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# Speciation and Mobilization of Toxic Heavy Metal Ions by Methanogenic Bacteria

Mark S. Foster, Shawn Conver, Allyson Rodabough, Tressia M. Dayton, John A. Koropchak, and Eric C. Niederhoffer

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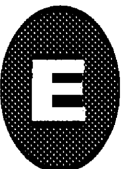

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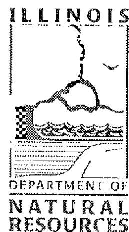


WMRC is a division of the Illinois Department of Natural Resources

Hazardous Waste Research and Information Center
One East Hazelwood Drive
Champaign, Illinois 61820

*HWRIC RR-066*

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## Preface

One of the many problems facing industrialized societies concerns the presence of toxic materials in our environment Toxic materials may be thought of as comprising inorganic (non-carbon-containing) and organic (carbon-containing) substances The ability of these materials to survive for long periods of time under a variety of conditions has caused us to examine both the potential interactions that occur with living systems and highly effective methods to store them or remove them from the environment

Many inorganic substances are essential for life Certain substances are toxic to specific organisms or to a range of life forms from simple bacteria to complex human beings The toxicity presents a challenge to us Can we continue to use toxic materials or should we pursue the development of alternative materials? How do we approach the significant levels of toxic materials already present in the soils and aquifers? Regardless of our choices , we will need to develop highly effective approaches to containment and long-term stabilization of storage matrices

Within this report, we have addressed the importance of basic scientific knowledge to the understanding of toxic metal interactions with anaerobic microorganisms The first portion of our work is concerned with the development of improved techniques for studying thermophilic microorganisms, the second section summarizes our results for methanogen mediated solubilization of inorganic matrices, the third section focuses on determining the relative sensitivity of methane-producing microorganisms to toxic heavy metals, while the fourth section concerns the specific physical and chemical interactions that define toxic metals and methanogens

Taken together, we believe that our studies demonstrate the importance of anaerobic microorganisms in the metabolism of essential and toxic metal ions The apparent sensitivity and toxicity of inorganic species should have profound influence on the future development of bioremediation and biostabilization technologies

## Acknowledgments

Our project officer, Pam Tazik, and research program manager, Jacqueline Peden, from the Hazardous Waste Research and Information Center made this past year and a half enjoyable by providing us with the resources to do the described studies and for minimizing the paper work necessary for conducting environmentally related research Pam was particularly cooperative in helping us meet deadlines and in allocating various portions of our research budget Larry Hawse (Office of Research Development and Administration, SIUC) has been a shining star and assisted us with contracts and modified agreements, which made our daily lives much easier

Mark S Foster and T Shawn Conver were the primary graduate assistants, they and the following data gatherers were responsible for carrying out many of the research ideas appearing in this document or in the scientific literature Tressia M Dayton, Allyson Rodabough, Karen A Diefenbach, Michelle L Fuller, Eugenia M Melko, Sandra S Szegedi, and Brian W Guzik

We thank Professor Gerard V Smith (Department of Chemistry and Biochemistry, SIUC) and his laboratory for demonstrating by gas chromatography that our active methanogen cultures were indeed producing methane from carbon dioxide and hydrogen Dr David P Nagle (Department of Botany and Microbiology, University of Oklahoma) was the catalyst that directed us to discuss our research results and plans with his colleague Dr Ralph S Tanner (Department of Botany and Microbiology, University of Oklahoma) Ralph provided us with several variations in the determination of cell densities, which were particularly useful when cultures contained colloidal sulfide precipitates, with valuable discussions relating to the isolation of mutant strains, and with the sense that it is good to be a generalist when working on interdisciplinary approaches to problems

We appreciate the release time given to us by the Department of Chemistry and Biochemistry and the Medical Biochemistry Unit in the School of Medicine for the purpose of delivering seminars and discussing our research results at more than a half dozen colleges and universities within the midwest region Finally, we are indebted to Marilyn Davis (ORDA, SIUC) for summarizing our research efforts in the Spring 1993 issue of *SIUC Perspectives*

**Table of Contents**


| | |
|---|---|
| **Preface** | iii |
| **Acknowledgments** | iv |
| **Tables** | vi |
| **Figures** | vii |
| **List of Abbreviations** | viii |
| **Abstract** | ix |
| **1.0 Introductory Material** | 1 |
| **2.0 Methodology** | 4 |
| **2.1 Strains and medium** | 4 |
| **2.2 Bottle assembly** | 4 |
| **2.3 Nephelo flask and stopper assembly** | 4 |
| **2.4 Cultivation of methanogens** | 4 |
| **2.5 Growth studies** | 5 |
| **2.6 Analytical determination of THM by ICP-AES** | 5 |
| **2.7 Solubilization of sulfide matrices** | 6 |
| **2.8 Whole cell binding of THM** | 6 |
| **3.0 Results** | 7 |
| **3.1 Cultivation of thermophilic methanogens** | 7 |
| **3.2 Solubilization of CdS** | 8 |
| **3.3 Growth studies** | 12 |
| **3.4 THM-binding by methanogens** | 15 |
| **4.0 Discussion** | 16 |
| **5.0 Conclusions** | 17 |
| **6.0 Recommendations** | 18 |
| **References** | 19 |
| **Glossary** | 21 |
| **Appendix A. Photographs of Assemblies** | 23 |

## Tables


Table 1 Relationship of nutritional requirements for $Fe^{2+}$, $Co^{2+}$, and $Ni^{2+}$ in methanogens to metal sulfide solubilities 2

Table 2 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation 8

Table 3 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation Multiple solids washings 9

Table 4 Methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation Contributions from glass bottles and solids washings 10

Table 5 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after high speed centrifugation separation 11

Table 6 Summary of CdS dissolution in MS medium 12

Table 7 Binding of $Cd^{2+}$ to washed whole cells of *M thermoautotrophicum* ΔH and *M thermoautotrophicum* Marburg 15

## Figures


| | | |
|---|---|---|
| Figure 1 | The $Fe^{3+}$-binding groups represented by hydroxamic acid, phenol, and catechol | 2 |
| Figure 2 | Typical growth (increase in Klett units) of *M thermoautotrophicum* ΔH (■) and *M thermoautotrophicum* Marburg (□) as measured with the nephelo flask assembly | 7 |
| Figure 3 | Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium plus 1 mM $S^{2-}$ (■ and □) and when supplemented with 100 µM $FeSO_4$ (● and O) | 13 |
| Figure 4 | Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) µM Cd excess. | 13 |
| Figure 5 | Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) µM Hg excess. | 14 |
| Figure 6 | Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) µM Pb excess or 100 µM Fe (◆ and ◇) excess. | 14 |
| Figure A-1 | Nephelo (side-arm) flask (left) and bottle assembly (right) used to cultivate thermophilic, strictly anaerobic methanogens | 23 |
| Figure A-2 | Single colonies of *M thermoautotrophicum* Marburg and *M thermoautotrophicum* ΔH as they appear on MS-Gelrite medium after 3 to 5 days incubation at 65°C | 24 |

## List of Abbreviations

**ATP** adenosine triphosphate
**CONWR** Crab Orchard Natural Wildlife Refuge
**EDTA** ethylenediaminetetraacetic acid
**EPA** Environmental Protection Agency
**GC** gas chromatography
**ICP-AES** inductively coupled plasma atomic emission spectrometer
**MESA** mercaptoethanesulfonic acid
**MOPS** 3-(*N*-morpholino)propane sulfonic acid
**O.D.** optical density
**THM** toxic heavy metals
**TRIS** tris-(hydroxymethyl)amino-methane

## Abstract

Methanogenic archaea were used as a model system for exploring the effects of toxic heavy metals (THM) on biological systems The three major goals were (*1*) to determine whether toxic heavy metals would affect the growth of methanogens, (*2*) to evaluate the ability of methanogens to solubilize inorganic precipitates of THM, and (*3*) to quantify the binding affinity of methanogen cell surfaces for THM

