	21	-364
12	-	-
13	25	-392
14	-	-
15	29	-
16	31	-377

0-8 cm: oxidized green-brown sediment of fine silt/sand (hole #1) gives way to an olive green layer just above patches of black (hole #3).

8-16 cm: black patches in a matrix at the top of the grey tailings layer (hole #5): tailings zone with predominantly black interior of core with fibrous material (hole #7).

17 cm: transition band from slate grey tailings to more brown colored natural sediments beneath (hole #9).

18-32 cm: brown colored natural sediments: no dark patches, no H_2S smell.

-: no readings taken.
[a]: Water depth 7 m.

Table 3.2 Oxidation-reduction potential (ORP), pH and temperature measurements for core #3 taken at Kam Lake

Hole#	Sediment Depth[a] (cm)	ORP (mV)	pH	T°C
1	-	-	7.38	20.1
2	3	-220	7.15	-
3	5	-290	7.00	-
4	7	-415	-	-
5	9	-400	6.95	-
6	11	-336	7.00	-
7	13	-363	7.11	19.0
8	15	-262	7.07	-
9	17	-281	-	-
10	19	-270	-	-
11	21	-	-	-
12	23	-	6.97	19.1
13	25	-	-	-
14	27	-	6.93	-
15	29	-	-	-
16	31	-	6.69	18.2

- : no readings taken.
[a] Water depth 6.5 m

Sediment samples that were inoculated into Media A and B from 1, 5, 9, 13, 19 and 29 cm depths, all showed growth. It is an interesting result that aerobic microbial growth occurred at all depths. The redox potentials of the sediment ranged from -225 to -425 mV and at this low redox potential, aerobes are not expected to thrive.

The anaerobes were also successfully cultivated from all depths. This is again surprising because Medium B has a redox potential of 130 mV.

Addition of sediment and the metabolism of bacteria in the anaerobic medium could be expected to lower the redox potential but only slightly, and not to the -300 mV necessary for strict anaerobes. Possibly the mixed anaerobic population comprises obligate anaerobes at the surface ranging to strict anaerobes at the bottom of the core. The anaerobes probably have oxygen-protective enzymes to enable them to survive in the oxygenated surface layer.

3.3 HG-GC-AAS

The hydride generation technique was used for the determination of arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide. The reducing system employed for arsine production is an alkaline potassium borohydride solution. It is customary to use an aqueous solution of KBH_4 (0.5-8% w/v) in NaOH medium. The reaction in acidic medium is:

$$As(OH)_3 + BH_4^- + H^+ \rightarrow AsH_3 + B(OH)_3 + H_2.$$

The excess of the reagent is decomposed by the acidic solution:

$$BH_4^- + 3H_2O + H^+ \rightarrow B(OH)_3 + 4H_2.$$

Two main types of apparatus have been used for the hydride generation. In the continuous flow technique,[28,46,49] a pump is used for the transport of sample solution and KBH_4 solution to a reaction coil where the hydride formation occurs. A continuous signal is observed by the AAS.

In the batch type apparatus,[59,60] the KBH_4 solution is mixed with the acidic sample in a reaction vessel. Reaction takes place immediately and the

arsines are swept by an inert carrier gas to the AAS. A discontinuous signal is observed by the AAS.

The batch type system was used in the present study to determine arsenic speciation. The reaction mixture is buffered to pH 0.5 with 4M HCl and the addition of KBH_4 reduced inorganic arsenic [As(III) + As(V)] to AsH_3, monomethylarsonic acid (MMAA) to monomethylarsine (MMA), dimethylarsinic acid (DMAA) to dimethylarsine (DMA), and trimethylarsine oxide (TMAO) to trimethylarsine (TMA). The reaction mixture is subsequently buffered to pH 6 with 1M citrate in 10% citric acid whereupon addition of KBH_4 converts arsenite [As(III)] only to AsH_3. The arsenate concentration can be determined from the difference between the arsine signals in the two buffered systems.

The generated arsines (Table 1.1) are collected by cryogenic trapping. Efficient and reproducible separation of the arsines is achieved by using a gas chromatographic column such as a silanized Porapak PS packed column[44] interfaced to the trap. Temperature programming is used to enhance the separation. The effluent from the GC is swept into a quartz cuvette of an AAS where it is combusted in a hydrogen-air flame.

Retention times are used for the identification of arsines and peak areas are used to calculate the concentration of As(III), As(V), MMAA, DMAA and TMAO in the sample solutions.

A typical chromatogram of the generated arsines is shown in Figure 3.1. Arsine (AsH_3) has a retention time of 0.96 min, monomethylarsine (CH_3AsH_2) of 1.66 min and dimethylarsine [$(CH_3)_2AsH$] of 2.41 min.

