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FINAL REPORT

Development of Biological and Chemical Methods for Environmental Monitoring of DOE Waste Disposal and Storage Facilities

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1.0 INTRODUCTION

Hazardous chemicals in the environment have received ever increasing attention in recent years. In response to ongoing problems with hazardous waste management, Congress enacted the Resource Conservation and Recovery Act (RCRA) in 1976. In 1980, Congress adopted the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), commonly called Superfund, to provide for emergency spill response and to clean up closed or inactive hazardous waste sites. Scientists and engineers have begun to respond to the hazardous waste challenge with research and development on treatment of waste streams as well as cleanup of polluted areas (e.g., Collins and Saukin, 1981; Exner, 1982; Brown et al., 1983; Schweitzer and Santolucito, 1984; Bhatt et al., 1985).

The magnitude of the problem is just now beginning to be understood. The U.S. Environmental Protection Agency (USEPA) National Priorities List, as of September 13, 1985, contained 318 proposed sites and 541 final sites (USEPA, 1985). Estimates of up to 30,000 sites containing hazardous wastes (1,200 to 2,000 of which present a serious threat to public health) have been made (Public Law 96-150). In addition to the large number of sites, the costs of cleanup using available technology are phenomenal. For example, a 10-acre toxic waste site in Ohio is to be cleaned up by removing chemicals from the site and treating the contaminated groundwater (Environ. Sci. Technol. 19:654). The federal government has already spent more than $7 million to remove the most hazardous wastes, and the groundwater decontamination alone is expected to take at least 10 years and cost $12 million. Another example of cleanup costs comes from the State of California Commission for Economic Development which predicts a bright economic future for the state except for the potential outlay of $40 billion for hazardous waste cleanup mandated by federal and state laws (Environ. Sci. Technol. 19:759).

Hazardous waste sites contain a variety of chemical wastes, and the same pollutants appear in many waste sites. In many cases, hazardous organics find their way into drinking water supplies, presenting a hazard to human health. The frequency of occurrence of some hazardous wastes in drinking water supplies (Council on Environmental Quality, 1980) is shown in Table 1.1. Contamination of groundwater by hazardous inorganic and organic compounds has caused the closing of many public wells in the last few years (Craun, 1984).

The cost of waste site remediation by conventional means depends on the system used, but all are extremely costly. Immediate solutions include removing contaminated soil to another location; however, that is not an ultimate solution because the wastes have not been eliminated, merely moved. Other techniques include washing contaminated soil with water, extracting it with organic solvents, or incinerating it at high temperature. Washing and extracting soil with solvents may not remove contaminants, however, because of their solubility properties and affinity for soil particles; extraction and incineration destroy the usefulness of soil; and all of these techniques require digging up and processing large amounts of soil.

TABLE 1.1
Inorganic and Organic Compounds Reported in Drinking Water Supplies at Concentrations Greater than the Limits Set By EPA

Compound	Percent of water supplies
Dichloroethylenes	23
1,1,1-Trichloroethane	19
1,1-Dichloroethane	18
Carbon tetrachloride	18
Trichloroethylene	14
Perchloroethylene	13
1,2-Dichloroethylene	7
Vinyl chloride	6
Methylene chloride	2
Mercury	5
Lead	19
Cadmium	11
Chromium	13
Silver	14

Wastewater or contaminated groundwater may be stripped of volatile organics by pumping the water through a spray nozzle and letting the contaminants evaporate. However, this technique also releases the contaminants to the environment and is limited by the volatility of the contaminants and the extent of their partitioning from soil into groundwater. All of these techniques are expensive.

Considering the potential number of sites and the projected costs of cleanup, research is needed to develop less costly treatment systems. An alternative treatment of contaminated waste sites can be provided by the development of biological degradation and immobilization systems, which degrade wastes to innocuous compounds or precipitate them into biologically unavailable forms. In some cases, these systems work as rapidly as chemical or physical treatments and are usually much less expensive. Various types of biological treatment systems have been used successfully for years to treat waste (Rodgers et al., 1985), but there has been little emphasis on *in situ* treatment (Wetzel et al., 1985). If systems could be developed that would treat contaminated soils and/or groundwater without having to remove them from the site, a great deal of money could be saved and more areas could be cleaned up.

This report describes efforts by the Oak Ridge Research Institute to isolate and develop microbial systems that have application for degrading, converting, precipitating, and/or absorbing organics, inorganics, and radionuclides from soil, sediment and/or water.

Microbial degradation of chemical wastes has been an important process for many years; it is the principal means of treating sewage. Organisms capable of degrading organic pollutants not ordinarily present at significant concentrations have been found in municipal sewage (Clark et al., 1979; Cook et al., 1983; Ebing and Schuphan,

1979; Gerike and Fischer, 1979, 1981; Ghisalba, 1983; Leisinger, 1983; Tabak et al., 1981). Degradative organisms seem to be enriched where pollutants have accumulated (Baya et al., 1986), and can be selected under conditions of limiting nutrients when the nutrient in question is a component of the hazardous waste (Balthazor and Hallas, 1986). Microbial treatment systems have also been described for removing combined forms of nitrogen (Focht and Chang, 1975), phosphate (Timmerman, 1979), and various heavy metals (Gale and Wixson, 1979; Hansen et al., 1984; Shumate et al., 1978; Strandberg et al., 1981; Zajic and Chiu, 1972) from wastewater.

Heavy metals occur naturally in soil and sediment. However, as a result of industrial pollution, increased concentrations of various heavy metals are reported in soil, sediment, and water. These increases may have human health and environmental consequences because several heavy metals are associated with environmental disasters and human diseases. The two processes most often used to remediate heavy metal contamination in soil, sediment, and water are removal and storage and acid or base extraction. Both methods are relatively expensive when compared to biological remediation. Biological systems have been identified that convert heavy metals to the metal sulfide form, reduce mercury to the elemental form, or absorb heavy metals to the cell wall. Further investigation of these systems as to their application to the environmental concerns at Department of Energy (DOE) facilities was the primary objective of the experimental studies discussed below. Organic chemicals occur naturally as byproducts of growth and metabolism by all living organisms, and most of them are also naturally degraded. The majority of biodegradation of organic waste products, both natural and synthetic, is done by microorganisms (Alexander, 1981). Many hazardous organic wastes are related chemically to naturally occurring chemicals (Cook et al., 1983), so it is not surprising that microorganisms capable of degrading hazardous organic wastes are fairly readily isolated (Ahmed and Focht, 1973; Cook et al., 1983; Fedorak and Westlake, 1984; Johnson and Talbot, 1983; Kong and Sayler, 1983; Tabak et al., 1981).

A variety of methods for treating hazardous waste sites contaminated with organic wastes have been proposed and tested. Some of these methods depend on physical removal of the compounds from groundwater, for example by evaporation while the groundwater is pumped from wells and sprayed into the air (air stripping). However, this treatment does not decrease the amount of the compound in the environment, it merely transfers it from groundwater to air, from which it is likely to reenter groundwater elsewhere. In addition, if the wastes are in soil, and especially if they are hydrophobic, they are likely to be tightly bound to soil particles and, thus, are not readily removed with the groundwater. Technologies for physical treatment of wastes are, in some cases, better developed than those for bioreclamation, but biological processes offer many advantages, among them lower requirements for energy input. Therefore, an intensive effort should be made to develop bioreclamation technologies as an economical way to remove hazardous organic wastes. Systems for testing hazardous organic wastes to assess their biodegradability have been proposed and discussed by a number of authors (Freitag et al., 1979; Gerike and Fischer, 1979, 1981; Gilbert, 1979; Shelton and Tiedje, 1984; Tiedje et al., 1986; Dolfing and Tiedje, 1987), and

field assessments of groundwater transport and degradation of hazardous organics have also been reported (Roberts et al., 1980).

Hazardous wastes accumulate predominantly as a result of various industrial and agricultural activities. They may occur in wastewater or in sludges or residues from manufacturing processes, in soil contaminated by spillage, run-off, or direct application of compounds, or in aquifers and other water supplies (Leisinger, 1983). Detoxifying contaminated sites by physical or chemical means is extremely costly. Biological detoxification may be less costly, but may also be very slow. Therefore, it is worthwhile to develop methods for enhancing biodegradation of organic pollutants and bioconcentration or bioconversion of toxic metals and radionuclides, so that polluted sites might be detoxified more quickly.

In situ biodegradation can be an effective means of removing organic compounds from soil by complete degradation to inorganic constituents or by transformation to non-hazardous compounds. Microbes in groundwater can migrate to localized concentrations of pollutants and attack them in situ, decontaminating the soil particles that serve as a reservoir for hazardous organic compounds. A variety of technologies are currently being developed for the delivery of organisms and nutrients to contaminated zones in soil (Biosystems, undated; Flathman and Githens, 1985; Gore, 1985; Heyse et al., 1985; Nyer, 1985; Wetzel et al., 1985). We plan to use and expand on these technologies to attempt to find optimal methods to treat selected waste sites.

We have surveyed polluted sites at the Y-12 facilities in Oak Ridge, Tennessee, for microbes capable of degrading toxic organics, converting or reducing heavy metal ions, or absorbing toxic inorganics and radionuclides. Several bacterial systems were isolated from these polluted sites and were shown to degrade chlorinated organics, convert ionic metals to the relatively insoluble metal sulfide, reduce the ionic forms of mercury, lead, and silver to the elemental forms, and absorb several inorganics and radionuclides. Results of these studies are discussed in the following sections.

1.1 OBJECTIVES

The overall objectives of these studies were to identify microbial systems that may have application to treating waste at Department of Energy facilities. These objectives were achieved by isolating bacteria from contaminated sites. The isolated bacteria were evaluated for their ability to biodegrade chlorinated organics, convert or reduce heavy metals, and/or adsorb inorganics and radionuclides. The organics, inorganics, and radionuclides chosen for these studies were based on the prevalence of these contaminants at the DOE facility. In some cases, selection was made based on the interest of DOE. For example, radioactive strontium is not a serious problem at the Y-12 facility, however, at other DOE facilities it is an environmental concern.

Specific objectives of the studies included:

Identification of sites of environmental concern.

Isolation of microbial populations from these sites (microbes isolated from soil, sediment, sludge, and water).

Identification of the isolated microbes.

Determination of the metabolic properties of the isolated bacteria for degrading chlorinated organics, converting inorganic ions to the metal sulfide, reducing inorganic ions to the elemental form, and adsorbing inorganic ions and/or radionuclides.

The succeeding chapters of this report describe specific studies performed. Chapter 2 discusses bacterial reduction of heavy metals; Chapter 3 deals with adsorption; Chapter 4 describes our studies on sulfide reduction; Chapter 5 details the problems of methylmercury in the environment and proposed ways to prevent its formation, and Chapter 6 discusses biodegradation of PCE. Chapter 7 provides information about the Historically Black Universities and Colleges program and its participation in the work performed under this contract. The Appendices contain manuscripts which have been accepted for publication and patents granted as a result of the work described herein.

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2.0 BACTERIAL REDUCTION OF HEAVY METALS

2.1 IDENTIFICATION OF CONTAMINATED SITES

With the help of Y-12 personnel, sites were identified containing the following contaminants: tetrachloroethylene and perchloroethylene, PCBs, uranium, mercury, and chromium. The sites included the S-3 Ponds, groundwater beneath the burial grounds, the oil farm, New Hope Pond, East Fork Poplar Creek, and Bear Creek. Sites with radioactive contaminants included White Oak Lake.

2.1.1 Isolation of Bacteria from Contaminated Sites

Sediment, soil, and water samples were collected from the above sites. From these samples, bacteria were isolated using conventional methods. After isolation, the bacteria were evaluated for their ability to degrade chlorinated organics, convert ionic heavy metals to the metal sulfide, reduce mercury, lead, cadmium, chromium (trivalent), silver, gold, and platinum to the elemental form, and adsorb the metals strontium, cesium, mercury, lead, cadmium, and silver.

2.1.2 Bacterial Reduction or Adsorption of Heavy Metals

Many microbial species contain genetic determinants of resistance to heavy metal toxicity. Resistance to the following metals has been observed: lead, cadmium, chromium, tin, zinc, cobalt, copper, titanium, boron, silver, nickel, arsenic, bismuth, thallium, and antimony (McHugh et al., 1975; Silver, 1981; Silver et al., 1981; Smith and Novick, 1972; Summers and Jacoby, 1977, 1978; Summers and Silver, 1978; Bopp et al., 1983; Bender and Cooksey, 1986). The resistance determinants are frequently associated with plasmids and transposons. Resistant microbes are observed frequently in hospitals and are frequently associated with antibiotic resistance (Foster, 1983). In the environment, however, antibiotic resistance is generally not observed in metal-resistant bacteria. It has been suggested that in the environment heavy metal-resistant bacteria may be found with great frequency at sites with heavy metal contamination, whereas the reverse may be observed at non-contaminated sites (Foster, 1983). Thus, heavy metal contamination may select for metal resistance. Several mechanisms have been proposed for metal resistance; they include cell membrane transport systems that exclude the heavy metal, heavy metal-induced synthesis of intracellular binding proteins such as metallothionein, intracellular reduction of the heavy metal via a metal reductase such as the mercury reductase, and adsorption of the heavy metal to the cell surface. The two systems we were able to identify in samples isolated from the above contaminated sites included heavy metal reduction and adsorption.

2.1.3 Heavy Metal Reduction

Heavy metals such as mercury, lead, and cadmium are toxic to bacteria because they bind avidly to sulfhydryl groups and inhibit macromolecule synthesis and enzyme action. Many enzymes have critical thiol groups and are sensitive to these metals. Other cellular processes are also inhibited by these metals, such as transcription and translation. Resistance to these metals may be a plasmid-determined property of gram-negative and gram-positive bacteria (Summers and Silver, 1978). The observation of these resistant bacteria in hospitals and the environment may be related to the use of these metals as disinfectants (i.e., silver and mercury) or as result of industrial pollution.

Several studies have shown that bacterial resistance to mercury is determined by enzymatic reduction of ionic mercury to the elemental form (Hg^{++}----->Hg^{o}). The enzyme referred to as a mercuric reductase is an intracellular FAD-containing enzyme which has been isolated and purified from several bacterial species (Summers and Sugarman, 1974). A bacterial plasmid has been isolated that codes for the enzyme and a mercury-specific transport system (Summers and Kight-Olliff, 1980). Some bacterial strains have been shown to contain a cytoplasmic enzyme that cleaves the mercury carbon bond of organomercurial compounds, resulting in the release of ionic mercury (Begley et al., 1986). The released mercury may be converted to elemental mercury. An overall scheme for these interconversions is shown in Figure 2.1. The mechanism associated with the reduction of ionic mercury has been well characterized and is easy to demonstrate because elemental mercury under normal atmospheric pressure will volatilize. Several studies have shown that mercury-resistant bacteria can grow in solutions containing 100 ppm mercury with conversion rates (i.e., Hg^{++} to Hg^{o}) of 1 ppm per hour. We have isolated a mercury-resistant bacteria from a mercury-contaminated site which grows in solutions containing 1,000 to 3,000 ppm mercury. This bacteria also appears to be resistant to the metals lead, silver, gold, platinum, cadmium, zinc, copper, and selenium. We have performed several experiments to characterize the mechanism of resistance to these metals. The results and conclusion are shown below.

Mercury-contaminated soil was isolated from the floodplains of East Fork Poplar Creek. Pseudomonas species were isolated from this soil using Pseudomonas isolation medium. After several transfers, the culture was plated onto Pseudomonas agar and the individual colonies sub-plated onto Pseudomonas agar. The isolated colonies were then grown in the medium shown in Table 2.1 for 24 hr. An aliquot of the culture was then transferred to the same medium containing mercury, lead, cadmium, selenium, copper, silver, platinum, or zinc at concentrations of 10 and 100 ppm. The isolated colonies were considered resistant if they grew to densities greater than 0.5 optical density units (optical density was measured at 600 nm) in a medium containing 100 ppm of metal. A total of 13 individual colonies was identified, but only one of the isolated colonies grew in the medium containing the individual metals (Table 2.2).

Figure 2.1
Bacterial Metabolism of Mercury

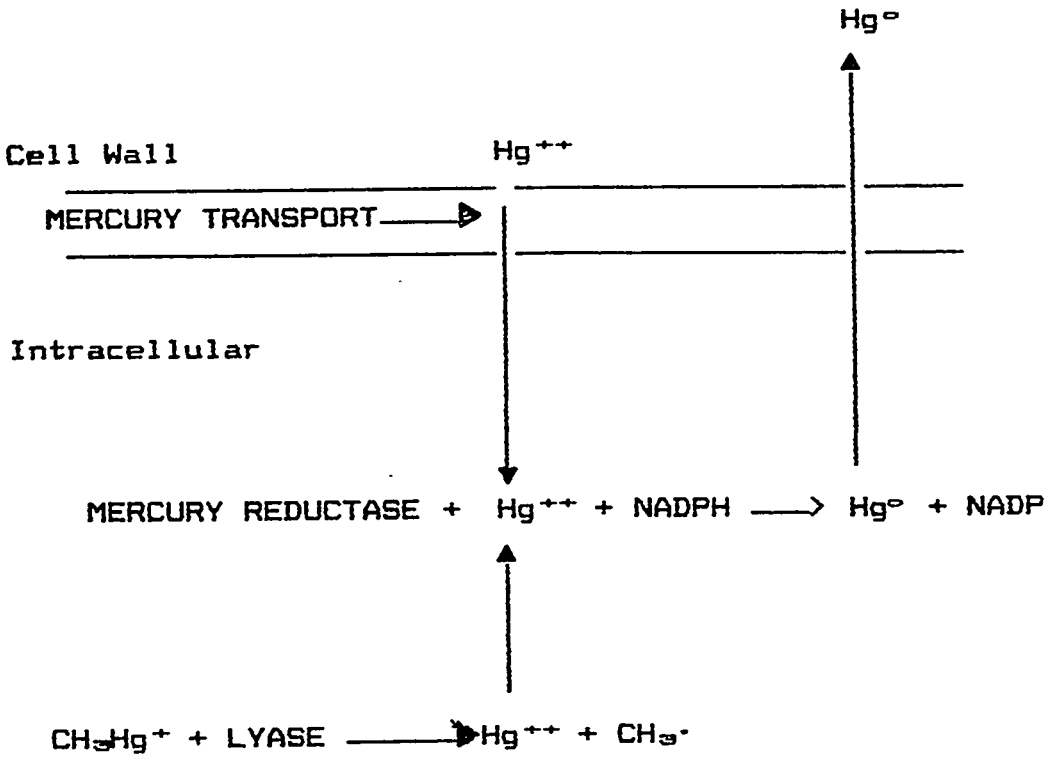


TABLE 2.1
Medium for Pseudomonas maltophilia

Potassium/Sodium Phosphate buffer pH 7.4
$(NH_4)_2SO_4$
Arginine
Methionine
$NaNO_3$ or NaCl
$MgSO_4$
Glucose
beta-Glycerophosphate
Alanine
Phenylalanine
Serine
Valine

TABLE 2.2
Growth of Pseudomonas Species
in the Presence of Heavy Metals

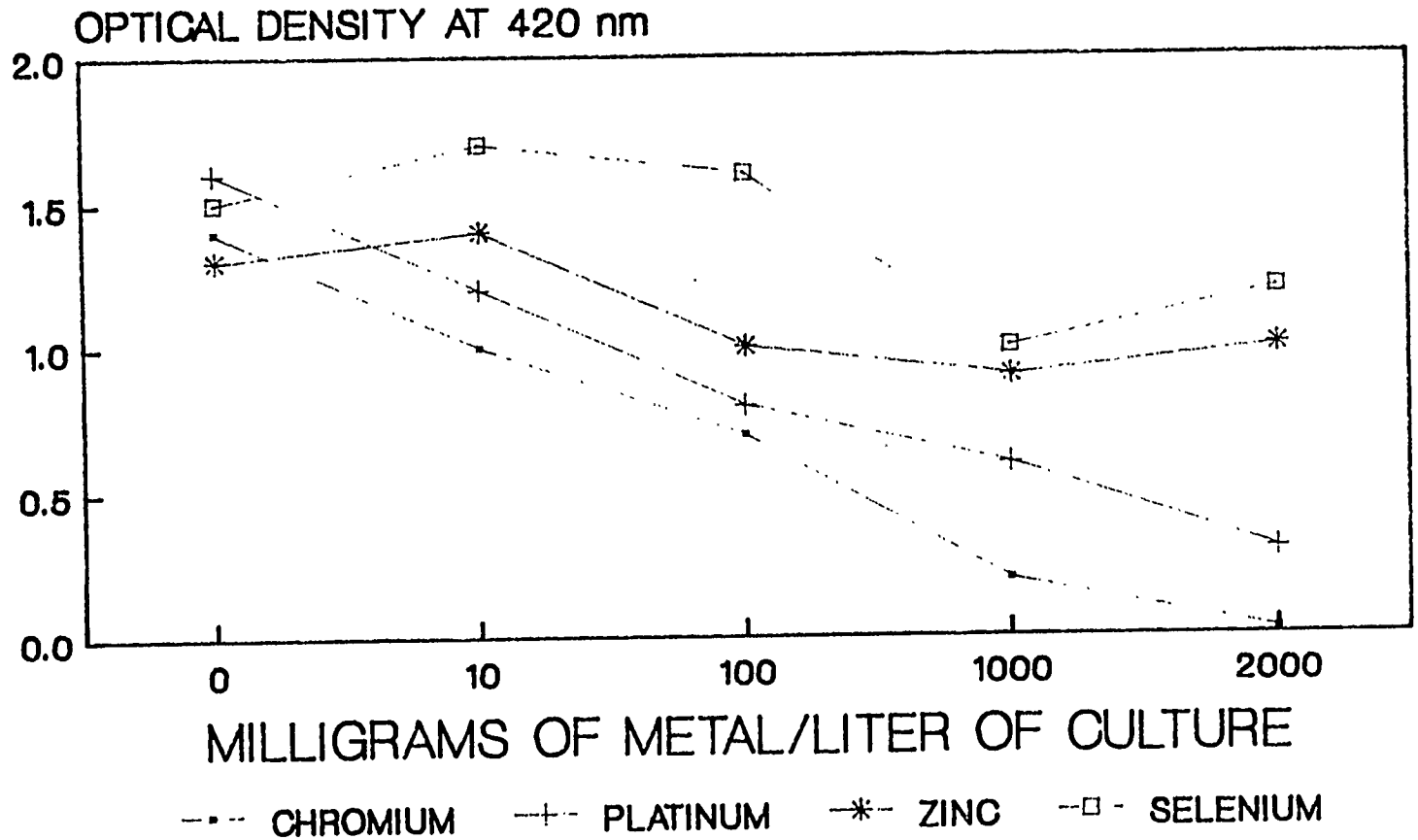
Heavy metal	Metal concentration 0	10	100	1000
Mercury chloride	13/13	3/13	1/13	1/13
Lead chloride	13/13	4/13	1/13	1/13
Cadmium chloride	13/13	1/13	1/13	1/13
Silver nitrate	13/13	5/13	1/13	1/13
Platinum chloride	13/13	1/13	1/13	0/13
Chromium chloride	13/13	3/13	1/13	0/13
Zinc chloride	13/13	7/13	3/13	2/13
Copper chloride	13/13	10/13	3/13	1/13
Sodium selenate	13/13	5/13	2/13	1/13

Individual metals were incubated in medium containing an isolated Pseudomonas colony (i.e., 13 different colonies) for 24 hr, and the optical density (OD) determined. Colonies in which the OD was less than 0.5 were considered non-resistant, whereas colonies with OD greater than 0.5 were considered resistant to the metal. The first number refers to the colonies with an OD greater than 0.5 and the last number to the total number of colonies used.

The isolated colony which showed the greatest resistance was identified as Pseudomonas maltophilia. At ORRI, this isolate is referred to as O_2. In addition to showing resistance to the above metals, this isolate is resistant to the antibiotics penicillin, ampicillin, and 6-aminopenicillanic acid. Results also indicate that the metal resistance can be amplified by the presence of ampicillin in the medium.

The effect of heavy metals on O_2 growth was determined in culture. Growth profiles of O_2 in the presence of metals are shown in Figures 2.2, 2.3, and 2.4. These experiments were performed by adding metal to a culture in nutrient broth and incubating the culture at 32°C for 24 hr. At various time intervals, the optical density at 600 nm was determined. Most metals were added in the chloride form, with the exception of silver and lead which were added as lead nitrate and silver nitrate. As shown in Figures 2.2 and 2.3, the O_2 organism

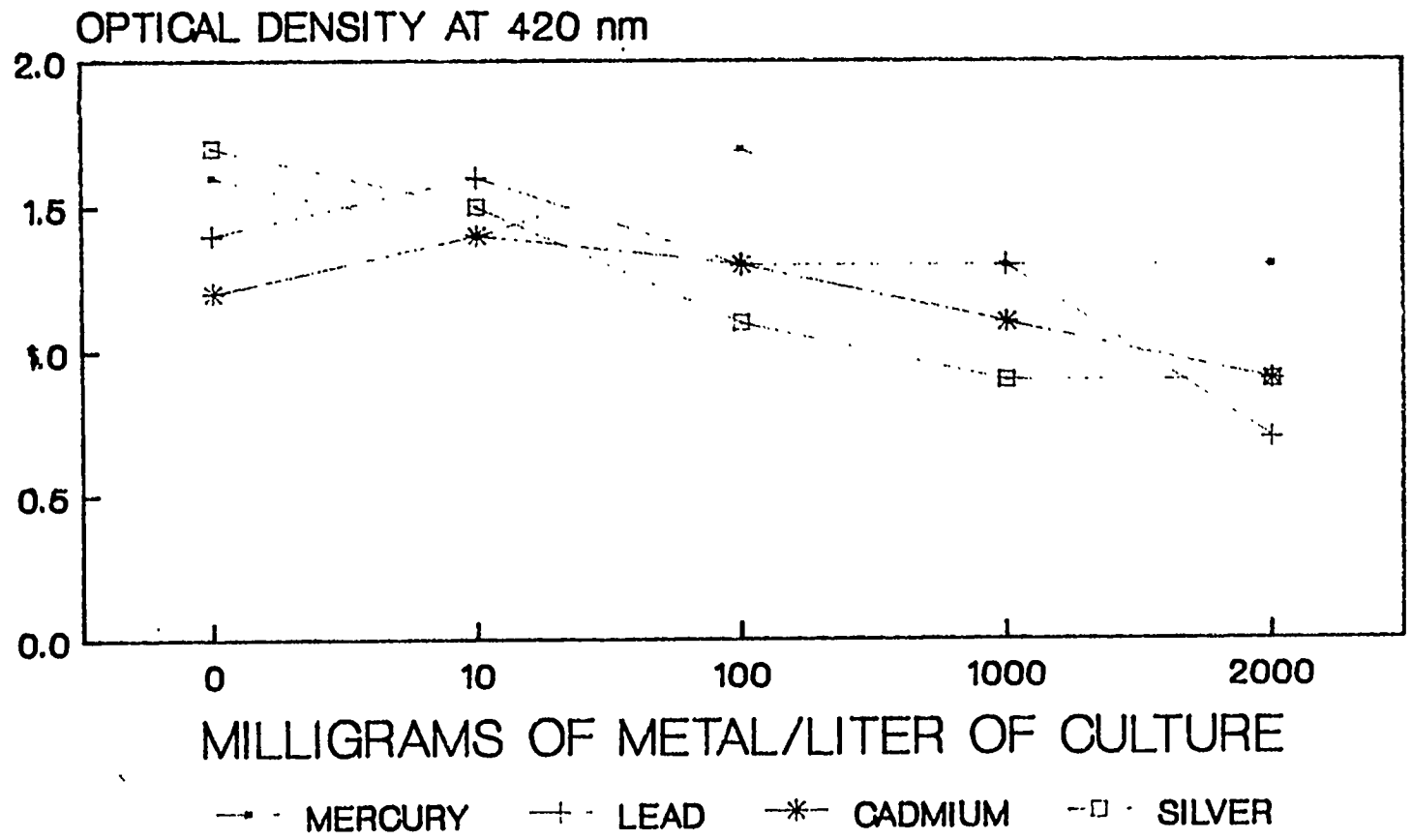
EFFECT OF Cr, Pt, Zn, AND Se ON THE GROWTH OF PSEUDOMONAS MALTOPHILIA



Pseudomonas maltophilia grown at 32oC for 24 h in the presence of metal was filtered and metal analyzed in filtrate

Figure 2.2

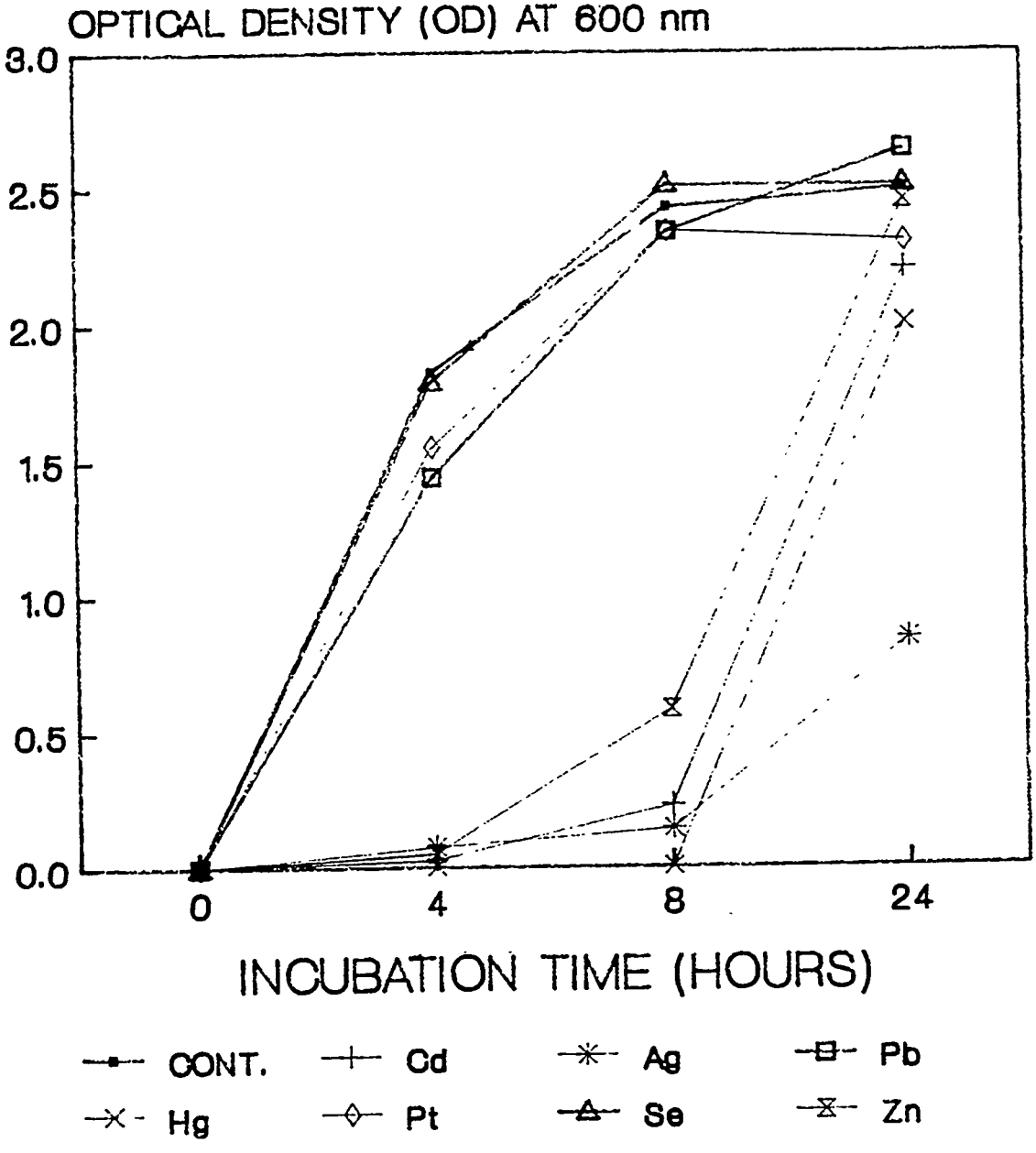
EFFECT OF Hg, Pb, Cd, AND Ag ON THE GROWTH OF PSEUDOMONAS MALTOPHILIA



Pseudomonas maltophilia grown at 32oC for 24 h in the presence of metal was filtered and metal analyzed in filtrate

Figure 2.3

EFFECT OF HEAVY METALS ON THE GROWTH OF PSEUDOMONAS MALTOPHILIA



Cells were grown in the presence of metal (concentration 100 ppm) and at various time intervals OD was determined

Figure 2.4

grew to confluency at metal concentrations ranging from 10 to 100 ppm. Even at metal concentrations of 1,000 to 2,000 ppm, O_2 cell viability remained high and growth was decreased by only 10 to 20% in most cultures, with the exception of cultures grown in the presence of chromium. Growth in this compound was decreased by 60 to 90% at high metal concentrations. Figure 2.4 shows the growth profiles as a function of time. These cultures were grown in the presence of 100 ppm metal and as shown (Figure 2.4), most cultures reached confluency following 8 hr of incubation. The exceptions were mercury, cadmium, zinc, chromium, and silver. The mercury, cadmium, and zinc cultures reached confluency at 24 hr, and the chromium and silver cultures never reached confluency. At 48 hr incubation, the culture with silver reached confluency. It would appear from these results that an inducible process is required prior to the growth of O_2 in the presence of mercury, cadmium, zinc, or silver. This suggestion was confirmed by harvesting the induced cells and growing them in the presence of metal and fresh medium. Confluency was reached in 8 hr for cells grown in the presence of mercury, cadmium, zinc, or silver.

The mechanism associated with the heavy metal resistance in this bacteria was investigated. As discussed above, several mechanisms are associated with metal resistance. We investigated the reduction of heavy metals to the elemental form, bacterial exclusion of heavy metal, and intracellular inducible binding proteins.

Because elemental mercury is volatile at normal atmospheric pressure we devised a system for measuring the conversion of ionic mercury to the elemental form. The system consisted of growing the O_2 in culture with ionic mercury at 32°C, continuous purging of the culture with air, and passing the air coming off the culture through a glass tubing containing gold filing. As the air coming off the culture passes over the gold filing, elemental mercury is amalgamated onto the gold. Elemental mercury was stripped from the gold with nitric acid and mercury determined using the mercury hydride method. As shown in Table 2.3, mercury levels in the culture decreased as the levels of mercury increased on the gold filings. At 24 hr incubation, the levels of mercury in the culture had decreased from 100 to 0.10 ppm. Approximately 70 mg of mercury was recovered on the gold filings. The original amount of mercury in culture was 100 ppm and the amount at 48 hr was 0.10 ppm, which suggests that almost all of the mercury was lost from the culture. We suggest that the difference in the amount leached from the gold filings and the amount lost from the culture (29.9 mg) is associated with the plating of mercury on a glass culture vessel wall (we assume that the plated mercury is elemental mercury). These results suggest that ionic mercury may be quantitatively converted by O_2 to the elemental form.

In the elemental form, the metals platinum, cadmium, lead, selenium, zinc, and silver have a high vapor pressure and, thus, would not be expected to volatilize at normal atmospheric pressure. Therefore, we would not anticipate the volatilization of these metals following the reduction of the ionic form to elemental metal. Density gradients were used to determine the presence of elemental metal in the culture grown with the ionic metal. Cells were grown in cultures containing each metal individually for 48 hr. An aliquot of

TABLE 2.3
Conversion of Hg^{++} to Hg^{o} by *Pseudomonas maltophilia*

Incubation time, hours	Conc. Hg^{++} in the culture mg/L		Mercury recovered on the gold filings, mg	
	Control	Exper.	Control	Exper.
0	96	98	0	0
8	99	88	0	9
16	93	48	0	21
24	97	4	0	35
48	95	0.1	0	5

Pseudomonas maltophilia was grown in culture with 100 ppm mercuric chloride at 32°C for 48 hr. The culture was bubbled with air, and the air coming off the culture was passed through a glass vessel containing gold filings. At various time intervals, mercury was determined in the culture and on the gold filings. Mercury amalgamated to the gold filings was removed with nitric acid and mercury determined in the nitric acid leachate. Mercury was analyzed using the mercury hydride method.

culture was removed and layered onto a renografin density gradient. The gradients were 10, 30, and 80% renografin. After the culture was layered onto the renografin, the sample was centrifuged for 30 min at 5,000 rpm. The concentrations of metal in the top layer, 10%, 30%, and bottom 80% renografin were analyzed by atomic absorption (graphite furnace). The results are shown in Table 2.4.