In summary, the methanogens studied were killed by > 25 μM $Cd^{2+}$, $Hg^{2+}$, or $Pb^{2+}$ The ICP-AES studies supported our findings of a small tendency for methanogens to solubilize cadmium, from cadmium sulfide, and a moderate ability to bind $Cd^{2+}$ at the cell surface Methanogens appeared to be capable of transporting THM in the environment

This work suggested that appropriate containment procedures for toxic wastes be developed Methanogens may be useful for incorporation into barrier technologies for the generation of clean water Finally, THM-resistant methanogens should be developed for their use in the degradation of toxic organic wastes that also contain THM to prevent the premature killing of the active biological agent

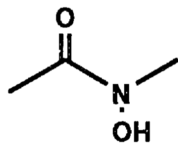
## 1.0 Introductory Material

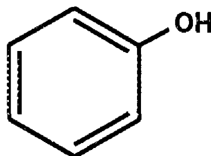
Southern Illinois possesses a large and wonderful collection of lakes and forests The Crab Orchard Natural Wildlife Refuge (CONWR) is one member of this group, but faces severe problems associated with contamination from organic and inorganic wastes caused by past industrial use of the area Several industries have posed potential problems with the contribution of toxic heavy metal (THM) species to the environment In this report, we will focus on cadmium, mercury, and lead, but it is important to understand that THM include silver, gold, chromium, copper, and many other alkali, alkali earth, transition and post-transition metals During and after World War II, explosives were produced at the CONWR sites However, the major sources of THM were derived from the processing and plating of metal parts Since 1947, when the War Department reassigned approximately 44,000 acres of land to the Department of the Interior and the U S Wildlife Service, we have been increasingly aware of the problems associated with THM Six specific sites within CONWR have been recognized to contain elevated levels of THM Of these six sites, five pose a threat to wildlife and in some instances to humans In 1987, the seriousness of the problem resulted in CONWR becoming the first national wildlife refuge to be designated a Superfund site Because THM can find their way into soils and subsurface locations, it is important to understand how THM species can be distributed in the environment by the action of soil-borne microorganisms

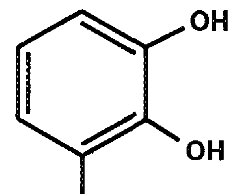
We have examined various aspects of THM contamination by studying the class of methane-producing archaea (Archaea are one of the three kingdoms of living things, the others are the Eubacteria and Eucarya) called methanogens (Jones, et al , 1987, Whitman, 1988) Methanogenic archaea synthesize methane ($CH_4$) from very simple feedstocks, including hydrogen ($H_2$) and carbon dioxide ($CO_2$), and are responsible for 80 to 90% of the $4 \times 10^{15}$ g of biogenic methane found in the atmosphere, which classifies them as an important energy source (Rogers & Whitman, 1991) In Europe, methanogenic consortiums form the heart of waste processing/energy cogeneration systems Much greater amounts of methane are produced by methanogens, but the bulk of the one-carbon product serves as a nutrient for methane-utilizing bacteria called methanotrophs (Schlegel, 1986) Interception of methane by methanotrophs attenuates the pronounced effect of methane to serve as a powerful greenhouse gas (Rogers & Whitman, 1991)

Our Earth has undergone many changes over the past billions of years (Zehnder & Stumm, 1988) For example, the waning of anoxic conditions lead to the introduction of dioxygen ($O_2$) and the evolution of aerobic life forms Although the process appears to have transformed a difficult situation into a compatible one, the process created a significant dilemma The oxygenated aqueous environments that resulted were characterized by iron-limiting conditions because of the inherently poor solubility of the ferric ion How then did aerobic life deal with this problem?

The specific uptake and utilization of iron (III) complexes by aerobic microorganisms has been documented (Crosa, 1989, Neilands, 1992, Winkelmann, et al , 1987) For example, *Escherichia coli* uses the siderophores (iron binders) aerobactin (a hydroxamic acid) and enterobactin (a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine) to facilitate the acquisition of $Fe^{3+}$ Ferric complexes with aerobactin and enterobactin are recognized by outer membrane receptors and then transported into the periplasm of the bacterium where the iron is apparently released by a combination of proton- and redox-mediated steps (Ecker, et al , 1986, Matzanke, et al , 1986) A survey of the types of siderophores used by various microorganisms reveals the persistent theme of hydroxamate, phenolate, and catecholate coordinating groups as is illustrated in Figure 1




Hydroxamic acid — Phenol group — Catechol group

Figure 1 The $Fe^{3+}$-binding groups represented by hydroxamic acid, phenol, and catechol

Members of the tribe *Proteeae*, which comprises *Proteus-Providencia-Morganella*, have been shown to utilize the siderophores described above and sequestering agents that result from the deamination of amino acids such as phenylalanine, tryptophan, and leucine (Drechsel, et al , 1993) Therefore, we should expect to discover a variety of complexation agents in the category of short chain and polar α-keto acids, such as phenylpyruvic acid, indolylpyruvic acid, α-ketoisocaproic acid, α-ketoisovaleric acid, and α-ketoadipic acid

By contrast, there are many unexplored questions involving metal ions such as $Co^{2+}$ and $Ni^{2+}$, which are important cofactors in methanogenesis (Jones, et al , 1987, Wackett, et al , 1989) Nickel uptake in *M bryantii* has been shown to be independent of membrane potential and intracellular ATP, but is somehow coupled to the movement of protons across the cell membrane (Jarrell & Sprott, 1982) However, in the acetogen *Clostridium thermoaceticum*, $Ni^{2+}$ uptake appears to be energy dependent and to involve both a low- and high-affinity transport system (Bryson & Drake, 1988, Lundie, et al , 1988) No evidence has been found for the production of catecholate- or hydroxamate-containing siderophores in methanogens (Hausinger, 1987) Methanogens may use different molecules to chelate or facilitate transport of its essential metal ions

Methanogens are strict anaerobes that can live as organized microbial mats (Belkin & Jannasch, 1989) in habitats such as bogs and waste heaps Methanogens can utilize a variety of sulfur sources and can grow quite well in high sulfide environments They usually require 0 1 to 3 mM x $10^{-3}$ M sulfide for optimal growth At the concentrations typically present in the culture medium, the sulfide should immediately combine with the reduced metal ions to form insoluble metal sulfides (as indicated in Table 1) unless there are mechanisms that kinetically inhibit the precipitation process

Table 1 Relationship of nutritional requirements for $Fe^{2+}$, $Co^{2+}$, and $Ni^{2+}$ in methanogens to metal sulfide solubilities

| Metal | Requirement (M) | Uptake (g/g cell dry weight) | $K_{sp}$[a] | Solubility[b] (M) |
|---|---|---|---|---|
| $Fe^{2+}$ | 3 to 8 x $10^{-4}$[c] | 1 to 3 x $10^{-3}$ | 3 7 x $10^{-19}$ | 1 x $10^{-15}$ |
| $Co^{2+}$ | 1 to 5 x $10^{-6}$ | 1 to 12 x $10^{-5}$ | 3 0 x $10^{-26}$ | 1 x $10^{-22}$ |
| $Ni^{2+}$ | 1 to 5 x $10^{-6}$ | 2 to 18 x $10^{-5}$ | 1 4 x $10^{-24}$ | 5 x $10^{-21}$ |

[a] $K_{sp} = [M^{2+}][S^{2-}]$

[b] solubility at 3 x $10^{-4}$ M sulfide; (Whitman, 1988)

[c] ferrous salts are usually added to a concentration of 1 to 2 x $10^{-5}$ M, (Balch, et al , 1979)