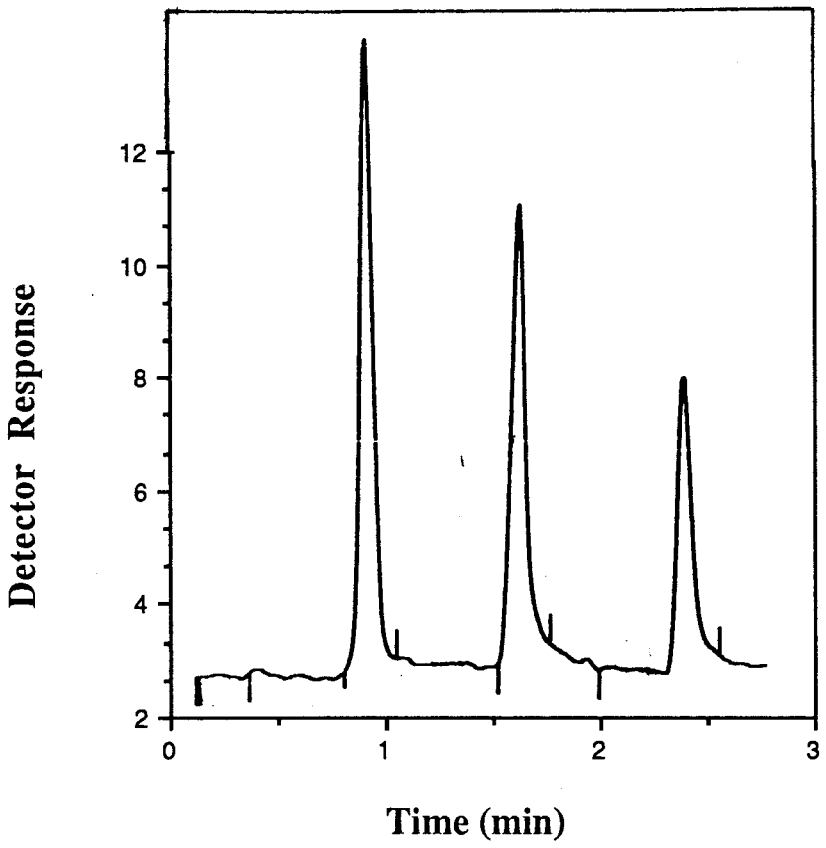


Figure 3.1 Chromatogram for 4 mL of a standard solution of 10 ppb As(V), MMAA and 20 ppb of DMAA.

The dimethylarsine signal from the HCl buffered reaction is the least reliable in terms of sensitivity and reproducibility, as has been previously reported.[44] This has been attributed to incomplete reduction, condensation in the transport tubes or incomplete atomization.[49]

Changing the acid system to 4M acetic acid (pH 2.5) improves the DMA signal (Figure 3.2) but the problem of long term reproducibility remains to be solved.

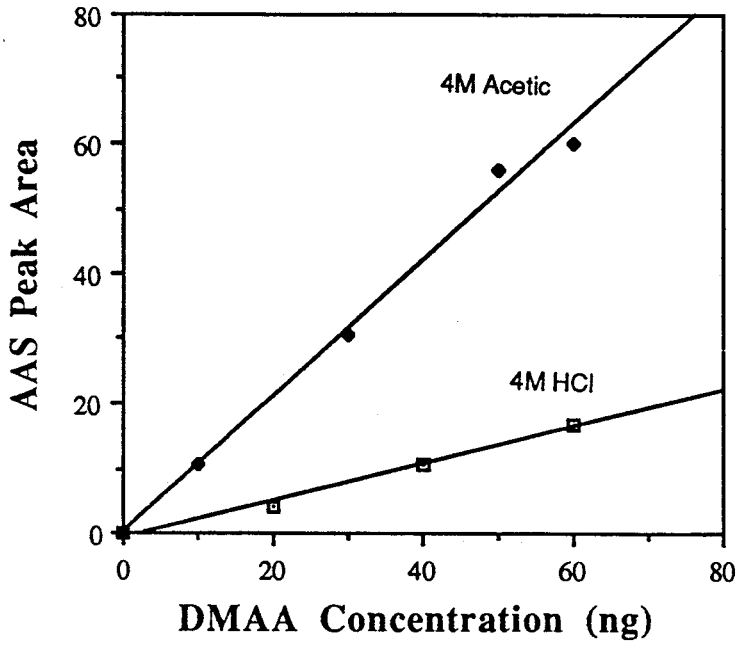


Figure 3.2 Comparison of effects of 4M HCl and 4M CH_3COOH on DMA signal.

3.3.1 Calibration And Limit of Detection.

Typical calibration plots of AAS peak area versus concentration (ppb) for inorganic arsenic [As(III) + As(V)], As(III), MMAA and DMAA are shown in Figures 3.3 - 3.6.