The density for each compound used in this study and for the elemental metal is shown in Table 2.4. As shown, the density of elemental metal, in most cases, was twice the density of the compound. Thus, a density gradient may provide a method for separating and quantitating the amount of ionic metal reduced to elemental metal. To determine if density gradients could provide information on the reduction of ionic metals to the elemental form, the O_e was incubated

TABLE 2.4
Heavy Metal Densities after Incubation with *Pseudomonas maltophilia*

Heavy metal	Metal density		Percent renografin			
			0 (top)	10	30	80 (bottom)
	Compound	Metal	Inorganic ion added to culture, percent of total			
Silver nitrate	4	11	2	5	13	75
Mercury chloride	5	14	0	3	8	84
Lead nitrate	5	11	1	7	17	70
Cadmium chloride	4	9	11	17	18	46
Zinc chloride	3	7	16	15	9	45
Sodium selenate	3	6	13	7	6	69
Platinum chloride	6	22	1	6	9	80

Pseudomonas maltophilia was incubated with the individual metal shown above at a concentration of 100 ppm for 48 hr at 32°C. An aliquot of the culture was then layered onto a renografin gradient and, after centrifugation for 30 min, an aliquot of each layer was removed and analyzed for metal content using the atomic absorption spectrophotometer. In the controls, the above metals were added individually to the culture, mixed, and an aliquot of this mixture was immediately layered onto the renografin gradient and centrifuged. The layers were isolated as above and analyzed for metal. In the controls, 99% of the added metal was observed in the top two layers. The results of the experimental samples are expressed as a percent of the total metal concentration in the culture at zero time.

with the individual metal for 48 hr as described in Table 2.4. In the controls, each metal was mixed with culture and immediately layered onto the renografin gradient and centrifuged. After isolating the five layers and analyzing them for the metal of interest, we observed in the top two layers 94% of the initial metal concentration in the culture. In contrast to the controls, 54 to 92% of the metals were observed in the bottom two layers of the experimental cultures. The metals silver, mercury, lead, and platinum showed the greatest concentration of metal in the bottom layer, and these metals show density differences of approximately 3. These results suggest an increase in the density of the metal compound following incubation with the O_2. This change in density may be related to the reduction of the ionic to the elemental form. However, these results may also

suggest that the change in density is the result of metal adsorption to the cell surface and/or absorption into the cell. To further explore the latter suggestion, cells were grown in the presence of the metals (10 ppm) for 48 hr. At 48 hr, a 10% solution of EDTA was added to each culture and incubated with shaking at room temperature for 6 hr. An aliquot of the culture was removed and centrifuged at 5,000 rpm for 15 min. After removing the supernatant, the pellet was resuspended in deionized water. The controls (i.e., at 0 time incubation) were treated the same as for the experimental samples except that the culture with metal was not incubated for the 48-hr period. The concentrations of metal in the supernatant and pellet were determined; the results are shown in Table 2.5.

Each metal was added to the culture at a concentration of 10 ppm and, as shown in Table 2.5, the recovery of metal averaged 98%. Prior to the 48-hr incubation and after the addition of EDTA and the 6-hr incubation (i.e., 0 time), metal concentrations in the supernatant and the pellet were analyzed. As shown, the metal concentration in the supernatant accounted for an average 90% of metal in the culture. However, after 48-hr incubation, 85% of the metals silver mercury, lead, selenium, and platinum in culture were associated with the pellet. For the metals cadmium and zinc, the pellet contained 45% of total metal present in the culture. Based on the results at 0 time and at 48-hr incubation, we suggest that metal reduction and/or absorption into the cells are the likely factors explaining the change in the density of metals following the incubation with O_2. A change in density as a result of the adsorption of metal to the surface of the cells does not seem likely because of the following: we assume that the EDTA would remove adsorbed metal from the surface of cells, resulting in lower concentrations of metal in the pellet and the reverse in the supernatant. In fact, for all metals studied we observed higher levels of metal in the pellet.

It is difficult to distinguish between metal reduction and absorption because both processes take place inside the cell. Thus, during the process of metal reduction we would assume <u>a priori</u> that the metal concentration inside the cells would be relatively high. However, when the ionic form of metal in culture is completely reduced, the cellular concentration should be relatively low. This assumes, of course, that the reduced metal is by some mechanism removed from the cell (see Figure 2.1 for mercury). In contrast, metal absorption would most likely involve an inducible binding protein such as metallothionein, which would result in high intracellular levels of metal (Kagi and Vallee, 1969). For example, most of the metals used in these studies are toxic to bacteria. Several bacterial species have evolved mechanisms for absorbing toxic metals into the cell and, once absorbed, the metal is chelated by a metallothionein-like protein. The chelated metal is thus prevented from interfering with the normal metabolism of the cell. Without this mechanism, the absorbed metal would most likely kill or inhibit the growth of the organism. Since microbial metallothionein-like proteins are inducible by metals, it may be possible to measure the level of binding proteins in the O_2 as a means of determining whether cellular absorption has occurred following exposure to the metals.

TABLE 2.5
Metal Adsorption and Absorption by *Pseudomonas maltophilia*

Metal	Total metal conc. in culture µg metal/mL culture	Metal conc. µg metal/culture			
		Supernatant		Pellet	
		0	48 hr	0	48 hr
Silver nitrate	9.5	8.9	1.7	1.0	7.7
Mercury chloride	9.8	9.3	1.4	0.9	8.7
Lead nitrate	10.0	9.7	1.3	0.7	7.9
Cadmium chloride	10.9	9.7	4.9	0.8	4.1
Zinc chloride	10.0	9.9	5.3	0.4	4.7
Sodium selenate	9.3	9.4	1.5	0.3	8.1
Platinum chloride	9.7	9.1	1.0	0.6	8.2

Each metal above was added to a culture containing *Pseudomonas maltophilia* and the metal concentration determined (total metal concentration (conc. in culture) prior to incubating the culture at 32°C for 48 hr. After incubation, a 10% solution of EDTA was added and the culture was incubated for an additional 6 hr. The culture was then centrifuged at 30,000 rpm for 15 min and the supernatant and pellet were isolated. After suspending the pellet in deionized water the concentration of metal was determined on the pellet and supernatant. The metal concentration was determined by atomic absorption using the graphite furnace.

Following centrifugation of a culture containing the O_2 and metal, we would anticipate that the reduced and absorbed metal would both be observed in the pellet. However, when the renografin gradients containing the metal-exposed O_2 culture were centrifuged, we observed a high concentration of metal in the bottom layer. In the top two layers with relatively low concentrations of metal, we observed a high density of the O_2 (determined by measuring the O_2 at 600 nm). Since the top two layers have low metal levels and a high density of O_2, we decided to isolate the O_2 from the top layers of the gradient and determine the presence of heavy metal-binding proteins. The O_2 was grown in the presence of metal for 72 hr at 32°C. After normalizing each culture to an optical density (OD) of 1.0 (determined at 600 nm), an aliquot was layered onto the renografin gradient and centrifuged. The top two layers were removed and centrifuged at 30,000

rpm for 10 min to collect the cells. The cells were dispersed into a small volume of Tris buffer and sonicated for 5 min. The sonicated cells were centrifuged at 5,000 rpm for 10 min and the supernatant removed and centrifuged at 5,000 rpm for 15 min. After dialysing and concentrating the supernatant, an aliquot was added to solution containing the metal of interest. This mixture was incubated at room temperature overnight and the mixture was acidified with a 10% solution of TCA. The acidified solution was centrifuged at 5,000 rpm for 20 min and the supernatant removed. After dispersing the pellet in deionized water, the metal concentration in the supernatant (i.e., the supernatant before centrifugation) and pellet were determined. The concentrated supernatant was acidified to precipitate proteins. The metallothionein protein is not precipitated by TCA and, thus, remains in the supernatant following centrifugation (Piotrowski et al., 1979). The results of these experiments are shown in Table 2.6.

The concentration of metal in the TCA pellet of the controls ranged from 0 to 36% of total metal added. In contrast, the concentration of metal in the pellet from the experimental samples ranged from 51 to 84% of total metal added. These results suggest that a TCA-precipitable protein was induced following the exposure to the above metals. The greatest induction occurred when the O_2 was exposed to the metals mercury, lead, and platinum. Zinc was the least responsive of the metals studied. It is clear from these results that these metals failed to induce in the O_2 a TCA-insensitive metallothionein-like protein. However, these metals appeared to induce metal-binding proteins that were sensitive to TCA. Thus, as the above metals are absorbed into the O_2, an apparent induced synthesis of metal-binding proteins appears to occur. Based on the results discussed above, it would appear that these metal-binding proteins are associated with the reduction of the above metal to the elemental form. Confirmation of this suggestion awaits further studies.

It is noted that the protein concentration in the bottom layer of the renografin gradient was low and represented less than 3% of the concentration observed in the top layers. That the protein concentration in the bottom layer was less than 3% of the concentration in the top layer suggests that metal observed at the bottom of the gradient was extracellular. This suggestion is based on the assumption that metal absorbed into the O_2 would be observed in the bottom layer following centrifugation, which would result in higher concentrations of protein in the bottom layer.

TABLE 2.6
Heavy Metal Binding Proteins in *Pseudomonas maltophilia*

	Metal conc. in			
	TCA mixture (total)	TCA supernatant	TCA pellet	Percent of total in the pellet
	µg metal/100 mL of supernatant			%
Silver nitrate				
Control	99	93	1	1
Experimental	97	41	58	60
Mercury chloride				
Control	100	88	9	13
Experimental	93	29	63	68
Lead nitrate				
Control	96	98	0	0
Experimental	100	28	71	77
Cadmium chloride				
Control	97	80	19	20
Experimental	98	23	78	80
Zinc chloride				
Control	100	91	8	0
Experimental	96	46	49	51
Sodium selenate				
Control	93	70	33	36
Experimental	91	27	76	84
Platinum chloride				
Control	94	96	0	0
Experimental	97	18	80	82

The O_2 was grown in the presence of each metal above for 72 hr at 32°C. After normalizing an aliquot of culture to an OD of 1, it was layered onto a renografin gradient and centrifuged. The top two layers were removed and centrifuged at 5,000 rpm for 10 min. The pellet was suspended in Tris buffer and this mixture was sonicated for 5 min. The sonicated cells were centrifuged at 5,000 rpm for 10 min and the supernatant was removed and centrifuged at 30,000 rpm for 15 min. After dialysing and concentrating the supernatant, 1 µg of metal/mL supernatant was added (i.e., the metal used in the original culture was added to the supernatant) along with a solution of TCA (to give a final TCA concentration in the supernatant of 1%). After mixing the samples they were left overnight at room temperature. After centrifuging the sample, the supernatant was removed and the pellet was dispersed in deionized water. The TCA mixture, the supernatant before and after centrifugation, and the dispersed pellet were digested in nitric acid and the metal concentration determined by atomic absorption using the graphite furnace. The controls were treated the same as the experimental samples except that they were not incubated for 72 hr. The TCA mixture refers to the TCA supernatant before centrifugation.

2.2 CONCLUSIONS

The results describing the reduction of mercury, lead, cadmium, selenium, silver, platinum, and zinc to the elemental form by Pseudomonas maltophilia are discussed above. These results suggest that these metals induce metal-binding proteins. This induction is associated with an increase in the density of each metal as demonstrated by renografin gradients. Since the density of the elemental metal is greater than the ionic form, we suggest that the results above show the reduction of seven metals to the elemental form by one bacterial species. The effect of this species on other metals is currently under investigation.

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3.0 ADSORPTION OF HEAVY METALS

The removal of dissolved metals from aqueous waste by microbes has been reported by several investigators. The microbial removal mechanisms include (1) adsorption to the cell surface, (2) bioaccumulation, (3) metal reduction, and (4) conversion to insoluble metal sulfides (Kurek et al., 1982; Witte et al., 1986; Nakahara et al., 1977a; Summers and Sugarman, 1974; Nakahara et al., 1977b; Tzesos and Volesky, 1981; Craig and Bartlett, 1978). The mechanisms associated with the various microbial species appear to be specific for the different metals. For example, mercury (Hg^{2+}) and cadmium (Cd^{2+}) are associated with bioaccumulation; lead (Pb^{2+}), selenium (Se^{2+}), silver (Ag^{1+}) and Hg^{2+} with metal reduction; uranium (UO_2^{2-}) and arsenic (As^{2+}) with cell surface adsorption; and Hg^{2+}, Pb^{2+} and Cd^{2+} with the formation of metal sulfides. Based on this information, it appears that when selecting microbial systems for aqueous waste treatment, careful consideration must be given to the microbial species and the type of metal to be removed.

We have recently investigated microbial strains with broad specificity for removing metals from aqueous waste. Studies were also performed to determine the removal efficiency for radionuclides by these strains. Of the strains studied, microbial spores showed broad specificity for removing heavy metals and the isotopes ^{85}Sr and ^{137}Cs from aqueous waste. Results showing the application of spores for treating aqueous waste containing toxic metals and radionuclides are described below.

3.1 ISOLATION

Bacillus megaterium was isolated from sediments contaminated with Hg, Pb, ^{90}Sr, and ^{137}Cs. The pure isolate was maintained at 4°C on agar plates containing nutrient broth made up at 1/10 strength.

3.1.1 Preparation of Spore Suspensions

A loop of surface growth from a slant was used to inoculate "seed" 400-mL flasks containing 100 mL of 50 mM KCl, 1 mM alanine, and 1 mM inosine for 2 to 12 hr. A 3-L flask containing 2 L of medium (ingredients in g/L; glucose, 2; $MgCl_2$, 0.1; NaCl, 3; NH_4Cl, 2; $Na_2HPO_4 \cdot 12H_2O$, 6; KH_2PO_4, 3; Na_2SO_4, 0.11) was inoculated from the seed flask at 10% by volume and incubated at room temperature with aeration for 36 hr. Nearly complete sporulation was achieved. The spores were harvested by centrifugation at 10,000 rpm at a flow rate of 0.8 L/min in a continuous flow centrifuge. The harvested spores were stored in deionized water at 4°C until used. For adsorption studies, spores were added to the metal mix or plating rinse waste to give a weight ratio of 1/10 (i.e., 100 g of spores (wet weight) per

1000 g (or mL) of rinse waste). Spore dry weight was determined by heating a known volume of a spore suspension for 24 hr at 105°C on watch glasses of known weight. After heating, the watch glasses were cooled and then re-weighed. The spore dry weight was approximately 20% of the spore wet weight.

3.1.2 Adsorption of Metals by Spores

A solution of metals was prepared containing the following compounds in mg/L: $Hg(NO_3)_2 \cdot H_2O$, 20; $CdCl_2$, 20; $Cr(NO_3)_3 \cdot 9H_2O$, 25; AsO_3, 20; Na_2SeO_3, 20; $Pb(NO_3)_2$, 25; $Sr(NO_3)_2$, 40; and $BaCl_2$, 40. The pH of this solution was adjusted to 7.1 with 5N ultra-pure HCl and the solution was filtered. Spores were incubated with the filtered metal mix at room temperature for various time intervals. Aliquots of the culture were removed, centrifuged at 30,000 rpm for 30 min or filtered, and the concentration of the above metals determined in the supernatant (or filtrate) and pellet by inductively coupled plasma spectrometry (ICP). The pellet (or filter) was digested in concentrated ultra-pure nitric acid prior to metal analysis by ICP.

3.1.3 Desorption of Metals from Spores

Desorption of metals from spores was accomplished as described by Bender and Marquis (1982). Briefly, spores from the adsorption studies were suspended in deionized water and titrated to pH 2 with 5N ultra-pure HCl. They were maintained at this pH for 3 hr at room temperature, heat shocked at 60°C for 30 min, centrifuged at 10,000 rpm for 10 min, and washed (3x) by centrifugation with deionized water. The supernatant after each wash was collected and pooled, and the metal concentration determined by ICP analysis.

3.1.4 Adsorption of Radioisotopes by Spores

In some studies, the radioisotopes strontium (^{85}Sr, specific activity 10.2 mCi/mg) and cesium (^{137}Cs, specific activity 87 mCi/mg) were added to a suspension of spores in deionized water or metal mix. The suspension was incubated at room temperature for various time intervals and aliquots were removed, filtered, and the radioactivity determined in the filter and filtrate by gamma spectroscopy.

3.1.5 Adsorption Studies of Plating Rinse Waste

The pH of plating rinse waste was adjusted to 7.1 with ultra-pure HCl and the resulting solution was filtered before use. The filtered plating rinse waste was added to a suspension of spores, and this mixture was incubated at room temperature for 24 hr. Aliquots of the mixture were filtered and the concentrations of metals in the filtrates and filters determined by ICP analysis. The filters were digested as described above.

3.2 RESULTS

In previous experiments, we observed that spores added to metal mix in a weight ratio of 1/10 (i.e., 100 g of spores (wet weight) per 1,000 g (or mL) of rinse waste) and incubated for 240 min gave maximum adsorption for the metals tested in this study. Accordingly, this ratio and incubation time were used in the adsorption studies discussed below. The adsorption by spores of heavy metals frequently associated with environmental pollution is shown in Table 3.1. The adsorption ranged from 43 to 92% for the metals shown. Spores adsorbed more Hg, Pb, and Cr than As or Cd. The cumulative amount of metals adsorbed per gram of spores (dry weight) was 2.5 mg or 2.5% of the total dry weight of spores. Adsorption by spores of the metals listed in Table 3.1 was not affected by pH in the range of 4.1 to 9.6. Furthermore, the treated spores subsequently germinated on nutrient agar, which would suggest that the spores are resistant to the toxic effects of these metals.

TABLE 3.1
The Adsorption of Dissolved Metals by Spores of *Bacillus megaterium*

Compound	Concentration, ppm	Initial µg metal per gram spore (dry weight) in filtrate, µg/g	Adsorbed initial metal remaining in filtrate, percent
$HgCl_2$	8.0	370	8
$CdCl_2$	20.0	560	44
As_2O_3	16.6	360	57
$PbCl_2$	10.0	450	10
$CrCl_3$	19.8	760	23

A metal mix was prepared containing the compounds shown above and added to a suspension of spores (weight ratio of spores wet weight) to metal mix 1/10). This suspension was incubated at room temperature for 240 min and filtered though a 0.45-µm filter. Metals were determined in the filter and filtrate by ICP analyses. The results are the mean of two experiments.

Results in Table 3.1 suggest that spores have multiple binding sites for heavy metals. In an attempt to determine the presence of other binding sites in spores, adsorption studies were performed using eight different metals. These metals were added to deionized water

and the pH adjusted to 7.1 with HCl. After filtering this mixture (0.45-μm filter), the metal concentrations were determined. Based on the amount of metal added to the deionized water, several metals in this mixture had limited solubility at this pH (Table 3.2). Nevertheless, the observed metal concentrations after filtration were sufficient to determine the available binding sites in spores for the metals shown in Table 3.2. The percentage of the initial metal concentration adsorbed by spores ranged from 15 to 100 for the eight metals studied. Relative to the initial concentrations, Hg, Pb, and Cr showed the highest percentage adsorption. In contrast, Sr and Ba showed the lowest adsorption. However, the absolute amount of these metals adsorbed (in μg/g dry weight of spores) was similar to that seen for the other metals (i.e., Hg, 510; Pb, 40; Cr, 100; As, 180; Cd, 610; Se, 720; Sr, 350; and Ba, 280). When the filtrate obtained after spore treatment was re-treated with new spores, the final concentrations observed in the filtrate for most metals were below the detection limits.

The radioactive isotopes of both strontium and cesium are associated with waste from the nuclear power industry. Their removal from aqueous waste is desirable because of the cost associated with storing radioactive waste. Results shown in Table 3.2 indicate that spores were effective in adsorbing strontium. In an attempt to expand this observation, the adsorption efficiency of spores for strontium and cesium was determined. As shown in Table 3.3, spores were more effective in adsorbing ^{85}Sr than ^{137}Cs. Maximum adsorption for both isotopes was achieved after 240 min of incubation. At 240 min of incubation, spores had adsorbed 99% of the available ^{85}Sr (specific adsorption was 63 ng ^{85}Sr/g dry weight of spores). The total capacity for spore adsorption of strontium in the absence of other competing metals (i.e., Ca and Ba) was 609 μg Sr/g dry weight of spores. The total capacity for spore adsorption of cesium was 2.7 ng Cs/g dry weight of spores. We have no evidence that other metals compete for cesium adsorption sites. We have observed that adsorbed strontium can be desorbed with acid (HCl) or molar concentrations of calcium chloride.

Results shown in Table 3.4 indicate that spores have a broad specificity for adsorbing dissolved metals in aqueous waste. For example, in addition to adsorbing the metals shown in Tables 3.1 and 3.2, spores adsorbed zinc, copper, aluminum, cobalt, nickel, and uranium from plating rinse waste. Complete adsorption of the metals aluminum, chromium, nickel, and copper was observed following an additional treatment with fresh spores. Table 3.4 also shows the extent to which the adsorbed metals could be desorbed from spores with HCl. Furthermore, we have observed that these acid-treated spores can be reused in metal adsorption studies.

3.3 DISCUSSION

Microbial systems have been and are currently being considered for treating aqueous waste containing a variety of toxic metals. However, unlike chemical and physical systems, microbial systems require careful consideration of the potential toxicity of the metals

TABLE 3.2
The Adsorption of Dissolved Metals by Spores of
Bacillus megaterium

Compound	Initial metal concentration ppm	Metal concentration after incubation: Spores (mg/100 g spores (wet weight))	Metal concentration after incubation: Filtrate mg/L	Percent adsorbed
$HgCl_2$	13.3	10.1	1.7	84
As_2O_3	7.0	3.6	3.1	51
$PbCl_2$	0.72	0.77	0	100
$CdCl_2$	18.0	12.2	7.7	64
$CrCl_3$	2.2	2.0	0	100
$BaCl_2$	38.1	5.5	29.0	15
$SrCl_2$	31.0	7.0	24.4	23
Na_2SeO_3	19.0	14.4	5.0	74

A metal mixture was prepared and added to spores to give a weight ratio of 1/10 (spores to metal mix). The suspension was incubated at room temperature for 240 min, filtered, and the above metals determined in the filter and filtrate by ICP. Results are the mean of three experiments.

in the waste to the microbes and of the physiological needs (i.e., nutrients) of the microbe(s) used in the treatment system. Consideration must also be given to the type of metal to be treated because microbes display specific mechanisms for detoxifying heavy metals. Thus, a variety of microbial mechanisms for removing heavy metals from aqueous waste have been reported. Various microbial species can effectively remove heavy metals from aqueous media by (1) cell surface adsorption, (2) chelation by thiol and carboxyl groups (contained in low molecular weight molecules, peptides and/or

TABLE 3.3
The Adsorption of Radioactive Strontium and Cesium by Spores of Bacillus megaterium

Compound	Initial concentration dpm	Exposure time min	Spores dpm/g of spore (wet weight)	Filtrate (dpm/10 mL)
$^{85}SrCl_2$	280,000	60	168,000 (60)	113,000 (40)
		120	244,000 (87)	33,000 (12)
		240	277,000 (99)	1,500 (1)
		480	274,000 (98)	2,700 (1)
$^{137}CsCl$	320,000	60	80,000 (25)	237,000 (74)
		120	76,000 (24)	229,000 (72)
		240	88,000 (28)	223,000 (70)
		480	82,500 (26)	244,000 (76)

Spores were added to a solution containing ^{85}Sr and ^{137}Cs (1 g of spores (wet weight)/10 mL of radioactive solution), incubated at room temperature, and aliquots removed at various time intervals. After filtering the mixture, radioactivity in the filtrate and filter was determined by gamma spectroscopy. Results are the mean of two experiments. Percent of added metal is shown in the parentheses.

proteins), (3) reduction of heavy metals, or (4) the conversion of heavy metals to metal sulfides. Since these mechanisms do not all exist in a single microbe, it has been proposed that several microbial strains be combined to treat aqueous waste contaminated with heavy metals. We have studied a bacterial strain that appears capable of removing a variety of heavy metals from aqueous waste. Furthermore,

the form of the organism active in our metal adsorption studies is the dormant spore, which does not require nutrients and appears resistant to the toxic effects of heavy metals.

The removal of heavy metals and radionuclides from aqueous waste by the spores of the bacterium Bacillus megaterium was investigated because spores have been shown to be relatively resistant to a variety of conditions that are frequently encountered in treating aqueous industrial waste (i.e., radiation, temperature, and extremes of pH) (Hsieh, 1960; Hills, 1949; Levinson and Hyatt, 1960). However, the adsorption efficiency of heavy metals and radionuclides in aqueous waste by spores remained to be reported. Results of the present studies show that spores effectively adsorbed those metals frequently associated with industrial aqueous waste, such as Hg, Pb, As, Cd, Se, Cr, Ba, and Sr. However, spores were more effective in adsorbing Hg, Pb, Se, and Cr than As, Cd, Ba, and Sr. The metal adsorption efficiency depended on the concentrations and types of metals present in the mixture. For example, in solutions containing only As, Cd, Ba, or Sr, spore adsorption of these metals was complete at concentrations of 20 ppm or less. If Ba was added to a solution containing Sr, the adsorption of Ba and Sr was correspondingly decreased. Similar observations of apparent competition for binding sites were made with the groups Hg, As, and Cd, Hg and As, Hg and Cd, and As and Cd. These results suggest that similar adsorption sites in spores exist for Ba and Sr and for Hg, As, and Cd. Results likewise suggest that Pb, Se, Cs, and Cr have adsorption sites which are different from those for Hg, As, Cd, Ba, and Sr.

Several investigators have observed that spores accumulate Sr and Ba (Levinson and Hyatt, 1960; Slepecky and Foster, 1959; Vinter, 1956). These metals accumulate in the absence of calcium, which is the metal that normally accumulates in sporulating bacteria. It has been shown that these metals accumulate in sporulating bacteria in conjunction with the synthesis of the spore coat constituent dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA). Furthermore, studies have shown that Ca, Ba, and Sr bind to the carboxyl groups of DPA (Halvorson, 1961). Studies have also shown that Sr will displace Ca bound to DPA. Thus, the relative concentration of Ca in aqueous waste may not affect Sr adsorption. However, we have observed that when the concentration of Ca is 10 times greater than that of Sr, spore adsorption of Sr is reduced by at least 50%. Thus, the aqueous levels of Ca and the concentration of DPA in spores will affect adsorption of Sr. Several investigators have reported that the thiol concentration in spores is relatively high, which may explain the efficiency of spores for adsorbing Hg, As, and Cd (Vinter, 1957, 1959). Increasing the concentrations of thiol-containing macromolecules in spores would increase the amounts of Hg, Cd, and As adsorbed, because thiol groups are associated with the chelation of these metals (Jocelyn, 1972). We are presently investigating nutritional methods that may increase the spore concentration of DPA and thiol-containing macromolecules.

Results from the present studies also showed that the adsorbed metals could be partially or completely desorbed by acid treatment. Furthermore, acid-treated spores could be reused for adsorbing metals after neutralization. It was also observed in this study that the spores were resistant to the toxic effect of the metals. Thus spores, unlike other microbial systems, are highly resistant to the toxic

effects of heavy metals and do not require nutrients for normal maintenance in an aqueous waste treatment system. Ion exchange resins are the most frequently used system for treating aqueous waste containing heavy metals. Because of the metal specificity of resins, a variety of resins may be necessary to treat a mixed metal waste. Thus, the adsorption specificity for the heavy metals and radionuclides will, in part, determine whether spores will be more effective than the various ion exchange resins. Studies are currently in progress to determine the specificity and efficiency of spores versus resins for the adsorption of heavy metals and radionuclides from aqueous waste. Operational costs for aqueous waste treatment using spores versus ion exchange resins are also being considered. Based on information on ion exchange resins and the results described above, it would appear that spores have a broader specificity for adsorbing heavy metals and the radionuclides of strontium and cesium.

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4.0 SULFIDE REDUCTION

The presence of large amounts of mercury in aqueous effluents from the Department of Energy's Y-12 plant has been thoroughly documented. The very large amounts of mercury and other metals discharged over a long period of time have resulted in contamination of East Fork Poplar Creek and the surrounding floodplain. Soil from the floodplain has been dug up from time to time and used as landfill for homes and gardens, which makes the concern for the environmental fate of this element even more acute.

The presence of mercury in the soil surrounding the creek makes the problem of remediating the contamination more intangible than if only the creek water were contaminated. This is because the possibility exists that the metal may be intermittently irrigated from the soil by rain and mobilized into surface water and groundwater. This process of mobilization presents the possibility that the metal could be taken up into crops and enter the food chain. In addition, multiple bioconversions and interactions of mercury with soil constituents may occur. A conversion of particular concern would be formation of one or more of the organic forms of mercury. These forms are water soluble, volatile, and highly toxic.

An ideal approach to the problem of mercury contamination at East Fork Poplar Creek would be to physically remove all the contaminated soil to a site where it would pose no environmental threat. Unfortunately, this solution is impractical because of the very large area involved and the probability of causing more environmental dissemination of the mercury in dust during the removal process. Clearly, the only practical remediation processes are those which can treat the contamination on site, by immobilizing and/or converting the metals to environmentally benign forms. However, such an approach requires knowledge of which chemical forms of mercury are present at the site and how they can be changed.

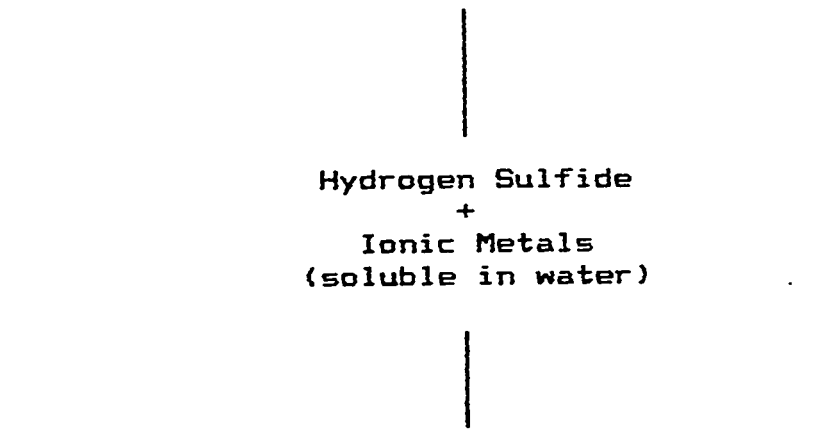
In this section, we describe the experiments we have carried out to discover the chemical forms of mercury that exist in the East Fork Poplar Creek floodplain. We also describe model and <u>in situ</u> experiments aimed at finding out how mercury is leached from soil and how these processes may be affected by conditions which would tend to not only immobilize the metal in the soil, but convert it to a stable, metabolically inert component. The approach we have used is based on the well-known ability of certain simple chemicals such as sodium hydroxide or sodium sulfide to precipitate metals from aqueous solution as either the hydroxide or sulfide (Perry, 1974). This approach to precipitating metals from solution has found widespread use in the treatment of aqueous effluents from chemical and metal treatment companies.

A problem with the direct application of compounds such as sodium hydroxide or sodium sulfide to soil is that these compounds themselves are harmful and, in the case of sodium sulfide, could release highly toxic hydrogen sulfide into the atmosphere. For this reason, we have explored the possibility of using H_2S generated in the soil by sulfate-reducing bacteria (Siegel, 1975; Postgate, 1984). These microbes are obligate anaerobes which are widespread at lower soil depths. A unique feature of their metabolism is the replacement of

oxygen with sulfur as a final proton acceptor in the process of oxidative phosphorylation. This is accompanied by the release of the reduced sulfur compound, H_2S, which is then directly available to undergo further chemical transformations in the presence of heavy metal ions, such as mercury, to form the insoluble and biologically inert metal sulfides (Ehrich, 1982; Norris and Kelley, 1979). This scheme is summarized in Figure 4.1.

Figure 4.1

The Relationship of Sulfate-Reducing Bacteria to Metal Sulfide Formation



Although much of the experimental work contained in this report has been focused on mercury and its biotransformations, many of the concepts may be applicable to other metals which may be released into the environment and contaminate soil. This is because, as shown in Table 4.1, many of the metal sulfides are extremely insoluble in water.

A limiting factor on the use of the sulfate-reducing bacteria as a source of hydrogen sulfide for interaction with mercury ions is that, in nutritionally inadequate conditions, the metabolism of these bacteria may be redirected to the formation of methyl mercury (Compeau and Bartha, 1985). To assure that the metabolism of sulfate-reducing bacteria is oriented toward sulfate reduction, we have hypothesized that it is essential to maintain the microbes in nutritionally adequate conditions by providing an external supply of sulfate and other nutrients such as lactate. Consequently, we have tested a

TABLE 4.1
Solubility of Metal Sulfides in Aqueous Solution

Metal		Solubility (µg/L)
Cadmium	(CdS)	0.00000087
Cobalt	(CoS)	0.000016
Copper	(CuS)	0.000000000000088
Lead	(PbS)	0.000043
Mercury	(HgS)	0.00000000000000011
Nickel	(NiS)	0.00011
Silver	(Ag_2S)	0.0000000084
Tin	(SnS)	0.000048

scheme, both in model and *in situ* systems, whereby powdered gypsum (calcium sulfate) was spread on the soil surface to ensure that the bacteria were maintained in nutritionally adequate conditions, and thus, metabolically oriented toward sulfate reduction. This approach had been suggested previously by Jernelov (1970) and by Craig and Bartlett (1978). The rates of mercury leaching were compared with those from similarly sized untreated soil samples or from plots receiving no treatment.

The findings resulting from this work are reported herein and are summarized as follows:

(1) All soil samples tested contained sulfate-reducing bacteria.

(2) In *in vitro* systems, sulfate reduction may be enhanced by the addition of sulfate.

(3) Column experiments suggest that mercury is difficult to elute from soil with water.

(4) Altered irrigation conditions can bring about release of mercury bound to soil particles.

(5) The amount of mercury eluted from soil columns is less in the presence of powdered sulfate than in controls.

(6) Field experiments involving the application of gypsum to soil plots and the measurement of mercury in lysimeters indicate that only a very small proportion of the total soil mercury

is mobilized into groundwater, but that the amount is significantly less in those plots treated with gypsum for 60 days than the levels in the same plots at zero time. By contrast, the mercury released from control plots was higher after 60 days than at zero time.

(7) Relatively low levels of mercury are solubilized from soil using the TCLP.

(8) Differential and/or sequential extraction of mercury from soil using nitric acid and then sodium sulfide suggests that, in most of the soil samples tested, much of the mercury is already in the mercuric sulfide form.

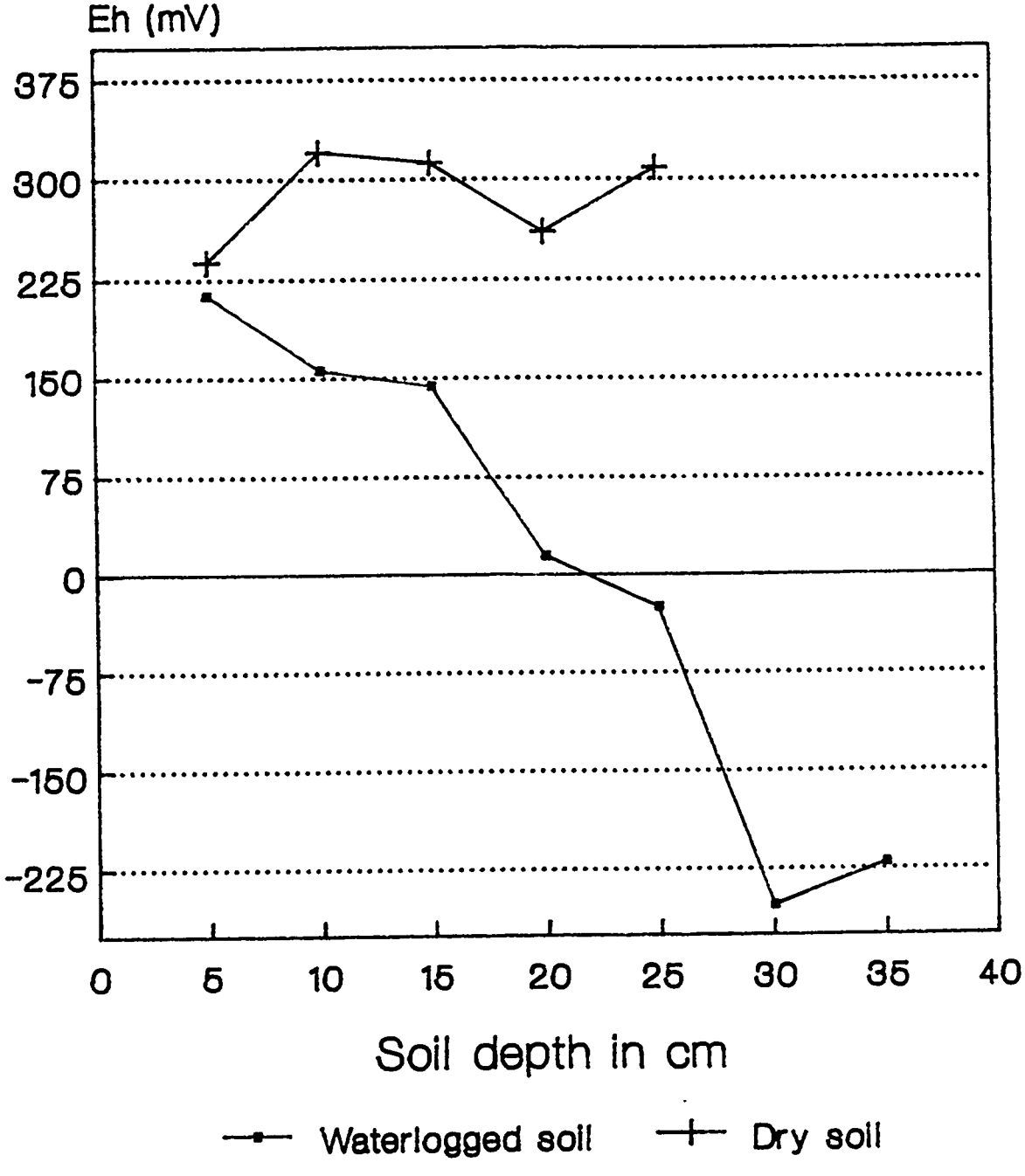
(9) Once formed in the soil, mercuric sulfide does not appear to undergo further chemical transformations.