Indeed, at the indicated sulfide concentration, the corresponding metal ion solubilities are predicted to be extremely small, precluding nutritionally relevant concentrations of these metal ions As such, methanogens scavenge $Fe^{2+}$, $Co^{2+}$, and $Ni^{2+}$ from stainless steel fermentor surfaces when not supplied with exogenous sources of metal ions Therefore, it would be reasonable to predict the existence of a specific uptake and transport system, one featuring a secreted low molecular weight molecule and a cell wall-associated polymeric component (Doyle, 1989, Ferris, 1989)

In the absence of high levels of sulfide, is it possible to use other oxidation states of sulfur to

drive cellular metabolism (Daniels, et al , 1986) Sulfur spans oxidation states from +6 such as that found in the sulfate anion ($SO_4^{2-}$), to +4 in sulfite ($SO_3^{2-}$), +2 in trithionate ($^-O_3SSSO_3^-$), 0 in elemental sulfur (S), and -2 in disulfide ($S^{2-}$) Interconversions of these species are well known and some involve the production of free sulfide The thermophilic strains that we employ are able to process thiosulfate, sulfite, and sulfide, but the fundamental biochemistry is not understood and would therefore be an attractive and worthwhile undertaking

The ability of microorganisms to survive and thrive in the presence of THM has attracted much attention (Collins & Stotzky, 1989) THM are remarkable in that there have evolved a variety of protection mechanisms (Silver, et al , 1989, Silver & Walderhaug, 1992) We can define the following four specific types of approaches for THM processing (1) *volatilization*, conversion of Hg(II) to $(CH_3)_2Hg$, (2) *extracellular complexing and precipitation*, coupling of sulfate reduction to sulfide and the formation of insoluble precipitates, (3) *binding to cell surfaces*, teichoic acids and phospholipopolysaccharides, and (4) *extracellular transport*, Cd export through coupling to membrane-associated ATPases (Brierley, et al , 1989)

This work was undertaken to identify and characterize some of the fundamental interactions between THM and methanogens Few studies have been reported on the role of THM in anaerobic metabolism Thus, our studies are important in defining some of the novel features of methanogen biochemistry and physiology as they apply to bioremediation and biostabilization technologies

## 2.0 Methodology

**2.1 Strains and medium.** Methanogen strains *Methanobacterium thermoautotrophicum* Marburg and *M thermoautotrophicum* ΔH were obtained from David R Boone of the Oregon Collection of Methanogens (Beaverton, OR) and Mary F Roberts of Boston College, respectively We used the MS medium described by Boone and coworkers (Boone, et al , 1989) but increased the buffer concentration to 0 1 M Liquid (MS) medium contained the following per liter 8 4 g $NaHCO_3$, 2 g trypticase peptone (Becton Dickinson Microbiology Systems, Cockeysville, MD), 2 g Bacto yeast extract (Difco Laboratories, Detroit, MI), 1 g $NH_4Cl$, 0 8 g $K_2HPO_4$, 1 g $MgCl_2$ $6H_2O$, 0 4 g $CaCl_2$ $2H_2O$, 0 001 g resazurin (Sigma Chemical Company, St Louis, MO) The medium was adjusted to ~ pH 7 1 with 6 M HCl Before use, the medium was completed with the addition of the following three reagents (*1*) 1 ml of a 1000-fold concentrate of trace minerals, which contained per 100 ml 0 5 g $Na_2EDTA$ $2H_2O$, 0 15 g $CoCl_2$ $6H_2O$, 0 1 g $FeSO_4$ $7H_2O$, 0 21 g $ZnSO_4$ $7H_2O$, 0 14 g $AlK(SO_4)_2$, 0 03 g $Na_2WO_4$ $2H_2O$, 0 02 g $CuCl_2$ $2H_2O$, 0 02 g $NiCl_2$ $6H_2O$, 0 01 g $Na_2SeO_4$, 0 01 g $H_3BO_3$, and 0 01 g $Na_2MoO_4$ $2H_2O$ adjusted to pH 7 1, then the trace minerals concentrate was mixed with 0 1 g $MnSO_4$ $H_2O$, (*2*) 10 ml of sterile 0 3 M mercaptoethanesulfonic acid (MESA, Sigma Chemical Company, St Louis, MO), and (*3*) 1 ml of a sterile 2 M stock (9 6 g $Na_2S$ $9H_2O$ in 20 ml $H_2O$) $Na_2S$, which was prepared and stored under anaerobic conditions Solutions were sterilized by filtration through a sterile membrane unit (Nalgene Labware Div , Nalgene/Sybron Corp , Rochester, NY) or in an autoclave for 5 minutes at 15 psi and 121°C Solid medium contained liquid (MS) medium plus Gelrite (Schweizerhall, Inc , South Plainfield, NJ) at 8 g per liter

**2.2 Bottle assembly.** The culture bottle assembly components (Figure A-1) were obtained from local distributors of scientific equipment and supplies or directly from the indicated manufacturer 125 ml to 1000 ml media bottles (Wheaton Industries, Millville, NJ), black plastic screw caps (33-430 thread) with open top (Wheaton Industries, Millville, NJ), and solid black rubber stoppers, #3 (< 1 liter bottle sizes) or #4 (1 liter bottle size)

**2.3 Nephelo flask and stopper assembly.** The 125- and 250-ml nephelo flasks (Kimble/Kontes, Vineland, NJ) were fitted with #5 or #7 solid black rubber stoppers, which were wired in place with a copper retainer that was fashioned for reversible release (Figure A-1) A ring of 18 or 20 gauge copper wire was fastened about the neck of the flask and two cross wires were strung over the top of the stopper to keep it in place The cross wires were positioned ~90 degrees to each other and anchored to the neck wire The retainer was removed by loosening the neck wire with a quarter-turn twist, a process that was repeated 6 times before the wire degraded The flask assembly was pressurized from 15 to 20 psi for routine incubations

**2.4 Cultivation of methanogens.** Caution Carefully examine each bottle for defects or signs of stress in the glass Do not use bottles with nicks or cracks Wrap intact bottles with rubber electrical tape to limit glass fragments in the event of an explosion Infrequent explosions have occurred during sterilization in the autoclave

The following detailed procedure was applied for the isolation of single colonies of *M thermoautotrophicum*

1 Sterile stocks of MS medium were prepared by dissolving the mineral salts and hydrolysates in purified $H_2O$ The pH of the medium was adjusted to ~ 7 1 with 6 M HCl and then quickly poured into storage bottles, which were then sealed with a rubber stopper and solid top screw cap (Figure A-1) These solutions were sterilized in an autoclave for 15 to 20 minutes at 15 psi and 121°C

2 In a typical application, 40 ml of freshly prepared nonsterile or sterile medium was added to a 125 ml bottle This was followed by the addition of 0 4 ml of 100-fold MESA, 0 04 ml of 1000-fold trace minerals, and 0 32 g of Gelrite to the bottle The bottle was sealed with a wetted rubber stopper and fitted with an open top screw cap to retain the rubber stopper during sterilization

3 The mixture was deoxygenated on a metal pressure/vacuum line by cycling 5 times between vacuum for 5 minutes and 20% $CO_2$-80% $H_2$ at 20 psi for 1 minute The internal bottle pressure was lowered to 10 psi with 2 brief cycles between vacuum and 100% $N_2$

4 The bottles were transferred to a Coy anaerobic chamber where care was taken to remove the caps and loosen the rubber stoppers The medium was mixed with 0 04 ml of 2 M $Na_2S$ $9H_2O$, the assemblies were sealed, and the solution allowed to change color from pink to light yellow This usually took 15 to 30 minutes at room temperature

5 The bottles were removed from the Coy chamber and the Gelrite mixture was dissolved (or dissolved and sterilized) by autoclaving for 15 to 20 minutes at 15 psi and 121°C

6 While the bottles were hot and the solution was still molten, a slant was formed by inclining the bottles against a test tube rack or some other convenient device The angle was such that the solution extended from the bottom to the top of the bottle