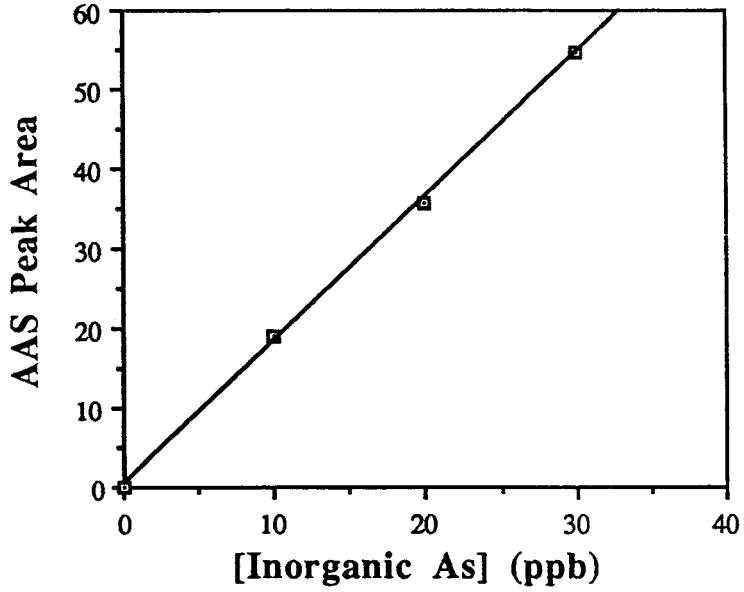


Figure 3.3 Calibration curve for the determination of total inorganic arsenic

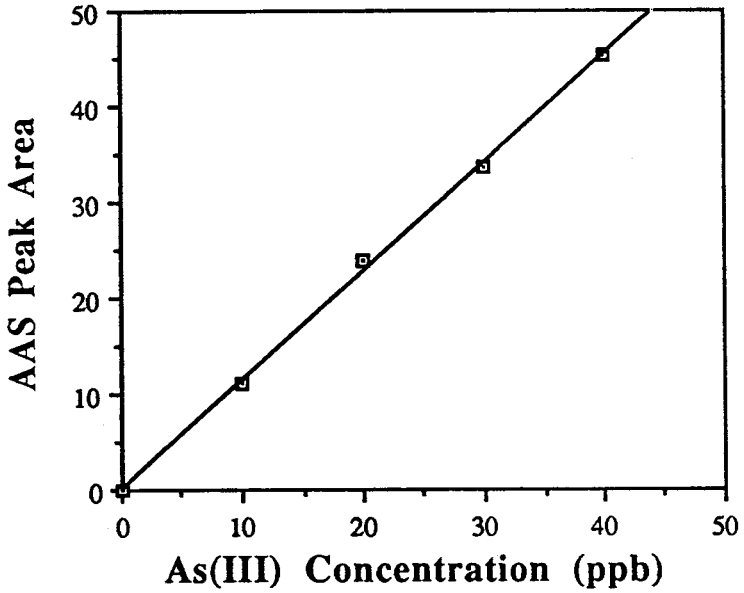


Figure 3.4 Calibration curve for the determination of arsenite

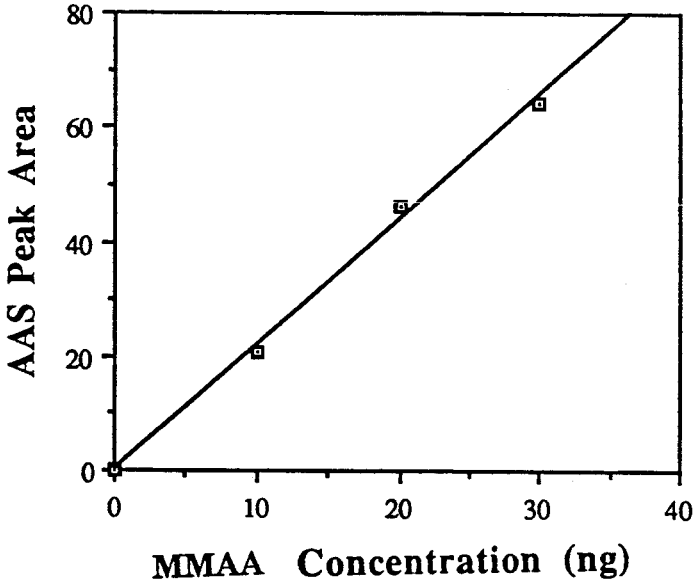


Figure 3.5 Calibration curve for the determination of MMAA

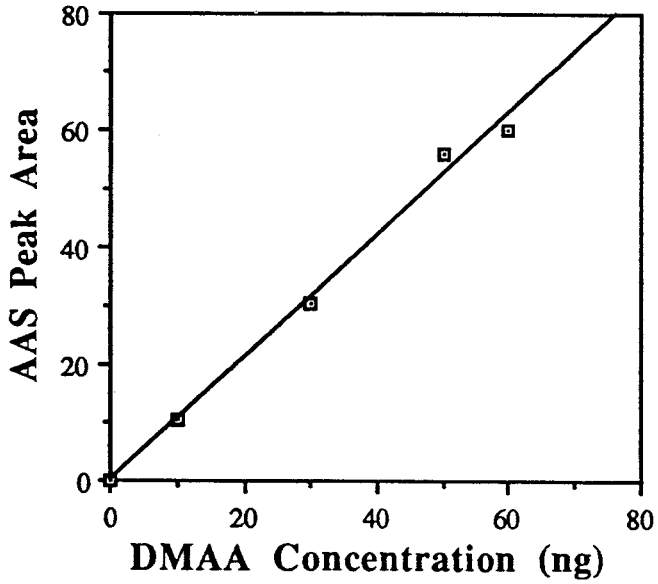


Figure 3.6 Calibration curve for the determination of DMAA

The calibration curves show a linear dependence of the AAS peak for the concentration range studied. The absolute limit of detection, defined as the analyte concentration producing a signal equal to the blank plus three standard deviation of the blank, is 0.12 ng (of As) for As(III), 0.25 ng for As(V) and the methylarsenic acids.

3.4 AEROBIC ARSENIC METABOLISM

According to Bright et al.,[51] the concentration of methylated arsenic species in porewater from a Kam Lake sediment core is highest (about 5.2 ppb As) at a depth of 8-13 cm. From Tables 3.1 and 3.2, this layer corresponds to the deposited tailings from the mine.

3.4.1 Effect of Arsenate

The mixed bacterial populations from 5, 9 and 13 cm depth were chosen for the initial assay of arsenic metabolizing activity. The inoculum used was standardized by spectrophotometric measurement of the turbidity of a cell suspension. The mixed populations were grown in arsenic augmented broth (50 ppb As(V) solution). Samples taken daily from a series of Evergreen-tube cultures over a period of 1-7 days were analyzed for arsenic species.

Figures 3.7 - 3.9 show the results of the initial experiment. The mixed population from the 5 cm depth (Figure 3.7) shows a pattern of methylation and demethylation. The concentration of added arsenate in the media decreases rapidly within 2 days, reaching zero by 5 days. The added arsenate

is transformed into MMAA and DMAA over the 7 days of incubation. The formation of DMAA increases from day 1 to day 4, giving way to a progressive decline on days 5-7. This pattern of variation indicates a shift from production to decomposition of DMAA, indicating that demethylating microorganisms are replacing the methylators in the mixed community.

The 9 cm and 13 cm microbial cultures show very small amounts of methylated arsenic species (Figures 3.8 and 3.9). The amount of added arsenate decreases with time with no apparent transformation to MMAA or DMAA.

Comparisons of the blank and control media (Figure 3.10) with the cultured media show that the mixed microorganism from the 5 cm depth have a profound influence on the biomethylation of arsenic.

From Figure 3.7, the amount of added arsenic (50 ppb As(V) solution, which is equivalent to approximately 95 units of peak area) is not recovered fully in the supernatant analyzed. The total arsenic concentration also varies from day to day (Figure 3.11). The observed decrease could be due to: (1) the added arsenic could be transformed and adsorbed on the bacterial cells and thus lost when the solid phase was discarded after centrifugation; (2) uptake of arsenic by the bacteria could cause the concentration to drop; (3) there are other species of arsenic in the supernatant that are undetected by the hydride generation technique; (4) formation of volatile arsines, that escape detection by the "snift test". The "snift test" is based on the intense, garlic-like odor of the arsines and arsine production can be evaluated by cautious sniffing of a sample of the headspace of the test tube.[30,44]