We conclude that, in much of the East Fork Poplar Creek floodplain, most of the mercury was already in the sulfide form, probably as a result of the action of sulfate-reducing bacteria over a long period of time. Thus, the significance of the use of sulfate-reducing bacteria to bioremediate a mercury-contaminated site may be best judged by their application to an "unstable," recently contaminated site, in which the thrust of the bioremediation effort would be to accelerate the formation of mercuric sulfide. In this case, based on the results reported here, we speculate that the extensive spreading of gypsum and other nutrients such as lactate on an "unstable" mercury-contaminated site may have the beneficial effect of shortening the period which would normally have been required to effect the transition from soil-bound mercuric ions to insoluble mercury sulfide.

4.1 EXPERIMENTAL

In situ treatment of contaminated soil requires that the microbes exist at the site, that nutrients be adequate, and that the products formed have the desired properties. We have assessed the ability of soil samples taken from the floodplain to support sulfate respiration. We found that all samples tested contained sulfate-reducing bacteria and adequate carbon sources, but in every case the concentration of endogenous sulfate was too low to allow significant production of hydrogen sulfide. When sulfate was added to these samples, sulfate reduction ensued. Sulfate reduction activity requires an anaerobic environment and, specifically, a low redox potential (Harter and McLean, 1965; Deverel et al., 1986), with available sulfate and labile carbon sources. The redox potential (Eh) in many soils tends to decrease with depth in the soil column. This is especially true in waterlogged or wet soils and sediments where oxygen diffusion to deeper soils is limited and in sludge ponds containing high concentrations of organic matter. In the East Fork Poplar Creek floodplain, soil cores in a waterlogged area showed a corresponding decrease in Eh whereas those from a drier region showed little decrease in Eh (Figure 4.2). Although sulfate reduction activity may

Redox potentials in dry v waterlogged Hg-contaminated soils at various depths.



Means of five samples per site

Figure 4.2

not be optimal in the dry soil site, sulfate reduction may go on in microniche environments and result in metal sulfide accumulation; these same reduced microniches would likely not affect the measured Eh of bulk soil samples.

The preliminary experiments described above indicate that sulfate-reducing bacteria were present in the soil samples, and that the addition of sulfate enhanced the production of hydrogen sulfide. The next series of experiments used soil samples removed from the East Fork Poplar Creek floodplain to construct soil columns which could be used as a model in leaching experiments to examine the question of the rate at which mercury might be mobilized from soil, and the ability of sulfate amendment to provide sulfate at reasonable depth without adversely affecting soil pH. The columns were treated with sulfate in various ways, leached with water, and various parameters of the leachate determined.

Soil columns were constructed of 4-inch diameter PVC pipe hung vertically. The bottom of each column was covered with a sheet of glass window screen held in place by heavy rubber bands, and a glass fiber filter was set on the screening.

Soil was collected from the floodplain of East Fork Poplar Creek, at ORRI station 12, on the Wayne Clark property. At this site, the soil contained approximately 1000 mg Hg/kg soil. The soil was collected in layers with as little disturbance as possible. The columns contained a total of 40 cm of soil: (from the bottom up) three 10-cm layers and two 5-cm layers. Descriptive parameters of the soil are listed in Table 4.2. Each column contained approximately 4 kg of soil.

TABLE 4.2
Properties of Soil in Columns

Parameter	Depth (cm) 0–5	5–10	10–20	20–30	30–40
pH (18 hr)[1]	7.23	7.21	7.21	7.25	7.66
Conductivity (μMho/cm[2])[2]	110	100	105	90	205
Moisture (%)[3]	39.3	36.0	32.7	35.7	35.1
Organic (%)[4]	12.8	12.6	11.8	11.2	10.3

[1]pH was measured on suspensions of soil in water.
[2]Measured on suspensions of soil in water.
[3]Determined by drying samples of soil at 110°C to constant mass.
[4]Determined as % of dry weight by ignition of dried soil.

The columns were arranged into three different sulfate treatment groups. The first group was an untreated control group. The second group received about 2 g of $CaSO_4$ on the surface (500 mg/kg soil), and the third received 500 mg $CaSO_4$/kg soil, mixed into each soil layer before the columns were constructed. The columns were further divided into three water treatment groups. The first group received water at a rate that approximated annual rainfall, which for columns of this size was 75 cc on Monday, Wednesday, and Friday of each week. The second group received water at approximately the rate of the previous year's rainfall. Daily rainfall records for 1985 obtained from ATDL were used to determine the amount of water to be added to columns in this group on treatment days. Effluents from these columns were collected in wide-mouth glass jars and sampled at intervals for analysis. The third group was sealed and filled with water and allowed to sit for long-term waterlogging studies.

The data summarized in this report focus attention on the columns which were eluted with 75 mL of water three times per week. Table 4.3 shows that there was little difference between treatments in the observed pH of the effluent.

TABLE 4.3
ph of Soil Column Effluents

Column	Number of samples	Average pH
H_2O	1	5.6
No sulfate	4	7.8
Surface addn.	5	7.9
Mixed	5	7.7

The key parameters measured in the eluates of the soil columns were sulfate and mercury. Sulfate was measured to assess the ability of surface-layered gypsum to provide sulfate at the depths at which the sulfate-reducing bacteria were likely to be found. Figure 4.3 shows clearly that there was a considerable difference in the rates of elution of sulfate from columns in which the gypsum was intimately mixed with the soil compared to those receiving surface-layered gypsum. In the former case, the appearance of sulfate in the eluate was immediate, with the majority eluted by 48 days. By contrast, in those columns which were treated with a surface layer of gypsum, sulfate did not begin to appear until about 30 days following the construction of the column (Figure 4.4). However the total amounts of sulfate liberated from the columns were similar.

Figure 4.4 shows the accumulating release of mercury from the soil columns. These data were obtained by measuring mercury in the column eluates using the ICP analyzer. The most important fact to emerge is that only a very small percentage of the total mercury was released from the soil. This may be judged by the measurements of endogenous

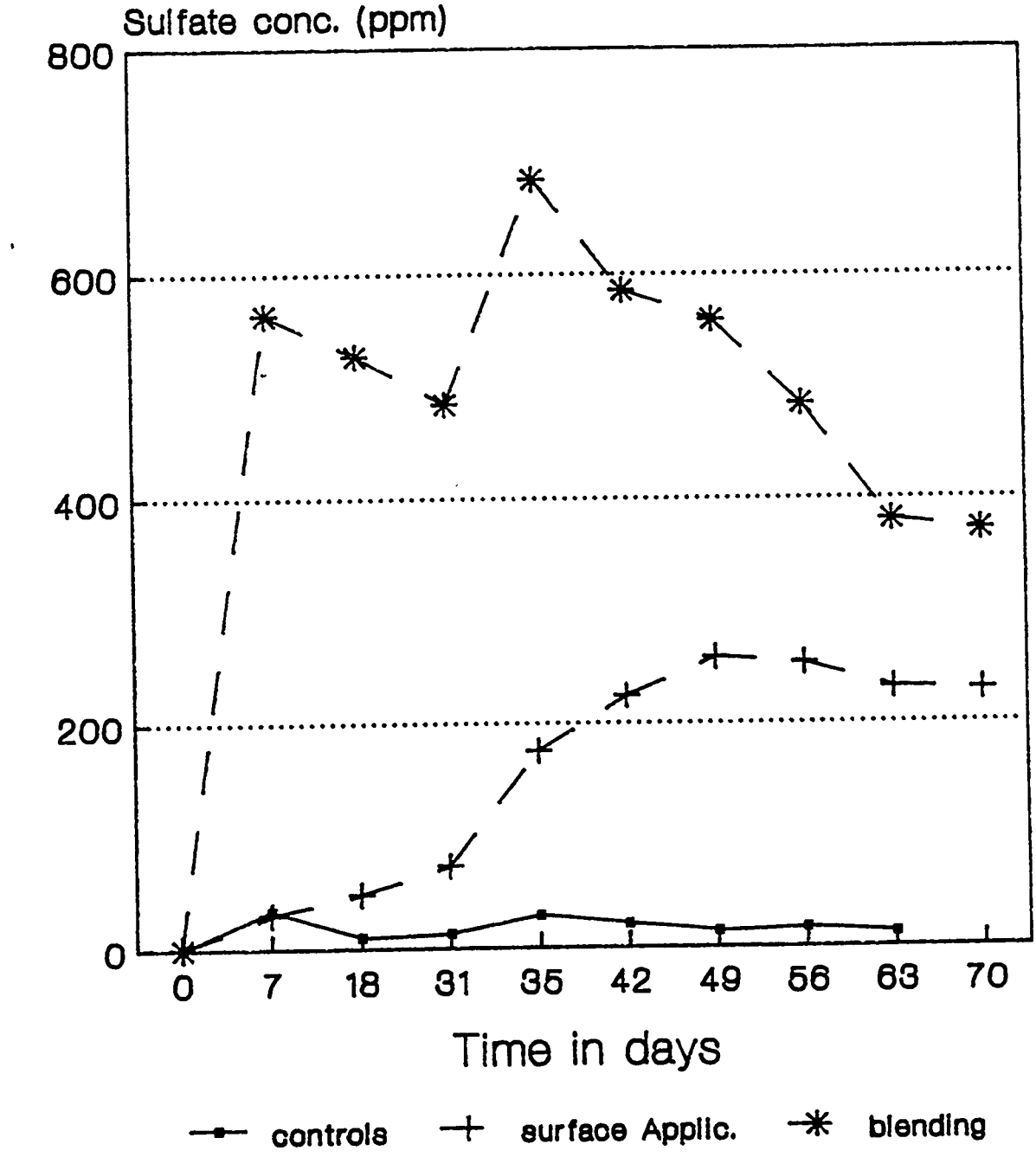

Water Leaching of Sulfate from
Sulfate-Amended Soil Columns
Sulfate conc. (ppm)
800
600
400
200
0
0
7
18
31
35
42
49
56
63
70
Time in days
controls
surface Applic.
blending
blending v surface application

Figure 4.3

Accumulated elution of mercury from soil columns. Effect of calcium sulfate.

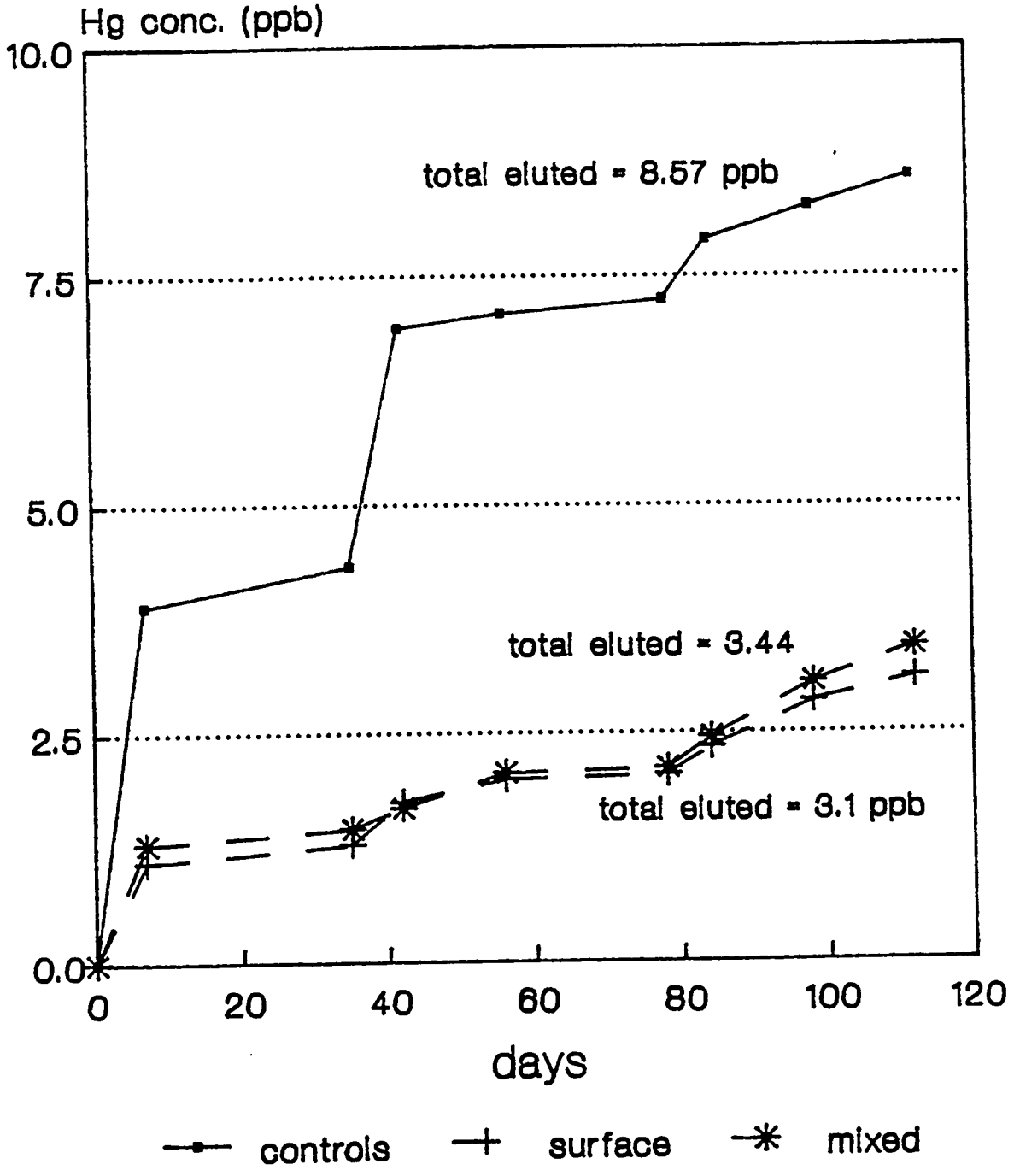


Figure 4.4

mercury in the soil samples, which gave an estimation that each of the columns initially contained approximately 4 g of mercury. The total amount of mercury eluted from the control column, for example, was only 0.643 µg, which represents 0.000016% of the total amount of mercury on the column.

It is also clear from Figure 4.4, however, that the amount of mercury released from the control columns was at least double that liberated from columns treated with powdered gypsum, either by intimate mixing or by layering along the surface. This result is consistent with the concept that application of gypsum to the soil may have an immobilizing effect on soil mercury. Analysis of mercury was also carried out on the eluates from the second group of columns which were only intermittently eluted with water. These elution conditions were chosen as a model for the rainfall pattern which existed in East Tennessee during 1985. A key observation from this phase of the experiment was that the eluates from these columns contained a considerable amount of sediment, in contrast to those of the regularly watered group. In addition, the concentration of mercury in the eluates was considerably higher. Thus, as shown in Table 4.4, the concentration of mercury in the intermittently watered, no-sulfate group was 40-fold higher than in the regularly watered group. Similarly, there was a 16-fold difference between the mercury concentrations of the eluates when comparing water treatments in the gypsum treated group. Interestingly, the concentration of mercury in the control eluates for this intermittently eluted group were 6-fold higher than in the sulfate-treated group. Again, this is consistent with the concept that sulfate remediation has the ability to immobilize mercury in soil and to retard the mobilization of the element into groundwater. However, when the particulate material was allowed to settle out or was filtered, the mercury concentration in the clear eluate was again very low, suggesting that the eluted mercury was insoluble and/or bound to soil particles.

TABLE 4.4
Particulate Nature of Mercury
Eluted from Soil Columns

H_2O addn.	Column	Total Hg (ng)	Soluble Hg (ng)	Sediment-bound Hg (ng)
Regular				
	No sulfate	160	123	37
	Surface addn.	60	54	6
	Mixed	56	35	21
Intermittent				
	No sulfate	6383	88	6295
	Surface addn.	1013	106	907

Taking all the data from the soil columns together provides a picture of mercury, and perhaps other heavy metals, being very tightly bound to the soil matrix. However, the extent to which the elements may be environmentally benign is unclear because there may still be

possibilities for the immobilized elements to react with soil components and undergo potentially harmful interconversions. The data from Table 4.4 suggest that sulfate remediation can affect and retard the liberation of these particulate mercury-containing components. If this is true, and if the product of sulfate remediation is the formation of metal sulfides, then this emphasizes the fact that formation of the metal sulfides is a worthwhile goal and valuable endpoint of the in situ remediation effort. However, even the particulate mercury eluted from the intermittently watered columns was a very small percentage of the total, which again allows the suggestion that the vast majority of the soil mercury was already in the immobile and biologically inert sulfide form. Further evidence of the immobility of mercury in the soil is provided by the EP toxicity test. In this procedure, soil was leached for 24 hr with enough acetic acid to maintain the pH of the suspension at 5. To determine how the leaching of metals from the soil columns was related to potential toxicity, soil suspensions were extracted with acetic acid and analyzed to determine heavy metal concentrations. The results of this analysis are shown in Table 4.5.

TABLE 4.5
Leaching of Heavy Metals from Soil
by the EPA Extraction Procedure

	Concentration in leachate (ppb)				
Metal	Blank[1]	Soil alone	Soil + $CaSO_4$[2]	Soil + Hg[3]	EPA tox. level
Arsenic	10	<10	<10	<10	5000
Beryllium	0.11	0.10	0.06	0.11	--
Cadmium	<10	<10	<10	<10	1000
Lead	<10	<10	<10	<10	5000
Mercury	0.05	0.05	0.13	564	200

[1] Distilled water, acidified with 12.5 mM acetic acid.
[2] $CaSO_4$, 500 mg/kg soil.
[3] $HgCl_2$, 1000 mg/kg soil = 50,000 ppb in extract.

These results show clearly that no significant amounts of heavy metal were leached from the soil samples. In fact, when the soil was spiked with additional $HgCl_2$, only about 1% of the newly added mercury was extracted.

The next phase of the study was to examine the effect of added calcium sulfate on mercury mobilization from plots of soil in the floodplain of EFPC. The experiments described below provide information over an extended period of time about the availability of sulfate and the mobility of mercury and other metals in amended soil; they could provide the basis for in situ treatment of contaminated soil at a relatively low cost.

The objective of this experiment was to determine the effect of $CaSO_4$ (gypsum) amendment on the movement of mercury in floodplain soils. The approach used was to add gypsum (Terra-Alba brand) to the soil in three amounts (none, 0.02 kg/m^2, and 0.20 kg/m^2) and to monitor levels of total mercury, sulfate, conductivity, and pH in water that has percolated through the soil. The groundwater samplers were lysimeters composed of a long glass tube with a fritted-glass bulb at the bottom through which water could enter when a vacuum was applied to the glass tube. The lysimeters were installed by removing a core of soil to about 35 cm deep and inserting the sampler into the hole. The hole was filled with a finely ground sand (microsil) to a level above the lysimeter and a bentonite seal (volclay) installed above the sand to prevent water from running directly down the tubes.

The site for this experiment was located on East Poplar Creek on DOE land behind the NOAA building in Oak Ridge, Tennessee, in an area where elevated levels of the metals of concern had been found. A randomized complete block design was used with each of the three treatments of $CaSO_4$ applied to a plot within each of four blocks (replicates). Within each plot, three lysimeters were installed. Treatments were randomly assigned to plots within blocks; samplers also were located randomly within plots. Plot size was 3 X 3 m with a 1-m buffer strip between plots.

The original soil cores removed for placement of the samplers were analyzed for the parameters listed above. Water was withdrawn from the samplers for analysis after 60 and 90 days. Water in the lysimeters was pumped up to the surface flask; the initial samples were discarded. Then a vacuum of approximately 25 cm of mercury was applied to the sampler and the freshly collected sample was used for analysis. Samples were preserved by the immediate addition of supra-pure nitric acid to reduce the pH below 2. Other samples which were not acidified were taken to measure pH, conductivity, and sulfate concentration.

Data on rainfall during the experiment came from a gauge maintained at the nearby NOAA facility. Tubes were installed in the soil near the plots to monitor the height of the water table.

Statistical handling of the sulfate and mercury concentrations in the lysimeters was by the Student's t-test of unpaired values when comparing group means at a single time point. When comparisons of means within a group but at different time points were made, the Student's t-test of paired values was used. Correlations between sulfate and mercury concentrations in the lysimeters were achieved by linear regression analysis, using the method of least squares.

Results shown in Table 4.6 indicate the levels of mercury and sulfate from soil isolated from the plots prior to the start of the experiment. Mercury concentrations varied from 288 to 868 ppm whereas sulfate levels were undetectable in most cases.

The extent to which spreading gypsum on the surface of the soil plots was able to provide sulfate at suitable depths to make available a respiration substrate for sulfate-reducing bacteria may be judged by the concentrations of sulfate which were released into groundwater and collected in the lysimeters. These data are summarized in Figure 4.5, and demonstrate considerable elevations in the sulfate concentrations after 60 and 90 days. Thus, the zero time and control levels of sulfate remained below 0.6 ppm, whereas the high

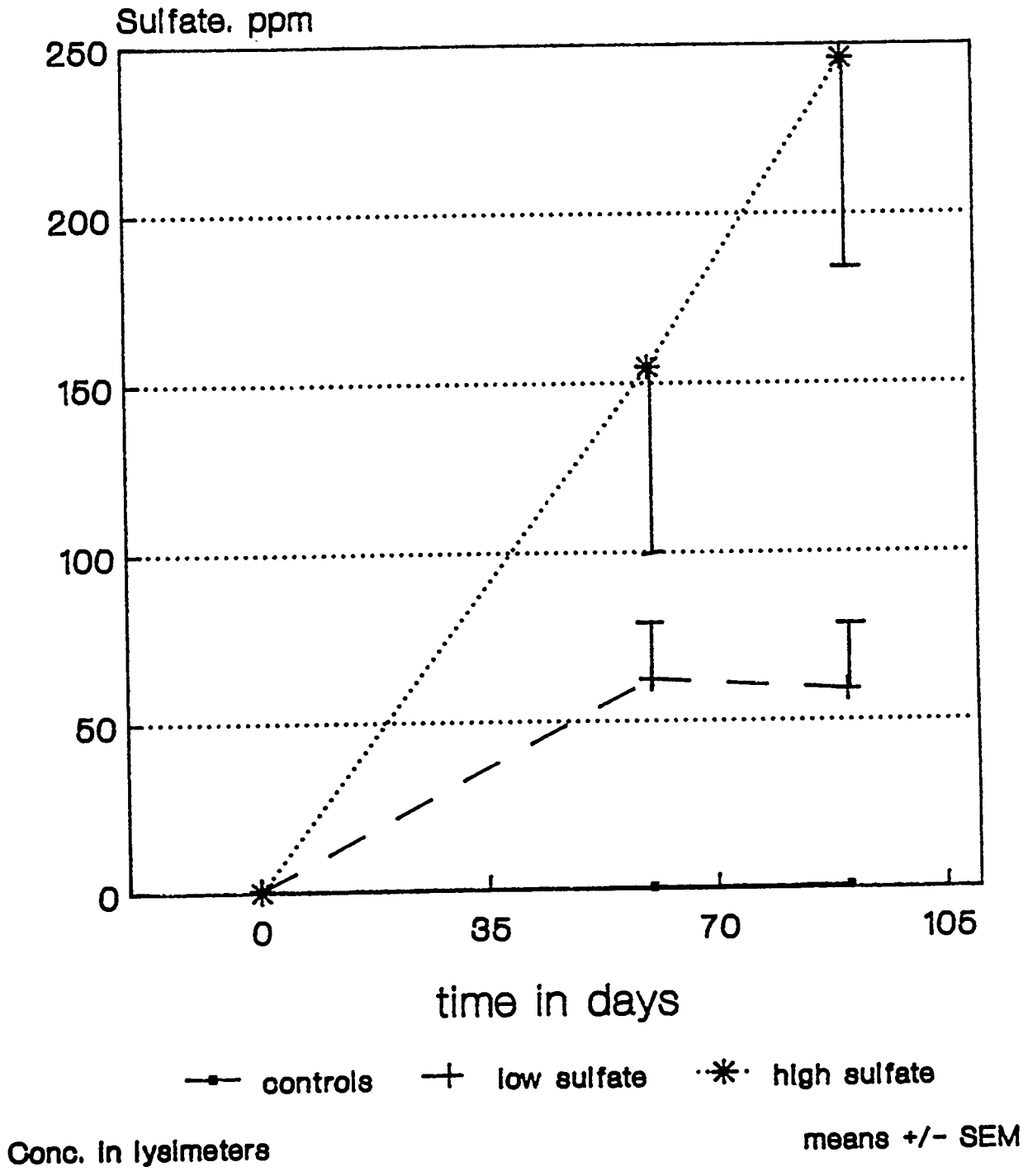

Gypsum (CaSO4) application to soil plots
Elution of sulfate into ground water
Sulfate. ppm
250
200
150
100
50
0
0
35
70
105
time in days
controls
low sulfate
high sulfate
Conc. in lysimeters
means +/- SEM

Figure 4.5

TABLE 4.6
Mercury Levels in Soil from the
Floodplain of East Fork Poplar Creek

Site ID No.	Soil mercury mg/kg	Soil SO_4 mg/kg
Ac	468 ± 230	0
Al	288 ± 72	13.2
Ah	425 ± 139	0
Bc	742 ± 266	0
Bl	379 ± 103	0
Bh	440 ± 121	0
Cc	683 ± 331	11
Cl	575 ± 267	0
Ch	868 ± 353	0
Dc	455 ± 146	0
Dl	385 ± 72	0
Dh	551 ± 217	11
	MEAN ± SD	

gypsum plots had mean sulfate concentrations in the lysimeters of 154.8 ppm and 245.8 ppm for 60 and 90 days, respectively. Plots receiving low gypsum levels had mean sulfate concentrations in the lysimeters of 62.3 ppm and 59 ppm after 60 and 90 days, respectively. These latter levels represent a 100-fold increase compared to zero time or control values.

The amounts of mercury released into groundwater and collected in the lysimeters were strikingly low compared to the overall levels of mercury present in the soil. For example, the maximum concentration of mercury in the lysimeters for any treatment group or time point was 2.2 ppb. These very low amounts of mercury recovered, by analogy with the limited release of mercury from the soil columns, again suggest that the overwhelming majority of the mercury was tightly bound in the soil, and could not be released from it by water leaching. An interesting question, however, is the extent to which the very low amounts of mercury which were released may be affected by the concentration of available sulfate. We examined the changes in mercury levels in groundwater which occurred within the plots due to sulfate remediation for 60 or 90 days compared to the levels of mercury released from the soil prior to the start of treatment. As shown in Figure 4.6, the consistently lower levels of mercury in the lysimeters of the high treatment group plots after 60 or 90 days compared to the zero time values demonstrates a statistically significant change due to treatment, using the Student's t-test for

Concentration of Mercury in Lysimeters.
Effect of Sulfate treatment for 90 days.

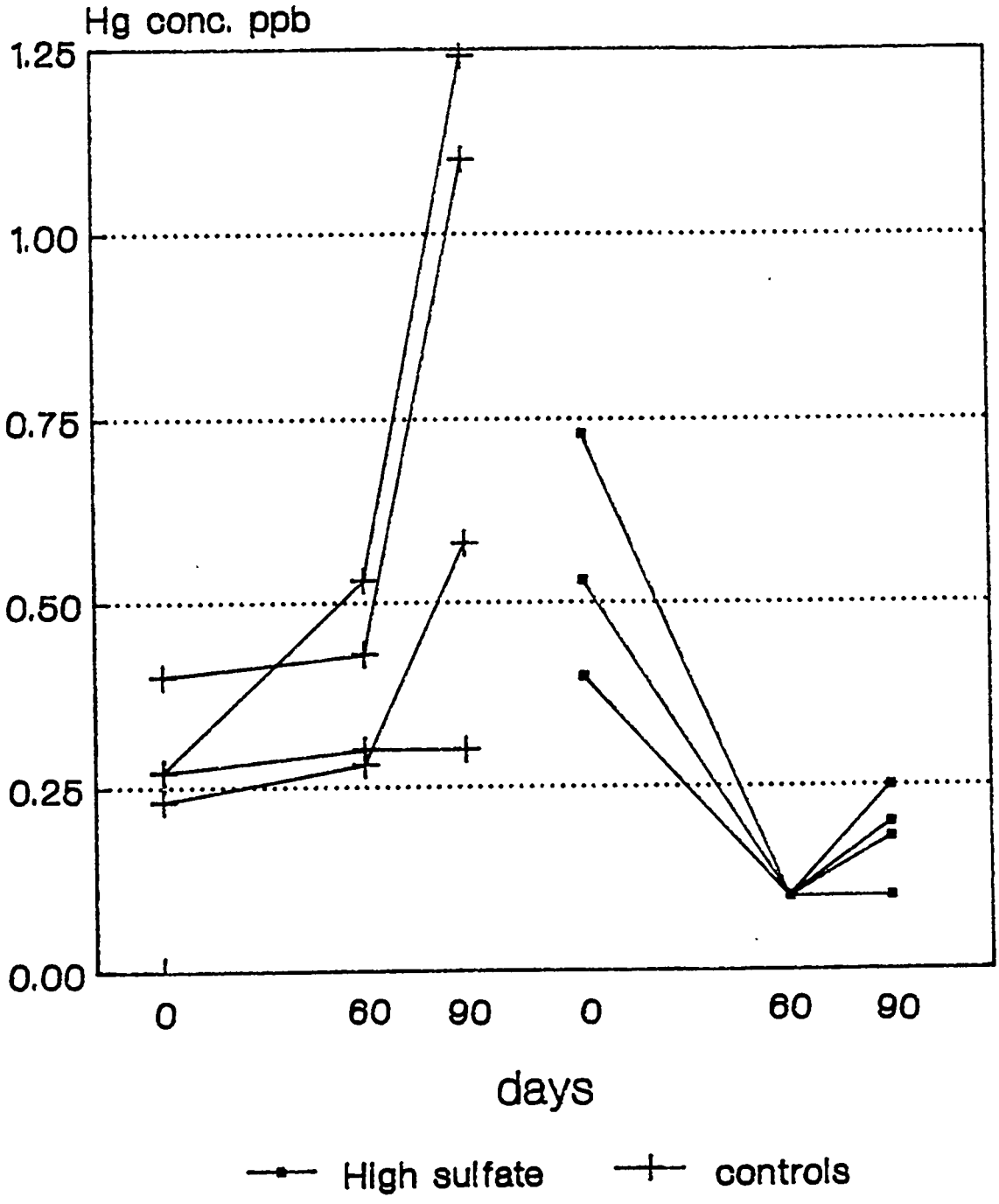


Figure 4.6

paired values. This downward trend in mercury concentrations is in contrast to that seen for the mercury concentrations in the lysimeters in control plots, where no statistically significant reductions were seen. In fact, there was an overall trend toward increasing mercury concentrations with time in the control plots, although this trend did not achieve statistical significance. Another statistical comparison which may be made of these data is that of the levels of mercury released into groundwater in the high sulfate plots at 60 or 90 days, with those levels released from control plots at the same time points. The mean levels of released mercury after 60 or 90 days due to high sulfate were significantly lower than those from control plots when compared by the Student's t-test for unpaired values.

Taken together, these data suggest that there may be an association between high levels of sulfate in the soil and the decreased liberation of soil-borne mercury into groundwater. That this association can occur lends weight to the concept that high levels of sulfate in the soil can affect the metabolism of sulfate-reducing bacteria, and that the resulting release of hydrogen sulfide may be the mechanism by which mercury release from the soil is retarded.

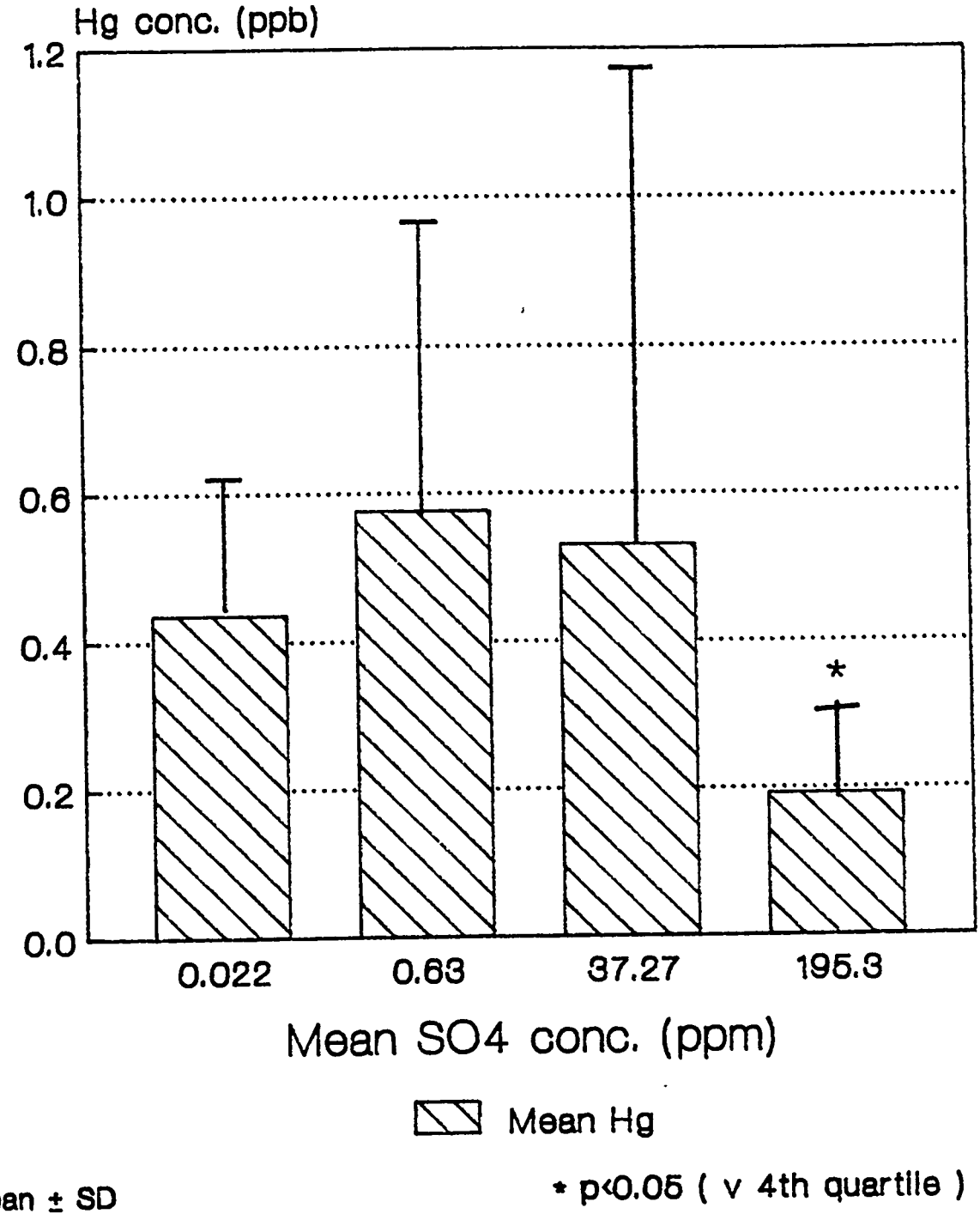
Further attempts have been made to clarify the extent of the possible association between the levels of mercury and sulfate in groundwater. In the first instance, the sulfate concentrations measured in the lysimeters for all treatment groups and time points were ranked in decreasing order, then divided into quartiles. The mean sulfate concentrations in the quartiles were 195.3, 37.27, 0.63, and 0.022 ppm, respectively. Figure 4.7 provides a comparison of the mean mercury concentrations in the quartiles and demonstrates that the average amount of mercury in the first quartile was significantly different from that in the fourth quartile with 95% probability, when compared using the Student's t-test of unpaired values. As might be expected, lower mercury levels were associated with the higher concentrations of sulfate.

Each data pair was also entered into a correlation matrix and subjected to linear regression analysis by the method of least squares. The correlation coefficient ($r = -0.376$) was statistically significant with 95% probability, although reference to Figure 4.8 suggests that the actual relationship may not be linear because of the wide spread of mercury concentrations observed at low sulfate concentrations. However, the data provide further supporting evidence of an inverse association between release of mercury into groundwater and soil sulfate levels, particularly at the higher concentrations.

If the application of sulfate to soil, in the form of calcium sulfate, has the effect of immobilizing at least part of the mercury contamination, presumably because of its conversion to mercuric sulfide, the question arises as to how important this process may be, in view of the fact that the overwhelming majority of the mercury was not released from the soil, even from the control columns or plots. It, therefore, became important to learn precisely what chemical forms the mercury in soil had assumed.