7 The medium was slowly cooled for a minimum of 30 minutes with the formation of a smooth surface

8 In order to remove as much residual water as possible, the bottles were transferred to the Coy chamber and the caps and rubber stoppers were removed from each bottle Because we have an incubator placed within the anaerobic chamber, the solid medium was allowed to sweat for 2 to 3 days at > 45°C (room temperature was used for a longer period of time) Any collected $H_2O$ was decanted into a beaker and the bottles were resealed for storage

9 A fresh methanogen culture was prepared and a sample of cells was streaked, picked, or spread on the surface of the solid medium Cells were transferred with an inoculating loop, a sterile toothpick or wood applicator, or an automatic pipetter Larger volumes were spread using a modified glass rod

10 The bottles were sealed and pressurized to 30 psi with 20% $CO_2$-80% $H_2$ and incubated in an oven set at 65 to 70°C We exchanged the gas phase once every day or every two days as necessary

11 Single colonies appeared within 2 to 5 days The anaerobic culture may be stored at room temperature for a minimum of 3 to 4 months

For liquid cultures, we followed steps 1 through 4 The Gelrite was omitted in step 2, 10 % inocula were used to prepare fresh cultures in step 9, and the head space was exchanged with 20% $CO_2$-80% $H_2$ every 12 hours as described in step 10 Cultures may be stored at room temperature for 6 months or longer with infrequent gas exchange, but we have found it useful to prepare fresh cultures every three to four months This can be done using serial cultures or using frozen glycerol stocks

**2.5 Growth studies.** A 10% inoculum from a starting culture of *M thermoautotrophicum* Marburg or *M thermoautotrophicum* $\Delta$H was transferred to 20 ml of fresh MS medium in 125 ml nephelo flasks The flasks were pressurized to 20 psi with 20% $CO_2$-80% $H_2$ and incubated with rapid shaking at 65°C The gas head space was replaced with fresh gas mixture every 12 hours Light scattering was followed with a Klett-Summerson colorimeter fitted with a red (#66) filter In order to determine the effects of essential and toxic metals on the sulfide-based growth of methanogens, we found it necessary to dissolve the mineral precipitates accompanying the cultures with a small volume of concentrated HCl A series of 5 ml cultures were prepared in Balch tubes and allowed to grow for 12 to 20 h until we measured an $A_{600}$ of approximately 0 5 to 0 7 on a 1 ml acidified sample (taken within the Coy chamber) The tubes were transferred to the Coy chamber and a calculated excess of sterile stocks of 0 5 M $Fe^{2+}$, $Cd^{2+}$, $Hg^{2+}$, or $Pb^{2+}$ were added to the samples The cultures were repressurized and allowed to grow for an additional 24 to 48 h, after which time the final $A_{600}$ was measured The viability of the resulting cultures were determined by subculturing the cells in fresh THM-free MS medium

**2.6 Analytical determination of THM by ICP-AES.** Elemental analyses were obtained using a Leeman Labs 2 5 Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES), including an echelle monochromator and Plasma Spec software for rapid sequential multielement analysis In general, analyses were conducted for a single element with each set of samples, however Samples consisted of liquid solutions of the growth medium containing some amount of Cd, or solids obtained from centrifugation of growth media The latter samples generally consisted of bacterial cells Although individual cells are small enough to be directly aspirated into the ICP-AES, the cells normally aggregate (flocculate) into larger clusters which tend to clog the nebulizer of the ICP sample introduction system Consequently, a digestion procedure

involving dissolution with sulfuric acid was required for these samples

Elemental analyses were conducted in accordance with EPA Method 6010, SW-846, 3'rd Edition, 1986 Blank levels were routinely determined for the growth medium and various reagents Matrix effects were evaluated by spiking the various matrices, followed by determination of recoveries No significant matrix effects were identified for the sample types studied within this work All determinations were made with reference to calibration standards prepared in accordance with standard protocols, that is, sub-ppm standards were prepared on the day of use from higher concentration standards prepared at least weekly All measurements were done at least in triplicate Solutions were prepared using analytical reagent grade or higher starting materials Dilutions were performed using deionized, distilled water containing 1% nitric acid for solution stabilization

**2.7 Solubilization of sulfide matrices.** Cadmium sulfide was prepared by mixing a slight (5%) stoichiometric excess of $Na_2S\ 9H_2O$ to $Cd(acetate)_2$ The resulting yellow precipitate was washed with water and ethanol and dried at room temperature for several days One millimole of CdS was mixed with cultures of methanogens, which were allowed to grow for several days The cells and other solids were separated from the culture medium by centrifugation at 3,000 to 7,000 x *g* for 20 min All solids were washed with fresh culture medium and the washings were combined or handled individually For selected studies, the centrifuge tubes and glass growth bottles were washed and the washings saved for analyses

**2.8 Whole cell binding of THM.** Sets of large scale (3 L) cultures of methanogens were prepared in MS medium After 3 to 5 days of growth, the cells were harvested by centrifugation at 7,000 x *g* in a Sorvel RC-5B preparative centrifuge The cells were washed several times with TE (10 mM TRIS, 0 1 mM EDTA) pH 8 buffer and then with 1 mM EDTA pH 8 A diluted cell suspension was prepared and a series of samples were combined with a standard analytical Cd stock (typically ~100 ppb) After incubation of the samples at 65°C for 1 h, the cells were separated from the supernatant by high speed centrifugation (~100,000 x *g*) in a Beckman L80 ultracentrifuge Samples of the supernatants and cells were analyzed by ICP-AES

## 3.0 Results

**3.1 Cultivation of thermophilic methanogens.** One of the most troublesome aspects of high temperature cultivation of strict anaerobes was preparation and storage of medium, either liquid or solid based formulations We have combined several concepts in our development of a simple method that allowed continuous monitoring of single colonies (Figure A-2) and routine production of liquid cultures (Figure A-1) (Balch, et al , 1979, Balch & Wolfe, 1976, Boone, et al , 1989, Harris, 1985, Hermann, et al , 1986, Miller & Wolin, 1974, Olson, 1992) It was useful to keep carbonate-based buffers in a sealed container in order to minimize the release of $CO_2$ and the subsequent change in pH Freshly autoclaved carbonate-based medium has a precipitate that redissolves upon standing at room temperature in one to two days Solutions that contained resazurin were initially blue and became pink after sterilization, which reflected a partial reduction of the redox dye by the organic components of the medium Sterile stocks, rather than freshly prepared nonsterile solutions, may be used to prepare growth medium Carbonate-based buffers were replaced by 0 1 M TRIS or MOPS buffers with similar success It was important to grow methanogens in liquid culture under the same conditions as found in the solid medium in order to achieve more rapid growth of colonies (Figure A-2) In fact, we have used static growth (Daniels, et al , 1986) to condition strains to changes in buffer composition or sulfur sources We found that the sulfur source could be replaced by a combination of 100 µM $S^{2-}$ and 4 mM $S_2O_3^{2-}$ or 0 5 mM $SO_3^{2-}$ without affecting growth of the organism As Figure 2 illustrates, cultivation in nephelo flasks provided a convenient method for determining growth parameters