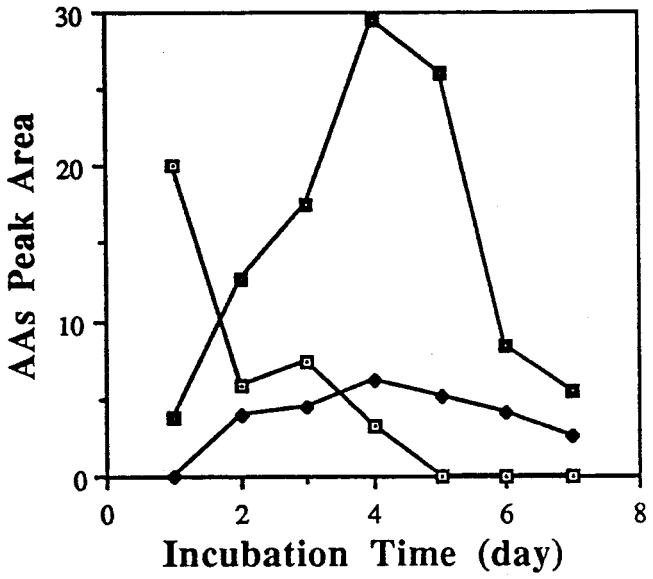


Figure 3.7 Inorganic As (❑), MMAA (◆) and DMAA (■) production by 5 cm aerobic heterotrophs from Kam Lake sediments

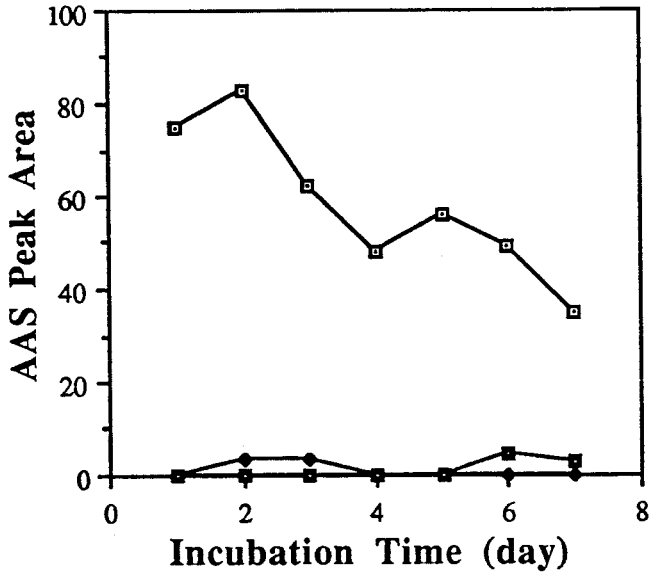


Figure 3.8 Inorganic As (❑), MMAA (◆) and DMAA (■) production by 9 cm aerobic heterotrophs from Kam Lake sediments

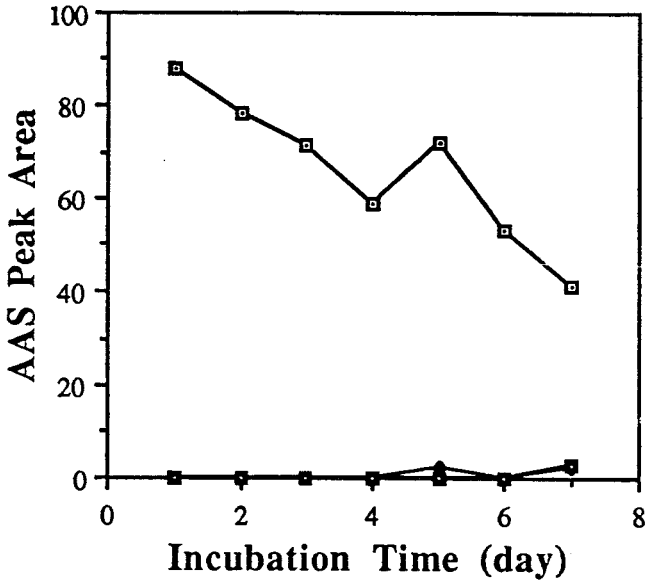


Figure 3.9 Inorganic As (□), MMAA (◆) and DMAA (■) production by 13 cm aerobic heterotrophs from Kam Lake sediments

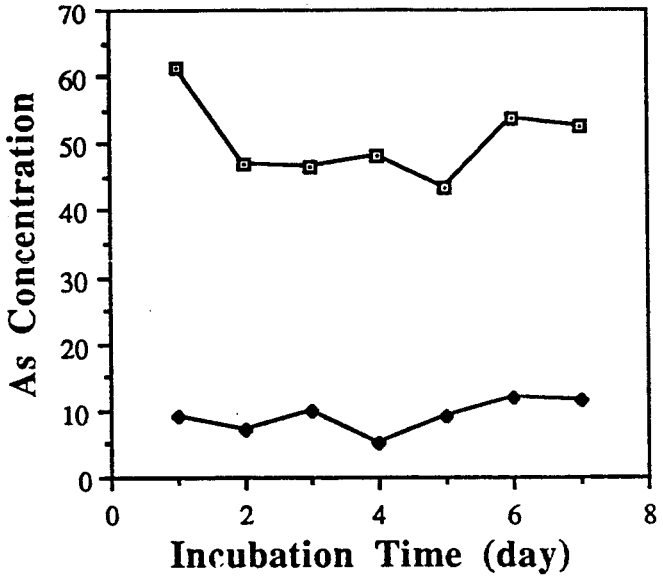


Figure 3.10 Production of AsH_3 in Blank (□) and Control (◆)

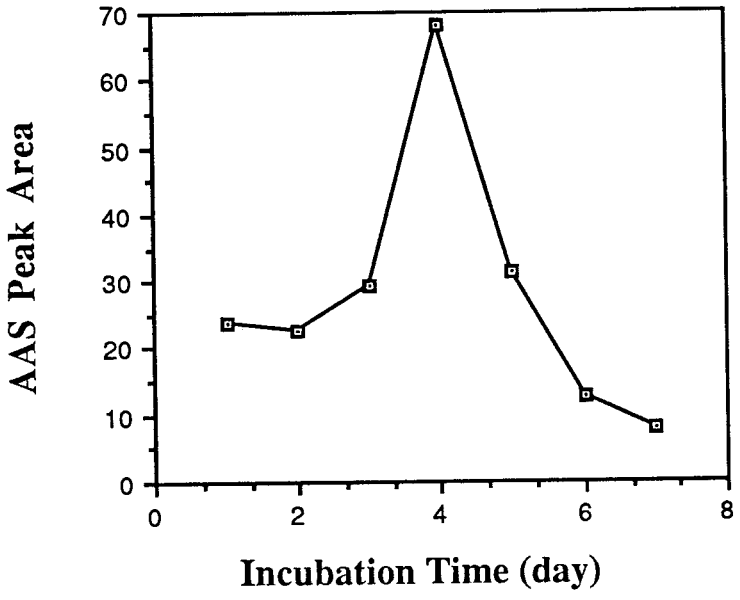


Figure 3.11 Day-to-day variation of total arsenic in the 5 cm aerobic heterotrophs incubation