To answer this question, we investigated possible approaches to the differential solubilization of the mercury components in the soil, in order to assess their contribution to the overall level of contamination. Specifically, we sought to learn how much of the total

Mean Hg conc. in lysimeters ranked by decreasing SO4 and sorted into quartiles



Mean ± SD

* p<0.05 (v 4th quartile)

Figure 4.7

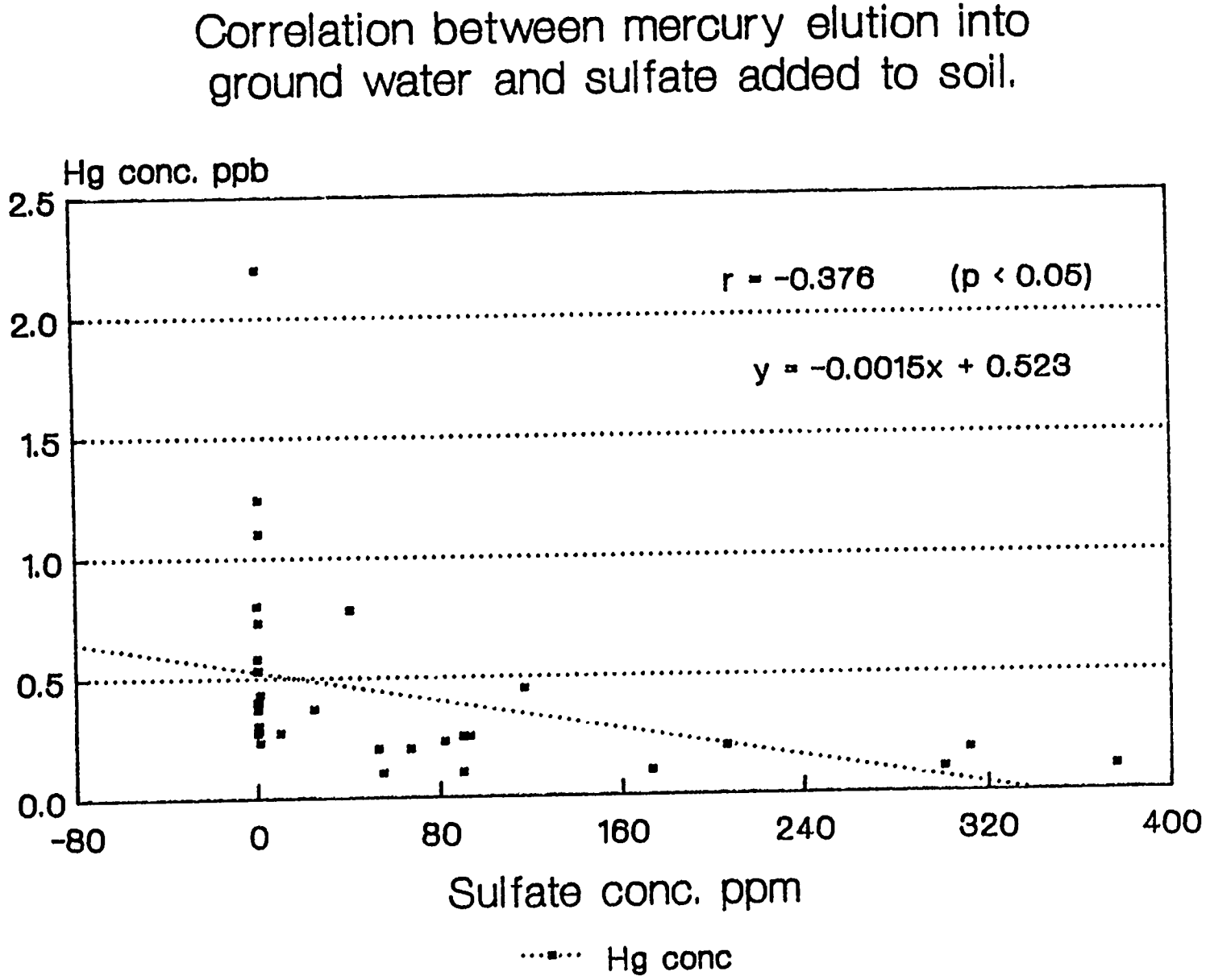

Correlation between mercury elution into ground water and sulfate added to soil.
Hg conc. ppb
2.5
2.0
1.5
1.0
0.5
0.0
r = -0.376 (p < 0.05)
y = -0.0015x + 0.523
-80
0
80
160
240
320
400
Sulfate conc. ppm
Hg conc

Figure 4.8

soil mercury was in the form of mercuric sulfide, in order to assess the overall significance of hydrogen sulfide formation to the *in situ* bioremediation of mercury-contaminated sites.

In all the experiments, we measured mercury by atomic absorption spectrophotometry using the cold vapor technique. The ability to unequivocally ascribe one or another fraction to a particular chemical component of mercury depends absolutely on the degree of discrimination of the extraction procedure used and its exclusivity for the target mercury compound. We used the ability of saturated sodium sulfide to solubilize mercuric sulfide as a means to differentiate between this and other mercury compounds, although it was first necessary to be assured that this compound would solubilize only the sulfide and not other mercury components. Our approach to this question was to take multiple aliquots of a heavily mercury-contaminated soil, and subdivide them into two groups. The first group was extracted sequentially with nitric acid, then with sodium sulfide, while the second group was extracted with these solutions in the reverse order. The results of this experiment are shown in Table 4.7, which indicates that nearly 80% of the total mercury in the soil was resistant to acid solubilization, but could be solubilized in sodium sulfide. This component was presumably mercuric sulfide. When the extractants were used in reverse order, the percentage of the total mercury released into the first extractant, i.e., sodium sulfide, was again nearly 80%. This result suggests that sodium sulfide solubilizes mercuric sulfide only, because if it had the ability to solubilize other components, the percentage recovery of the total mercury would have been higher where sodium sulfide was the first extractant used.

With the knowledge that the sodium sulfide solubilization procedure was exclusive for mercuric sulfide, we were able to apply this solubilization technique to a large number of mercury-contaminated samples taken from various sites. These data are summarized in Table 4.8 and indicate that sodium sulfide solubilized approximately 90% of the total mercury in the soil. This strongly suggests that it is this percentage of the total mercury which is in the mercuric sulfide form, presumably as a result of the action of sulfate-reducing bacteria over a long period of time.

If much of the soil mercury is immobile because it has been converted to the sulfide form, the question arises as to how long the process of biotransformation to sulfide takes to occur if left unassisted. This is a particularly relevant question in relation to "unstable" sites, which are afflicted with recent spills and which may require immediate action.

TABLE 4.7
The Sequential Extraction of Mercury from Soil
Using Nitric Acid and Sodium Sulfide

Creek mile	First extraction nitric acid	Second extraction sodium sulfide	Total Hg extracted	Total Hg in the digest
	µg Hg/gram dry weight soil			
10.6	53	198	251	264
	50	255	295	315
	11	102	113	124
	373	983	1,356	1,410
	27	86	113	100
	First extraction sodium sulfide	Second extraction nitric acid	Total Hg extracted	Total Hg in the digest
	µg Hg/gram dry weight soil			
10.6	477	44	521	611
	189	66	254	304
	313	28	341	319
	1,529	140	1,669	1,883
	159	45	204	209

Soil samples were collected from the above sites, mixed, and divided in half. Half of each sample was digested and the other half was sequentially extracted with nitric acid and then sodium sulfide (or the reverse). After extracting for 24 hr, samples were centrifuged and the supernatant was removed and filtered. Mercury was determined in the filtrate and soil digest by atomic absorption (cold vapor technique). Results are the mean of three experiments.

TABLE 4.8
Sodium Sulfide Extractable Mercury in Soil:
A Comparison of Sodium Sulfide Extractable
Mercury in Soil to Mercury Levels in a Soil Digest

Creek mile	Sample No.	Total mercury in soil digest (µg Hg/gram dry weight soil)	Total mercury in Na_2S extract (µg Hg/gram dry weight soil)	Percent extracted
10.8				
	1	170	135	79
	2	138	116	84
	3	158	148	94
	4	1,097	885	81
	5	909	656	72
	6	1,003	970	97
	7	1,485	1,228	83
	8	1,273	1,146	90
	9	1,380	1,052	76
	10	19	20	100
	11	641	622	97
	12	421	438	100
	13	460	495	100
	14	60	63	100
	15	2,920	2,970	100
				MEAN 88±7.1
11.7				
	1	116	115	99
	2	181	149	82
	3	114	130	100
	4	1,410	1,914	100
	5	2,461	1,680	68
	6	1,289	1,442	100
	7	2,013	1,273	63
	8	1,996	2,006	100
	9	1,563	1,306	84
	10	1,685	1,273	76
	11	2,961	3,030	100
	12	2,200	2,015	92
	13	98	122	100
	14	2,562	2,492	97
	15	3,003	3,772	100
				MEAN 92±11.8

Table continued on next page.

TABLE 4.8 (Contd.)
Sodium Sulfide Extractable Mercury in Soil:
A Comparison of Sodium Sulfide Extractable
Mercury in Soil to Mercury Levels in a Soil Digest

Creek mile	Sample No.	Total mercury in soil digest	Total mercury in Na_2S extract	Percent extracted
		μg Hg/gram dry weight soil		
13.7				
	1	45	40	89
	2	9	11	100
	3	43	39	91
	4	1.8	2.	100
	5	2,339	1,757	75
	6	1,797	1,856	100
				MEAN 93+10

Soil samples were collected from the above sites and mixed; part of the sample was digested and the other part was extracted with Na_2S. Mercury was determined in the soil digest and the Na_2S extract. Results in the percent extraction column are given as the mean + SD. The coefficients of variation between samples were X-10.8, 8.4; X-11.7, 12.9; and X-13.7, 11.

We recently carried out an experiment to address this question by preparing a soil sample which had been spiked with $^{203}HgCl_2$. Equivalent sections of this soil sample were extracted into either water, nitric acid, or sodium sulfide after 5 days incubation. Aliquots of the extraction supernatants were counted on a gamma counter.

Table 4.9 shows the results from this experiment and indicates that almost none of the radioactive mercury was leachable with water or sodium sulfide. By contrast, 95% of the added radioactivity was recovered in the nitric acid. These data suggest that, during the 5-day incubation period, the radioactive mercury had become physically bound to soil particles but had not been converted to the sulfide form. This result indicates the very slow nature of the conversion to sulfide in unamended soils.

The demonstration that almost all of the soil-borne mercury was in the sulfide form provides very strong inferential evidence to suggest that the sulfate-reducing bacteria may have played a key role in the conversion of the potentially harmful and reactive forms of mercury to the environmentally benign (and water-insoluble) mercuric sulfide. A question of importance, however, relates to how inert the mercuric sulfide is, once formed. We have endeavored to answer this question by synthesizing radioactively labelled mercuric sulfide, and incubating it in various types of soil for periods of up to 6 weeks.

TABLE 4.9
The Extraction of Radioactive Mercury from Soil Using Deionized Water, Nitric Acid, or Sodium Sulfide

Creek mile	Radioactive Hg added dpm/g dry weight soil	Water soluble	Nitric acid soluble	Sodium sulfide soluble
		Total Hg extracted from a gram of soil dpm/g dry weight soil		
10.8	24,000	240 (1.0 %)	22,892 (95 %)	316 (1.3 %)
11.7	24,000	256 (1.0 %)	23,356 (97 %)	232 (1.0 %)

An aqueous solution of radioactive mercuric chloride (24,000 dpm of ^{203}Hg) was added to soil (1 gram) from the above sites and incubated at room temperature for 5 days. After incubation, samples were centrifuged and the supernatant removed. The above extraction solutions were added to the soil samples and this mixture was incubated overnight. After incubation, the samples were centrifuged and the supernatant was removed and filtered and the radioactivity determined in the pellet and filtrate. The counts observed in the supernatant of the first incubation were less than 1% of the total counts added. Results are the mean of three experiments.

At 0, 2, 4, and 6 weeks, aliquots of soil were sequentially extracted into water, nitric acid, then sodium sulfide, and the soluble products counted on a gamma counter. This allowed us to assess the possibility that mercuric sulfide, once formed, might be subject to some form of further biotransformation or mobilization in soil. A demonstration that the labelled mercuric sulfide remained largely inert would clearly establish the formation of metal sulfides in soil as a worthwhile environmental goal.

Radiolabeled HgS was synthesized by incubating stoichiometric amounts of $^{203}HgCl_2$ and Na_2S in water, and washing the precipitated ^{203}HgS several times with water. The concentration of ^{203}HgS in the final suspension was determined by counting a sample in a gamma spectrometer.

Soil samples were collected from three locations in the East Fork Poplar Creek floodplain, representing a variety of soil types and moistures. Subsamples were weighed, dried to constant weight, and reweighed to determine moisture content. A portion of each soil sample was autoclaved to sterilize the soil. Soil was suspended in sterile 125-mL flasks in 30 mL of sterile distilled water at a concentration of 33% (w/w, based on dry weight of soil), and

sufficient ^{203}HgS was added to bring the concentration of $^{203}Hg^{++}$ to 100 ppm. The slurries were incubated under the conditions indicated in Table 4.10. A control set containing no soil was also included, as was a slurry containing the soluble salt $^{203}HgCl_2$ instead of ^{203}HgS.

TABLE 4.10
Conditions for Soil Slurries Containing ^{203}HgS
(conditions of incubation, soil slurry, 10 g dry wt/30 g slurry; 100 ppm HgS, approx. 10^6 cpm ^{203}Hg/g slurry)

Set	Source	Group	Treatment
A	Deionized water	1	Sterile, aerobic[1]
B	Soil, creek mile 14.6	2	Sterile, anaerobic[2]
C	Soil, creek mile 13.75	3	Incubated 4°C[3]
D	Soil, creek mile 10.9	4	Aerobic[1]
		5	Anaerobic[2]
		6	Plus hydrogen peroxide[4]
		7	$HgCl_2$ replacing HgS

[1]Aerobic conditions were maintained by shaking the slurries at approximately 100 reciprocations per minute on a platform shaker at room temperature.

[2]Anaerobic conditions were maintained by incubating the slurry in a Coy anaerobic chamber with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2.

[3]Slurries were incubated without shaking in a refrigerator.

[4]Slurries were incubated without shaking at room temperature.

At weekly intervals, 3% H_2O_2 was added to bring the final concentration to 0.1%. Immediately after they were prepared and then at weekly intervals, the slurries were thoroughly mixed and sampled for analysis. The analysis procedure is summarized in Table 4.11 and was as follows:

Water-soluble: 1-mL samples of slurry were removed in triplicate from the flask to 13 x 100 mm screw-cap tubes. One mL of water was added to each tube, and the tubes were counted in a gamma spectrometer. The samples were then centrifuged for 10 min at approximately 2000 x g. The supernate was filtered through a 0.45-micron membrane filter, and 1 mL was counted. The residue was washed twice by suspension in 5 mL of distilled water, followed by centrifugation and removal of the supernate.

Acid-soluble: The residue was resuspended in 2 mL 10N HNO_3 and incubated overnight at room temperature without shaking. The total suspension was counted in the gamma counter, centrifuged, and filtered as above, and 1 mL of supernate was counted. The residue was again washed twice with 5 mL distilled water as above.

Neutralized acid-soluble: The acid filtrates from each slurry were combined, and 1 mL of the filtrate was neutralized with 1 mL 10N NaOH which, in most cases, caused a brown precipitate to form. The sample was again counted, centrifuged, filtered, and 1 mL of filtrate was counted.

Sodium sulfide-soluble: The washed acid residue was resuspended in 2 mL saturated Na_2S solution and incubated overnight at room temperature without shaking. It was then counted, centrifuged, and filtered, and 1 mL of filtrate was counted. In almost every case, the fraction of ^{203}Hg solubilized by Na_2S was greater than 1, probably indicating that counting efficiency was greater in the filtrate than in the soil slurry.

Calculations: The fraction of ^{203}Hg solubilized at each fractionation step was calculated by dividing twice the radioactivity in 1 mL of filtrate by the total radioactivity of the 2-mL suspension. This calculation gives the fraction of residual ^{203}Hg dissolved at each fractionation step rather than the fraction of the total. The fraction of total radioactivity recovered was calculated by dividing the fraction of the residue by the fraction the residue should theoretically represent, i.e., 1 - (soluble counts).

TABLE 4.11
Conditions of Extraction

1 ml slurry added to 1 mL water, counted in gamma counter. Centrifuged, filtered, 1 mL counted (H_2O.
Residue resuspended in 2 mL 10N HNO_3, counted in gamma counter. Centrifuged, filtered, 1 mL counted (Acid).
Residue resuspended in 2 mL sat. Na_2S, counted in gamma counter. Centrifuged, filtered, 1 mL counted (Na_2S).
1 mL Acid added to 1 mL 10N NaOH, counted in gamma counter. Centrifuged, filtered, 1 mL counted (Acid (neutralized)).

Table 4.12 presents the means and standard deviations of all treatment groups, both aerobic and anaerobic, for each soil source and extraction condition. As may be judged from the relatively low

standard deviations, there were no appreciable effects of the different incubation conditions on the solubility behavior of the labelled mercury. The table presents the results of calculating the percent of ^{203}Hg in each sample relative to the initial amount of ^{203}Hg. This correction is small except in the cases in which

TABLE 4.12
Percent Recovery of ^{203}Hg Relative to Initial Radioactivity (mean ± S.D.)*

Set	H_2O percent extracted	Acid percent extracted	Acid (neutral) percent extracted	Na_2S percent extracted
		Week 0		
Water	0.13 (±0.02)	3.09 (±0.75)	0.47 (±0.11)	114.03 (±38.96)
CM 14.6	0.29 (±0.19)	8.32 (±0.33)	0.55 (±0.02)	114.51 (±4.38)
CM 13.75	0.08 (±0.05)	1.67 (±0.26)	0.09 (±0.00)	116.12 (±4.39)
CM 10.9	0.06 (±0.03)	6.86 (±1.26)	0.54 (±0.10)	105.88 (±4.43)
CM 10.9, $HgCl_2$	0.19	45.33	2.72	51.99
		Week 2		
Water	0.19 (±0.06)	4.24 (±1.39)	0.82 (±0.88)	124.94 (±16.31)
CM 14.6	0.55 (±0.70)	10.45 (±2.07)	0.88 (±0.46)	108.91 (±3.97)
CM 13.75	0.34 (±0.43)	1.64 (±0.49)	0.13 (±0.03)	115.42 (±3.78)
CM 10.9	0.11 (±0.12)	14.46 (±5.69)	0.91 (±0.37)	95.20 (±6.95)
CM 10.9, $HgCl_2$	0.07	62.46	2.05	44.74

Table continued on next page.

TABLE 4.12 (Cont'd)
Percent Recovery of ^{203}Hg Relative to Initial Radioactivity (mean ± S. D.)*

Set	H_2O percent extracted	Acid percent extracted	Acid (neutral) percent extracted	Na_2S percent extracted
		Week 4		
Water	0.19 (±0.16)	1.55 (±1.27)	0.35 (±0.41)	133.81 (±12.8)
CM 14.6	1.91 (±3.49)	13.74 (±1.06)	0.37 (±0.07)	108.79 (±5.76)
CM 13.75	0.10 (±0.11)	2.20 (±0.40)	0.13 (±0.07)	119.91 (±8.63)
CM 10.9	0.03 (±0.04)	16.19 (±8.25)	0.92 (±0.39)	98.50 (±9.26)
CM 10.9, $HgCl_2$	0.02	58.44	2.71	53.42
		Week 6		
Water	0.79 (±1.10)	3.47 (±1.27)	0.45 (±0.41)	167.41 (±12.35)
CM 14.6	0.42 (±0.61)	20.37 (±4.31)	1.75 (±0.48)	87.69 (±4.96)
CM 13.75	0.31 (±0.27)	4.10 (±0.53)	0.30 (±0.19)	104.11 (±4.58)
CM 10.9	0.08 (±0.09)	33.52 (±5.90)	2.18 (±0.91)	64.62 (±12.74)
CM 10.9, $HgCl_2$	0.05	95.47	9.86	5.15

CM = creek mile.
* Aerobic and anaerobic combined means.

acid-soluble material represented a significant fraction of the initial radioactivity. Because the correction was so small, the correction was made only for Na_2S extracts.

These experiments showed clearly that ^{203}HgS, when added to soil which is subsequently slurried, does not become readily soluble in water. A limited amount eventually becomes associated with some acid-soluble, neutral-insoluble material, especially in the case of

dry soils. In all cases, the amount of free or associated soluble ^{203}Hg is very low. The results indicate that aerobic conditions are not significantly more likely than anaerobic conditions to solubilize HgS from soil slurries and, in fact, solubilization of HgS does not appear to be significantly greater in slurries in which bacterial activity is possible than it is in sterile or chilled slurries, in which bacterial activity is minimal or nonexistent.

We conclude from these experiments that the solubility of Hg from HgS added to soil is very low and remains low, even though the Hg present may become associated with some components of the soil. Apparently neither bacterial action nor the extent of aeration of the soil has a significant effect on the solubility of Hg added to the soil as HgS. Dry soils appear to accumulate some form of Hg-binding, acid-soluble material found to a lesser extent in the more moist soil sample studied. This component may be some form of decomposition product of humic acid or other organic components of the soil, which is formed only under the aerobic conditions which predominate in dry soils, rather than under the anaerobic conditions expected in waterlogged soils. Alternatively, it may be a product which is formed but rapidly degraded by natural soil processes under anaerobic or moist conditions but not under dry conditions.

4.2 DISCUSSION

The presence of large concentrations of mercury and of other heavy metals in the soil constitutes a potential environmental hazard because of the possibility that these metals may enter surface water and groundwater, be taken up into crops, and thus enter the food chain. Many heavy metals are toxic to living plants and animals because they bind to sulfhydryl groups in enzymes and other proteins, with a resulting alteration of tertiary structure with the loss of metabolic activity.

Heavy metal contamination in soil exists in the East Fork Poplar Creek floodplain as a result of discharges of untreated aqueous waste into the streams leading to the creek from the DOE Y-12 plant. The problem is particularly acute in the case of mercury, large amounts of which were released into the creek over a long period. Unfortunately, mercury is one of the more toxic of the heavy metals which is known to have harmful biological effects. In addition, it has the potential to undergo chemical or biological transformation to other highly toxic forms such as methyl mercury.

Many analytical surveys have been carried out by or on behalf of DOE, to learn the extent of the problem of mercury contamination of the East Fork Poplar Creek floodplain. As a result of these studies, a picture has emerged of many square miles of floodplain contaminated with concentrations of mercury ranging from levels of several hundred ppm to "hot spots" with mercury concentrations of up to 4,300 ppm.

These essentially descriptive data have not addressed the problem as to what immediate or long-term threat these levels may pose to the general population of Oak Ridge. Potential threats could be the appearance of large concentrations of mercury in groundwater or evidence of the biotransformation of the element to the highly toxic organic forms.

Oak Ridge Research Institute, under its contract with DOE to develop biological waste treatment systems and concepts, has undertaken to discover the environmental fate of the released mercury by answering the question as to what chemical forms the element may assume in the soil. We have also carried out studies focused on the possible role that sulfate-reducing bacteria may play in the immobilization of newly released mercury.

It is well known that sulfide has the ability to precipitate heavy metals from aqueous solution. The production of hydrogen sulfide by sulfate-reducing bacteria has been investigated to learn whether the process can be harnessed to cause bioconversion of soil-borne mercury to the immobilized and probably biochemically inert sulfide form. Release of hydrogen sulfide from sulfate-reducing bacteria and the subsequent conversion of soil mercury to mercury sulfide would be highly beneficial for two reasons. First, the newly formed mercuric sulfide might be expected to be very unreactive and therefore biochemically inert, although the overall concentrations of mercury in the soil would remain high. Second, orienting the organism's metabolism to sulfate reduction, aided by the provision of high concentrations of sulfate and other nutrients, would reduce the possibility of formation of methyl mercury.

One of the most obvious conclusions to come out of our studies is that we have found it extremely difficult to directly test our main hypothesis using soil from East Fork Poplar Creek. This is because in both soil column experiments and <u>in situ</u> experiments, almost all of the mercury was found to be immobile in the soil, even from control columns and plots which received no sulfate remediation. These results strongly suggest that almost all of the mercury was in some soil-bound or precipitated form to begin with. Interestingly, sulfate addition to the columns or soil plots was associated with a reduction of mercury release, compared to the already low levels seen in the controls. These reductions are consistent with our overall concept of promoting sulfate reduction to immobilize heavy metals. However, they raise the question as to whether any kind of remedial action in the soil may be necessary at all, if the process can go to completion in unamended contaminated sites. This point is answered by the failure of sodium sulfide to solubilize more than a small proportion of $^{203}HgCl_2$ which was added to the soil and incubated for 5 days. This result suggests that the unamended bioconversion of heavy metals to their sulfide forms may be extremely slow, perhaps taking several years or decades. The ready solubilization of the radioactive metal in nitric acid but not in water suggests that an initial event in the interaction between heavy metals and soil is the binding of the inorganic metal ions to soil particles. This is consistent with the findings of other research groups (see, for example, Khanna and Stevenson, 1962; Rashid, 1974). Further transformations, whether to the sulfide or organic forms, may take place very slowly, and depend on bacterial metabolism. In the case of sulfate-reducing bacteria, the metabolic pathways which operate depend on the nutritional status of the organisms (Compeau and Bartha, 1985). Sulfate reduction in unamended soils might be expected to occur very slowly, during which time the possibility might exist for direct mobilization of the bound mercury in particulate form if the watering conditions changed. Alternatively, harmful bioconversions could occur, depending on the

presence of other species of microorganisms in the soil or of sulfate-reducing bacteria in nutritionally inadequate conditions.

Because of the difficulty of directly testing our main hypothesis using soil samples from East Fork Poplar Creek, we have attempted to answer the question of the environmental fate of mercury by assaying a number of soil samples collected from this site for the different chemical components of mercury. The method used was based on measuring total mercury in various extraction solutions. For this reason, it became essential to ensure that the different extractants used were highly specific for their target component. This was especially true for sodium sulfide, because it was vital to demonstrate that its specificity was for only mercuric sulfide. To answer this question, we carried out a series of experiments in which aliquots of soil were incubated with first nitric acid, and then sodium sulfide, or by the same chemicals but in reverse order. The percentage of the total soil mercury in the sodium sulfide eluates was approximately 79% for each phase of the experiment, which suggests that sodium sulfide is likely to be highly specific for mercuric sulfide. Based on this finding, we were then able to extract a number of soil samples from different sites with sodium sulfide and show that, on average, 90% of the soil mercury was extractable by sodium sulfide. Thus, the mercury was probably almost all in the sulfide form at this site.

These analysis results answer, in part, the question as to the importance of sulfate-reducing bacteria to the immobilization of soil mercury. We can now speculate that, although the <u>in vitro</u> and <u>in situ</u> soil elution experiments demonstrated the unavailability of most of the soil mercury for aqueous leaching, it seems likely that the soil mercury was already in the sulfide form when the soil was collected. This conversion suggests that sulfate-reducing bacteria do indeed have a crucial role in the modification and immobilization of mercury released from the soil, but that, without the direct addition of nutrients, it can be an extremely slow process. Once mercuric sulfide has been formed in the soil, however, it is likely to be extremely resistant to further chemical transformations. This is suggested by the fact that, in the soil samples measured, this component consistently made up 90% of the total mercury contained in the soil. In addition, the radioactive mercuric sulfide added to soil did not undergo any discernible transformations under any of the incubation conditions tested. Clearly, acceleration of the formation of mercuric sulfide in soil is a worthwhile goal in efforts to remediate environmental contamination due to mercury or other heavy metals.

In order to <u>directly</u> demonstrate the applicability of calcium sulfate treatment to the problem of heavy metal immobilization in soil, it would be necessary to choose a site where there had been recent heavy metal contamination, and where the majority of the mercury was still in the inorganic form, whether bound to the soil particles or in solution. In such circumstances, the ultimate fate of the mercury would depend on the availability of sulfate-reducing bacteria, and on their nutritional state. By maintaining gypsum and non-gypsum treated plots within a site, and by measuring groundwater and soil cores for mercuric ions and methyl mercury, we would expect to obtain a direct demonstration of the applicability of our technology to an "unstable" mercury-contaminated site by the retarded

liberation of mercury into groundwater from amended plots compared to controls. Further evidence would be provided by a comparison of the rates of mercuric sulfide formation in the different plots, which should be higher in those receiving the gypsum treatment.

Oak Ridge Research Institute has recently proposed such a course of study to the USEPA and is seeking to set up a joint demonstration as part of the USEPA SITE 004 environmental remediation demonstration program.

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5.0 FORMATION OF METHYLMERCURY IN THE ENVIRONMENT

In this chapter, we describe the experiments carried out and the conclusions resulting from our work related to methylmercury on sites in the East Fork Poplar Creek floodplain. In the sections which follow, we report on the levels of methylmercury found and compare them with the levels of total mercury found at these same sites. We also report the results of laboratory experiments which demonstrate the rapid formation of methylmercury from mercuric ions when methylcobalamine is available, but the almost total absence of methylmercury formed from methyl sulfide under these conditions.

5.1 BACKGROUND

5.1.1 Mercury Pollution in Oak Ridge

The estimate of 250,000 lb of mercury released from the Y-12 Plant may be conservative because of uncertainties in inventories and record keeping. Furthermore, certain buildings in the Y-12 facility continue to discharge several million liters of mercury-contaminated water per month. Although a significant percentage of this mercury may be removed from the effluent by filtration, it is recognized that an unknown proportion of the mercury has been and continues to be released into the environment (U. S. DOE, 1986).

Wastewater is released initially into New Hope Pond, located east of the Y-12 facility, which acts as a primary holding and settlement pond. The water is subsequently discharged into East Fork Poplar Creek, south and west of the city of Oak Ridge and adjacent to both commercial and residential areas.

It is clear that the wastewater discharges from the Y-12 Plant continue to contaminate the floodplain of East Fork Poplar Creek with mercury, plus other materials such as thorium, chromium, zinc, and a variety of organic compounds. The use of soil from this floodplain as landfill and topsoil for residential gardens has resulted in a potential environmental problem of uncertain proportions.

In 1983, the Oak Ridge Task Force (U. S. DOE, 1986) began to collect toxicological and environmental data to evaluate the long-term public health impact of the pollution. This effort involved analysis of mercury in soil and sediment samples taken from various sites throughout the city by different agencies within the task force. Although these sites include some private residences, the Oak Ridge Civic Center, and some commercial property, the primary focus of the analytical effort was along the floodplain of East Fork Poplar Creek.

In studies of topsoil taken from different residential areas, a large number of samples was determined to have levels of contamination greater than 12 ppm of mercury. This value is an interim guidance level established by the State of Tennessee as the maximal recommended level for mercury in soil. Results of analyses of mercury from sites in local residential areas are given in Table 5.1. The West End Water Treatment Plant is another site from which large numbers of samples

TABLE 5.1
Total Mercury in Soil Samples Taken from Residential Property in Oak Ridge*

Area	No. of samples	Range of Hg conc. (ppm)	No. > 12 (ppm)
Fairbanks Rd.	40	0.02 - 15.0	1
Illinois Ave.	334	0.02 - 4300	174
Robertsville Rd.	139	0.02 - 69	44
Scarboro	63	0.03 - 21	7
Woodland	15	0.05 - 0.35	0

* A summary of the soil sampling in the Oak Ridge community for 1986 (data taken from U.S. DOE, 1987).

were taken. In soil from this site, 136 samples out of 242 had mercury levels greater than 12 ppm, with values up to 3,000 ppm being observed.

In two zones (one 9.2 to 12 km and the other 13.5 to 15.85 km upstream from the point the stream reenters the DOE reservation), concentrations of up to 3,000 ppm were observed, with more than 50% of the samples taken at depths of less than 50 cm having concentrations greater than 100 ppm.

A further major focus of the studies initiated by the task force was to assess the cost-effectiveness of various remediation options. ORRI initiated studies to monitor mercury levels at sites along the floodplain of East Fork Poplar Creek and to assess the range of microflora which were associated with these soil samples. This approach was based on the concept that bacteria present in soil samples which are highly contaminated with mercury may have undergone selection pressure and, thus, possess unique metabolic features which allow the organism to survive in a hostile environment. If these metabolic features include a mechanism for degrading or immobilizing the pollutant, it may be possible to use these microorganisms to develop a system for the bioremediation of the contaminated sites by creating conditions that would enhance the particular metabolic pathway. At the heart of this concept is the belief that the use of effective bioremediation procedures to clean up a toxic site would be cheaper and less environmentally disturbing than mechanical and/or incineration methods.

If microbial metabolic interaction with environmental mercury could have a beneficial potential, the possibility cannot be ignored that other microbial interactions with mercury may have a harmful

potential, as in the formation of hazardous organomercurials, such as methylmercury. Many microbial systems in soils, lakes, and sediments have been shown to be capable of supporting the formation of methylmercury. Its bioaccumulation in fish has been shown to be a significant route of intake in man. We have attempted to examine the extent of *in situ* conversion to methylmercury by its extraction and analysis from soil samples taken from various sites along East Fork Poplar Creek.

The analyses of methylmercury and mercuric sulfide focused attention on the impact of microbial metabolism on environmental interconversions and pointed to the possibility that, although the overall level of mercury contamination is high, the possible environmental hazard that it represents may be low. This is because of the near total existence of the mercury in the metabolically inactive sulfide form. Because sulfate-reducing bacteria play a role in both the production of mercuric sulfide and methylmercury, depending on the nutritional environment, we have developed a technology for the bioremediation of mercury-contaminated sites based on the optimization of the metabolism of the sulfate-reducing bacteria. We can channel their metabolism to produce H_2S, which subsequently interacts with inorganic mercury and precipitates as mercuric sulfide.

5.1.2 Toxic Effects of Methylmercury

It has long been recognized that mercury and other heavy metals, such as lead and cadmium, have harmful effects on living systems. These effects are due to the ability of the metals to interact with important biochemicals such as proteins, altering their tertiary structure and, thus, their bioreactivity within cells. For example, mercury can interact with proteins by covalently binding through the sulfhydryl residues to form a metalloprotein which might be expected to have different reactive properties from the native protein if the binding is at or near the active site. A review by Clarkson (1977) considered the importance of this question, and noted the ease with which elemental mercury is taken in as a vapor through the lungs and passed into the blood stream. Once in the blood, elemental mercury can quickly cross the blood/brain barrier or be transported into the red blood cells. It is then oxidized to the mercuric form by the enzyme catalase. The mercuric moiety is capable of binding to proteins. The effects of mercuric ions have been shown to be particularly important in liver and kidney tissue in animals, which are the key target organs for inorganic mercury toxicity.

In contrast to the mode of toxicity of inorganic mercury, the toxicity of methylmercury is primarily focused in the central nervous system. D'Itri and D'Itri (1978) gave a dramatic summary of some of the recent poisoning events involving methylmercury which have contributed to our present state of knowledge concerning the interaction of this chemical with biological systems. With this understanding has come the recognition that the primary route by which people take in methylmercury is through eating fish. Fish have a unique ability to bioaccumulate methylmercury in muscle, thus posing a possible hazard to those communities which use fish as their staple diet. It is also recognized that the very dependence of some communities on fish in their diet means that the problem of

methylmercury intake can never be totally eradicated. It is for this reason that environmental health experts have sought to establish guidelines for acceptable intake levels of methylmercury, and to learn how the body clears methylmercury following uptake.

One of the earlier attempts to study the interaction of methylmercury on human beings involved feeding some Swedish volunteers fish containing methylmercury containing $^{203}HgCl_2$. These experiments showed that approximately 95% of the label was absorbed, and that its half-life in the human body was approximately 74 days (Miettinen, 1973).

The most famous instance of natural methylmercury poisoning occurred in Japan, in Minamata Bay, where the local inhabitants consumed large amounts of shellfish which had taken up and bioconverted mercury, from heavy-metal-laden industrial waste, into methylmercury. Initial symptoms included numbness of lips and limbs, tunnel vision, and lack of muscular coordination. These dysfunctions became more acute and spread to other systems before death ensued. Children were seen to be particularly at risk from this disorder, developing symptoms which resembled cerebral palsy. The ease with which methylmercury crosses the placenta was also demonstrated by the birth of affected offspring to mothers who had shown no signs of poisoning.

In addition to acute symptoms, a range of chronic and subchronic symptoms of mercury poisoning have been identified. In some cases, there were no clear symptoms until an autopsy indicated that the structure of the brain had degenerated. The victims appeared to have undergone premature aging associated with other degenerative diseases such as cancer, atherosclerosis, and diabetes.

Takeuchi (1982) gave a detailed account of the pathology of methylmercury poisoning arising out of this outbreak. He reports that the metal becomes distributed in the cerebral cortex resulting in neurotoxic effects, as well as cytotoxic effects on epithelial and parenchymatous tissue. He considered that the acute and subacute effects of the poison were probably due to hypoxic and anoxic effects resulting in edema of the perivascular space. Neurotoxic effects <u>per se</u> were more likely to be important in chronic poisoning cases. Elhassani pointed out, however, that there is no known antidote to the toxic effects of methylmercury on the central nervous system (1982).

Two other famous cases of methylmercury poisoning involved the use of organomercurial fungicides on seed and grain. This resulted in the deaths of thousands of seed-eating birds in Sweden. Farmers were also affected during the sowing season, with a number of fatalities. The largest epidemic from eating treated seed grain occurred in Iraq, in 1971, where organomercurial-coated grain was used to make flour. The use of this flour to make bread by poor people in rural areas resulted in widespread brain damage, blindness, and paralysis. The problem was then further exacerbated by feeding the grain to domestic animals and cattle, who rapidly became sick. The dumping of much excess grain in the Tigris river likewise killed or poisoned a large proportion of the local fish population. In the final analysis, official figures recognized 6,530 victims of this disaster, 459 of whom died.

A recent article by Tollefson and Cordle (1986) focused on the basic facts observed from these instances of poisonings, and attempted to collate what is so far known about the body's capacity to withstand

methylmercury poisoning. A multinational team of researchers studied the victims of the Minamata poisoning incident and, with the knowledge of the ratios of mercury found in hair and blood, hypothesized that the lowest level of mercury in blood which was associated with toxic effects was 220 ppb, and in hair, 50 ppm.

The FAO/WHO (1972) established a "Tolerable Weekly Intake" of 300 µg of total mercury and 200 µg of methylmercury per person. This corresponds to an "Acceptable Daily Intake" (ADI) of 0.41 µg/kg body weight. Tollefson and Cordle (1986) considered that the total body burden would become eventually steady after about 1 year, at approximately 100 times the daily intake. They estimated that a steady daily intake of 300 µg per day would give a blood concentration of 200 ppb at the steady state.

Other workers, using information gathered in studies of the Iraq poisoning incident, suggested that toxic effects of methylmercury may become apparent with a body burden of 240 µg/kg (Clarkson et al., 1976). It was at this level that the incidence of parathesia in the victims was greater than in the background population.