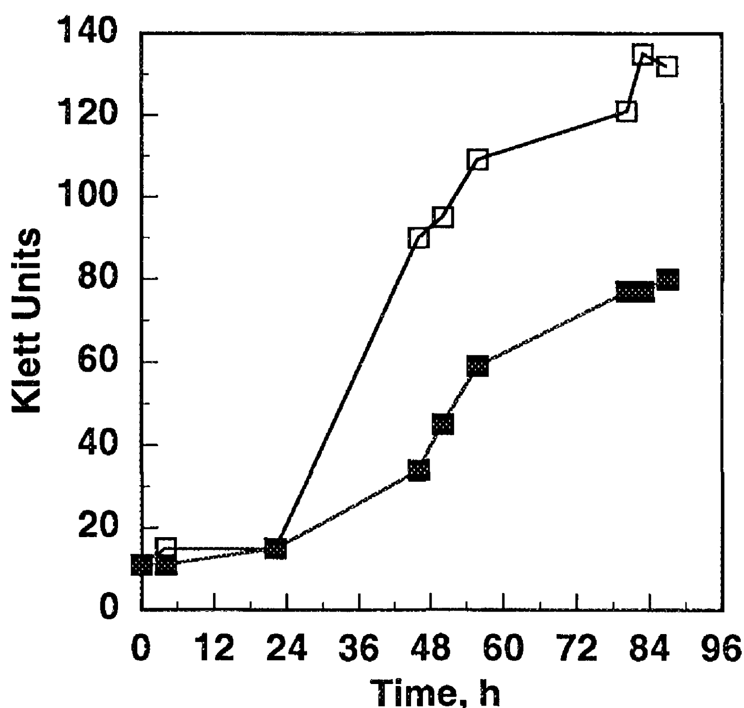



Figure 2 Typical growth (increase in Klett units) of *M thermoautotrophicum* ΔH (■) and *M thermoautotrophicum* Marburg (□) as measured with the nephelo flask assembly

Bottles sizes varied from 60 ml serum bottles, which required a hand crimper, thick rubber septa, and aluminum seals, to a 1 L storage bottle, which used a #4 rubber stopper Rubber stopper presented the greatest problem related to maintenance of anaerobic conditions Old used black rubber stoppers were replaced by new ones after approximately 12 uses in

order to insure tight seals in the bottles It was important to secure the rubber stoppers in place with an aligned cap that grabbed the glass threading on the bottle and allowed approximately 3/8 to 1/4 in of the rubber stopper to extend above the lip of the bottle This allowed for easy removal of the rubber stopper during manipulations in the Coy chamber and for sufficient sealing during sterilization

Solid medium was dried at temperatures greater than 45°C to reduce the amount of water that accumulated on the surface during incubation An alternative method was to include a small amount of sterile Drierite in the bottom of the bottle Bottles were typically incubated with the Gelrite side up (normal inverted position for petri dishes) to minimize these problems

Single colonies (Figure A-2) appeared on MS-Gelrite medium within 2 to 5 days at 65°C when freshly grown cultures were used to inoculate the surface Otherwise, it took substantially more time for the appearance of single colonies from older cultures Because these were thermophilic microorganisms, there was minimal opportunity for contamination by other organisms

Proper application of sterile anaerobic technique has provided an improved method for the routine cultivation of thermophilic methanogens The incorporation of Gelrite extends the usefulness to aerobic and anaerobic hyperthermophiles such as *Pyrococcus furiosus* We have used these bottle and flask techniques to understand how THM interact with methanogens and this forms the bulk of our report

**3.2 Solubilization of CdS.** A series of experiments were performed to assess the ability of active methanogenic cultures to solubilize CdS, a prototypical inorganic THM matrix Tables 2 through 6 summarize the distribution of Cd species among soluble and insoluble forms We determined appropriate backgrounds for the absorption and adsorption of Cd onto a variety of surfaces Our largest errors were initially in the determination of Cd present in the liquid phase As cells were washed in MS medium or buffers, they became cleaner, less adherent to surfaces, and thus more resistant to packing (pelleting) at moderate centrifugal forces Early studies were dominated by a high Cd background due to carry over of particulates Overall the data were consistent with a slight ability of *M thermoautotrophicum* ΔH and *M thermoautotrophicum* Marburg to solubilize CdS The level of Cd was relatively low and presented no threatening environment to the microorganism (see growth section below) However, should methanogenic cultures reach high cell densities, there presents a possibility of biological transport of THM in the environment

Table 2 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation

Solids

-after dissolution in $H_2SO_4$

| | [Cd] in 100 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 58 3 | 5 83 |
| *M t* ΔH + Cd | 45 3 | 4 53 |
| *M t* Marburg + Cd | 53 2 | 5 32 |

Liquids

| | [Cd] in 50 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 8 00 | 0 400 |
| *M t* ΔH + Cd | 6 15 | 0 308 |
| *M t* Marburg + Cd | 0 611 | 0 036 |

Mass Balance

| | Solids, mg | Liquids, mg | Total, mg | Cd added, mg[a] | % Recovery[b] |
|---|---|---|---|---|---|
| MS medium + Cd | 5 83 | 0 400 | 6 23 | nd[c] | nd |
| *M t* ΔH + Cd | 4 53 | 0 308 | 4 84 | nd | nd |
| *M t* Marburg + Cd | 5 23 | 0 036 | 5 27 | nd | nd |

[a] calculated from the amount of CdS added [b] % recovery = (total/Cd added) x 100 [c] nd, not determined

Table 3 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation Multiple solids washings

**Solids**

| | [Cd] in 100 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | nd[a] | nd |
| *M t* ΔH + Cd | 16 9 | 1 69 |
| *M t* Marburg + Cd | 35 4 | 3 54 |

**Liquids *supernatant***

| | [Cd] in 20 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 2 90 | 0 0580 |
| *M t* ΔH + Cd | 2 89 | 0 0578 |
| *M t* Marburg + Cd | 1 67 | 0 0334 |

**Liquids *washing #1***

| | [Cd] in 5 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 6 78[b] | 0 136 |
| *M t* ΔH + Cd | 3 09 | 0 0155 |
| *M t* Marburg + Cd | 1 01 | 0 00505 |

**Liquids *washing #2***

| | [Cd] in 5 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | nd | nd |
| *M t* ΔH + Cd | 2 59 | 0 0130 |
| *M t* Marburg + Cd | 3 78 | 0 0189 |

**Mass Balance**

| | Solids, mg | Liquids, mg | Total, mg | Cd added, mg[c] | % Recovery[d] |
|---|---|---|---|---|---|
| MS medium + Cd | nd | 0 194 | 0 194 | 2 3 | >8 |
| *M t* ΔH + Cd | 1 69 | 0 0863 | 1 776 | 2 3 | 77 |
| *M t* Marburg + Cd | 3 54 | 0 0574 | 3 597 | 2 3 | 156 |

a nd, not determined
b in 20 ml
c calculated from the amount of CdS added
d % recovery = (total/Cd added) x 100

Table 4 Methanogen-mediated solubilization of CdS as analyzed by ICP-AES after low speed centrifugal separation Contributions from glass bottles and solids washings

Solids

| | [Cd] in 100 ml, μg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 20 7 | 2 07 |
| *M t* ΔH + Cd | 20 5 | 2 05 |
| *M t* Marburg + Cd | 20 1 | 2 01 |

Liquids *supernatant*

| | [Cd] in 50 ml, μg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 0 127 | 0 00635 |
| *M t* ΔH + Cd | 0 07 | 0 0035 |
| *M t* Marburg + Cd | 0 034 | 0 0017 |

Liquids *solids washing*

| | [Cd] in 25 ml, μg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 0 183 | 0 00458 |
| *M t* ΔH + Cd | 0 147 | 0 00368 |
| *M t* Marburg + Cd | 0 142 | 0 00355 |

Liquids *growth bottle washing*

| | [Cd] in 20 ml, μg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 0 179 | 0 00358 |
| *M t* ΔH + Cd | 0 230 | 0 0046 |
| *M t* Marburg + Cd | 0 401 | 0 00802 |

Mass Balance

| | Solids, mg | Liquids, mg | Total, mg | Cd added, mg[a] | % Recovery[b] |
|---|---|---|---|---|---|
| MS medium + Cd | 2 07 | 0 0145 | 2 085 | 2 3 | 92 |
| *M t* ΔH + Cd | 2 05 | 0 0118 | 2 062 | 2 3 | 90 |
| *M t* Marburg + Cd | 2 01 | 0 0133 | 2 023 | 2 3 | 88 |

[a] calculated from the amount of CdS added
[b] % recovery = (total/Cd added) x 100

Table 5 Summary of methanogen-mediated solubilization of CdS as analyzed by ICP-AES after high speed centrifugation separation.