Only the 5 cm microbial population shows strong methylation ability. From the original core profile (Table 3.1), the 5 cm depth is at the border of the tailings layer and not in the tailings (9-16 cm) where the arsenic levels are anticipated to be highest. Probably some reaction (possibly microbial) takes place in the tailings layer that causes arsenic to be mobilized to the layers above, where the microbial population then transforms the inorganic arsenicals to methylarsenicals. The thickness of the tailings layer varies from one core sample to another and the arsenic methylators are probably present in the layer overlying the mine tailings.

Following the results of the initial assay, the mixed population from the 5 cm depth was used for further investigation of the biotransformation of arsenic. The protein in the cultured medium caused severe foaming in the reaction vessel during the speciation by hydride generation and addition of 2-propanol, as an anti-foam agent, did not alleviate the problem. The culture medium was reduced to half strength TSB in subsequent experiments to alleviate this problem. The medium appears to be more favorable for the growth of arsenic methylators.

3.4.2 Effect of DMAA

Because mixed microbial populations from 5 cm depth methylate the added arsenate to MMAA and DMAA, we would expect the microbes to demethylate any added DMAA to MMAA and inorganic arsenic. Instead, as shown in Figure 3.12, no MMAA is detected in the supernatant over a period of 1-11 days. The DMAA concentration fluctuates from day-to-day giving a

complex zigzag pattern with no hint of a trend. This pattern appears to indicate that DMAA is taken up by the mixed populations at a different rate each day, either transforming it and releasing it back to the solution or storing it inside the cells.

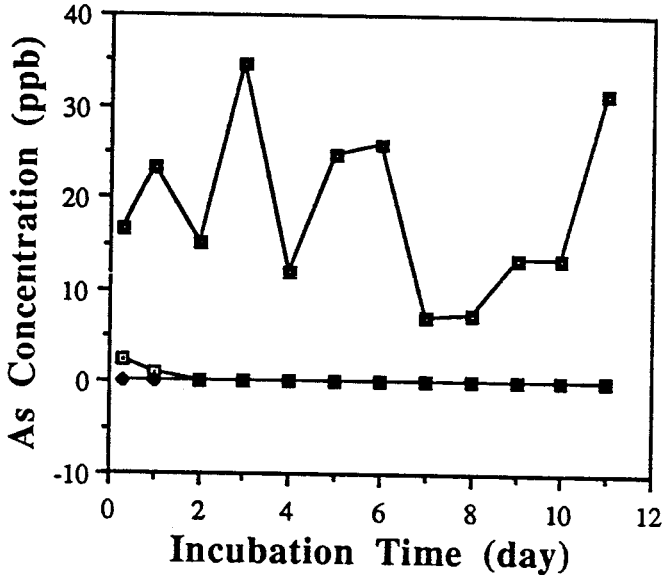


Figure 3.12 Production of AsH_3 (□), CH_3AsH_2(◆) and $(CH_3)_2AsH$ (■) by the 5 cm aerobic heterotrophs in Medium D

The DMAA concentration decreases with no evidence of transformation to MMAA or inorganic arsenic. The amount of inorganic arsenic in the supernatant is zero on day 2. However, the amount in the blank (Figure 3.13) is ten times higher, over the 11 days study period.

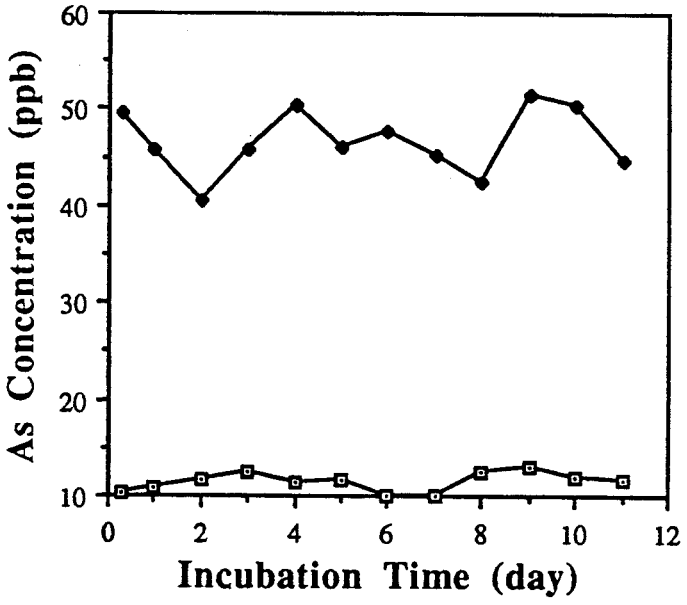


Figure 3.13 Production of AsH_3 (□) from Blank and $(CH_3)_2AsH$ (◆) from Control media

Trimethylarsine oxide is produced on days 5-11 (Figure 3.14) from DMAA. (Due to the difficulty of quantification, the amount of trimethylarsine is reported in terms of AAS peak area).