Consideration of the chronic and subchronic effects of methylmercury poisoning suggests that a more realistic ADI will be lower than 0.41 µg/kg body weight. Taking into account the many uncertainties in the data correlating body burden with toxicity and, allowing a margin of safety, Tollefson and Cordle suggest that a Maximum Tolerable Level for methylmercury in the diet should be 30 µg per day, a value also arrived at by Sherlock et al. (1984), who studied the elevation of mercury in human blood resulting from the chronic ingestion of fish containing methylmercury. This level of intake would result in a mercury level in blood of 20 ppb and 5 ppm in hair.

Other diagnostic features have been investigated as being early indices of methylmercury toxicity. Woods' research group (1977, 1984) demonstrated the increase of urinary uroporphyrin and copophyrin in humans and rats receiving low levels of methylmercury, but not inorganic mercury.

Children and unborn babies appear to be especially vulnerable to the effects of methylmercury poisoning. In Minamata, 6% of the children born between 1955 and 1959 had cerebral palsy, although their parents were unaffected (Koos and Longo, 1976). Furthermore, Inskip and Piotrowski (1985) have shown that the level of methylmercury in fetal blood is twice that in maternal circulation. Marsh et al. (1981) studied the mercury levels in children of victims of the Iraqi poisoning incident and found that subjects were free of neurological deficiencies with a concentration of mercury in the hair of less than 68 ppm. They considered 68-180 ppm to be the critical concentration for the onset of neurological signs.

The question of the late onset of symptoms of methylmercury poisoning has occupied a number of research groups. In 1973, in the Agano area of Niigata, Japan, new cases of methylmercury poisoning were reported several years after the consumption of contaminated fish had ceased (Tsubaki et al., 1978). Similarly, Iraqi infants were found with neurological and developmental dysfunctions after they had initially been thought to be free from toxic effects (Amin-Zaki et al., 1979).

These data, taken together, suggest that the threshold level of methylmercury intake associated with chronic and subchronic toxic

effects may be very low, despite several demonstrations that certain fish-eating populations appear to have unusually high levels of methylmercury in their blood (e.g., up to 82 ppb) without displaying any overt symptoms of poisoning (Turner et al., 1980; Marsh et al., 1974).

Based on the data presented here, the FDA has established a Maximal Regulatory Amount for methylmercury in fish meat of 1 ppm, which is considerably above the levels of methylmercury detected in marine species off the coasts of the United States, and also in excess of the maximum values seen in fish caught in the Great Lakes (Simpson et al., 1974).

5.1.3 Environmental Interconversions of Mercury

The previous section outlined how the uptake of mercury into living cells and organ systems constitutes a threat to the life and well-being of that system, because of the ready interaction of the metal with proteins and its ability to affect the active site of enzymes and, thus, uncouple certain metabolic pathways. In animals, the liver, and more especially the kidney, are primary target organs for mercury and its toxic effects. These effects are exacerbated by the very slow clearance rate of the metal, which allows the possibility that bioaccumulation will occur as a result of continued exposure. Methylmercury also has a long half-life in living tissues and may, thus, also bioaccumulate with harmful consequences to the organism.

In this section, we summarize the wide range of chemical interactions which mercury can undergo in the environment, and emphasize the key role which microbial metabolism plays in these interconversions. The occurrence of methylmercury has been studied *in situ* in lakes, sediments, and soils, together with *in vitro* incubation studies using materials frequently taken from these same matrices, to define the potential of a matrix to support methylmercury formation. In general, studies using the latter approach have demonstrated that an environmental matrix may have a considerable potential for formation of methylmercury because of the metabolism of its microbial population, in contrast to the low or nearly absent levels of methylmercury observed in the same sample by direct measurement. Although environmental levels may be low, this potential for methylmercury formation should not be ignored, because of the implication that an environmental site may have the ability, in certain circumstances, to form significant amounts of the highly toxic substance. Furthermore, the near total absorption of methylmercury by animals, and its long half-life, imply that even very small levels of methylmercury in the environment may constitute a significant biohazard.

Figure 5.1 summarizes the major interconversion pathways which mercury may undergo in the environment, and emphasizes the key role which microbial metabolism plays in many of these transformations. The size of the arrows in the figure indicates the probable relative

Environmental interconversion of Mercury and microbial formation of Methylmercury

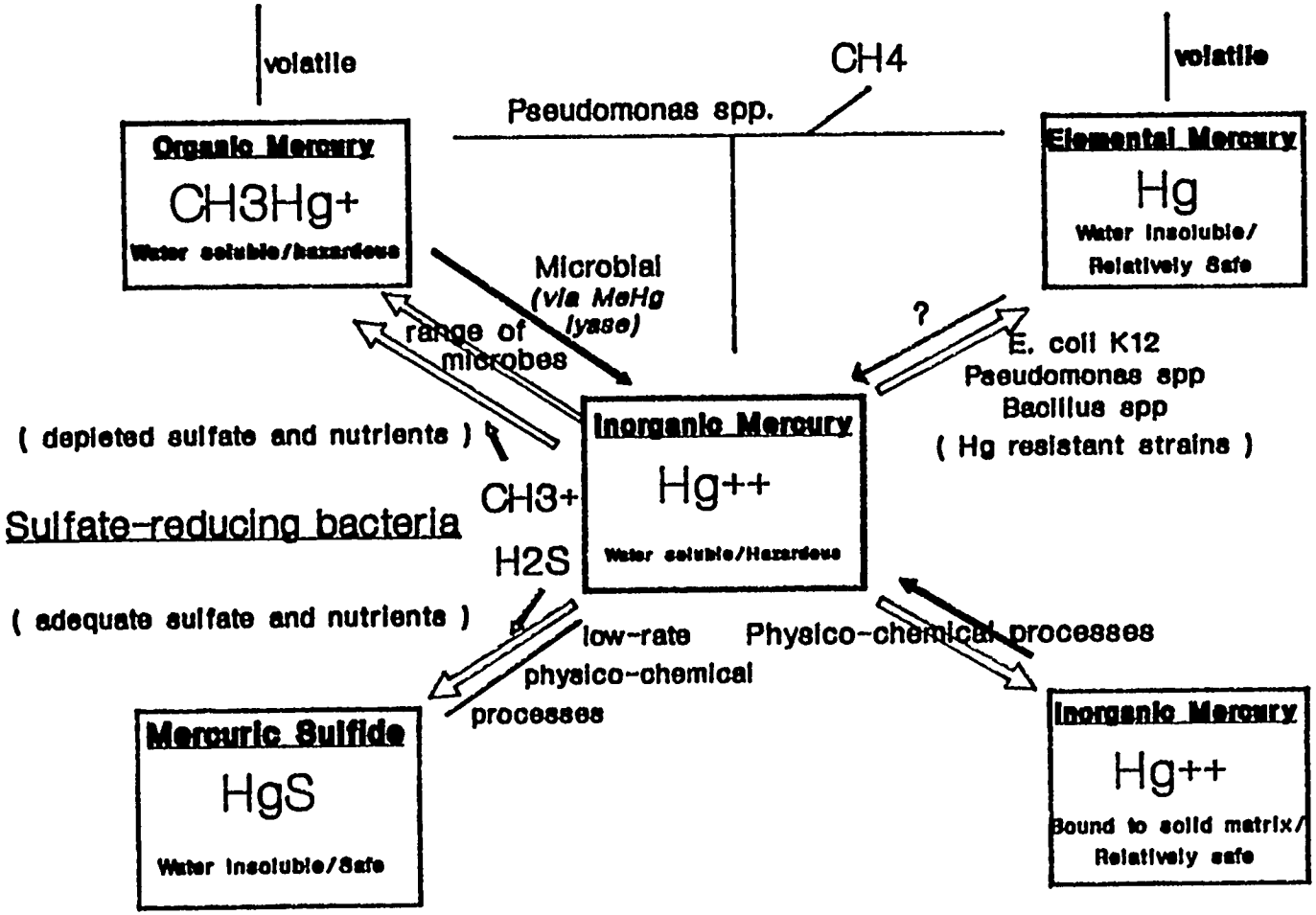


Figure 5.1

importance of that pathway. Clearly, the starting point of pollution, soluble inorganic mercury, is extremely unstable in soil and will be converted almost immediately into one or more of the other forms. Clearly also, some end points are more desirable than others. It might be expected that the most hazardous forms of mercury are the water-soluble forms which are readily absorbed by animals. Methylmercury is water-soluble and, as such, might constitute a greater biohazard than inorganic mercury which, although also water-soluble, is only absorbed by intestinal epithelium to a limited extent. Insoluble forms of mercury, particularly those which do not undergo any further significant interconversions and are known to have very low rates of absorption, such as mercuric sulfide, might be expected to constitute a more benign end product of mercury interconversions. Large concentrations of mercury in soil may not constitute a significant biohazard if the metal is sedimented in the sulfide form.

Fagerstrom and Jernelov (1972) discussed the relative importance of many of these environmental mercury interconversions, and pointed out that mercuric ions will rapidly bind to a variety of substrata, such as carbohydrates, proteins, and inorganic matrices such as clay. However, the bound mercuric ions can be released from the substratum in certain circumstances, such as if the redox potential decreases. In addition, if the mercuric ions are bound to soil particles, the particle in its entirety may be mobilized during flooding, which would allow the inorganic mercury, although bound to a solid matrix, to undergo further interactions and perhaps become released from its support medium.

The conversion of water-soluble inorganic mercury to the elemental form has been demonstrated *in vitro* by a number of research groups. In all cases, this conversion occurred under the influence of microbial metabolism. A wide variety of microorganisms appear to have this ability, which is often found in discrete bacterial strains with demonstrable mercury resistance, such as *E. coli* K12 (Hansen et al., 1984). In another example, Timoney et al. (1978) described a mercury-resistant strain of Bacillae which can grow in up to 20 ppm mercury. The mercury resistance of this organism is associated with a plasmid which also encodes for ß lactamase formation, thereby conferring Ampicillin resistance on the organism. Other genera of bacteria which feature mercury resistance and the ability to reduce inorganic mercury to the elemental form include the Pseudomonads. Olsen et al. (1979) found nearly 900 mercury-resistant strains of marine bacteria, mostly Pseudomonads, and demonstrated, in the Pseudomonad strain K62, that this resistance is associated with the enzyme mercuric reductase, which is encoded on a plasmid. It is this enzyme which effects the conversion of inorganic mercury to the elemental form.

As shown in Figure 5.1, the elemental form of mercury may be considered to be one of the less harmful end points of mercury interconversion, because of its low solubility in aqueous media and

its expected low rate of absorption. The possibility also exists that the elemental form of mercury may become released into the atmosphere because of its volatility.

Much of the earlier work on the microbial formation of methylmercury was carried out by Wood and co-workers (1968), who identified some of the possible mechanisms and intermediates involved in transfer of methyl groups to mercury. For example, it is clear that the rate-limiting step in methylmercury formation is the production, by a microorganism, of a methyl-donating cofactor. This cofactor can interact spontaneously with mercuric ions to rapidly produce stoichiometric amounts of methylmercury. The chemical form of the transferred methyl group varies depending on the cofactor (Ridley et al., 1977). Thus, the methylcorrinoid and methylcobalamine cofactors transfer the methyl group as a carbanion, CH_3^-, whereas Sadenosylmethionine and N^5-methyltetrahydrofolate transfer the methyl group as a cation, CH_3^+. The factors which govern the rates and amounts of formation of these cofactors are not clearly understood at this time, nor are the subcellular sites and mechanisms whereby they interact with the metal. However, a considerable amount of understanding has been gained concerning the chemical interaction between methylcobalamine and mercury. This interaction proceeds rapidly and spontaneously by electrophilic attack by mercury on the cobalt moiety, with heterolytic cleavage of the Co-C bond, and transfer of the carbanion to the more oxidized state of the element, according to the following equation:

$$CH_3^- + Hg^{++} = CH_3Hg^+$$

with the concomitant formation of the methyl-depleted aquocobalamine derivative. Wood's group (1968) have also pointed out the possibility of further substitution occurring to form dimethylmercury, which is volatile and highly unstable.

Furutani and Rudd (1980) attempted to examine the environmental importance of these pathways by measuring the potential of microbe-containing environmental samples to methylate mercury. To do this, they obtained water and sediment samples from a mercury-contaminated lake and measured the potential of these samples to support methylmercury production by their incubation in the presence of $^{203}HgCl_2$. Considerable rates of methylation were observed, which were enhanced 15-fold in lake water and 315-fold in sediment floc, when tryptic soy broth was added to the incubations. These workers also used the same assay to demonstrate the possibility of mercury methylation occurring in the intestines of fish as a result of methyl donation from cofactors produced by the microflora (Rudd et al., 1980). This demonstration has important implications relating to the assimilation of methylmercury by man, whose main route of methylmercury uptake is from fish. The suggestion that methylmercury levels in fish may be due, in part, to its synthesis in the intestines of fish, as well as the result of bioaccumulation of preformed methylmercury, implies that even very low levels of inorganic mercury in lakes and rivers may constitute an environmental hazard to man through the subsequent synthesis and bioaccumulation of methylmercury. Clarkson's group (Nordberg et al., 1985; Goyer et al., 1985) considered the implications that acid rain may have on the production of methylmercury in lakes. They report an increased mobilization of

not only mercury, but also lead and cadmium into organometallic forms as a result of acid rain. These compounds can then bioaccumulate in fish.

Spangler and co-workers (1973) pointed out the interesting contradiction that, although many lakes and sediments possess endogenous microbes capable of synthesizing methylmercury, the level of this compound in these samples rarely seems to exceed background. They suggested that, although methylmercury may indeed be formed, it may not accumulate because of the presence of other bacteria which can demethylate it to reform elemental or inorganic mercury. Various microbial species and strains have been shown to have this capability, such as Clostridium cochlearium and several Pseudomonads. A common feature of this mechanism is the formation of methane as a byproduct of demethylation.

Bartha's group focused attention on the question of which of the many genera of bacteria capable of methylating mercury are likely to be most important environmentally. They studied various interactions undergone by mercury in a polluted estuarine sediment, with particular care being taken to preserve the anaerobic nature of the sample in the time between collection and the establishment of incubation conditions (Compeau and Bartha, 1984). They found that the rates of methylation were favored in conditions of low redox potential and salinity, and inhibited by the reduction of sulfate to sulfide. Hydrogen sulfide also appeared to have a role in the formation of dimethylmercury from the monomethyl derivative, with subsequent rapid volatilization and/or breakdown to form elemental mercury.

Later research efforts by this group considered in more detail the question of how the different metabolic pathways which the sulfate-reducing bacteria can use to convert mercury can be controlled. Using in vitro incubation of the sediment samples in a bioreactor under anaerobic conditions, they added various nutrients and specific inhibitors to the system and studied the production of methylmercury which resulted. Briefly, the initial demonstration that methylmercury production is enhanced by the addition of pyruvate to the medium suggests that the bacteria can metabolize fermentatively in the absence of sulfate. Conversely, enrichments with lactate and sulfate promoted the production of sulfide from sulfate, as demonstrated by the sedimentation of ferric sulfide in the medium, formed from endogenous ferric sulfate. There was a concurrent inhibition of mercury methylation. Another inhibitor, sodium molybdate, was found to promote methanogenesis by the inhibition of both sulfate reduction and methylmercury formation.

Bartha's group identified the key organism in their system as being Desulfovibrio desulfuricans and speculated that, during early growth, mercury methylation may occur when the amount of hydrogen sulfide formed is low (Compeau and Bartha, 1985). Limiting amounts of endogenous sulfate, and the presence of carbon sources which can be utilized in the absence of sulfate, would also favor methylation. Where sulfate and other nutrients are adequate, the production of hydrogen sulfide occurs rapidly, with the consequent sedimentation of inorganic mercury as the sulfide. The presence of sulfide in the medium also serves to further inhibit the process of mercury methylation.

This demonstration of the multivectorial nature of the metabolism of sulfate-reducing bacteria may be a key finding. These bacteria are

widespread in anaerobic soils and sediments and, thus, might be important in the interconversions of mercury species at local sites. In those circumstances, in situ bioremediation efforts could be focused on the creation of environmental conditions which are favorable for the promotion of mercuric sulfide formation. This would have the dual effect of removing mercury from the biosphere by sedimentation and inhibiting methylmercury formation by the increase in sulfide. Mercuric sulfide, which is water-insoluble, biologically stable, poorly absorbed, and relatively non-toxic to animals, thus represents the most desirable end point of the many possible interconversions of mercury outlined in Figure 5.1.

5.2 EXPERIMENTAL

5.2.1 Field Sampling

Soil samples for total mercury and methylmercury analysis were collected along six transects across the East Fork Poplar Creek floodplain. Transects were laid out perpendicular to the direction of creek flow (along a magnetic N-S compass line in most cases) and were identified by their proximity to surveyed stakes from previous studies. Six sites were sampled along each transect; three sites on each side of the creek were generally sampled. At each sample site, a surface (0-5 cm) and bottom (20-25 cm) soil sample was collected. Soil samples were taken by scraping the side walls of a small pit with a clean wooden tongue depressor. Approximately 100 g of soil was scraped directly into polyethylene sampling bags. Samples were then returned to the laboratory and processed within 2 hr of sampling. Preliminary studies showed that the differences between subsampling soil samples in the field and in the laboratory were insignificant. In the laboratory, soil samples were well-homogenized in the sample bags prior to removal of subsamples for the analysis of total mercury and methylmercury. Additional samples were also removed and air-dried at room temperature for 48 hr to determine their dry weight.

5.2.2 Extraction and Analysis of Mercury Components

5.2.2.1 Total Mercury

One g of each soil sample was digested in a 250-mL flask containing 5 mL of sulfuric and 10 mL of nitric acid (ultra pure). An insulated condenser was then fixed to the top of the flask and the system placed on a hot plate with the temperature set to 350°C. Samples were heated for 4 hr, or until the nitric acid evaporated. A 10-mL aliquot of perchloric acid was then added to the flask and the temperature increased to 450°C. The sample was digested for 15 hr (or until the perchloric acid evaporated), then cooled before the condenser was rinsed with deionized water. After digestion, the volume was adjusted to 250 mL with deionized water and the mercury determined using the cold vapor technique.

5.2.2.2 Methylmercury

Methylmercury was extracted from the homogenized soil samples using the method described by Furutani and Rudd (1980). The procedure is based on the initial extraction of total organics into toluene, followed by back extraction of methylmercury into aqueous thiosulfate. A further partition step extracted the methylmercury into benzene for analysis by gas chromatography. One-g portions of the soil samples were mixed with 1 mL of distilled H_2O in screw-cap test tubes, then acidified by the addition of 40 µL of 4N HCl. One-hundred µL each of 0.5M $CuSO_4$ and 3M NaBr in 11% sulfuric acid were added plus 1 mL of toluene. After thorough mixing, the samples were centrifuged to produce two phases; the upper toluene phase was collected. This phase (0.5 mL) was mixed with an equal volume of 0.0025M sodium thiosulfate in 20% ethanol, and a further vigorous mixing followed by phase separation step was carried out. The lower, mainly aqueous phase containing the methylmercury was carefully harvested, and 0.2 mL of this solution was mixed with 140 µL of 3M KI and 400 µL of benzene. The mixtures were again homogenized vigorously followed by centrifugation to yield two phases. A small volume of the upper benzene phase containing the methylmercury was collected into a teflon-sealed gas chromatography vial.

Methylmercury, in benzene extracts of soil samples, was measured by gas chromatography using a method based on that described in the AOAC Handbook (1984). A 6-ft Silar 10C column was maintained at 155°C in a Hewlett Packard 5880 gas chromatograph, with Argon/Methane carrier gas flowing at 30 mL per min. The injector and detector (ECD) temperatures were 200°C and 300°C, respectively.

The column was routinely preconditioned by maintaining a temperature of 200°C overnight, followed by one or more injections of mercuric iodide at 160°C. The reduction of the detector signal strength following this pretreatment indicated that the column was ready for use.

Up to 10-µL volumes of benzene extracts and 1-ppm standard solutions of methylmercury iodide in benzene were injected onto the column.

5.2.3 Formation of Methylmercury In Vitro

5.2.3.1 Methylmercury Formation from $^{203}HgCl_2$ Via Methylcobalamine

One-hundred µl of $^{203}HgCl_2$ was added to a solution of $HgCl_2$ in water containing 1 mmole/L of mercury. This solution was initially counted on a Searle model 1185 gamma counter and found to contain 118,868 cpm/mL. To 500-µL aliquots of this solution was added 50 µL of an aqueous solution containing 10 mmole/L of methylcobalamine. Replicate incubations were established as controls containing water instead of methylcobalamine solution. One set of test samples and its controls were incubated for 1 hr at room temperature, and the other was incubated at 4°C. After incubation, 25 µL of 4N HCl was added to each tube to stop the reaction prior to extraction and analysis. Three distinct extraction procedures were examined for each treatment group. These were: (1) mixing with an equal volume of benzene, (2) mixing with an equal volume of toluene, (3) mixing with an equal volume of toluene, after the addition of 100 µL of 0.5M $CuSO_4$ and 1 mL of 3M NaBr in 11% sulfuric acid. After thorough mixing, the tubes

were centrifuged to separate the organic phases, a small aliquot of each being counted on the gamma counter as before.

5.2.3.2 Methylcobalamine Interaction with $HgCl_2$ and HgS

In this experiment, 1-mL aliquots of 1-mmole/L solutions of $HgCl_2$ and HgS (as a dispersion) in water were treated with 100-µL aqueous solutions of 10-mmole/L methylcobalamine. Other incubations contained the addition of 1 g of soil. Control incubations were also established containing no mercuric salts. After incubation at room temperature for 1 hr, the samples were extracted as described in Section 5.2.2.2., and 2-µL aliquots of the benzene extracts were examined for methylmercury formation by gas chromatography as described above.

5.2.3.3 Interconversion of ^{203}HgS to Other Forms of Mercury by Incubation with Soil

As a preliminary to this experiment, 10 mL of 50-mmole/L $HgCl_2$ in water, containing an aliquot of $^{203}HgCl_2$, was incubated for 1 hr with excess NaS to form ^{203}HgS, which was precipitated as a black sediment. This was harvested by centrifugation and washed with several changes of water. The washings were counted for residual radioactivity on the gamma counter and, when background levels had been achieved, the ^{203}HgS was dispersed in a small volume of water.

Aliquots of this dispersion were mixed with soil samples and with soil that had been sterilized as a control. Incubation of these mixtures was carried out in both aerobic and anaerobic conditions for a total period of 6 weeks, during which aliquots from the mixtures were taken for analysis at 0-, 2-, 4-, and 6-week intervals. These samples, taken in triplicate, were extracted for methylmercury, inorganic mercury, mercuric sulfide, and total mercury to assess the possibility that long-term mobilization of the HgS moiety may have occurred due to the presence of soil microorganisms. Methylmercury was measured by gas chromatography following extraction into benzene, as previously described. Inorganic mercury, mercuric sulfide, and total mercury were analyzed by the cold vapor technique after extraction, which, for the inorganic and sulfide forms, consisted of a sequential extraction procedure featuring, first, the incubation of samples overnight in concentrated nitric acid and, second, the extraction of acid-resistant mercuric sulfide into saturated sodium sulfide.

5.3 RESULTS

5.3.1 G.C. Analysis of Methylmercury

5.3.1.1 Standard Curve of Methylmercury Iodide

The method for determining methylmercury in benzene extracts is based on the conversion of the extracted methylmercury, in the intermediate thiosulfate phase, to the iodide form. This step is incorporated into the method because the ECD detector is considerably more sensitive to iodide than to the other halides. Standardization of the analysis was, therefore, carried out using freshly made dilutions of methylmercury iodide in benzene.

This standardization process was done as a preliminary step whenever methylmercury analysis runs were conducted, necessitated in part by the column preconditioning steps which were an essential component of the procedure. These periodic injections of mercuric iodide might be expected to alter the column conditions substantially from day to day.

The standard solutions were freshly made to eliminate the possibility that evaporation of the organomercurial would give a diminished signal response over a period of time. We observed that injections of the same standard solution over a period of days gave a gradually diminished response with time.

Figure 5.2 gives examples of two separate standard curves of methylmercury iodide dilutions run on different days. Both curves display a strong correlation between the amount of methylmercury injected and the peak area, as well as a closely similar slope.

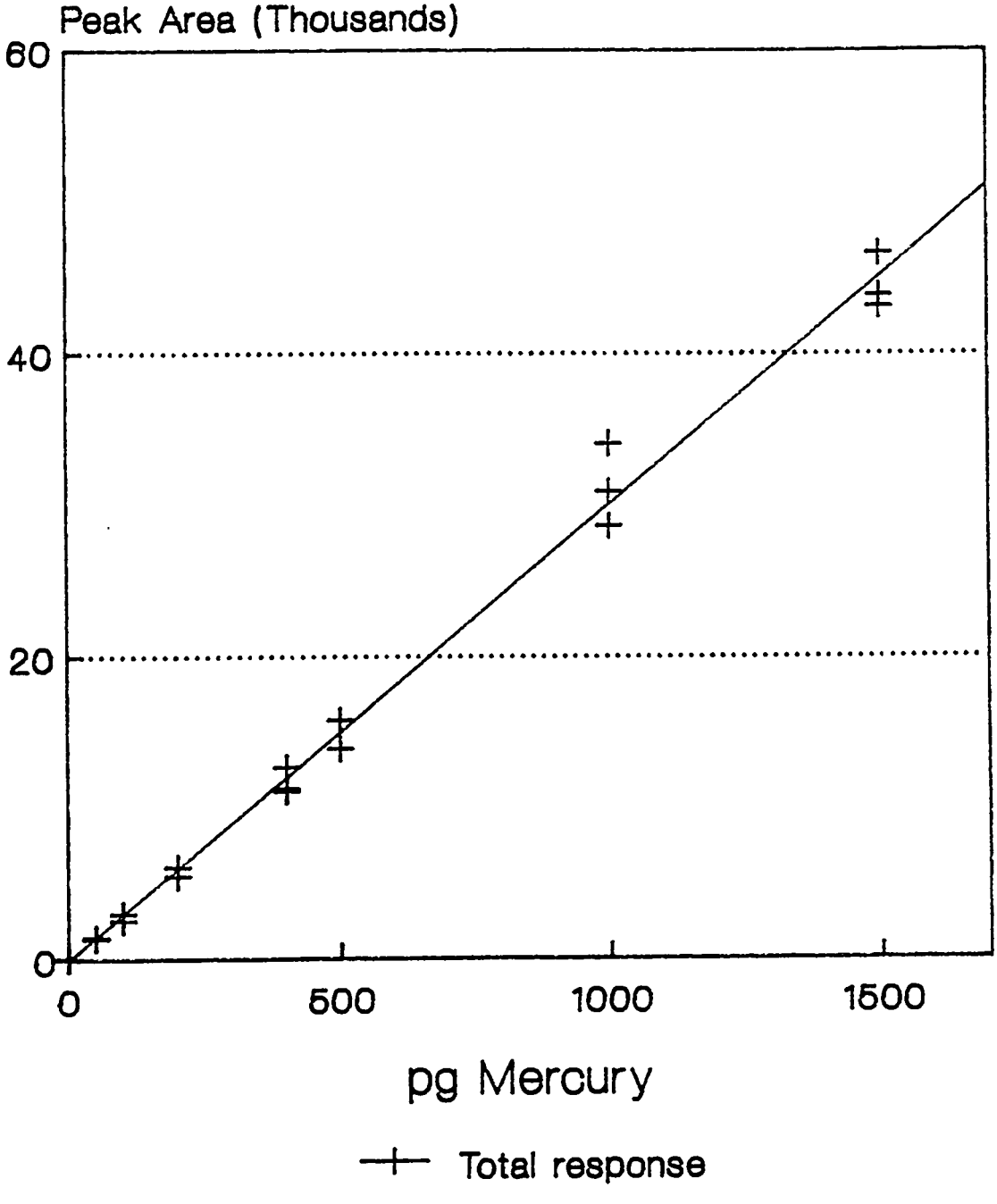
5.3.1.2 Lower Limit of Detectability of Methylmercury in Soil

Figure 5.3 shows the level of signal response of the ECD detector to a known concentration of methylmercuric iodide in benzene, compared to that of a benzene extract of mercury-rich soil prepared as described in Section 5.2.2.2.

In the figure, a 5-µL injection of 1 ppm methylmercuric iodide in benzene achieved a response of 53,806 area units compared to 2,302 area units from 5 µL of soil extract. This indicates a concentration of 43 pg/µL in the soil benzene extract, which converts to a concentration of 86 µg/g (ppb) of methylmercury in the original soil sample.

Because 10 µL of benzene extract was found to be the maximum volume that could be conveniently injected into the GC column, we would expect a peak volume of 4,604 area units to be obtained from that volume of benzene extract equivalent to a methylmercury concentration of 86 ppb in soil. If we take an area of 500 units as being the lowest usable degree of response, we obtain an equivalent value of 9.34 ppb, from which we take an approximation to 10 ppb as being the lower limit of detectability of methylmercury in soil using these methods.

METHYL MERCURY STANDARD CURVE
Methyl mercuric iodide



Silar 10-C column, ECD detector

Figure 5.2

Gas chromatography of Methylmercury. Extraction of MeHg+ from soil.

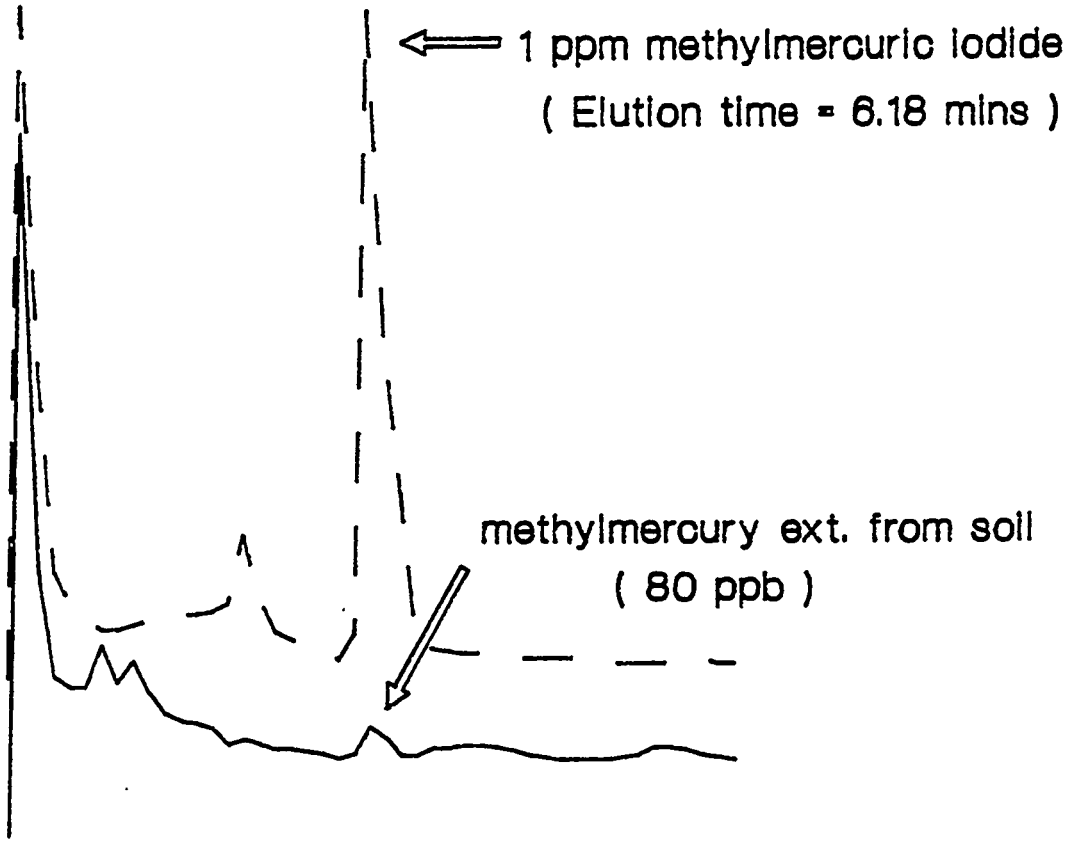


Figure 5.3

5.3.1.3 Extraction Efficiency for Methylmercury in Soil

In an experiment to assess the efficiency of the methylmercury extraction procedure, a solution of 1 ppm methylmercury in water was extracted using the toluene/thiosulfate/benzene method, in the presence or absence of added soil. Small aliquots of both the initial toluene and final benzene phases were assayed for methylmercury.

Figure 5.4 shows that when 2 µL of the toluene extracts or 4 µL of the benzene extracts was injected onto the column, near quantitative recovery of the organomercurial was obtained, irrespective of the presence or absence of added soil. These data suggest that, with thorough mixing, the extraction procedure for methylmercury from soil is quantitative.

During the course of analyses of soil samples from the East Fork Poplar Creek floodplain for methylmercury, different batches of soil samples were routinely spiked with accurately determined aqueous methylmercury solutions to give a continuing check on extraction efficiency throughout the course of the experiment. With few exceptions, recoveries in excess of 80% were obtained.

5.3.2 Levels of Total Mercury and Methylmercury along the East Fork Poplar Creek Floodplain

In the first experiment, soil samples were collected from various depths at sites previously identified as being especially high in total mercury concentration. These sites are 9.2 to 11.5 km, and 13.5 to 15.85 km, upstream from the point East Fork Poplar Creek reenters the Oak Ridge reservation, respectively. Consistent with the ORTF report for 1986, these zones are hereafter referred to as Reach 2 and Reach 4.

Table 5.2 summarizes the concentrations of total mercury and methylmercury observed at various depths at these sites, and shows that the methylmercury present represents a very small percentage of the total. This is especially true for the 30-cm-deep sample at Reach 2, where a concentration of total mercury in excess of 2,000 ppm was observed. Methylmercury was not detectable in this sample, and thus was considered to have a concentration of less than 10 ppb, which would represent a concentration of less than 0.0004% of the total.

In the second experiment, six transects were established at lines across the creek, with three in Reach 2 and three in Reach 4. Six sites were chosen along each transect for the collection of soil samples, three on either side of the creek. Duplicate soil samples were taken from each site, sealed into a plastic bag, then homogenized by hand. Commencement of extraction for total mercury and methylmercury was less than 2 hr after collection. For comparative purposes, results are expressed per unit dry weight, determined by oven drying a known wet weight of soil until no further weight changes occurred (Tables 5.3a and 5.3b).

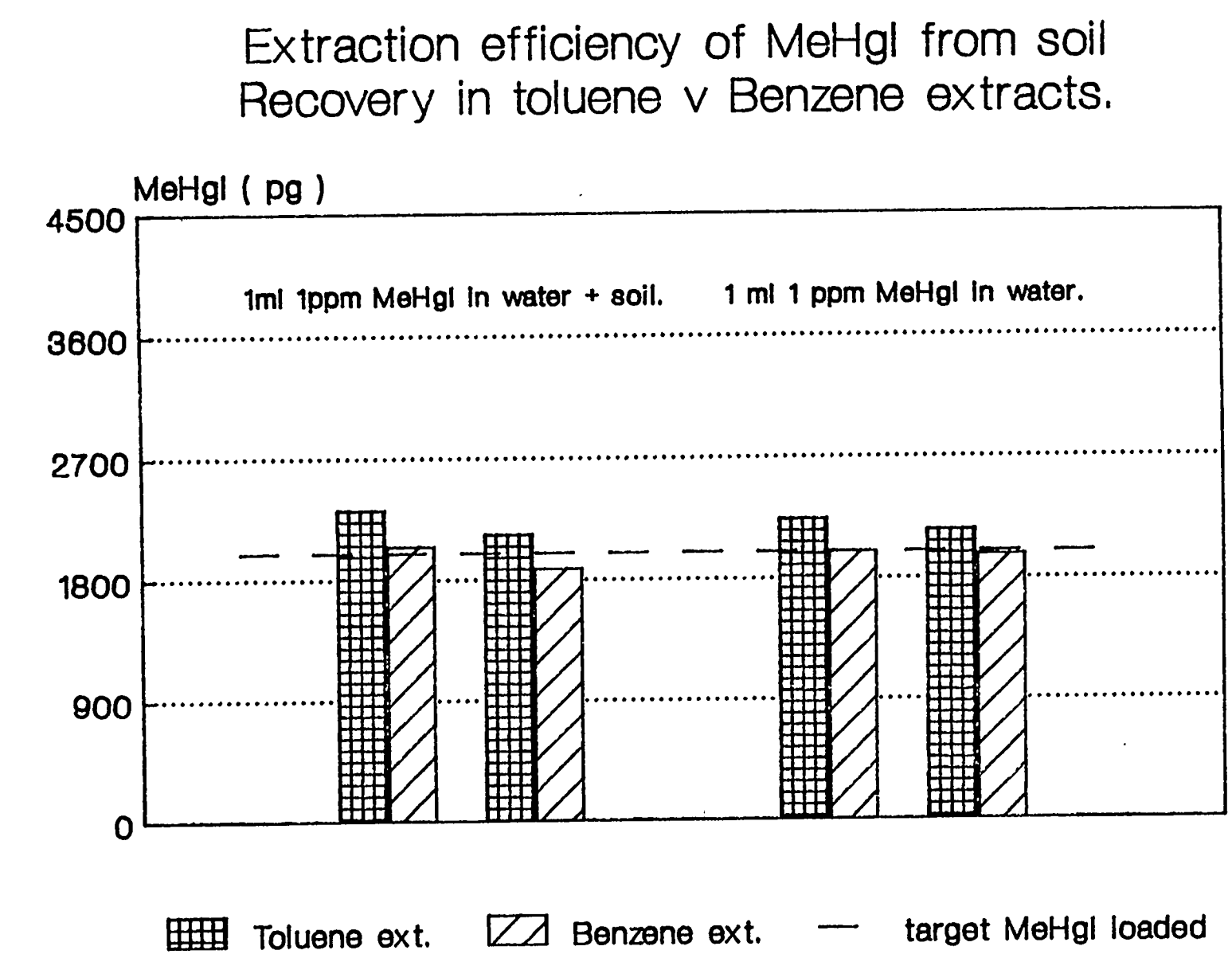

Extraction efficiency of MeHgI from soil
Recovery in toluene v Benzene extracts.
MeHgI (pg)
4500
3600
2700
1800
900
0
1ml 1ppm MeHgI in water + soil.
1 ml 1 ppm MeHgI in water.
Toluene ext.
Benzene ext.
target MeHgI loaded
ECD detector

Figure 5.4

TABLE 5.2
Concentration of Methylmercury in EFPC Floodplain Soil

Sites*	Depth (cm)	MeHgI (ng/g)	Total Hg (μ/g)	Percent MeHgI of total
Reach 2	0-5	<10	109	<0.009
	15-20	<10	226	<0.0044
	25-30	<10	2314	<0.0004
Reach 4	0-5	58	316	0.018
	15-20	85	1274	0.007
	25-30	<10	47	<0.02

*Soil was collected from two sites at the depths shown, and assayed for total Hg using the cold vapor technique, following complete acid digestion. Methylmercury (as MeHgI) was assayed by gas chromatography of benzene extracts of the soil samples prepared as described in Section 5.2.2.2. This involved an initial extraction into toluene, followed by back extraction into aqueous thiosulfate containing ethanol, then finally into benzene. Control experiments demonstrated that this procedure gave near quantitative recovery of methylmercury from spiked soil samples.