Solids

| | [Cd] in 100 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 25.0 | 2 5 |
| *M t* ΔH + Cd | 20 0 | 2.0 |
| *M t.* Marburg + Cd | 19 0 | 1 9 |

Liquids *supernatant*

| | [Cd] in 100 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 0 65 | 0 065 |
| *M t* ΔH + Cd | 0 01 | 0 001 |
| *M t* Marburg + Cd | 0 10 | 0 010 |

Liquids *solids washing*

| | [Cd] in 50 ml, µg/ml | Total Cd, mg |
|---|---|---|
| MS medium + Cd | 0 12 | 0.006 |
| *M t* ΔH + Cd | 0 08 | 0 004 |
| *M t* Marburg + Cd | 0 10 | 0 005 |

Mass Balance

| | Solids, mg | Liquids, mg | Total, mg | Cd added, mg[a] | % Recovery[b] |
|---|---|---|---|---|---|
| MS medium + Cd | 2 5 | 0.071 | 2 571 | 2.33 | 110 |
| *M t.* ΔH + Cd | 2 0 | 0.005 | 2 005 | 2 33 | 86 |
| *M t* Marburg + Cd | 1 9 | 0 006 | 1.906 | 2.72 | 70 |

[a] calculated from the amount of CdS added

[b] % recovery = (total/Cd added) x 100.

Table 6 Summary of CdS dissolution in MS medium

Solids

| | [Cd] in 100 ml, μg/ml | Total Cd, mg |
|---|---|---|
| Sample #1 | 23 2 | 2 32 |
| Sample #2 | 20 2 | 2 02 |
| Sample #3 | 25 7 | 2 57 |
| Sample #4 | 24 6 | 2 46 |
| Sample #5 | 25 5 | 2 55 |

Liquids *supernatant*

| | [Cd] in 50 ml, μg/ml | Total Cd, mg |
|---|---|---|
| Sample #1 | 0 28 | 0 0126 |
| Sample #2 | 0 27 | 0 012 |
| Sample #3 | 0 36 | 0 0162 |
| Sample #4 | 0 55 | 0 0248 |
| Sample #5 | 0 23 | 0 104 |

Liquids *solids washing*

| | [Cd] in 25 ml, μg/ml | Total Cd, mg |
|---|---|---|
| Sample #1 | 3 521 | 0 070 |
| Sample #2 | 2 581 | 0 052 |
| Sample #3 | 2 221 | 0 044 |
| Sample #4 | 2 051 | 0 041 |
| Sample #5 | 3 911 | 0 078 |

Mass Balance

| | Solids, mg | Liquids, mg | Total, mg | Cd added, mg[a] | % Recovery[b] |
|---|---|---|---|---|---|
| Sample #1 | 2 32 | 0 0826 | 2 402 | 2 917 | 82 |
| Sample #2 | 2 02 | 0 0640 | 2 084 | 3 020 | 69 |
| Sample #3 | 2 57 | 0 0602 | 2 630 | 3 204 | 82 |
| Sample #4 | 2 46 | 0 0688 | 2 529 | 3 297 | 77 |
| Sample #5 | 2 55 | 0 0514 | 2 601 | 3 022 | 86 |

[a] calculated from the amount of CdS added
[b] % recovery = (total/Cd added) x 100

**3.3 Growth studies.** Methanogens grown on the semidefined MS medium were able to attain an $A_{600}$ of ~ 1 to 1 5 (Figure 3) We observed similar behavior in cells that were cultivated in the presence of 100 μM excess $Fe^{2+}$ (Figure 3) Should $Fe^{2+}$ have effectively removed all the available $S^{2-}$ from the medium and created a sulfur-limited environment, cell growth would have ceased and we would have measured insignificant increases in the $A_{600}$ after the addition of $Fe^{2+}$ stock By contrast, when cultures were supplemented to 50 μM with THM such as $Cd^{2+}$ (Figures 4), $Hg^{2+}$ (Figure 5), and $Pb^{2+}$ (Figure 6), the growth of the cultures ceased and the resulting cells were unable to grow when placed in fresh medium, suggesting that THM acted to kill methanogens Similar results were observed with higher concentrations of THM (results not shown) The minimum net amount of THM was determined to be ~ 25 μM for $Cd^{2+}$, $Hg^{2+}$, and $Pb^{2+}$ (results not shown), when cells were provided with 1 to 2 mM $S^{2-}$ Note that we found it necessary to conduct these studies in the presence of sufficient $S^{2-}$ to generate adequate growth and thus needed to add a small stoichiometric excess of $Cd^{2+}$, $Hg^{2+}$, or $Pb^{2+}$ to achieve the indicated levels As can be observed in Figure 5, we needed to apply careful technical skill to address $Hg^{2+}$ growth inhibition because of a persistent precipitation problem that interfered with the acid solubilization employed to minimize adventitious light scattering We replaced the S source with a combination of $S^{2-}$ and $SO_3^{2-}$ or $S_2O_3^{2-}$ and found that THM were still toxic to the cells at similar concentrations The primary difference in the approaches is in the requirement of small excesses of THM with $S^{2-}$ because of the precipitation of metal sulfides

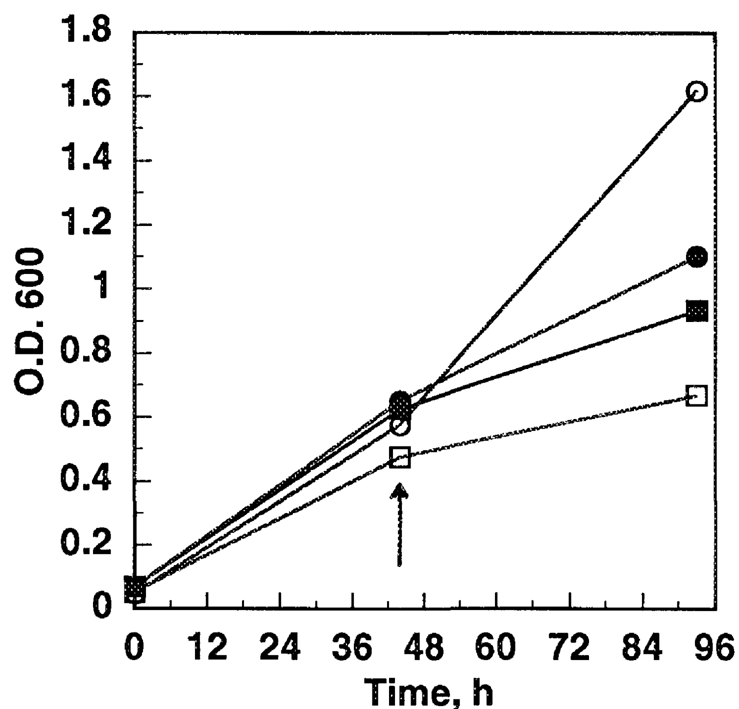



Figure 3 Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium plus 1 mM $S^{2-}$ (■ and □) and when supplemented with 100 μM $FeSO_4$ (● and O) Arrow indicates time of Fe addition