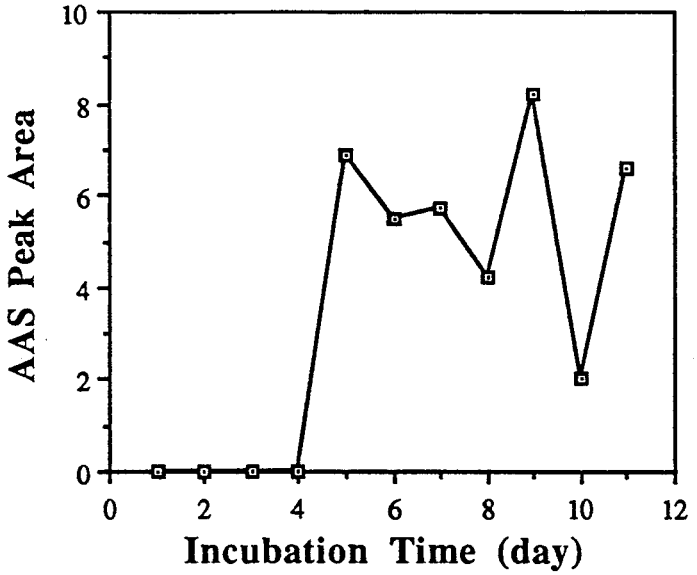


Figure 3.14 Trimethylarsine production from 5 cm microbial population in Medium D

3.4.3 Summary of Aerobic Metabolism

Microbial communities in sediments consist of co-existing species that interact with each other in a complex way. Microorganisms isolated from the layer overlying contaminated mine tailings are able to biomethylate arsenic.

Addition of an external arsenic source (50 ppb As(V) solution) to this mixed community reveals a pattern of methylation and demethylation

suggesting a successive change between the methylating microbes and demethylating microbes, as shown in Figure 3.7.

Addition of 50 ppb DMAA shows biomethylation to TMAO by the mixed microbes. No demethylation is observed. The reason for these observations is not understood at this time.

Further work needs to be done to quantify the total arsenic in the solid and liquid phase. Probably there is an uptake by the microbes and storage inside the bacterial cells, and this could account for the above discrepancy.

3.5 ANAEROBIC ARSENIC METABOLISM

The anaerobic "arsenate-reducer" produces a heavy yellow precipitate after 14 days of incubation. The yellow color of the precipitate indicates that it might be elemental sulfur, formed by the reduction of sulfate. The low (0.04 mM) sulfate concentration in Medium B could just be sufficient to maintain the metabolic activity of the microorganisms. Moreover there is an almost inexhaustible supply of sulfate in the Kam Lake sediment which contain approximately 0.33 mM sulfate in its porewater.[51] The sulfate reduction is usually carried out by dissimilatory sulfate-reducing bacteria, and the "arsenate-reducer" would have to compete successfully with the sulfate-reducing bacteria for the available substrates.

A second likely product is an arsenic sulfide compound. Kam Lake contains high levels of arsenic, hence, it is possible that the mixed microbial population uses arsenate as a terminal electron acceptor. In the presence of

large amount of sulfur and in acidic medium, As_2S_3 and As_4S_4 precipitate out.

3.5.1 Medium B

The arsenic concentration in Medium B was analyzed by AAS (Table 3.3). There appears to be a 10% loss of arsenic after sterilization. The medium is autoclaved in an air-tight Pyrex bottle, so the likelihood of loss by volatilization is minimized. To avoid the problem, whatever the cause, the arsenate solution should be filter-sterilized in future preparations of media to prevent its loss.

Table 3.3 Arsenic concentration in Medium B

	Flame-AAS	HG-GC-AAS		
	[As] g/L	$[As]_T$	[As(III)] g/L	[As(V)]
Before autoclave	1.07	1.02	0.05	0.97
After autoclave	0.98	0.91	0.04	0.86

3.5.2 Characterization of the Anaerobic Metabolic Product

3.5.2.1 Mass Spectrometry

The mass spectrum of a freeze-dried precipitate was obtained at both low and high resolution EI-MS50 (Figure 3.14). The spectrum shows peaks

at m/z of 428 (As_4S_4), 396 (As_4S_3), 300 (As_4), and other peaks with combinations of As and S. The result shows that the molecules dissociates prior to ionization and thus the observed mass spectrum is that of the decomposition products. The results rule out the possibility that the precipitate is elemental sulfur or arsenic.

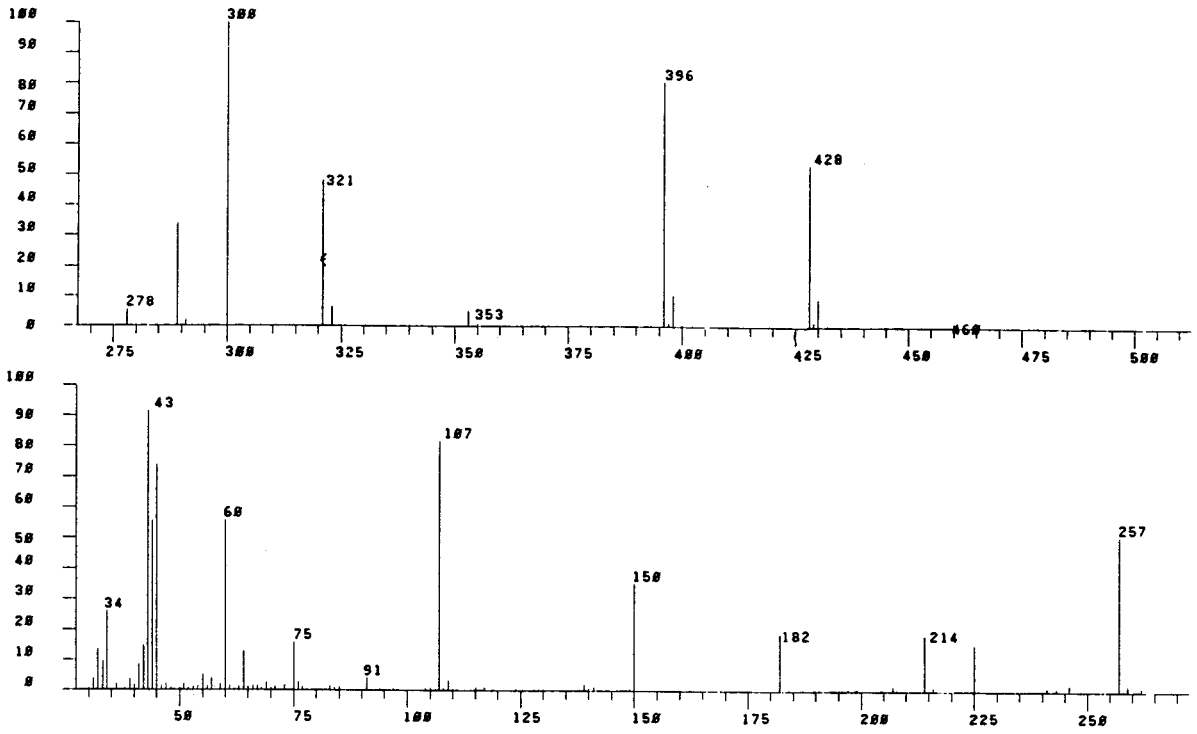


Figure 3.15 Mass spectrum of yellow precipitate. Spectrum obtained at 330°C

3.5.2.2 Elemental Analysis

Elemental analysis was done by Mr. Peter Borda, Chemistry Department, UBC. Table 3.4 shows the result of the analysis. The presence of arsenic interfered with the sulfur determination.