5.3.3 In Vitro Formation of Methylmercury

5.3.3.1 Formation of Organic Mercury Via Methylcobalamine from $HgCl_2$

This experiment, described in detail in Section 5.2.3.1, was designed to demonstrate the conversion of mercuric ions to organic forms of mercury, dependent on the presence of methylcobalamine. The efficiencies of slightly modified extraction procedures were also examined.

The results, shown in Table 5.4, suggest that there was a ready conversion of cationic mercury to an organic form. This conversion was both rapid and temperature-sensitive and depended on the presence of the methyl donor, methylcobalamine. From this, one may infer that the organomercurial product was probably methylmercury.

TABLE 5.3a
Analysis of Methylmercury in Soil Samples
from the EFPC Floodplain

REACH 2

Transect	Creek mile	Sample	Total Hg (ppm)	MeHgI (ppb)	% Hg as MeHgI
1	10.4	E 1-A	19	<10	<0.05
		1-B		<10	
		2-A	57	<10	<0.02
		2-B		<10	
		3-A	100	19.6	0.02
		3-B	192	<10	<0.005
		4-A	142	<10	<0.007
		4-B	152	<10	<0.007
		5-A	406	16.8	0.004
		5-B	37	<10	<0.027
		6-A	24	<10	<0.042
		6-B	15	<10	<0.067
2	10.8	C 1-A	46	<10	<0.02
		2-A	56	<10	<0.02
		3-A	184	<10	<0.005
		3-B	194	<10	<0.005
		4-A	76	<10	<0.013
		4-B	213	<10	<0.005
		5-A	172	<10	<0.006
		5-B	380	<10	<0.003
		6-A	187	<10	<0.005
		6-B	411	<10	<0.002
3	11.2	B 1-A	287	<10	<0.003
		1-B	166	<10	<0.006
		2-A	255	<10	<0.004
		2-B	78	<10	<0.012
		3-A	728	<10	<0.001
		3-B	126	<10	<0.008
		4-A	114	<10	<0.008
		4-B	369	<10	<0.003
		5-A	85	<10	<0.012
		5-B	148	<10	<0.007
		6-A	122	<10	<0.008
		6-B	179	<10	<0.006

TABLE 5.3b
Analysis of Methylmercury in Soil Samples
from the EFPC Floodplain

REACH 4

Transect	Creek mile	Sample	Total Hg (ppm)	MeHgI (ppb)	% Hg as MeHgI
4	13.5	D 1-A	94	<10	<0.01
		1-B	98	<10	<0.01
		2-A	222	<10	<0.005
		2-B	5	<10	<0.2
		3-A	46	<10	<0.02
		3-B	8	<10	<0.13
		4-A	200	<10	<0.005
		4-B	286	<10	<0.003
		5-A	247	<10	<0.002
		5-B	288	<10	<0.003
		6-A	610	14.5	0.002
		6-B	190	9.6	0.028
5	13.7	E 1-A	116	<10	<0.009
		1-B	27	<10	<0.04
		2-A	248	<10	<0.004
		2-B	126	<10	<0.008
		3-A	329	<10	<0.003
		3-B	78	<10	<0.012
		4-A	117	<10	<0.009
		4-B	196	<10	<0.005
		5-A	144	14.7	0.01
		5-B	144	<10	<0.007
		6-A	110	<10	<0.009
		6-B	27	<10	<0.04
6	14.4	F 1-A	3	<10	<0.33
		1-B	5	<10	<0.2
		2-A	6	<10	<0.17
		2-B	6	<10	<0.17
		3-A	35	33.4	0.095
		4-A	1.5	<10	<0.67
		4-B	1.6	<10	<0.63
		5-A	2.3	<10	<0.43
		5-B	0.5	<10	<2.0
		6-A	1.7	<10	<0.59
		6-B	0.82	<10	<1.22

Total mercury and methylmercury concentrations were determined as described in Sections 5.2.1. and 5.2.2. The sample locations (creek mile) were determined by reference to a TVA map of the creek. Concentrations are given on a dry weight basis. In most cases, the results reflect the lower detection limit of the method for measuring methylmercury (10 ppm).

TABLE 5.4
Conversion of $^{203}HgCl_2$ to Organic Forms of Mercury by Incubation with Methylcobalamine

Extraction	Incubation conditions: +Methylcobalamine (cpm)	Incubation conditions: -Methylcobalamine (cpm)
	ROOM TEMPERATURE	
Benzene	12998	1157
Toluene	12154	1309
Toluene (after $CuSO_4$ and NaBr)	12618	64
	4°C	
Benzene	7502	931
Toluene	7441	N/D
Toluene (after $CuSO_4$ and NaBr)	7262	62

Aliquots of 0.5 mL of 1 mmole/L of Hg as $HgCl_2$, containing 59,434 cpm of $^{203}HgCl_2$, were incubated with 50 µL of a 10-mmole/L solution of methylcobalamine, or with 50 µL of water as a control. After 1 hour at room temperature, or at 4°C, the mixtures were extracted using equal volumes of benzene, toluene, or toluene plus $CuSO_4$ and NaBr as described in Section 5.2.3.1. The extracts were counted on a Searle Type 1185 gamma counter.

The presence of $CuSO_4$ and NaBr in the extraction sequence was shown to be important in determining the specificity of the method for its target product. Thus, the extracts not containing these components contained higher background counts, suggesting that some non-specific transfer of inorganic mercury may have occurred in these extracts. By contrast, those control incubations treated with $CuSO_4$ and NaBr provided solvent extracts with counts at near background levels.

5.3.3.2 Formation of Methylmercury Via Methylcobalamine from $HgCl_2$ and HgS

This experiment was carried out to determine whether the organomercurial compound formed in the previous experiment was methylmercury. Thus, non-isotopic forms of the mercurial salts were used as substrates, and the identity of the product was confirmed by gas chromatography. The ability of HgS to act as a substrate for methylmercury formation, either by itself or in soil (in possible association with other mercury components), was examined by the solvent extraction of mixtures of methylcobalamine and, separately, HgS, HgS plus soil, and soil alone. Results from this experiment are summarized in Table 5.5. They confirm the identity of the organomercurial formed from $^{203}HgCl_2$ in the previous experiment as being methylmercury. Eighty-one percent of the $HgCl_2$ was converted to methylmercury during the 60-min incubation. The results also point to the possibility of methylmercury formation from HgS via methylcobalamine, albeit to a limited extent (<0.2). Methyl donation is also demonstrated in soil in vitro in these studies by the addition of soil to an aqueous solution of methylcobalamine. This formation may be from endogenous mercuric sulfide or from other forms of inorganic mercury in the soil.

5.3.3.3 Long-Term Interconversions of HgS

In this experiment, a sample of ^{203}HgS was made by the addition of an equimolar solution of NaS and $HgCl_2$ containing added $^{203}HgCl_2$ (see Table 5.5). The sedimented ^{203}HgS was washed several times with H_2O, until the counts in the washings had returned to background levels. The radiolabelled HgS was then mixed with various aliquots of soil and incubated for 6 weeks. Incubation conditions included (a) room temperature/aerobic, (b) room temperature/anaerobic, (c and d) room temperature/sterilized soil, aerobic and anaerobic, (e) 4°C/aerobic, and (f) room temperature/H_2O_2 present. Aliquots of the incubations were taken at 0 time and at 2-week intervals thereafter. These aliquots were extracted for total mercury, for inorganic mercury using concentrated nitric acid, and for HgS using saturated sodium sulfide. The mercury in these extraction media was assayed using the cold vapor technique. Other aliquots were extracted for methylmercury using the previously described toluene/thiosulfate/benzene procedure. The benzene extracts were counted in the gamma counter as an index of organomercurial formation from $^{203}HgCl_2$.

In contrast to the experiments using HgS for a methyl acceptor from methylcobalamine, no radioactivity above background, indicative of methylmercury formation, was evident in any of the benzene extracts at any time in this experiment. This suggests that, in these incubations, HgS was not converted to methylmercury by the microbes in the soil, even after an extended period of incubation. This may be because the appropriate microorganisms were not present, or because

TABLE 5.5
Conversion of $HgCl_2$ and HgS to Methylmercury
by Incubation with Methylcobalamine

Substrate	Area units	Vol injected (µL)	ppm	Percent recovery	Elution time (min)
$HgCl_2$	927882	1	161	81	5.95
HgS	1683	1	0.29	0.15	5.96
Soil	1815	1	0.31		5.97
HgS + soil	4813	1	0.84		5.97
Soil control	4685	10	0.08		5.97

Aliquots of 1 mmole/L $HgCl_2$ and 1 mmole/L HgS were incubated with equimolar amounts of methylcobalamine at room temperature for 1 hour, then extracted into benzene as described in Section 5.2.2.2. Except for an extract of soil alone, where 10 µL was used, 1-µL volumes of these extracts were injected onto the column. A 1-ppm standard of methylmercuric iodide in benzene, when injected onto the column in a volume of 1 µL, obtained a response of 11,516 area units. Thus, a methylmercury concentration of 100 ng/µL would give a response of 1,151,600 area units, for the same degree of sensitivity (100 ng/µL is the concentration of methylmercury expected if there had been quantitative conversion of inorganic mercury to methylmercury by methylcobalamine). The percentage conversion of inorganic mercury to methylmercury was therefore determined by comparison of the actual peak area obtained in experimental samples with this theoretical value for the peak area expected from 100 ng/µL methylmercury in benzene.

the conditions for conversion did not exist. These data, taken with those obtained for the other mercurial species, are consistent with the concept that mercuric sulfide is an extremely stable compound environmentally, and, once formed, is unlikely to undergo ready conversion to other molecular forms of mercury.

5.4 DISCUSSION

5.4.1 The Significance of Environmental Mercury Deposition on the Food Chain

The studies described in this section were designed to answer some basic questions about the nature of the possible interactions which may have occurred between the mercury released from the DOE Y-12 facility and the surrounding environment. These studies were given impetus by the discovery that some local sites contained large concentrations of mercury and, thus, might present an environmental hazard if these deposits entered the food chain.

We have focused on possible interactions which may have occurred between the released mercury and endogenous microorganisms. We speculate that the presence of a large concentration of a biocontaminant, such as mercury, may have acted as a selection pressure to enrich the contaminated environment with those microbes which can at least tolerate or, at best, metabolize the mercury. The ability to metabolize mercury implies the conversion of the metal to less mobile or bioactive forms, as a form of protection for the microorganism against the metals's toxic effects. However, in the case of mercury, the possibility also exists that microbial metabolism may convert the inorganic metal to methylmercury. This is an extremely hazardous compound because of its toxicity, its ready absorption by animals, and the ease with which it can accumulate in fish. Clearly, there is a need to know what the implications of the releases of mercury from Y-12 may be in terms of methylmercury formation, its possible bioaccumulation, its appearance in the food chain, and its consequent hazard to human beings. In addition, the appearance of large amounts of free inorganic mercury in the environment would also constitute a biohazard, because it also is toxic to animals, can bioaccumulate, and can undergo transformations in the environment to other forms of mercury such as methylmercury.

5.4.2 Microbial Impact on Mercurial Interconversions in Soil

The basic questions arising out of the mercury releases are:

1. Where is the mercury?
2. How much is there of it?
3. What chemical state is it in?
4. Can it be mobilized or metabolically converted to other forms of mercury by bacteria?
5. Can it enter the food chain?
6. Is any of it now in the form of methylmercury?
7. Can methylmercury be formed in the soil under certain conditions?

One of the key facts to come out of this study is that, at all environmental sites tested, the level of methylmercury in the soil was extremely low. In the majority of samples, the concentration of methylmercury was below the level of detectability of the analysis method, which approximated to a concentration in soil of 10 ppb. This was true even for sites where the deposition of total mercury was very high (>3000 ppm). At the few sites where any positive levels of

methylmercury were identified, the highest concentration observed was 85 ppb, corresponding to 0.0007% of the total mercury at that particular site.

Initial consideration of these low levels of methylmercury in the soil allowed speculation that the possibility of methylmercury constituting a significant biohazard as a result of the Y-12 mercury releases may have been remote. However, it is clear that there are many microbial populations in the environment which have the ability to demethylate methylmercury, most notably the methanogenic Pseudomonads (Spangler, 1973). Thus, the possibility cannot be ignored that, during the lifetime of the contamination, methylmercury production may have occurred, with subsequent breakdown of the organomercurial to elemental or inorganic mercury.

In addition, the very high levels of mercuric sulfide which are evident in these samples suggest that sulfate-reducing microorganisms must be an integral part of the environmental microflora. The presence of these microbes in soil also suggests that these sites may have methylmercury-producing potential, because the sulfate-reducing bacteria have also been shown to be one of the most important microbial producers of methylmercury in certain conditions (Compeau and Bartha, 1985). If the deposition of mercury had been accompanied by the existence of only limited amounts of environmental sulfate or nutrients, it is possible that methylmercury may have been produced in considerable amounts by the same bacteria usually responsible for the production of the environmentally benign mercuric sulfide.

Microbial interconversions of mercury to the methyl form proceeds in two basic stages. First, the bacteria produce the methyl donor, such as methylcobalamine or S-adenosylmethionine and, second, there is a rapid and spontaneous interaction between the mercuric form of the metal and the donor, with the transfer of the carbanion to the metal. It is not clear how, or at what subcellular site, this interaction occurs _in vivo_. However, _in vitro_ in the studies described here, the conversion has been shown to be near stoichiometric after incubation for 1 hour at room temperature. Clearly also, enzymic involvement in the _in vitro_ interconversion was not necessary.

Unpublished experiments in our and other laboratories have shown that the addition of ionic mercury to the soil is followed rapidly by the adsorption of the metal to insoluble particles in the soil such as clay, proteins and/or carbohydrates. In consequence, only a small percentage of the mercury may be leached from the soil, either _in vitro_ from soil columns, or _in vivo_ through groundwater irrigation into buried lysimeters. However, the possibility exists that the physicochemical forces involved in immobilization of the mercuric ion may be reversed under certain conditions (Fagerstrom and Jernelov, 1972). This would provide a substrate for methylation. In addition, the possibility cannot be excluded that bacteria may be able to methylate the adsorbed mercuric ion.

We have presented the evidence for concluding that greater than 90% of the mercury in the East Fork Poplar Creek floodplain sites is in the form of mercuric sulfide. The near total conversion of the mercury to this form argues strongly that there has been interaction between the mercury and sulfate-reducing bacteria, in conditions favorable for the production of the metallic sulfide and unfavorable for the production of methylmercury. These conditions are an adequate supply of sulfate and nutrients.

Conversion of the mercury to the sulfide form represents a highly desirable outcome, because the compound is known to be biochemically inactive, neither absorbed by, nor toxic to, animals, and immobile in the environment.

In the experiments described here we have examined the possibility, *in vitro* and *in vivo*, that mercuric sulfide itself may serve as a substrate for the formation of methylmercury. The complete absence of any formation of organic mercury from ^{203}HgS after 6 weeks of incubation with soil in various conditions, and the very low percentage formation of methylmercury from HgS via methylcobalamine, both suggest that mercuric sulfide is unlikely to represent a significant substrate for the formation of methylmercury at environmental sites. This fact, plus the recognition that any reconversion to the ionic form via physicochemical forces is probably very slow, argues strongly that the existence of environmental mercury as the sulfide represents the least environmentally harmful form in which the metal can exist, and also the most stable.

5.4.3 An Approach to Bioremediation at Unstable Mercury-Contaminated Sites

A primary concern of environmentalists, when the extent of the mercury releases in Oak Ridge became apparent, was the uncertainty as to how much of the deposited metal had entered the food chain since the onset of the pollution, and how much of the mercury, now present, might enter the food chain in the future. Key aspects of this potential biohazard would be the possibility of the mercury being converted to the toxic and readily absorbed organic form, methylmercury. Bioconversion of mercury to methylmercury would potentially set up a chain of events leading to the occurrence of the toxic substance in crops and fish, where it could accumulate to levels which would cause a health hazard to animals and man.

If the mercury depositions at the East Fork Poplar Creek sites, and at other locations in Oak Ridge, were considered to constitute an environmental hazard, the question would arise as to what steps were available for dealing with the problem.

It is reasonable to assume that the physical removal of the mercury by digging up the contaminated sites would be a last resort because of high cost, disruption to people's lives, and the inevitable possibility of spreading airborne mercury-contaminated dust beyond the confines of the affected site. For these reasons, it was important to learn how much of the deposited mercury was in the methylmercury form, and what other chemical species of mercury were present at affected sites.

The extreme difficulty of leaching mercury from soil by water irrigation, and the discovery that almost all of the mercury was in the sulfide form, are facts which form the basis for the concept that the mercury now present in the soil may be stable. This means that it is insoluble in water, difficult to mobilize by irrigation or leaching, not converted to other forms of mercury, and not bioreactive. If all these conditions are true, the mercury contamination need not be dug up or in any other way remediated, because the formation of mercuric sulfide is an environmentally satisfactory end point in itself. It suggests that the formation of mercuric sulfide is a desirable goal in efforts which may be needed to

clean up a mercury spill in the future, when conditions are still unstable.

It is possible to speculate that, when a contamination event occurs involving inorganic mercury at an environmental site, the metal immediately becomes the substrate for a large number of physicochemical changes and microbial enzymatic reactions (see Figure 5.1). Some of these changes are reversible, whereas others are the precursors of other possible physicochemical or enzymatic conversions. The overwhelming conclusion to emerge from our studies on the mercury deposited at the East Fork Poplar Creek sites is that the metabolism of the endogenous sulfate-reducing bacteria is the key component in determining whether the mercury is channelled into harmless products such as mercuric sulfide, or into toxic products such as methylmercury. If channeling the metabolism of these microorganisms is so pivotal to the ultimate fate of the mercury, it seems important to try to develop approaches which would seek to ensure that the metal is converted to the less biohazardous product.

We at ORRI have proposed a technology which could be used to treat sites contaminated with mercury. It is based on the application to the contaminated sites of solid calcium sulfate (gypsum) as a substrate for sulfate reduction, and the further addition of necessary nutrients to enhance the metabolic activity of these bacteria in the direction of mercuric sulfide formation and away from methylmercury formation.

We have carried out preliminary experiments using this remedial approach on the East Fork Poplar Creek site. Although the mercury deposited at this site was probably in the sulfide form already, as indicated in this report, the value of these studies may be judged by the information obtained concerning the fate of sulfate, methodological difficulties, effects on water quality in East Fork Poplar Creek, and cost.

As stated above, enhancement of hydrogen sulfide production by the sulfate-reducing bacteria, with its subsequent interaction to sediment inorganic mercury as the sulfide, is additionally beneficial because it will quickly immobilize any available mercuric ions which might otherwise serve as a substrate for methylmercury formation. It will also realign the metabolism of the sulfate-reducing bacteria away from the formation of methyl donor compounds such as methylcobalamine and thus reduce the possibility of methylmercury formation.

A key feature of the various aspects of the work carried out at ORRI under its contract with DOE, has been to develop the idea of using the metabolism of bacteria, to breakdown, immobilize, or transform environmentally hazardous substances, and to use this power in the conceptualization and fabrication of microbial waste treatment systems. At the heart of this concept is the ability of bacteria to adapt their metabolism to withstand and perhaps use a chemical which would normally be regarded as toxic. Thus, we have developed biosorption media to remove cations from aqueous waste, discovered strains of bacteria which can reduce metal cations to the elemental form, and demonstrated the <u>in situ</u> immobilization of cationic mercury as the sulfide. The system involved in this conversion is clearly not dependent on high technology, and has the advantage that the pollutant may be remediated <u>in situ</u>. This direct approach has a low cost, is not equipment-intensive, and will not disturb the environment. These features argue strongly for its further investigation as a possible

approach to the quick response procedures necessary in bioremediating a site polluted with mercury.

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6.0 ASSESSMENT OF PCE BIODEGRADATION BY MICROBES

In the study described in this chapter, environmental samples from a series of monitoring wells adjacent to the Department of Energy's Y-12 Plant in Oak Ridge, Tennessee, were evaluated for trichloroethylene (TCE) and perchloroethylene (PCE) contamination. Both TCE and PCE are highly toxic and exhibit a strong resistance to chemical degradation. A bacterial strain capable of growing in the presence of high concentrations of PCE was isolated and identified. Preliminary experiments were carried out to assess the ability of this bacterial strain to degrade PCE and to determine under what conditions the organism can be propagated.

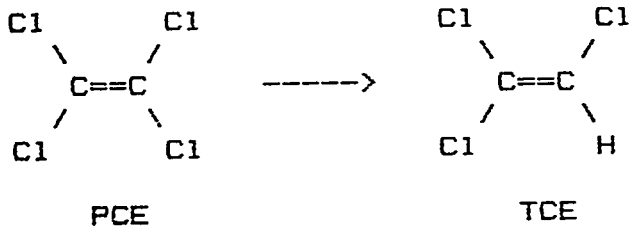
6.1 RATIONALE

The samples provided by Y-12 came from wells which have been shown to contain significant levels of PCE, TCE, and dichloroethylene (DCE), whereas the tanks leaking into the soil near these wells contained only PCE. PCE is stable to chemical decomposition. Therefore, it seemed reasonable that some biological activity in the soil was responsible for dechlorination of the compounds. The goal of this project was to isolate bacteria which are capable of complete mineralization of PCE, TCE, DCE, and vinyl chloride. Direct selection for the ability to use PCE as the sole carbon source was applied to the samples. However, the occurrence of complete mineralization could not be assured by the ability to tolerate PCE and to gain energy from its metabolism. In the absence of complete mineralization, complete dechlorination of the solvent was considered to be an acceptable remedial alternative, ethylene being considerably less toxic than its halogenated derivatives. Therefore, we also assessed the dechlorination of compounds which are derivatives of other compounds shown to be acceptable substrates for bacterial metabolism. These compounds were: monochloroacetic acid, which should yield acetate, a readily metabolized carbon source; 3-chlorobenzoic acid, which can be metabolized by a halogenase-related plasmid-coded pathway in Pseudomonads; and 4-chlorobenzoic acid, which can be metabolized by a chromosomal pathway in Pseudomonads.

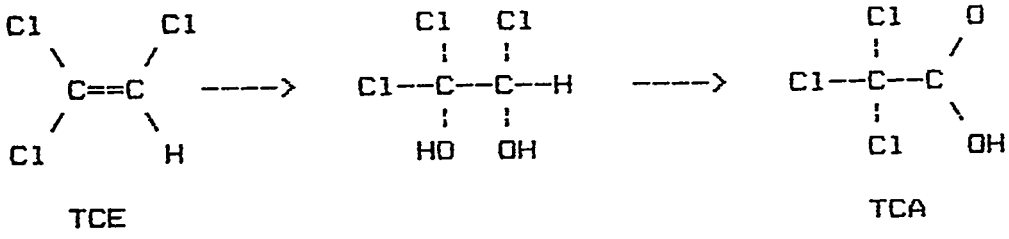
6.2 METHODS

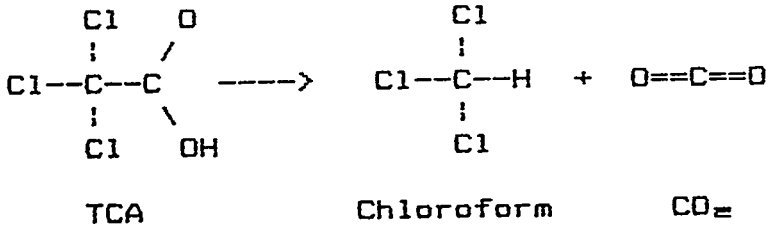
It was necessary to analyze samples for the presence of several chemical species, including PCE, TCE, chloroform, and trichloroacetic acid (TCA). The latter compounds could be generated by metabolism of PCE by dechlorination (Reaction 1), possibly followed by oxidation or epoxidation and rearrangement (Reaction 2). Two methods were used to measure PCE and its metabolites. These were dynamic headspace gas chromatography and direct automatic injection gas chromatography. These methods are described below. The same conditions were used for quantitation of TCE, chloroform, and other reaction products.

Reaction 1



Reaction 2





This section includes a description of growth media and enumeration methods used for culturing bacteria from the well samples.

6.2.1 Perchloroethylene

PERCHLOROETHYLENE

Formula: C_2Cl_4
M.W. 165.8
B.P. 121°
d. 1.623 g/mL

SYNONYMS: TETRACHLOROETHYLENE

Instrumentation: TRACOR MODEL 550 GAS CHROMATOGRAPH
TEKMAR MODEL 4000 DYNAMIC HEADSPACE CONCENTRATOR

Technique: DYNAMIC HEADSPACE GAS CHROMATOGRAPHY, FLAME IONIZATION DETECTOR

Analyte: PERCHLOROETHYLENE

Injection volume: 2-100 µL

Trap: Tekmar Tenax trap

Temperature: Injection 230°
Detector 250°
Column 60° 4 min
8°/min to 235°
235° 10 min (more as necessary)

Carrier: N_2, flowmeter on 9.5

Column: Supelco
Stainless steel, 1/8 in x 8 ft
1% SP-1000 on 60/80 Carbopack B

Calibration: Solutions of PERCHLOROETHYLENE in METHANOL

Range: Lower, 2 µg/sample @ Atten.= 8
Upper, 100 mg/sample @ Atten.= 1024

REAGENTS:
1. Perchloroethylene, chromatographic quality
2. Methanol, chromatographic quality
3. Nitrogen, 99.999%
4. Hydrogen, purified
5. Compressed air, zero grade

EQUIPMENT:
1. 5-mL capacity screw-cap tubes or 1-dram vials
2. Teflon-lined caps for tubes or vials
3. Volumetric flasks, 5 mL and 50 mL
4. Syringes, 10 µL and 250 µL gas-tight

SAMPLE PREPARATION:

1. Prepare two clean 5-mL screw-cap tubes or 1-dram sample vials for each sampler. Rinse each twice with methanol and allow to drain.

CALIBRATION AND QUALITY CONTROL:

2. Prepare a calibration standard solution by adding 50 mg (30.8 µL) PCE to a 50-mL volumetric flask and filling to the mark with methanol (1 mg/mL). Mix and transfer 0.25 mL (using a large gas-tight syringe) to a 5-mL volumetric flask. Fill to the mark with methanol (0.05 mg/mL).

3. Calibrate daily with at least five working standards including (for attenuator set at 8) 0.05, 0.15, 0.3, 0.45, and 0.6 µg (using a standard solution of 0.05 mg/mL in methanol, add 1.0, 3.0, 6.0, 9.0, or 12 µL to 2 mL H_2O).

4. Prepare a calibration graph of peak height vs amount of perchloroethylene.

MEASUREMENT:

5. Set the gas chromatograph as indicated above. Purge time is 15 min. With a 15-min purge, there is a 4-min dry purge to remove almost all of the methanol from the Tenax trap.

6. Rinse syringe by filling and discarding the sample solution three times. Waste sample can be discarded into activated charcoal in a beaker or flask. Add the desired volume of sample to 2 mL of water in a clean 13x100 mm screw-cap tube and attach to the dynamic headspace concentrator.

7. Run analysis cycle and determine peak height. Under the conditions listed, methanol elutes at about 2 min at 60°, and perchloroethylene elutes at about 16 min, at 210°.

CALCULATIONS:

8. Using the calibration chart, determine the mass (µg) of perchloroethylene in each peak. Calculate the mass in each sample by dividing by volume injected and multiplying by the volume of extract, e.g.:

$$W\ (mg) = [\text{Sample } \mu g/\text{sample } \mu L] \times 5.0\ mL$$

PERCHLOROETHYLENE

Formula: C_2Cl_4
M.W. 165.8
B.P. 121°
d. 1.623 g/mL

SYNONYMS: TETRACHLOROETHYLENE

Instrumentation: HEWLETT-PACKARD MODEL 5880 GAS CHROMATOGRAPH

Technique: DIRECT AUTOMATIC INJECTION GAS CHROMATOGRAPHY, ^{63}Ni ELECTRON CAPTURE DETECTOR

Analyte: PERCHLOROETHYLENE

Injection volume: 2-100 µL

Temperature:

Injection	230°
Detector	230°
Column	120° 2 min
	10°/min to 240°
	240° 4 min

Carrier: N_2, flow 20 cc/min

Column: Supelco
Stainless steel, 1/8 in x 8 ft
1% SP-1000 on 60/80 Carbopack B

Calibration: Solutions of PERCHLOROETHYLENE in METHANOL

Range: Lower, 2 µg/sample
Upper, 100 mg/sample

REAGENTS:

1. Perchloroethylene, chromatographic quality
2. Methanol, chromatographic quality
3. 95% Argon/5% Methane

EQUIPMENT:

1. 5-mL capacity screw-cap tubes or 1-dram vials
2. 1-cc crimp-top sample bottles with Teflon septa
3. Teflon-lined caps for tubes or vials
4. Volumetric flasks, 5 mL and 50 mL
5. Syringes, 10 µL and 250 µL gas-tight

SAMPLE PREPARATION:

1. Pipette samples into sample bottles and seal with Teflon septa.

CALIBRATION AND QUALITY CONTROL:

2. Prepare a calibration standard solution by adding 50 mg (30.8 µl) PCE to a 50-mL volumetric flask and filling to the mark with methanol (1 mg/mL). Mix and transfer 0.25 mL (using a large gas-tight syringe) to a 5-mL volumetric flask. Fill to the mark with methanol (0.05 mg/mL).

3. Calibrate daily with at least five working standards including (for attenuator set at 8) 0.05, 0.15, 0.3, 0.45, and 0.6 µg (using a standard solution of 0.05 mg/mL in methanol, add 1.0, 3.0, 6.0, 9.0, or 12 µl to 2 mL H_2O).

4. Prepare a calibration graph of peak height vs amount of perchloroethylene.

MEASUREMENT:

5. Set the gas chromatograph as indicated above. Set autosampler to rinse the syringe three times, and pump five times. Sample size is 1-2 µL.

6. Analyze standards and unknowns, using automatic baseline reset mode (Mode 0). Construct a calibration chart, using the peak areas observed for each standard concentration.

CALCULATIONS:

7. Using the calibration chart, determine the mass (µg) of perchloroethylene in each sample. Calculate the concentration in each sample by dividing by volume injected.

The characteristic retention times of the various compounds are given in Table 6.1.

TABLE 6.1
Retention Times of Halogenated Hydrocarbons in Two Gas Chromatographic Analytical Systems

Compound	Retention time (min)	
	System 1[1]	System 2[2]
Dichloroethylene	9.8	4.3
Chloroform	10.4	4.7
Trichloroethylene	15.4	8.2
Perchloroethylene	20.9	12.7

[1]Dynamic headspace chromatography (TRACOR 550)

[2]Direct injection (HP 5880)

6.2.2 Trichloroacetic Acid (TCA)

A method to measure TCA in urine by dynamic headspace gas chromatography was described by Senft (1985). This method relies on the conversion of TCA by heat to chloroform and CO_2, followed by gas chromatographic determination of the chloroform concentration. This method was adapted for the measurement of TCA in culture supernates after growth of bacteria in the presence of PCE.

Samples containing TCA are placed in screw-cap tubes with Teflon-faced seals. The samples are heated in a water bath for 105-120 min at 85°C, chilled, and analyzed for chloroform by gas chromatography by the techniques described above. Figure 6.1 shows the rate of conversion of TCA to chloroform when solutions containing 50 µg TCA were heated at 85°C. Figure 6.2 gives the concentration response of this system. In this procedure, chloroform peak heights were proportional to amount of TCA added to reaction tubes before heating; no chloroform was observed in unheated tubes with the same amounts of TCA.