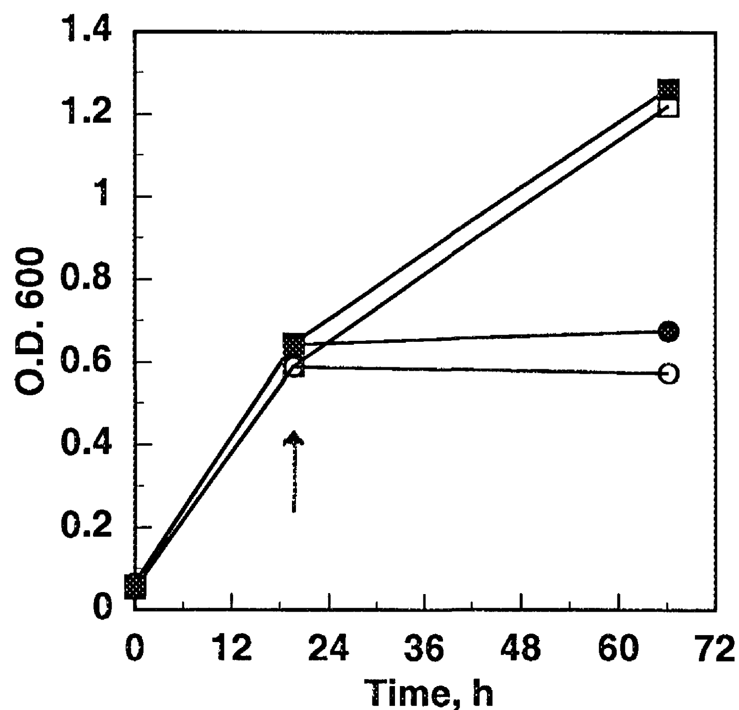



Figure 4 Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) μM Cd excess Arrow indicates time of Cd addition

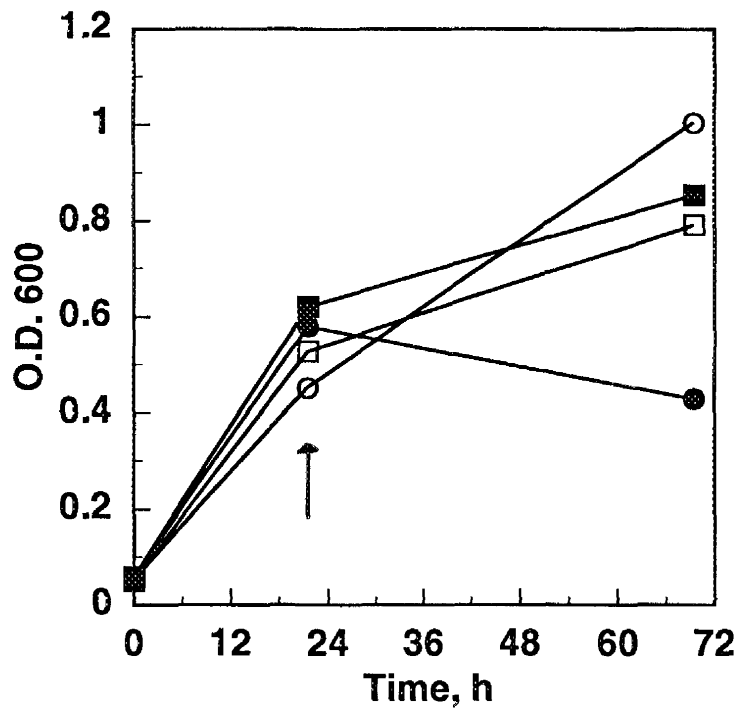



Figure 5 Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) µM Hg excess Arrow indicates time of Hg addition

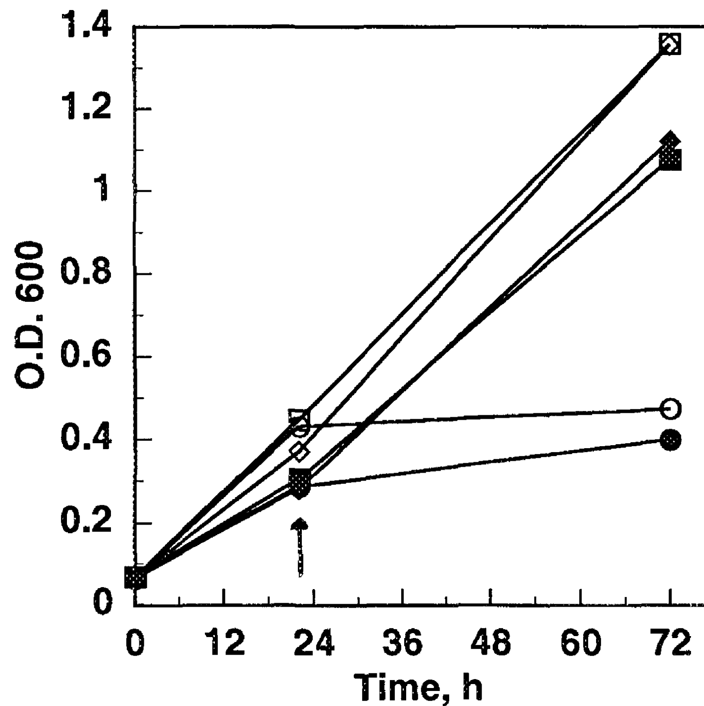



Figure 6 Growth of *M thermoautotrophicum* ΔH (closed symbols) and *M thermoautotrophicum* Marburg (open symbols) in MS medium supplemented with 1 mM $S^{2-}$ and 0 (■ and □) and 50 (● and O) µM Pb excess or 100 µM Fe (◆ and ◇) excess Arrow indicates time of Pb addition

**3.4 THM-binding by methanogens.** Because methanogens that were cultivated in the presence of ~25 µM $Cd^{2+}$, $Hg^{2+}$, or $Pb^{2+}$ were unable to grow, we reasoned that THM were able to enter growing cells and render them nonviable As a preliminary step to killing, THM would have to bind to the outer surface of the microorganism prior to entering the cell The effective binding interaction could be weak to very strong, but chances of detecting these interactions were optimized by selecting an extremely sensitive technique Well-washed cells of *M thermoautotrophicum* ΔH and *M thermautotrophicum* Marburg were incubated in the presence of a standardized analytical Cd stock and then separated into an aqueous and solid phase by high speed centrifugation The results (Table 7) of these studies support a significant but moderate binding interaction

Table 7 Binding of $Cd^{2+}$ to washed whole cells of *M thermoautotrophicum* ΔH and *M thermoautotrophicum* Marburg

| | $Cd^{2+}$ Concentration, ppb | |
|---|---|---|
| Sample | Trial #1 | Trial #2 |
| $Cd^{2+}$ Stock | 138 | 96 |
| *M t* ΔH supernatant | 86 | 85 |
| *M t* ΔH cells | nd[a] | 13 |
| *M t* Marburg supernatant | 110 | nd |
| EP $H_2O$ | 3 | nd |

[a] nd, not determined

## 4.0 Discussion

Methanogens, as a representative member of the anaerobic microbial world, were sensitive to the presence of THM in their local environment Under controlled laboratory conditions in the presence of 1 to 2 mM $S^{2-}$, we found that the thermophilic methanogens *M thermoautotrophicum* ΔH and *M thermoautotrophicum* Marburg contributed little to the solubilization of a typical THM matrix and that the composition of the growth medium served a more important role in freeing up the metal ion However, if $S^{2-}$ levels were decreased and a suitable sulfur source was substituted, solubilization of THM matrices could become significant, especially at lower pH conditions of growth

Several members of the prokaryotic kingdom possess an efficient system to handle the presence of THM (Silver, et al , 1989, Silver & Walderhaug, 1992) An impressive example of this is the selective export machinery of *Alcaligenes eutrophus*, which fails to discriminate essential from toxic metals during import to the cell, but selectively exports $Cd^{2+}$ from the cytosol to the surrounding fluid by the action of an ATPase The two thermophilic methanogens lack this type of protection and thus are subject to the harmful effects of Cd, Hg, and Pb Low levels of THM, ~ 25 μM for $Cd^{2+}$, $Hg^{2+}$, or $Pb^{2+}$, were sufficient to inhibit growth and to eliminate viable cells that could be subcultured in fresh THM-deficient medium We found that studies on solid medium supplemented with large excesses of $Cd^{2+}$ yielded viable cells, suggesting to us that surfaces and microbial mat communities may play an important role in mediating the harmful effects of THM