Table 3.4 Elemental analysis of yellow precipitate

	C	H	N	S
% found	4.88	1.60	1.60	29.3
% expected (for As_2S_3)	-	-	-	40

3.5.2.3 Wet Digestion of Freeze-dried Yellow Precipitate and Flame-AAS Analysis

The amount of precipitate formed by the "arsenate-reducer" in the 10 mL test tube was small. The freeze-dried precipitate from the mixed populations of 5, 9 and 29 cm were combined for digestion. The precipitate is slightly soluble in nitric acid but a more elaborate digestion procedure that utilizes nitric acid, sulfuric acid and hydrogen peroxide was used. The precipitate dissolves completely and AAS analysis shows that the combined precipitate contain 59.2% As. (Calculated for As_2S_3: 60% As).

3.5.2.4 SEM + EDX

A flat surface of the precipitate on the carbon coated stub was scanned at 6.00K magnification. The scanning area of the sample must be smooth to enable the generated x-rays to reach the detector. Two or three different areas on the same stub were analyzed. Table 3.5 summarizes the results.

Table 3.5 SEM + EDX analysis of the yellow precipitate obtained from Kam Lake microorganism.

Sample	Weight Percent		
	As	S	Ca
5 cm	57.0 ±2.6	38.2 ±2.0	4.83 ±0.7
9 cm	57.9 ±2.9	42.1 ±2.9	0.0
29 cm	56.1 ±0.9	40.1 ±0.5	3.70 ±0.4
Medium B + H_2S	58.0 ±2.4	42.0 ±2.4	0.0
As_2S_3	57.6 ±4.3	42.4 ±4.3	
As_4S_4	70.3	29.3	

Standards of As_2S_3 and As_4S_4 were similarly analyzed. The combined results indicate that the yellow precipitate is probably As_2S_3 with some "included" calcium and organic compounds.

3.5.3 Arsenic Speciation of Supernatant

The supernatant media from cultures that produced the yellow precipitate was analyzed by HG-GC-AAS to determine arsenic speciation (Table 3.6). No methylarsenic acids were detected; approximately 76% of

arsenic exists as As(V) and 24% as As(III). Flame-AAS was also performed to compare total arsenic.

Table 3.6 Flame-AAS and HG-GC-AAS of supernatant medium after anaerobic growth of Medium B

Supernatant	HG-GC-AAS (g/L)			Flame-AAS (g/L)
	As_T	As(III)	As(V)	As_T
5 cm	-	-	-	0.52
9 cm	0.38	0.09	0.29	0.58
29 cm	0.22	0.09	0.13	-

- not analyzed.

A difference in total arsenic concentration of about 35% was observed between the two methods. The discrepencies could be due to the series of dilutions (10^4 dilution) of the sample that were necessary for arsenic concentration to be within the working range of the HG-GC-AAS. Flame-AAS is not as sensitive as the HG-GC-AAS and no dilution was necessary.

3.5.4 Summary of Anaerobic Metabolism

The mixed anaerobic microbes from Kam Lake sediment produce a yellow precipitate which is identified as As_2S_3 from elemental and SEM + EDX analysis. The sulfide is likely to be an artifact, produced from either As(V) or As(III) when microbial growth produces a localized acidic

environment in the medium. In an independent experiment it was found that hydrogen sulfide does not precipitate any arsenic sulfide from the media unless the media is acidified.

The source of sulfide is unknown. Presumably the microbes produce sulfide from the reduction of sulfate. The sulfate reduction is usually carried out by the sulfate-reducing bacteria, but these are unlikely to be present in this media which has a redox potential of 130 mV. The sulfate-reducers need media with a low redox potential (about -300 mV).

Speciation of arsenic in the supernatant shows 76% As(V) and 24% As(III), with no methylarsenic acids. Thus the mixed anaerobes do not methylate the added arsenate although they may be reducing As(V) to As(III). Otherwise the microbes could be arsenic tolerant and be particularly insensitive to arsenic, with no uptake or metabolism of the arsenate added.

CHAPTER IV

BIBLIOGRAPHY

1. National Research Council. *Arsenic*; National Academy of Sciences; Washington, DC, **1977**; p16.

2. Sanders, J.G. *Marine Env. Res.* **1980**, *3*, 257.

3. Boyle, R.W.; Jonasson, I.R. *J. Geochem. Explor.* **1973**, *2*, 251.

4. Andreae, M.O. In: *Organometallic Compounds in the Environment*; Craig, P.J. Ed.; Wiley: New York, **1986**; p198.

5. Kipling, M.D. In: *The Chemical Environment: Environment and Man*; Lenihan, J., Fletcher, W.W. Eds.; Blackie, **1977**; p93.