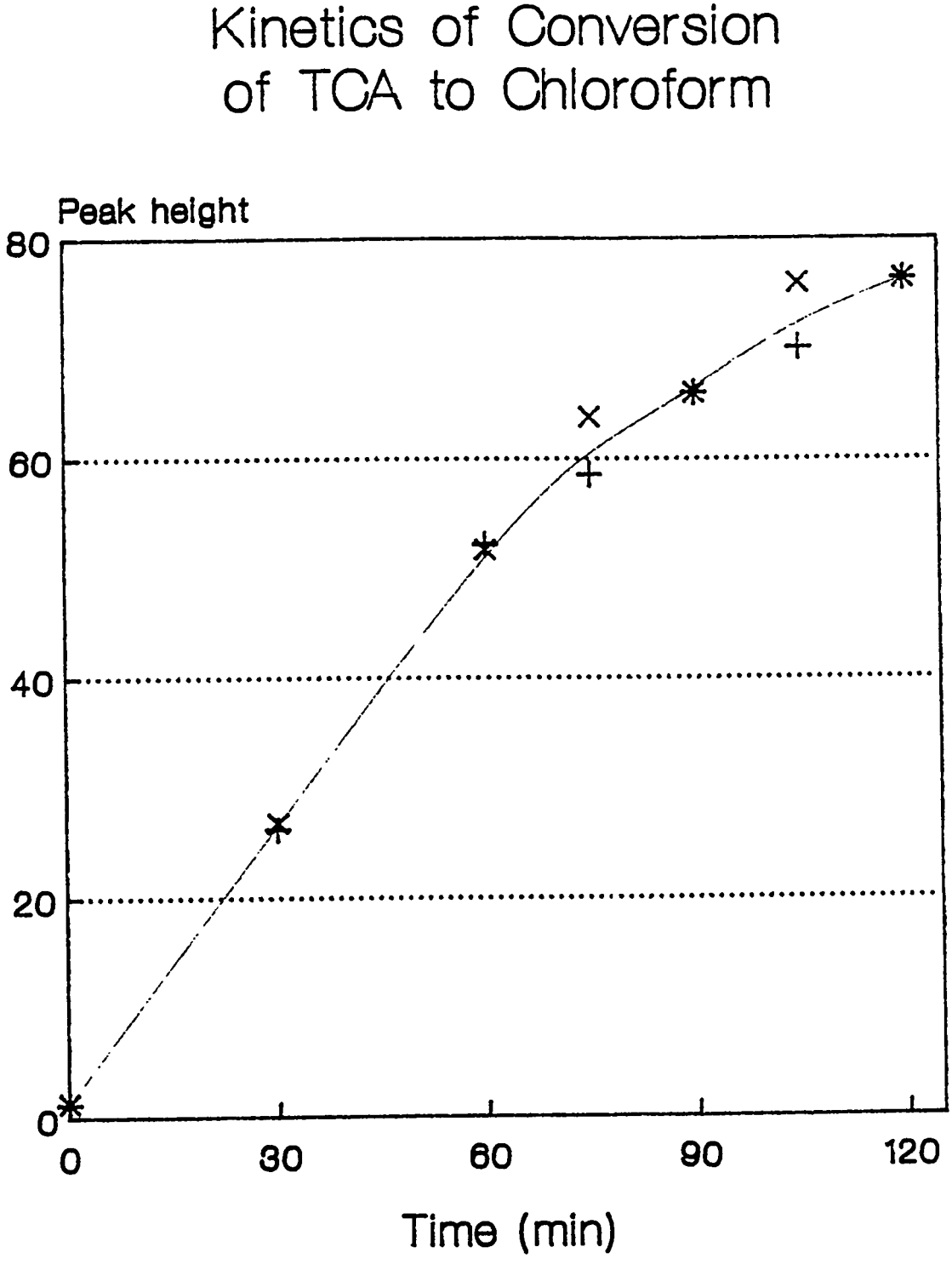

Kinetics of Conversion
of TCA to Chloroform
Peak height
80
60
40
20
0
0
30
60
90
120
Time (min)

Figure 6.1

Concentration Response of Conversion of TCA to Chloroform

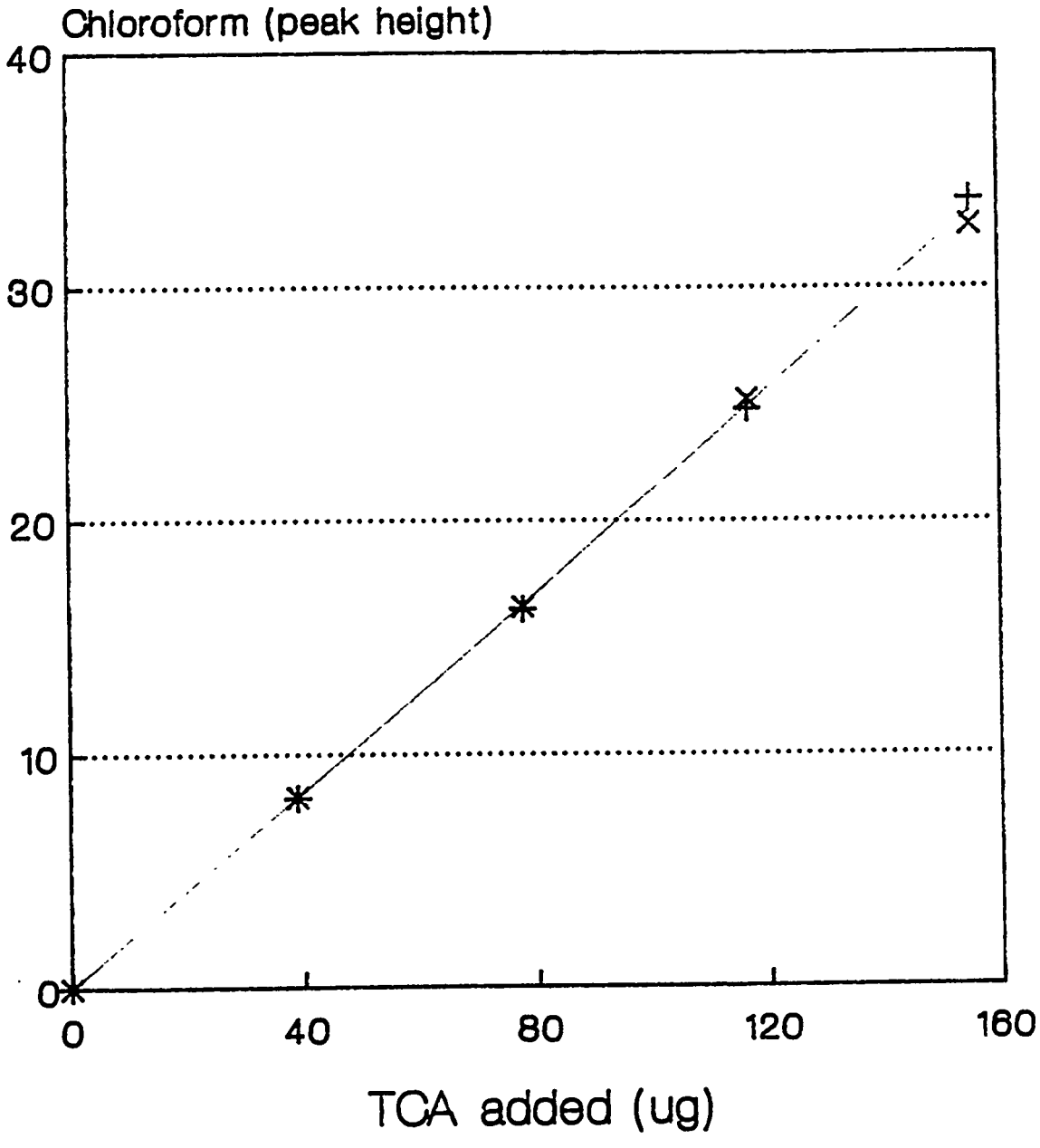


Figure 6.2

6.2.3 Growth Media

R2A is a semi-defined growth medium designed for the enumeration of bacteria from natural samples (Reasoner and Geldreich, 1985). It contains (grams per liter):

Yeast extract	0.5
Proteose peptone #3	0.5
Casamino acids	0.5
Glucose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
K_2HPO_4	0.3
$MgSO_4 \cdot 7H_2O$	0.07
Distilled water	1 l

Adjust pH to 7.2

MSVT is a defined salts medium made as follows:

Prepare and sterilize separately

MS base (autoclave):

1.0 g $FeSO_4$	
NH_4Cl	0.5 g
KCl	0.2 g
$MgSO_4$	0.2 g
NaCl	0.1 g
KH_2PO_4	0.44 g
K_2HPO_4	2.0 g
Distilled water	996 mL

Vitamin stock solution (filter-sterilize):

Calcium pantothenate	4.0 mg
Thiamine	4.0 mg
Pyridoxine	4.0 mg
Nicotinic acid	4.0 mg
p-Amino benzoic acid	2.0 mg
Riboflavin	2.0 mg
Distilled water	100 mL

Trace element stock solution (filter-sterilize): KNO_3 50.0 mg

$MnCl_2$	10.0 mg
$Co(NO_3)_2$	38.0 mg
$ZnCl_2$	10.0 mg
$CaCl_2$	2.0 mg
H_3BO_3	1.9 mg
Na_2MoO_4	1.0 mg
Na_2SeO_3	2.8 mg
$NiCl_2$	5.0 mg
Distilled water	100 mL

Biotin stock solution (filter-sterilize):

Biotin	24.0 mg
Distilled water	100 mL

Calcium-copper stock solution (filter-sterilize):

$CaCl_2$	0.15 g
$CuSO_4$	2.0 mL of 2.5 mg/100 mL solution
Distilled water	98 mL

Mix aseptically

MS base	996 mL
Trace elements stock	0.2 mL
Vitamin stock	2.0 mL
Biotin stock	164 µL
Calcium-copper stock	2.0 mL

SM is another defined salts medium (Billen et al., 1971), which contains (per liter):

K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
Na citrate	1.0 g
NH_4Cl	0.8 g
$MgCl_2 \cdot 6H_2O$	0.33 g
Na_2SO_4	14.0 mg
Distilled water	1 L

6.2.4 Enumeration of Bacteria

Bacteria in water samples were enumerated by platecount techniques. The samples were diluted in sterile water and spread on agar plates containing R2A agar medium (Reasoner and Geldreich, 1985). Colonies were counted after 24 to 48 hr incubation at room temperature.

6.2.5 Growth of Bacteria

Samples from wells containing PCE and other organic pollutants were inoculated into selective media and indicator media to select for the ability to metabolize chlorinated organics as sole carbon sources and for the ability to cometabolize chlorinated organics. Selection was done for utilization of PCE, monochloroacetic acid (MCA), 3-chloro- and 4-chlorobenzoic acid (3-CBA and 4-CBA), and 4-nitrochlorobenzene (CNB).

The protocol for inoculation of the bacteria under different conditions is given below:

Dilute 200 mL of water samples with 22 mL of ten-fold concentrated MSVT stock and dispense into tubes or bottles containing the desired substrates as follows:

1. Control
   Add 20 mL to a serum bottle containing no other additions.

2. YE Control
   Add 20 mL to a serum bottle with 0.02 mL of 10% yeast extract.

3. Acetate-PCE cometabolism
   Add 20 mL to a serum bottle with 0.02 mL of 10% yeast extract and 0.4 mL 10% sodium acetate, and 20 µL PCE.

4. PCE utilization - YE supplement
   Add 20 mL to a serum bottle with 20 µL PCE and 0.02 mL 10% yeast extract.

5. MCA utilization - YE supplement
   Add 20 mL to a serum bottle with 0.4 mL of 10% MCA and 0.02 mL 10% yeast extract.

6. 3-CBA utilization
   Add 20 mL to a serum bottle with 0.02 mL of 10% yeast extract and 0.2 mL of 10% 3-CBA in ethanol.

7. 2-CBA utilization
   Add 20 mL to a serum bottle with 0.02 mL of 10% yeast extract and 0.2 mL of 10% 2-CBA in ethanol.

8. Dechlorination of CNB
   Add 5 mL to a tube containing 0.1 mL 10% sodium acetate, 5.0 µL 10% yeast extract, and 50 µL 0.10% CNB in ethanol.

9. Acetate enrichment
   To 20 mL add 0.4 mL 10% sodium acetate and 0.02 mL 10% yeast extract. Incubate until growth is observed. Use this culture to reinoculate for tests above if necessary.

6.3 EXPERIMENTAL

6.3.1 Characterization of Potential Degradation Products of PCE

The goal of these experiments was to isolate and characterize microorganisms which could carry out partial or complete biodegradation of halogenated organic solvents. Initial consideration was given to the kinds of compounds which might appear in growth medium as a result of degradation of PCE. Potential pathways for biodegradation of PCE might include successive dechlorination (to TCE, DCE, vinyl chloride, and ethylene), oxidation of the double bond with formation of alcohol or diol, oxidation with elimination of chloro groups, or addition of acid or base to the double bond. Oxidation of TCE by mammalian cells has been observed to lead to formation of a diol product, followed by rearrangement, with the ultimate formation of TCA (Senft, 1985). Therefore, TCA was also considered to be a potential degradation product of PCE, by a reaction which would have to include elimination of one of the chloro groups.

In order to be sure that we could identify a variety of degradation products of PCE in subsequent experiments, an initial study was done to characterize reaction products of PCE. A number of potential degradation products and intermediates were characterized chromatographically so they could be identified if they appeared during experiments on biodegradation of PCE. Standards containing PCE, TCE, and DCE were mixed and chromatographed as described in the Methods section for the analysis of PCE. Figure 6.3 shows a tracing

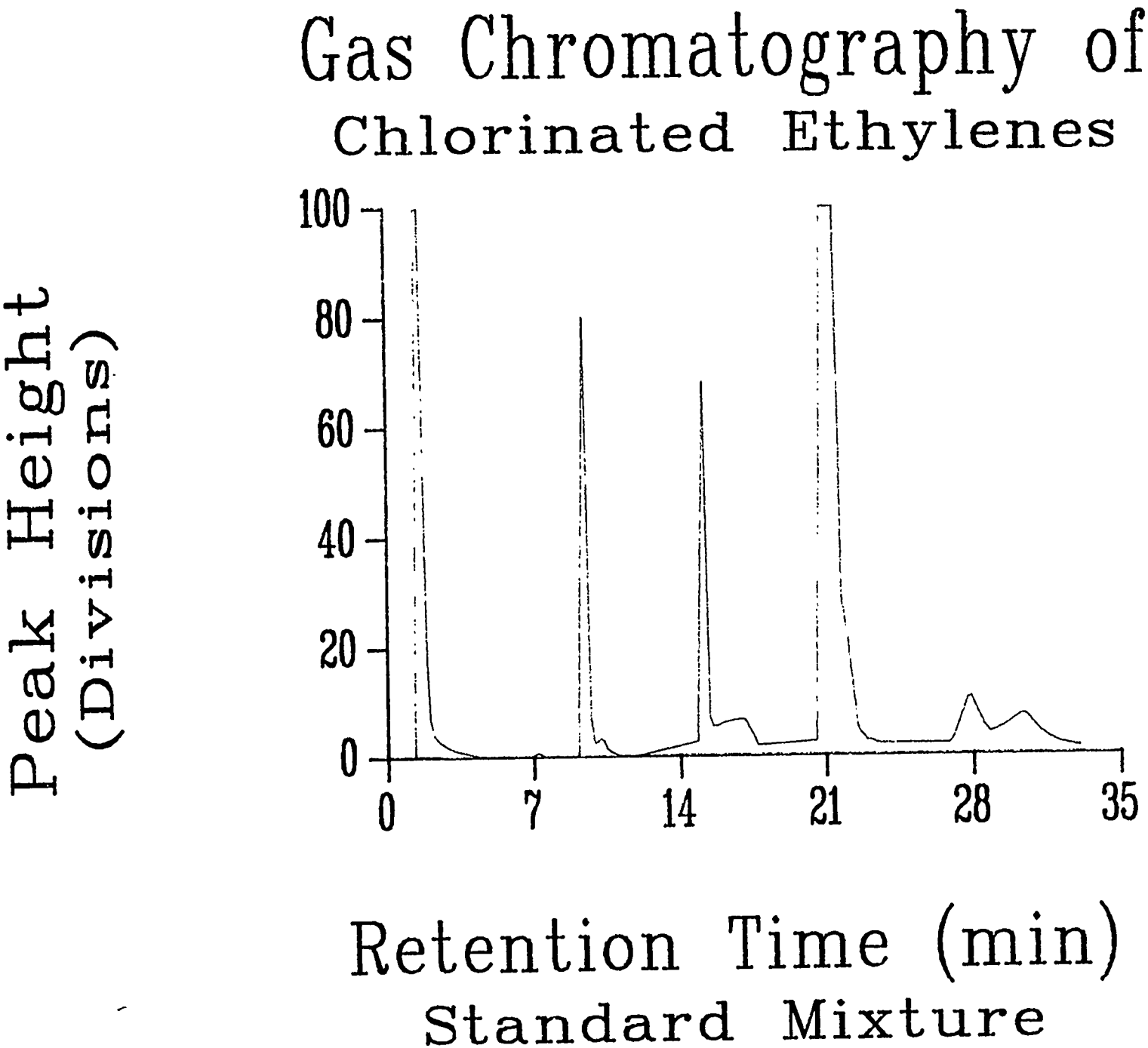

Gas Chromatography of
Chlorinated Ethylenes
Peak Height
(Divisions)
100
80
60
40
20
0
0
7
14
21
28
35
Retention Time (min)
Standard Mixture

Figure 6.3

of a typical chromatographic analysis of these standards. PCE, TCE, and DCE are well-separated in this analysis, but vinyl chloride elutes in the solvent peak. Oxidation products of PCE were generated by incubating PCE with concentrated sulfuric or hydrochloric acid at 60°C for 48 hr. The aqueous fraction was then analyzed by dynamic headspace gas chromatography. The results showed that a substantial amount of the PCE was converted to an apparently more polar compound, probably sulfate, alcohol, or diol, which eluted later than PCE, and a small amount of an early-eluting material was also formed (Figure 6.4).

Similarly, a mixture of PCE and water was irradiated with approximately 5 x 10^6 rads of radiation from a ^{60}Co gamma source, and a mixture of PCE and 1M $KMnO_4$ was incubated for 7 days at room temperature. The aqueous layers of these mixtures were then analyzed by dynamic headspace gas chromatography. In the case of radiation, radiolysis of water would be expected to yield a number of reactive species; the likely addition product would be alcohol or diol, formed by the addition of the hydroxyl radical to the double bond of the PCE molecule. As a result of the permanganate reaction, epoxide formation would be expected to occur, followed by formation of a diol and perhaps subsequent rearrangement and elimination of chloro groups. Figure 6.4 shows the results of these experiments. In this figure, the tracings are offset to facilitate comparison of the profiles. In each case, addition products were observed, although in very small amounts in the case of radiolysis products. Two early-eluting products were formed as a result of permanganate reaction, one of which chromatographed identically with the early-eluting addition product of acid treatment.

The characteristic retention times of chlorinated ethylenes and the observed addition products as measured by dynamic headspace gas chromatography are listed in Table 6.2.

These experiments demonstrated that products of addition to the double bond of PCE can be detected. Acid addition appears to have formed small amounts of dichloroethylene and an unidentified early-eluting material, as well as a substantial amount of slow-eluting adduct, probably an alcohol or a sulfate. Permanganate addition formed two early-eluting compounds, one of which appears to be identical to the early-eluting acid product. Conditions which would have allowed detection of the slow-eluting addition compounds were included in subsequent analyses of PCE biodegradation products.

6.3.2 Site Characterization

Water samples were removed by Y-12 personnel from 11 monitoring wells at the Y-12 Plant on March 10, 1986. Measurements of temperature, pH, dissolved oxygen, and conductivity were made in the field and reported to us when the water samples were delivered. These values are reported in Table 6.3, along with our assignment of sample numbers.

Water samples were diluted and plated on R2A agar plates (Reasoner and Geldreich, 1985) for the enumeration of total aerobic heterotrophic bacteria. These values ranged from undetectable at a 10^{-2} dilution to 10^6 organisms per mL. (By definition, clean water contains fewer than 10^3 total heterotrophs per mL.) There was no

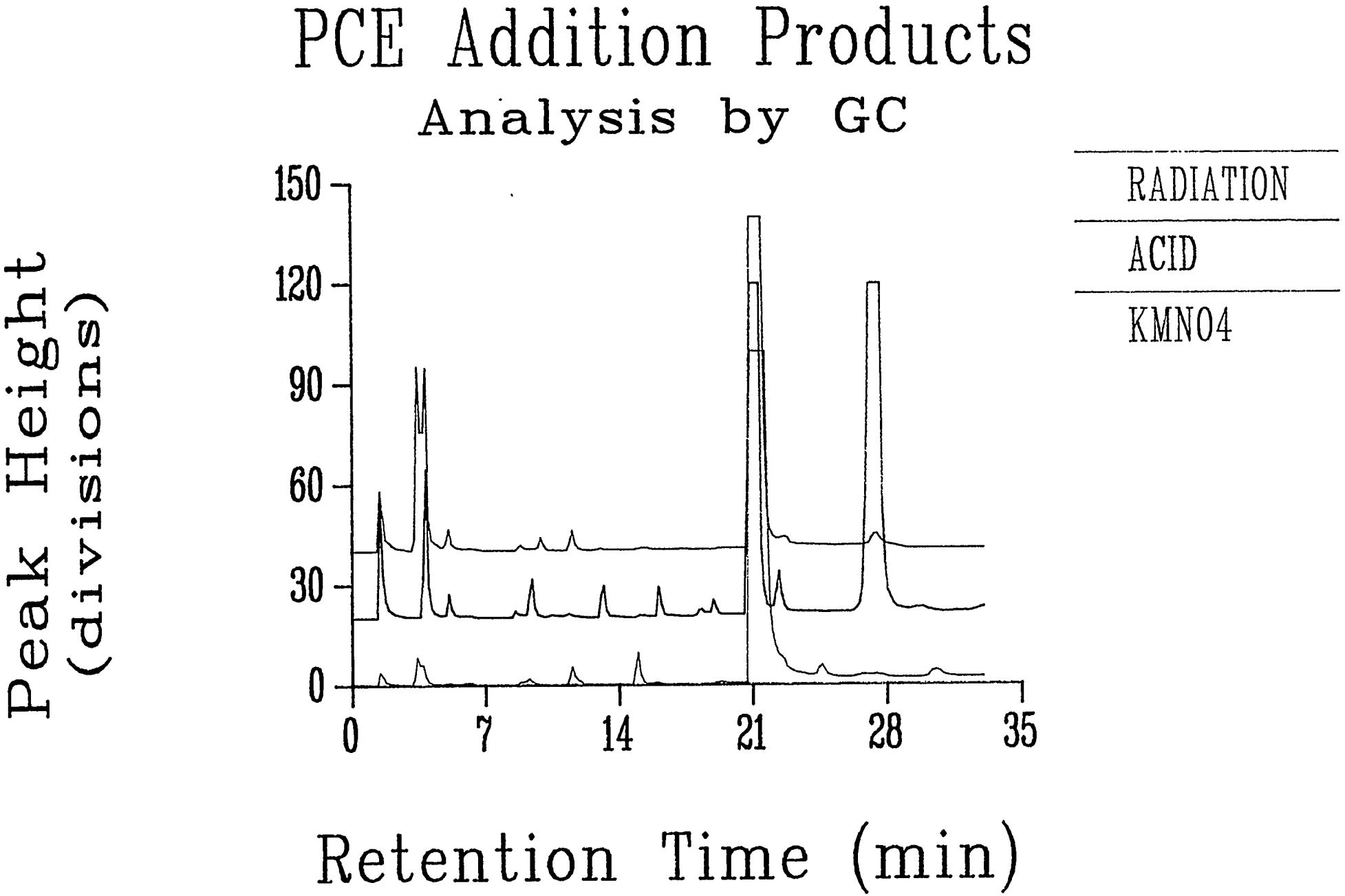

PCE Addition Products
Analysis by GC
RADIATION
ACID
KMNO4
Peak Height
(divisions)
150
120
90
60
30
0
0
7
14
21
28
35
Retention Time (min)

Figure 6.4

TABLE 6.2
Retention Times of Halogenated Hydrocarbons and PCE Reaction Products Analyzed by Gas Chromatography

Compound	Retention time (min)
Dichloroethylene	9.4
Chloroform	10.4
Trichloroethylene	15.2
Perchloroethylene	20.9
PCE addition products	
Acid addition	3.9, 9.4, 27
Permanganate addition	3.45, 3.9

apparent statistical correlation of the number of bacteria or the logarithm of the number of bacteria with other measured parameters in the water samples. Significant concentrations of PCE and TCE were not detected in the samples by gas chromatography (dynamic headspace concentration method), but trace amounts of these compounds could have been present initially and lost by adsorption to the walls of the sample bottle.

6.3.3 Solubility of PCE in Detergent Solutions

PCE and similar organic solvents are extremely poorly soluble in water, so disposal or degradation processes that are based on reactions in water solutions are not very efficient. In particular, biological degradation systems are hampered by low availability of poorly soluble substrates. In addition, it is difficult to make accurate measurements of rates of degradation if the total amount of substrate is greater than the soluble concentration. Therefore methods of increasing the solubility of PCE in water would be helpful in studies of biodegradation. In addition, bacterial cell membranes contain high quantities of lipids, which are essential for the proper structure and functioning of the membranes and, thus, for the survival and metabolism of the cell. PCE and similar solvents are soluble in lipids and could dissolve in cell membranes, disrupting their structure enough to kill the cells or disrupt their energy metabolism or other metabolic processes. The experiments described here were done to determine whether the addition of various organic solvents and detergents might increase the solubility of PCE in water-based media without increasing the toxicity of the PCE-containing media.

TABLE 6.3
Characterization of Water Samples
from Monitoring Wells at Y-12

Sample No.	Well No.	D.O.	Temp. (°C)	pH	Conductivity (µS/cm)	Aerobes per mL
1	GW-8	2.5	15.6	6.8	0	1.50×10^{4}
2	GW-14	3.2	12.8	7.5	0.2	5.95×10^{2}
3	GW-15	3.4	10.4	7.2	0.3	8.39×10^{5}
4	BG-18	3.1	17.4	7.4	0.3	3.50×10^{3}
5	GW-20	2.7	15.3	7.9	0.1	1.00×10^{6}
6	GW-27	3.1	16.9	6.7	0.4	1.60×10^{4}
7	DR-29	3.2	14.0	6.8	0.8	8.65×10^{4}
8	GW-59	5.7	14.8	7.6	0.1	7.45×10^{4}
9	GW-71	2.5	14.1	11.8	0.7	$<1.00 \times 10^{2}$
10	GW-79	4.5	14.7	7.8	0.1	4.46×10^{5}
11	GW-94	4.1	15.9	9.3	0	1.14×10^{5}
	Mean	3.45	14.72	7.89	0.27	2.36×10^{5}
	Std. dev.	0.92	1.85	1.42	0.25	3.46×10^{5}

We hypothesized that, in addition to increasing the availability of PCE to the cells, solubilizing agents might also provide the additional benefit of reducing the toxicity of the solvent to the growing cells. In this study, a number of agents were used that are known to increase the miscibility of polar and nonpolar compounds and are not highly toxic to bacterial cells. These compounds were ethanol, dimethylsulfoxide (DMSO), Triton X-100, and Tween-80.

The growth medium used was MSVT + sodium acetate + 0.01% yeast extract. Various organic solvents or detergents were added, along with 0.1% (v/v) PCE. Parallel cultures were incubated either uninoculated or inoculated with subcultures from sampling wells (Table 6.3). The inoculated cultures were assessed for toxicity and, in some cases, for degradation of PCE. Cultures containing the test compounds but not PCE were also inoculated with bacteria to assess the toxicity of the test compound alone.

PCE concentrations were measured by dynamic headspace gas chromatography, as described in the Methods section, except that the temperature was held constant at 210° C. Samples of 1 µL were diluted in 2.0 mL of glass-distilled water in screw-cap tubes. The tubes were then attached to the head space concentrator, purged with nitrogen for 6 min, and injected into the column by thermal desorption from the concentrator's trap. Under these conditions an injection peak was observed at 1.0 min, and PCE eluted at 4.5-4.8 min. With an output attenuator setting of 16, 1 µL of PCE-saturated water gave a peak area of 86.8 sq mm (height x width at 1/2 max height) and 1 µg of PCE gave a peak area of 392 sq mm. The latter value was used to calculate the concentration of PCE in the experimental samples.

The effects of the various additions on solubility of PCE are given in Table 6.4.

TABLE 6.4
Solubility of PCE in Growth Medium with Various Solvent Additions

Addition	Conc. (v/v)	Peak Ht (mm)[1]	Half max. width (mm)	Area (sq mm)	ppm PCE[2]
None		28.0	3.1	86.8	233
DMSO	5.0%	29.3	3.4	99.8	268
Ethanol	5.0%	23.8	3.4	80.9	218
Tween-80	0.5%	56.0	3.2	179.2	482
Triton X-100	0.5%	123.5	3.1	382.9	1030

[1]Peak height was normalized to the height of the injection peak.

[2]Calculated relative to a standard containing 1 µg PCE in methanol. No peaks were observed at the position of PCE when solvent controls containing no PCE were chromatographed.

These results demonstrate that the solubility of PCE in aqueous media can be significantly increased by the addition of certain detergents to the medium. The increase in solubility could make PCE in water more available to bacteria for biodegradation. In addition, the increased solubility might be important in situations where PCE is hydrophobically bound, for example to soil particles. Addition of the appropriate detergent to water being used to flush contaminated soil could be expected to increase the amount of PCE leached from a spill site.

6.3.4 Isolation of PCE-Resistant Bacteria

The purpose of this project was to isolate and characterize naturally occurring microorganisms which are capable of degrading PCE. Water samples from monitoring wells in the Y-12 Plant were used as the source of the organisms; these wells show contamination with PCE and

other volatile organics. The rationale was first to select organisms resistant to the toxic effects of PCE, and then to screen these for the ability to degrade PCE, either as a nutrient source or by cometabolism.

Selective and indicator media were inoculated from the well samples as indicated in the Methods section. Cultures were grown in 60-cc serum bottles sealed with Teflon septa. Initially, 20-mL samples were incubated at room temperature for 10-14 days. Cultures in which the bacteria grew were saved for isolation and further analysis. Some cultures in which 3-chlorobenzoate was the primary carbon source turned dark after several days, indicating degradation of the benzoate. The ability to grow with the indicated substrates as the major carbon source was confirmed by subculture of the bacteria. In addition, several of the samples were subsequently shown to be able to degrade biphenyl, the parent compound of polychlorinated biphenyls. The results of these experiments are summarized in Table 6.5.

Except for the control (#1), all cultures contained 10 ppm yeast extract and 100 ppm of the indicated substrate. All cultures showed little or no growth in the controls, indicating minimal carryover of nutrients, and sparse growth in all yeast extract controls (#2).

1. Control - no addition
2. YE Control - 10 ppm yeast extract only
3. Acetate-PCE, 100 ppm each
4. Perchloroethylene, 100 ppm
5. Monochloroacetate, 100 ppm
6. 3-Chlorobenzoic acid, 100 ppm
7. 2-Chlorobenzoic acid, 100 ppm
8. Chloronitrobenzene + acetate, 100 ppm each
9. Acetate, 100 ppm

Bacteria were streaked onto R2A agar and individual colonies were observed for morphological types. Cultures grown in the presence of excess PCE yielded mainly flat, matte colonies characteristic of the genus Bacillus. Several of the colonies were restreaked, and the isolated bacterial strains were tested further. Bacterial strains were identified on the basis of morphology, gram character, motility, and biochemical properties, using Bergey's Manual (Sneath et al., 1986) as the reference.

6.3.5 Protective Effect of Detergent for PCE-Sensitive Cells

The potential application of detergents to increase solubility and, thus, possible biodegradation of PCE depends in part on the toxicity of PCE to the cells. The suggestion was made above that the addition of detergent to bacterial culture media might reduce the potential partitioning of PCE to the lipids of the bacterial cell membrane, and thus reduce the toxicity of PCE to the cells. To test this hypothesis, an experiment was done to test the protective effects of one of the detergents against inhibition of growth of bacterial

TABLE 6.5
Growth of Bacteria from Monitoring Wells
in Selective and Indicator Media

Sample No.	Well No.	Extent of growth in condition number						
		3	4	5	6	7	8	9
1	GW-8	±	±	–	+	+	+++ YLO	+
2	GW-14	++	+++	+++	++ BR	++	++	++
3	GW-15	+++ BK	++	++ YLO	+ BK	++ BK	++ BK	++ BK
4	BG-18	++	++	– YLO	–	±	+	+
5	GW-20	++	+	–	+	–	+++	–
6	GW-27	++	+++	–	–	–	++	–
7	DR-29	++	–	–	++ YLO	–	+++	–
8	GW-59	++	+	–	–	±	±	–
9	GW-71	++	±	±	± YLO	± YLO	++ YLO	–
10	GW-79	++	± YLO	± YLO	± YLO	± YLO	±	+
11	GW-94	++	–	+	–	±	±	–

Pigments and colored products produced as indicated:
BR = brown, BK = black, YLO = yellow

– = No growth; ± = barely turbid; + = sparse growth;
++ = moderate growth; +++ = heavy growth

strains found in the monitoring wells. In this experiment, strains of bacteria from the sampling wells that were shown not to grow rapidly in R2A with 0.1% PCE were incubated in R2A alone or with 0.5% Tween-80, 0.1% PCE, or both PCE and Tween-80. The extent of growth was measured by determining the optical density of each culture. After 48 hr at room temperature, the results were as shown in Table 6.6.

TABLE 6.6
Protective Effects of Tween-80:
Growth of Bacteria in R2A with Additions

Well No.	Optical density (600 nm)			
	Control	TW-80	PCE	TW-80+PCE
2	1.23	1.09	0.27	0.70
5	1.12	0.97	0.62	0.60
6	1.54	1.90	0.50	0.60
8	0.44	0.54	0.11	0.17
9	0.46	0.64	0.06	0.18
11	1.46	1.79	0.30	0.60

These results indicate that, in general, Tween-80 was not significantly toxic to the bacteria. Tween-80 had some protective effect against the toxic effects of PCE when the bacteria were sensitive to PCE, but that the protection was somewhat limited. The bacteria from Well 5 grew reasonably well in the culture containing PCE, and this culture did not grow more extensively when Tween-80 was added along with the PCE. This may indicate that PCE-resistant strains are not as susceptible as PCE-sensitive strains to solubilization of essential cell components by PCE, and are thus not protected additionally by the presence of detergent. Overall, the protection of cells by detergent from the toxic effects of PCE was only marginal, indicating that the method will have limited practical application for growth of PCE-sensitive cells in the presence of high concentrations of PCE.

6.3.6 Identification of Bacillus W8

One of the PCE-resistant isolates, designated W8, was chosen as a representative and examined in some detail. Studies were done to determine its growth requirements and substrates. Its general properties are summarized in Table 6.7. The organism appears to be a variety of Bacillus, and some of its properties match those of some of the Bacillus species described in Bergey's Manual. However, none of the species of Bacillus listed in Bergey's Manual matched all of the properties of W8. It proved to be able to grow in a simple mineral salts medium without added trace elements or vitamins, and to grow on mineral salts with citrate as the sole carbon and energy source. Glucose is a poor but usable energy and carbon source; yeast extract and vitamin-free Casamino acids are better substrates. Inspection of old nutrient agar streaks of the organism revealed the presence of spores, indicating that it belongs to the genus Bacillus. The organism grew in nutrient broth containing saturating concentrations of PCE, indicating that it is resistant to the toxic effects of that compound.

TABLE 6.7
Properties of Bacillus W8

* Gram-positive spore-forming rod
* Facultatively anaerobic
* Catalase-positive, oxidase-negative, urease-negative
* Nitrate reduced to nitrite but not to N_2
* Grows on glucose, citrate, or yeast extract
* Vitamins not required for growth
* Does not grow at 50°C
* Grows in the presence of excess PCE

6.3.7 Resistance of Bacillus W8 to PCE

The toxicity of PCE to Bacillus W8 was compared to its toxicity to Escherichia coli B/R and a Pseudomonas species. Toxic effects were measured on cells grown to stationary phase in tryptone broth. The cells were diluted and plated on nutrient agar to determine the initial viable titer. A saturating amount of PCE (100 µL/mL) was added to each culture, and the cell suspensions were vigorously mixed for 15 seconds. The cells were sampled again, diluted, and plated on nutrient agar to determine the surviving viable titer. The results (Table 6.8) showed that the resistance of Bacillus W8 is several orders of magnitude greater than that of the other bacterial strains tested. E. coli and Pseudomonas are Gram-negative and therefore have a significantly different cell wall composition and structure than the Gram-positive Bacillus W8. This difference contributes to differential sensitivity to antibiotics and detergents, and could result in a difference in sensitivity to PCE. However, because another strain of Bacillus, Bacillus subtilis 168, has been observed not to grow in the presence of excess PCE, the resistance of Bacillus W8 is not likely to have been a result of the Gram-positive cell wall per se. It appears likely that the resistance is a property selected for in the PCE-containing environment of the organism.

6.3.8 Biodegradation of PCE

Cultures were grown in 20 mL of MSVT with 20 µL PCE and various carbon sources, sodium acetate, 3-chlorobenzoate (3CBA), and 2-chlorobenzoate (2CBA).

TABLE 6.8
Comparison of Resistance of Bacillus W8 and Other Bacterial Strains to PCE

Strain	CFU/mL Initial	CFU/mL Final	Survival (%)
Escherichia coli B/R	2.3×10^{7}	< 50	$< 2.0 \times 10^{-4}$
Pseudomonas sp.	6.7×10^{8}	1.5×10^{3}	2.2×10^{-4}
Bacillus W8	2.2×10^{7}	2.5×10^{6}	11.3

Cultures that showed growth were subcultured, and PCE degradation was determined as the disappearance of PCE measured by gas chromatography. Two samples have shown reduced levels of PCE after incubation. One, from sampling well GW-59, showed degradation of PCE in MSVT + 0.01% yeast extract + 0.1% 3CBA and 0.1% PCE. Another, from sampling well GW-20 showed degradation in chloride-free MSVT + 0.002% yeast extract + sodium acetate + 0.1% PCE. In this case, it is possible that growth of the organism depends on dechlorination of PCE to release Cl^- for cell metabolism. This possibility should be investigated further.

As described above, Bacillus W8 appeared to be able to degrade PCE. The experiments described below were carried out with this strain to evaluate further its potential to degrade halogenated solvents. Cells of Bacillus W8 were grown overnight in MSVT containing 0.1% yeast extract as the carbon source. They were centrifuged and washed with MSVT, and then resuspended at a 1:20 dilution in serum bottles containing 20 mL of MSVT + 0.01% yeast extract + 0.1% 3-CBA. PCE was added to a final concentration of 100 ppm, and the serum bottles were sealed with Teflon septa. Controls contained either no cells or no PCE. The bottles were incubated at 30° C, and at weekly intervals 200-µL samples were taken and diluted into 2 mL of water for analysis by dynamic headspace gas chromatography. The results of this experiment (Figure 6.5) indicated that after about 2 weeks of incubation, the concentration of PCE in the medium decreased, until at 4 weeks it was not detected. In contrast, the bottles containing no bacteria did not show a reduction in PCE concentration.

In a similar experiment, the effect of carbon source on degradation of PCE and TCE was tested. Cells of Bacillus W8 were grown overnight in SM + 0.1% yeast extract. They were then centrifuged and washed with SM, and resuspended at a 1:20 dilution in serum bottles containing 20 mL of tryptone broth, SM alone, or SM + 0.1% yeast extract or casamino acids. PCE or TCE was added at a final concentration of 100 ppm in methanol (final methanol concentration 1000 ppm). The bottles were sealed with Teflon-lined septa and incubated at 30° C. At weekly intervals, 200-µL samples were taken and diluted into 2 mL of water for analysis by dynamic headspace gas

Degradation of PCE by Bacillus W8 in MSVT

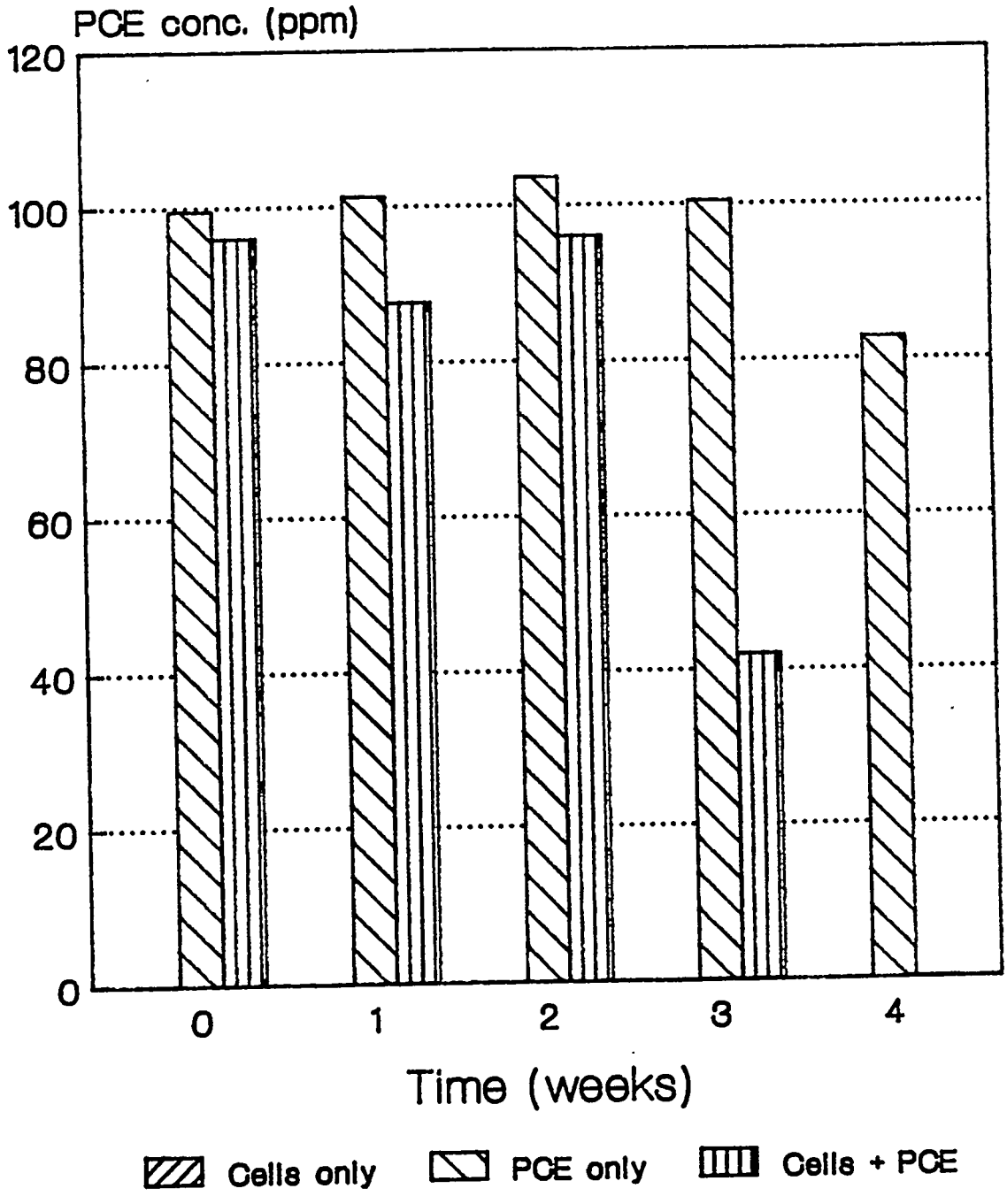


Figure 6.5

chromatography. The results showed degradation of the solvents in all of the media. Examples of the degradation of PCE and TCE in the various media are given in Figures 6.6 and 6.7. These results show that degradation of the compounds occurred more rapidly in the presence of a rich energy source such as the amino acid mix (CAA), yeast extract (YE), or Tryptone broth than in SM alone. The results indicated that both PCE and TCE were degraded in a mineral salts medium (SM) containing only citrate as the only additional carbon source. No degradation of chlorinated hydrocarbons has been observed without an additional carbon source.