Because THM arrest cell growth and probably kill them, specific binding of THM to methanogens was studied Our results support a physical association of THM with outer surfaces of methanogens, probably through some combination of polysaccharide and protein complex formation (Brierley, et al , 1989, Doyle, 1989, Ferris, 1989) The details of these specific interactions are currently under investigation in our laboratories

## 5.0 Conclusions

Methanogenic archaea define a class of microorganisms that are important in the global ecology of carbon and sulfur cycles and useful in the degradation of harmful organic compounds The interaction of these life forms with THM has dramatic consequences, which we may need to consider in the development of biotechnologies devoted to waste management Methanogens are sensitive to THM above a threshold value of ~>25 µM Although methanogens appear to have a minor role in the solubilization of THM matrices, they are well adapted to transport THM ions through general binding capabilities of their outer cell surfaces

## 6.0 Recommendations

Microorganisms are intimately involved in environmental processes The accumulation of toxic materials presents a challenge in the design and application of biological as well as non-biological approaches to stabilization and remediation of affected sites (Lowe, et al , 1993, Springael, et al , 1993, van der Meer, et al , 1992) For biological methodologies, we must be concerned with the specific interactions of THM with the microorganism and the effects of these agents on their viability For non-biological methodologies, it will be important to consider the structural integrity of the material in light of potential interactions with processing microorganisms

Our results from the past 16 months of studies clearly indicate that methanogens present a small contribution to the solubilization or release of THM from stabilized matrices However, we should not rejoice in this fact because, under the appropriate conditions, methanogens are well suited for the distribution of THM throughout the environment, as may occur through classical microbial-soil routes The moderate binding of THM by methanogens demonstrates their potential role as carriers, which could lead to increased problems Certainly, we must be aware of proper containment procedures, especially those that can prevent the introduction of methanogens (or other anaerobes) into THM sites

In a positive light, methanogens may be useful for incorporation into barrier technologies that have been developed with algae and fungi for the removal of inorganic metals from ground waters (Brierley, et al , 1989) One advantage of methanogen use stems from their utility as energy producers ($CH_4$ production), which would reduce the net cost of cultivating large quantities of the microorganism

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## Glossary

**$\alpha$-keto acid** molecule with carbonyl group adjacent to carboxylic acid functionality

**acetogen** microorganism that excretes acetate upon metabolism of $CO_2$ and $H_2$

**aerobactin** hydroxamate-containing siderophore

**alkali earth element** group IIa elements, which include magnesium, calcium, strontium, barium, and radium

**alkali element** group Ia elements, which include lithium, sodium, potassium, rubidium, and cesium

**anaerobic** metabolism in the absence of dioxygen

**anoxic** in absence of dioxygen

**aerobic** metabolism in presence of dioxygen

**aquifer** system of water source and sink

**archaea** kingdom of life, shares properties with bacteria and eucarya

**autoclave** device that sterilizes materials

**bacteria** kingdom of life, characterized by small size and lack of nucleus and organelles

**biogenic** of biological origin

**bioremediation** biological-based clean up of toxic materials

**biostabilization** biological-based containment of toxic materials

**catechol** 2,3-dihydroxybenzene

**cofactor** small molecule or ion that assists a protein or enzyme

**cogeneration** formation of two products from one metabolic pathway

**complexation** covalent-like association between elements

**deamination** replacement of amino functional group with hydrogen atom

**enterobactin** catecholate-containing siderophore

**essential** useful and necessary to living organism

**eucarya** kingdom of life, characterized by separate organelles and enclosed nucleus

**exogenous** outside source

**thermophile** microorganism that thrives at temperatures from 60 to 80°C

**hydroxamic acid** hydroxylated nitrogen atom adjacent to carbonyl group

**hyperthermophile** microorganism that thrives at temperatures greater than 80°C

**inoculum** a sample of growing cells that is used to initiate the growth of a larger culture of the same microorganism

**inorganic** molecule based upon non-carbon elements

**mat** an organized community of microorganisms similar to a biofilm

**membrane potential** voltage difference across a semipermeable double layer

**metabolism** the breakdown and buildup of complex molecules

**methanogen** microorganism that metabolizes $CO_2$ and $H_2$ to produce $CH_4$ as a method of obtaining ATP

**methanogenesis** process of forming methane from simple precursors

**methanotroph** microorganism that metabolizes $CH_4$ to produce ATP

**microorganism** a small organism such as a bacterium, yeast, fungi, algae, etc

**nephelo flask** a side-arm equipped flask

**organic** carbon based molecule

**oxidizing agent** a substance that removes electrons from another substance

**precipitation** formation of an insoluble material

**receptor** a surface feature that recognizes a class of molecules

**reducing agent** a substance that donates electrons to another substance

**sequestering agent** molecule that possesses the ability to bind another molecule with some selectivity

**siderophore** a molecule that has a high binding affinity and selectivity for $Fe^{3+}$

**solubilization** the process of dissolving a substance

**stabilization** a mode of keeping a material intact

**sterilization** the use of a physical or chemical process to destroy all microbial life

**toxic heavy metal** metal and metal-like element with atomic number greater than 30 that is harmful to living systems

**transition metal** group 3 to 12 elements

**transport system** mode of moving a molecule or species from one place to another

**uptake** method of transporting a molecule or species from the external environment to the interior of a cell

**volatilization** process that causes phase change from solid to gas

## Appendix A. Photographs of Assemblies

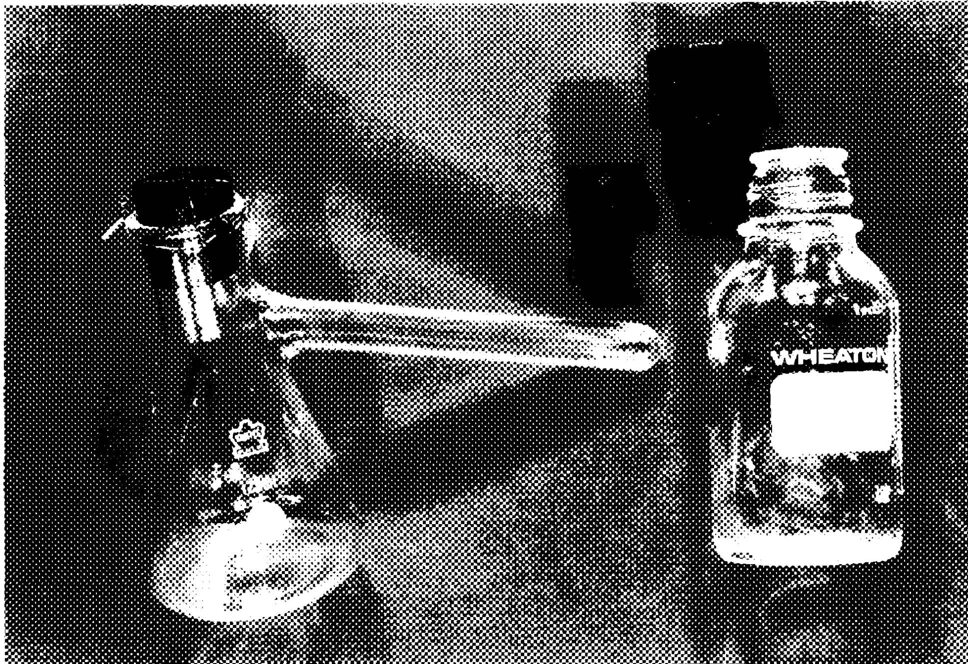


Figure A-1 Nephelo (side-arm) flask (left) and bottle assembly (right) used to cultivate thermophilic, strictly anaerobic methanogens.

Figure A-2. Single colonies of *M. thermoautotrophicum* Marburg and *M. thermoautotrophicum* ΔH as they appear on MS-Gelrite medium after 3 to 5 days incubation at 65°C.