6. World Health Organization. *Environmental Health Criteria:18, Arsenic*; WHO, Geneva, **1981**, p174.

7. Hanaoka, K.; Motoya, T.; Tagawa, S.; Kaise, T. *Appl. Organomet. Chem.* **1991**, *5*,427.

8. Peterson, M.L.; Carpenter, R. *Mar. Chem.* **1983**, *12*, 295.

9. Stumm, W.; Morgan, J.J. In: *Aquatic Chemistry*; Wiley; New York, **1970**, p583.

10. Cherry, J.A.; Shaikh, A.U.; Tallman, D.E.; Nicholson, R.V. *J. Hydrol.* **1979**, *43*,373.

11. Tsunogai, S.; Sase, T. *Deep Sea Res.* **1969**, *16*, 489.

12. Johnson, D.L. *Nature*, **1972**, *240*, 44.

13. Freeman, M.C. *N.Z.J. Mar. Freshwater Res.* **1985**, *19*, 272.

14. Johnson, D.L.; Braman, R.S. *Deep Sea Res.* **1975**, *22*, 503.

15. Scudlark, J.R.; Johnson, D.L. *Estuarine Coastal Shelf Sci.* **1982**, *14*, 693.

16. Turner, A.W. *Australian J. Biol. Sci.* **1954**, *7*, 452.

17. Jensen, S.; Jernelov, A. *Nature*, **1969**, *223*, 753.

18. Gavis, J.; Ferguson, J.F. *Water Res.* 1972, *6*, 989.

19. Challenger, F. *Chem. Rev.* **1945**, *36*, 315.

20. du Vigneaud, V.; Cohn, M.; Chandler, J.P.; Schenk, J.R.; Simmonds, S. *J.Biol. Chem.* **1941**, *140*, 625.

21. Challenger, F.; Lisle, D.B.; Dransfield, P.B. *J. Chem. Soc.* **1954**, 1760.

22. Cullen, W.R.; McBride, B.C.; Reglinski, J. *J. Inorg. Biochem.* **1984**, *21*, 45.

23. Cullen, W.R.; McBride, B.C.; Manji, H.; Pickett, A.W.; Reglinski, J. *Appl. Organomet. Chem.* **1989**, *3*, 71.

24. Cullen, W.R.; McBride, B.C.; Pickett, A.W. *Appl. Organomet. Chem.* **1990**, *4*, 119.

25. McBride, B.C.; Merilees, H.; Cullen, W.R.; Pickett, A.W. *ACS Symp. Ser.*. **1978**, *82*, 94.

26. Cox, A.P.; Alexander, M. *Bull. Environ. Contam. Toxicol.* **1973**, *9*, 84.

27. Shariatpanahi, M.; Anderson, A.C.; Abdelghani, A.A. *Trace Subst. Environ. Health* **1982**, *16*, 170.

28. Wong, P.T.S.; Chau, Y.K.; Luxon, L.; Bengert, G.A. *Trace Subst. Environ. Health*, **1977**, *11*, 100.

29. Ferguson, J.F.; Gavis, J. *Water Res.* **1972**, *6*, 1259.

30. Cullen, W.R.; Reimer, K.J. *Chem. Rev.* **1989**, *89*, 713.

31. Ehrlich, H.L. *Economic Geol.* **1963**, *58*, 991.

32. Ehrlich, H.L. *Economic Geol.* **1964**, *59*, 1306.

33. Bosecker, K. In: *Sulfur, its Significance for Chemistry, for the Geo- and Cosmosphere and Technology*; Muller, A.; Krebs, B. Eds. Elsevier: **1984**, *5*, 331.

34. Howard, A.G.; Arbab-Zavar, M.H. *Analyst,* **1981**, *106*, 213.

35. Andreae, M.O. *Anal. Chem.* **1977**, *49*, 820.

36. Howard, A.G.; Arbab-Zavar, M.H.; Apte, S. *Mar. Chem.* **1982**, *11*, 493.

37. Reimer, K.J.; Thompson, J.A.J. *Biogeochemistry,* 1988, *6*, 211.

38. Andreae, M.O. *Deep-Sea Res.* 1978, *25*, 391.

39. Braman, R.S.; Johnson, D.L.; Foreback, G.C.; Ammons, J.M.; Bricker, J.L. *Anal. Chem.* **1977,** *49*, 621.

40. Sadana, R.M. *Anal. Chem.* **1983,** *55*, 304.

41. Gohda, S. *Bull. Chem. Soc. Jpn.* 1975, *48*, 1213.

42. Cullen, W.R.; Dodd, M. *Appl. Organomet. Chem.* **1989,** *3*, 401.

43. Hansen, S.H.; Larsen, E.H.; Prits, G.; Cornett, C. *Anal. Atomic. Spect.* in press.

44. Reimer, K.J. *Appl. Organomet. Chem.* **1989,** *3*, 475.

45. Ebdon, L.; Walton, A.P.; Millward, G.E.; Whitfield, M. *Appl. Organomet. Chem.* **1987,** *1*, 427.

46. Andreae, M.O. *Anal. Chem.* **1977,** *49,* 820.

47. Odanaka, Y.; Tsuchiya, N.; Matano, M.; Goto, S. *Anal. Chem.* **1983,** *55,* 929.

48. Arbab-Zavar, M.H.; Howard, A.G. *Analyst,* **1980,** *105*, 744.

49. Anderson, R.K.; Thompson, M.; Culbard, E. *Analyst,* **1986,** *111*, 1143.

50. Hinners, T. A. *Analyst,* 1980, *105*, 751.

51. Bright, D.A.; Coedy, B.; Dushenko, W.D.; Reimer, K.J. Draft report, **1991.**

52. Vogel, A.I. *A Textbook of Qualitative Chemical Analysis*; Longmans.

53. Schenk, In: *Handbook of Preparative Inorganic Chemistry*; Brauer, G. Ed. **1965**, *1*, p603.

54. Balch, W.E.; Fox, G.E.; Magrum, L.J.; Woese, C.R.; Wolfe, R.S. *Microbiol. Rev.* **1979,** *43*, 260.

55. Herbett, R.A. In: *Methods in Aquatic Bacteriology*; Austin, B. Ed. Wiley: New York, **1988**, p17.

56. Hungate, R.E. *Methods in Microbiol.* **1969,** *3B*, 117.

57. Ljungdahl. L.G.; Wiegel. J. In: *Methods in Industrial Microbiology and Biotechnology*: Demain, A.L.; Soloman, N.A. Eds. **1986**, p84.

58. Wagemann, R.; Snow, N.B.; Rosenberg, D.M.; Lutz, A. *Arch. Environ. Contam. Toxicol.* **1978,** *7*, 167.

59. Thompson, K.C.; Thomerson, D.R. *Analyst,* **1974,** *99*, 595.

60. Dedina, J.; Rubeska, I. *Spectrochim. Acta* **1980,** *35B*, 119.