In none of the gas chromatographic analyses was there evidence of volatile intermediate metabolites, particularly dehalogenated intermediates. It has been shown that halogenated aromatics and TCE may be degraded by epoxidation, followed by hydrolysis, which results in hydroxyl groups being added to the double bond to yield a glycol. This could then rearrange, perhaps with a spontaneous dechlorination to TCA. If this or a similar mechanism is responsible for the observed degradation of PCE, a buildup of toxic vinyl chloride should not be expected to occur during degradation of PCE.

A method to measure TCA in urine by dynamic headspace gas chromatography was described by Senft (1985). This method relies on conversion of TCA by heat to chloroform and CO_2, followed by chromatographic determination of chloroform concentration. The application of this method to the determination of TCA concentrations in bacterial culture is described in the Methods section.

Preliminary analysis of samples suggested that after a sample of the culture that had been grown in medium with PCE was heated, the amount of chloroform increased, as would occur if the cells or medium contained TCA. Surprisingly, the amount of PCE observed in these samples also increased (data not shown). A further experiment was performed in which the culture was centrifuged and chromatographic analysis was carried out on both the supernatant and the resuspended cells (Table 6.9). Both cells and medium appeared to contain significant amounts of chloroform even without heating to convert TCA. The heated supernatant showed an increase in the amounts of both chloroform and PCE, whereas the resuspended cells appeared to contain less precursor of chloroform (presumably TCA). In this experiment, relatively little PCE was observed in the heated cells, although the amount of PCE purged from the cells increased by a factor of 5 as it did in the unfractionated culture. These experiments suggest that the cells accumulate some amount of PCE which is held in a non-volatile form, perhaps as a lipid complex in the cell membrane or as an excreted lipid or lipid-protein complex, and that PCE may be metabolized to a variety of products, including chloroform and probably TCA.

Degradation of PCE by Bacillus W8 with Added Carbon Sources

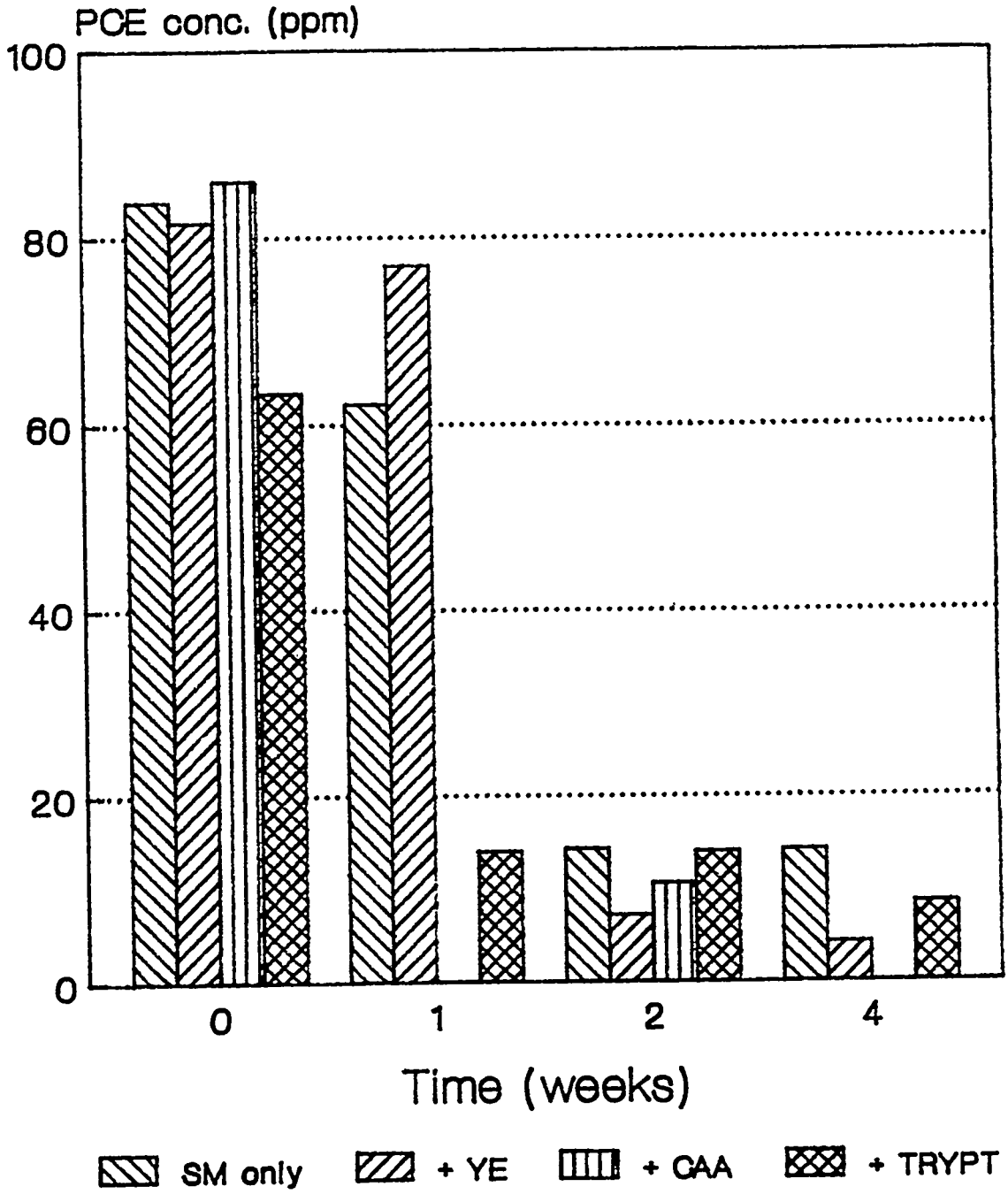


Figure 6.6

Degradation of TCE by Bacillus W8 with Added Carbon Sources

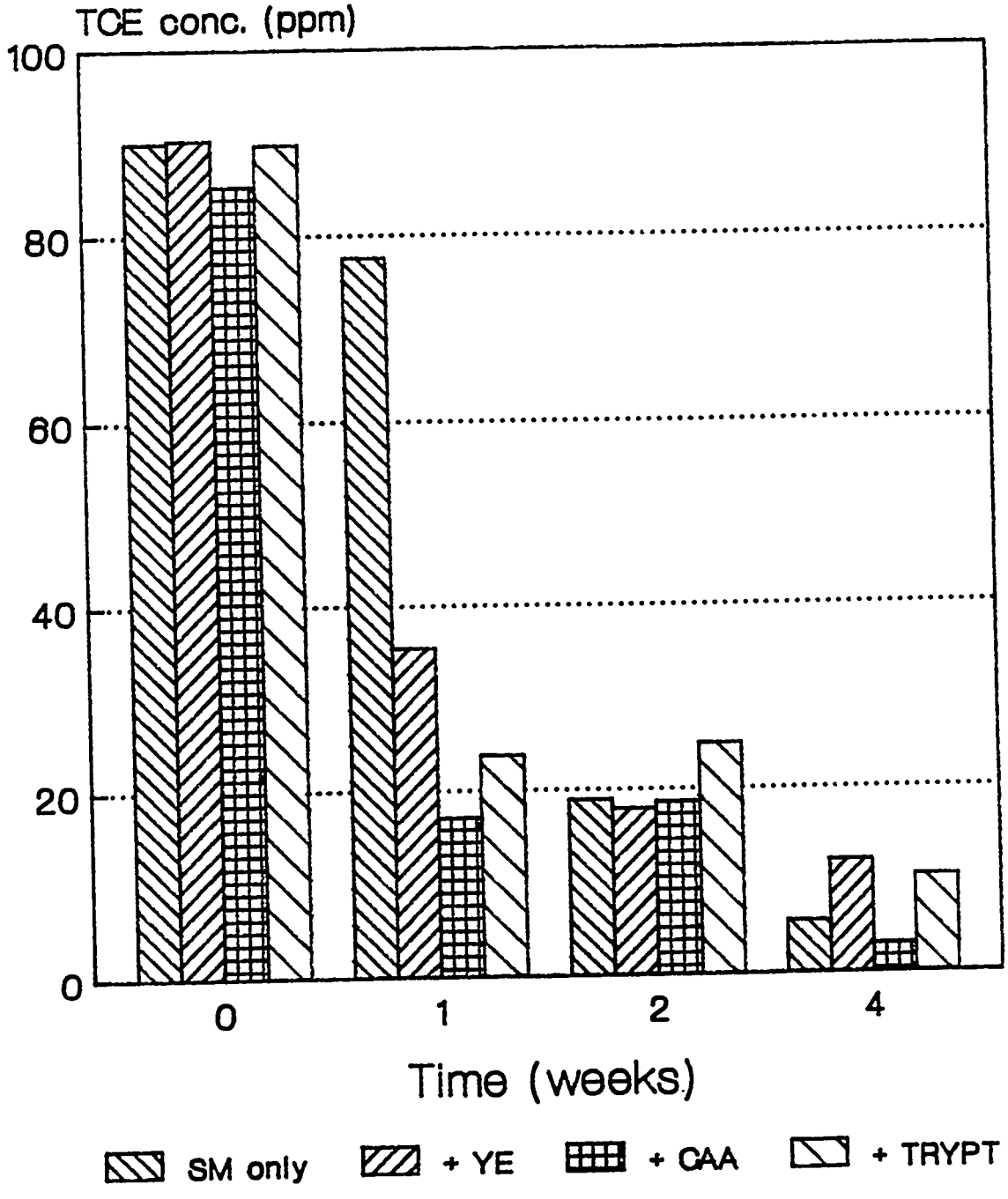


Figure 6.7

TABLE 6.9
Distribution of PCE and $CHCl_3$ in a Bacterial Culture

Condition	PCE ppm	$CHCl_3$ µg
Culture[1], unheated	14.2	1.14
Culture, 85° 150 min	77.2	0.99
Supernatant[=]		
Unheated	0.3	1.12
Heated 85° 150 min	14.1	2.07
Cells resuspended		
Unheated	1.1	1.03
Heated 85° 150 min	5.5	1.27

[1]A culture of Bacillus W8 growing in MSVT + 0.1% yeast extract and 0.1% 3-chlorobenzoate was diluted 50-fold into MSVT with 0.1% yeast extract and 100 ppm PCE. After 2 weeks of incubation at 30°C, a 200-µL sample of the culture was diluted with 2 mL H_2O and analyzed by dynamic headspace gas chromatography, with the attenuator set at 32.

[=]1 mL of culture was centrifuged to pellet the cells, and the cells were resuspended in 1 mL MSVT. Cells and supernatant were analyzed by dynamic headspace gas chromatography.

6.4 CONCLUSIONS

Monitoring wells in the Y-12 Plant in which PCE had been detected were screened to determine whether they contained bacteria resistant to the toxic effects of PCE and with the metabolic capability to degrade PCE and TCE. All of the wells surveyed contained some bacteria capable of growing in the presence of PCE. These bacteria were grown in mixed cultures in mineral salts media with various carbon sources, and several of them showed the ability to metabolize other chlorinated substrates. Most of the bacteria isolated in the presence of PCE had morphological characteristics indicating that they were probably of the genus Bacillus. One of these isolates was positively identified and named Bacillus W8. It was shown to be resistant to saturating concentrations of PCE, and measurements of PCE concentrations in culture media indicated that the organism is capable of degrading PCE.

Compounds formed by oxidation or irradiation of PCE were characterized by gas chromatography. These compounds had longer retention times than the chloroethylenes, implying that they were more

polar, as would be expected of alcohol or diol addition products. None of these compounds were observed in cultures of PCE-degrading bacteria in the presence of PCE. However, accumulation of chloroform was observed and accumulation of small amounts of trichloroacetic acid was inferred, suggesting a degradative pathway that includes either dehalogenation followed by hydration/hydroxylation and rearrangement, or hydration/hydroxylation followed by rearrangement and elimination of a chloro group, yielding a precursor (perhaps a diol) for further degradation to trichloroacetic acid and chloroform.

We conclude that PCE-resistant organisms can be selected for in well water (and probably soils) in which the concentration of PCE is high, and that bacteria can be isolated from these sources which have the capability of degrading PCE and TCE. It should be possible to enhance the ability of these organisms to degrade halogenated solvents *in situ* by a variety of methodologies, including nutritional enhancement to increase the total number of bacteria, *in vitro* cultivation of solvent degraders and their reinoculation into the contaminated wells, or use of the bacteria as biological sorbents or filters. There is no evidence that bacterial spores can degrade PCE, but it is clear that sporulating organisms, in particular *Bacillus* W8, can survive exposure to high concentrations of PCE and, thus, can maintain viability in groundwater or soil during periods of exposure to high solvent concentrations, so that resumption of growth and metabolism could occur after the solvents had dispersed to a sufficiently low concentration.

Detergents added to growth media increased the solubility of PCE and to some extent protected PCE-sensitive bacteria from the toxic effects of the solvent. Detergent addition did not appear to increase the degradation of PCE by bacteria *in vitro*, when PCE was readily available in solution. However, it is possible that *in situ*, detergents might increase microbial degradation of PCE by enhancing the dissolution of PCE bound hydrophobically to soil particles, making the solvent more readily accessible to the bacteria.

6.5 REFERENCES

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7.0 SUBCONTRACTS WITH HISTORICALLY BLACK COLLEGES AND UNIVERSITIES

As part of the requirement of contract No. DE-AC05-84OR21492 the Oak Ridge Research Institute established subcontracts with Kentucky State University, Meharry Medical School, and Tuskegee University. Through the subcontracts these Historically Black Colleges and Universities (HBCU) provided research support to the overall objectives of the prime contract. Specific research objectives of each HBCU are shown below and the final reports is enclosed.

HBCU	Specific Objectives
Kentucky State University:	Identification and Biochemical Characterization of Soil Microorganisms
Meharry Medical School:	Identification of Mercury Detoxification Mechanisms in Bacteria
Tuskegee University:	Genetic Characterization of Bacterial Strains Isolated from Contaminated Soil and Streams

Final Report

for

Subcontract No. ORRI-85-2

between

Oak Ridge Research Institute

and

Kentucky State University

by

Al R. Harris

ORRI Isolate # 1004(35)

Preliminary/Final Report

Organism Bacillus circulans

Date Received 3/10/87

Date Reported 6/4/87

GRAM STAIN: Medium short fat gram positive rods, arranged singly and in short chains

3% KOH	GRAM POS	CITRATE	-
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	+	STARCH HYDROLYSIS	+
PIGMENT	NONE	GROWTH AT 65°C	-
GROWTH AT 25°C/37°C/42°C	+/+/NT	PHENOL RED GLUCOSE	ACID
OF GLUCOSE	INERT	PHENOL RED LACTOSE	ACID
OF MANNITOL	INERT	PHENOL RED SUCROSE	ACID
OF SUCROSE	INERT	PHENOL RED MANNITOL	ACID
OF LACTOSE	INERT	PHENOL RED MALTOSE	ACID
OF MALTOSE	INERT	pH in VP broth	5.48
OF XYLOSE	INERT		
GROWTH ON MACCONKEY	-		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	+		
TRIPLE SUGAR IRON SLANT	K/A/-		
INDOLE	-		
METHYL RED	+		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	Beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

11820/28

ORRI Isolate # 1010(41)

Preliminary/Final Report

Organism Bacillus circulans

Date Received 3/10/87

Date Reported 6/4/87

GRAM STAIN: Medium short fat gram positive rods, arranged singly and in short chains

3% KOH	GRAM POS	CITRATE	-
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	+	STARCH HYDROLYSIS	+
PIGMENT	NONE	GROWTH AT 65°C	-
GROWTH AT 25°C/37°C/42°C	+/+/NT	PHENOL RED GLUCOSE	ACID
OF GLUCOSE	INERT	PHENOL RED LACTOSE	ACID
OF MANNITOL	INERT	PHENOL RED SUCROSE	ACID
OF SUCROSE	INERT	PHENOL RED MANNITOL	ACID
OF LACTOSE	INERT	PHENOL RED MALTOSE	ACID
OF MALTOSE	INERT	pH in VP broth	5.60
OF XYLOSE	INERT		
GROWTH ON MACCONKEY	-		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	+		
TRIPLE SUGAR IRON SLANT	K/A/-		
INDOLE	-		
METHYL RED	+		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	Beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

11820/29

ORRI Isolate # 1011(42)

Preliminary/Final Report

Organism Bacillus circulans

Date Received 3/10/87

Date Reported 6/4/87

GRAM STAIN: Medium short fat gram positive rods, arranged singly and in short chains

3% KOH	GRAM POS	CITRATE	-
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	+	STARCH HYDROLYSIS	+
PIGMENT	NONE	GROWTH AT 65°C	-
GROWTH AT 25°C/37°C/42°C	+/+/NT	PHENOL RED GLUCOSE	ACID
OF GLUCOSE	INERT	PHENOL RED LACTOSE	ACID
OF MANNITOL	INERT	PHENOL RED SUCROSE	ACID
OF SUCROSE	INERT	PHENOL RED MANNITOL	ACID
OF LACTOSE	INERT	PHENOL RED MALTOSE	ACID
OF MALTOSE	INERT	pH in VP broth	5.69
OF XYLOSE	INERT		
GROWTH ON MACCONKEY	-		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	+		
TRIPLE SUGAR IRON SLANT	K/A/-		
INDOLE	-		
METHYL RED	+		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	Beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

11820/30

ORRI Isolate # 1012(43)

Preliminary/Final Report

Organism Bacillus circulans

Date Received 3/10/87

Date Reported 6/4/87

GRAM STAIN: Medium short fat gram positive rods, arranged singly and in short chains

3% KOH	GRAM POS	CITRATE	-
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	+	STARCH HYDROLYSIS	+
PIGMENT	NONE	GROWTH AT 65°C	-
GROWTH AT 25°C/37°C/42°C	+/+/NT	PHENOL RED GLUCOSE	ACID
OF GLUCOSE	INERT	PHENOL RED LACTOSE	ACID
OF MANNITOL	INERT	PHENOL RED SUCROSE	ACID
OF SUCROSE	INERT	PHENOL RED MANNITOL	ACID
OF LACTOSE	INERT	PHENOL RED MALTOSE	ACID
OF MALTOSE	INERT	pH in VP broth	5.35
OF XYLOSE	INERT		
GROWTH ON MACCONKEY	-		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	+		
TRIPLE SUGAR IRON SLANT	K/A/-		
INDOLE	-		
METHYL RED	+		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	Beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

11820/31

ORRI Isolate # 1013(44)
Preliminary/Final Report
Organism Bacillus circulans

Date Received 3/10/87
Date Reported 6/4/87

GRAM STAIN: Medium short fat gram positive rods, arranged singly and in short chains

3% KOH	GRAM POS	CITRATE	-
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	+	STARCH HYDROLYSIS	+
PIGMENT	NONE	GROWTH AT 65°C	-
GROWTH AT 25°C/37°C/42°C	+/+/NT	PHENOL RED GLUCOSE	ACID
OF GLUCOSE	INERT	PHENOL RED LACTOSE	ACID
OF MANNITOL	INERT	PHENOL RED SUCROSE	ACID
OF SUCROSE	INERT	PHENOL RED MANNITOL	ACID
OF LACTOSE	INERT	PHENOL RED MALTOSE	ACID
OF MALTOSE	INERT	pH in VP broth	5.37
OF XYLOSE	INERT		
GROWTH ON MACCONKEY	-		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	+		
TRIPLE SUGAR IRON SLANT	K/A/-		
INDOLE	-		
METHYL RED	+		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	Beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

11820/32

Kentucky State University Frankfort, Kentucky 40601

May 20, 1987

Dr. Nathaniel Revis, President
Oak Ridge Research Institute
113 Union Valley Road
Oak Ridge, TN 37830

Dear Dr. Revis:

Enclosed please find final reports for isolates #1005-1009. The final results for isolates #1004 and 1010-1013 will be forwarded shortly. Below is a brief description of the methodology used to identify each organism.

#1005 - This isolate is morphologically and biochemically consistent with the characteristics of Klebsiella oxytoca. The API 20E system concurs with this result.

#1006 - This isolate is morphologically and biochemically consistent with the characteristics of Klebsiella oxytoca except for the positive reaction for ornithine decarboxylase. Based on that reaction, API 20E called this organism Serratia odorifera 1, but based on morphological characteristics of Klebsiella species, especially the lack of motility of this isolate, we have determined it to be Klebsiella oxytoca. Serratia species is positive for motility.

#1007 - This isolate is morphologically and biochemically consistent with the characteristics of Klebsiella oxytoca. The API 20E system concurs with this result.

#1008 - This isolate is morphologically and biochemically consistent with the characteristics of Klebsiella pneumoniae. This isolate differs from the previous three in that the indole reaction is negative. The API 20E system concurs with this result.

#1009 - This isolate is morphologically and biochemically consistent with the characteristics of Klebsiella pneumoniae. The indole reaction of this isolate is negative, which differentiates it from Klebsiella oxytoca.

If you have any questions do not hesitate to call.

Sincerely,

Al R. Harris

Al R. Harris
Associate Professor
Division of Math and Sciences

ORRI Isolate # 1000 (31)

Preliminary (Final Report)

Organism Pseudomonas putida

Date Peceived 1/9/87

Date Reported 2/9/87

GRAM STAIN: Gram negative coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	+
OXIDASE	+	GLUCONATE	+
PIGMENT	SLIGHT YELLOW	ACETAMIDE HYDROLYSIS	+
GROWTH AT 25°C/37°C/42°C	+/+/-	6.5% SALT BROTH	+
OF GLUCOSE	OXIDATIVE	FLAGELLA	polar, multitrichous
OF MANNITOL	K		
OF SUCROSE	K		
OF LACTOSE	K		
OF MALTOSE	K		
OF XYLOSE	A		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	+		
NITRATE REDUCTION	-		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	K/NC/-		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUEP	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

13550

ORRI Isolate # 1001 (32)

Preliminary/Final Report

Organism Pseudomonas putida

Date Received 1/9/87

Date Reported 2/9/87

GRAM STAIN: Gram negative coccobacilli arranged singly, occasionally paired

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	+
OXIDASE	+	GLUCONATE	+
PIGMENT	SLIGHT YELLOW	6.5% SALT BROTH	-
GROWTH AT 25°C/37°C/42°C	+/+/-	FLAGELLA	polar, multitrichous
OF GLUCOSE	OXIDATIVE		
OF MANNITOL	K		
OF SUCROSE	K		
OF LACTOSE	K		
OF MALTOSE	K		
OF XYLOSE	A		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	+		
NITRATE REDUCTION	-		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	K/NC/-		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	beta		
ARGININE DIHYDROLASE	+		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

13550

ORRI Isolate # 1002 (33)
Preliminary (Final Report)
Organism Pseudomonas stutzeri

Date Received 1/9/87
Date Reported 2/9/87

GRAM STAIN: Small-medium gram negative rods/coccobacilli arranged singly some paired

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	-
OXIDASE	+	GLUCONATE	-
PIGMENT	NONE	6.5% SALT BROTH	+
GROWTH AT 25°C/37°C/42°C	+/+/+	FLAGELLA	polar, monotrichous
OF GLUCOSE	OXIDATIVE		
OF MANNITOL	K		
OF SUCROSE	K		
OF LACTOSE	K		
OF MALTOSE	NC		
OF XYLOSE	NC		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	-		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	K/NC/-		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

13550

ORRI Isolate # 1003 (34)
Preliminary Final Report
Organism Alcaligenes denitrificans

Date Received 1/9/87
Date Reported 2/9/87

GRAM STAIN: Small gram negative coccobacilli arranged singly, some larger gram neg

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	+
OXIDASE	+	GLUCONATE	-
PIGMENT	NONE	6.5% SALT BROTH	-
GROWTH AT 25°C/37°C/42°C	+/+/-	FLAGELLA	peritrichous
OF GLUCOSE	INERT		
OF MANNITOL	K		
OF SUCROSE	K		
OF LACTOSE	K		
OF MALTOSE	K		
OF XYLOSE	K		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	+		
NITRATE REDUCTION	+		
UREASE	-		
MOTILITY	+		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	K/NC/-		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	-		
HEMOLYSIS ON 5% SBA	beta		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	-		
PHENYLALANINE DEAMINASE	-		

13550

Isolate 1000: This organism was identified by the API 20E System as "Excellent ID, Fluorescent Pseudomonas sp." The organism did indeed produce a fluorescent pigment on Pseudomonas F. Agar. Further differentiation between Pseudomonas fluorescens and Pseudomonas putida was made based on the negative reaction in gelatin liquefaction.

Isolate 1001: This organism was also identified by API 20E as "Excellent ID, Fluorescent Pseudomonas sp." All conventional biochemical results were identical to isolate #1000 with the exception of growth in 6.5% NaCl broth. According to Revised Tables from the Identification of Unusual Pathogenic Gram Negative Bacteria, CDC, 1983, the salt broth reaction for Pseudomonas putida is variable.

Isolate 1002: Inoculation of this isolate onto an API 20E strip yielded a "Very good ID" for Pseudomonas stutzeri. Conventional biochemical tests were consistent with this identification with exception of the production of acid from maltose. Colonial morphology of this isolate is consistent with that of Pseudomonas stutzeri in that dry, wrinkled colonies were produced on R2A and TGY Agars.

Isolate #1003: This isolate was biochemically inert with regards to acid production from carbohydrates and was motile by peritrichous flagella, both characteristic of Alcaligenes species. Alcaligenes denitrificans is quite similar to Alcaligenes faecalis. Speciation was based on dentrification, lack of growth at 42^{o}C and colonial morphology consistent with that of A. denitrificans.

ORRI Isolate # 1005(36)
Preliminary/Final Report
Organism Klebsiella oxytoca

Date Received 3/10/87
Date Reported 5/20/87

GRAM STAIN: Gram variable coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	-	STARCH HYDROLYSIS	-
PIGMENT	NONE		
GROWTH AT 25°C/37°C/42°C	+/+/NT		
OF GLUCOSE	FERM		
OF MANNITOL	ACID		
OF SUCROSE	ACID		
OF LACTOSE	ACID		
OF MALTOSE	ACID		
OF XYLOSE	ACID		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	+		
MOTILITY	-		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	A/A, gas		
INDOLE	+		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	+		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	+		
PHENYLALANINE DEAMINASE	-		

Note 8
Motility results on preliminary reports for isolates #1005-1009 are incorrect due to typographical errors. These organisms are non-motile and this result is reflected on the final reports.

11820/19

ORRI Isolate # 1006(37)

Preliminary/Final Report

Organism Klebsiella oxytoca

Date Received 3/10/87

Date Reported 5/20/87

GRAM STAIN: Gram variable coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	-	STARCH HYDROLYSIS	-
PIGMENT	NONE		
GROWTH AT 25°C/37°C/42°C	+/+/NT		
OF GLUCOSE	FERM		
OF MANNITOL	ACID		
OF SUCROSE	ACID		
OF LACTOSE	ACID		
OF MALTOSE	ACID		
OF XYLOSE	ACID		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	+		
MOTILITY	-		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	A/A, gas		
INDOLE	+		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	+		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	+		
PHENYLALANINE DEAMINASE	-		

11820/20

ORRI Isolate # 1007(38)
Preliminary (Final Report)
Organism Klebsiella oxytoca

Date Received 3/10/87
Date Reported 5/20/87

GRAM STAIN: Gram variable coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	-	STARCH HYDROLYSIS	-
PIGMENT	NONE		
GROWTH AT 25°C/37°C/42°C	+/+/NT		
OF GLUCOSE	FERM		
OF MANNITOL	ACID		
OF SUCROSE	ACID		
OF LACTOSE	ACID		
OF MALTOSE	ACID		
OF XYLOSE	ACID		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	+		
MOTILITY	-		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	A/A, gas		
INDOLE	+		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	+		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	+		
PHENYLALANINE DEAMINASE	-		

11820/21

ORRI Isolate # 1008(39)

Preliminary/Final Report

Organism Klebsiella pneumoniae

Date Received 3/10/87

Date Reported 5/20/87

GRAM STAIN: Gram variable coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	-	STARCH HYDROLYSIS	-
PIGMENT	NONE		
GROWTH AT 25°C/37°C/42°C	+/+/NT		
OF GLUCOSE	FERM		
OF MANNITOL	ACID		
OF SUCROSE	ACID		
OF LACTOSE	ACID		
OF MALTOSE	ACID		
OF XYLOSE	ACID		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	+		
MOTILITY	-		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	A/A, gas		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	+		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	+		
PHENYLALANINE DEAMINASE	-		

11820/22

ORRI Isolate # 1009(40)

Preliminary/Final Report

Organism Klebsiella pneumoniae

Date Received 3/10/87

Date Reported 5/20/87

GRAM STAIN: Gram variable coccobacilli arranged singly and in clumps

3% KOH	GRAM NEG	CITRATE	+
CATALASE	+	GROWTH ON CETRIMIDE	N/A
OXIDASE	-	STARCH HYDROLYSIS	-
PIGMENT	NONE		
GROWTH AT 25°C/37°C/42°C	+/+/NT		
OF GLUCOSE	FERM		
OF MANNITOL	ACID		
OF SUCROSE	ACID		
OF LACTOSE	ACID		
OF MALTOSE	ACID		
OF XYLOSE	ACID		
GROWTH ON MACCONKEY	+		
GROWTH ON SS AGAR	N/A		
NITRATE REDUCTION	+		
UREASE	+		
MOTILITY	-		
GELATIN LIQUEFACTION, 22°C	-		
TRIPLE SUGAR IRON SLANT	A/A, gas		
INDOLE	-		
METHYL RED	-		
VOGES-PROSKAUER	-		
ESCULIN HYDROLYSIS	+		
HEMOLYSIS ON 5% SBA	gamma		
ARGININE DIHYDROLASE	-		
ORNITHINE DECARBOXYLASE	-		
LYSINE DECARBOXYLASE	+		
PHENYLALANINE DEAMINASE	-		

11820/23

Final Report

for

Subcontract No. ORRI-85-4

between

Oak Ridge Research Institute

and

Meharry Medical School

by

Julius H. Jackson and Robert C. Blake, II

SUMMARY

During the reporting period the laboratory supplies and equipment necessary to conduct the project have been purchased and put in place. A full-time, postdoctoral research associate is carrying out most of the technical aspects of the work. The research associate required a six-month period of training and adjustment to prepare to complete the objectives of the project as previously outlined. The Statement of Work in the subcontract serves as the principal guide for the work conducted. According to the objectives of the subcontract, we began work on objective nos. 4 and 5, and have recently begun objective no. 1. We have examined the six bacterial isolates from the mercury contamination site at Poplar Creek and sent to us by ORRI. We have examined each of these for plasmid content, isolated plasmid and chromosomal DNA, experimented with different cultural conditions for large scale cell preparation, and begun large scale preparations for purification of mecuric reductase from each isolate. Our analysis of the plasmid DNA yields evidence for the presence of a nuclease activity that co-purifies with DNA preparations and thus prevents restriction enzyme analysis and mapping. We have communicated with Dr. Anne Summers to obtain two Gram negative mer probes. During the ensuing period of this contract year, we expect to complete a Southern analysis of the plasmid and chromosomal DNA contents of these strains by use of the mer probes and thus to complete objectives 4 and 5. Additionally, we expect to characterize mecuric reductase activities in these strains as described in objective 1. This project is currently on schedule and should be completed as projected within the time frame specified on the original subcontract. A summary of the original objectives and a more detailed experimental report follow.

OBJECTIVES

Resistance to mercury and organomercurials in microorganisms occurs by detoxification mechanisms. Detoxification is achieved by volatilization of mercury following activity of organomercurial lyase and/or mercuric reductase enzymes. In most bacteria thus far studied, these enzymes are inducible and encoded by plasmid genes. The goals of this study are: 1) to determine the mechanism(s) by which a community of bacteria isolated from Hg-contaminated soil may achieve resistance to Hg; 2) to determine whether a community of naturally selected resistant organisms may be manipulated to convert Hg to insoluble complexes and maintain these complexes as a potential decontamination mechanism; and 3) to determine whether standard, non-recombinant DNA genetic techniques may be used to increase the production of detoxifying enzymes to an extent useful in environmental decontamination. The initial objectives to achieve the first two stated goals are:

1. **Assay sample isolates from the community of mercury-resistant (Hg^r) bacteria found at a toxic waste site at Oak Ridge, Tn., to measure organomercurial lyase and mercuric reductase enzyme activities.** Bacterial isolates will be screened to confirm whether Hg^r is broad or narrow spectrum. Lyase and reductase enzyme levels will be measured by standard methods to correlate enzyme levels with the spectra of resistance.

2. **Determine the level of Hg^r among sulfate-reducing bacteria not exposed to high levels of mercury, and compare to Hg^r of similar organisms isolated from the Oak Ridge site.** The principal question is whether high production of H_2S causes precipitation of HgS or similar insoluble complexes sufficiently to protect the bacteria from mercury toxicity even in the absence of organomercurial lyase and mercuric reductase.

3. **Measure the extent to which and the rates at which Hg^r soil organisms, e.g. Thiobacillus sp., can oxidize HgS complexes to form soluble mercuric salts and organomercurials.** Significant activity of this type might counter the potential utility for production of insoluble mercury complexes as a decontamination mechanism. Oxidation of HgS will form the basis to test the influence of other metals, e.g. Fe^{2+} as part of a HgS complex, upon the rate of reoxidation of the insoluble salts of mercury.

4. **Search for sequences homologous to the mer probe in the community of bacterial isolates from a mercury contamination site at Oak Ridge, Tn.** The gram-negative bacterial community of isolates will be probed for the presence of Hg^r genes by the colony hybridization technique, or by probe hybridization to sheered DNA preparations from crude extracts. Mercury-resistant and mercury-sensitive strains will be screened.

5. **Determine whether mercury-resistance is of plasmid or chromosomal origin in the Oak Ridge-site strains.** Plasmids from strains with DNA which hybridizes with the mer probe will be isolated to detect whether the mer probe hybridizes with plasmid DNA, chromosomal DNA, or both. If the mer probe hybridizes to DNA of mercury-sensitive strains, it may reveal sequences used to evolve mercury-resistance.

EXPERIMENTAL REPORT

Bacterial isolates were collected from the community of mercury resistant bacteria found at a toxic waste site at Oakridge, TN. They were screened for various properties, including Gram staining characteristics, and out of 6 isolates, 3 were Gram negative rods and designated as nos. 1, 2 and 02, while the other 3 were Gram positive rods and designated as nos. 3, 4 and 5. All of the strains grew in R2A medium at 30°C. Four of the isolates (1, 2, 02, and 5) grew in Luria broth which is a convenience in preparation and is commonly used to grow enterics for DNA isolation.

In preparation to search for sequence homology with the mer probe, plasmid DNA was isolated from all strains by the use of the alkaline lysis method of Birnboim and Doly (1) from 1.5 ml volumes of cell cultures. The sizes of plasmids were estimated by use of molecular size standards in agarose gel electrophoresis of the DNA preparations. Plasmid sizes associated with the strains were: strain 1, 16 kilobase pairs; strain 2, 25 kilobase pairs; strain 02, 13.5 kilobase pairs; strain 3, 15 kilobase pairs; strain 4, 12 kilobase pairs; and strain 5, 25 kilobase pairs.

Large-scale plasmid DNA preps were done according to Maniatis et al (2) from all the strains by treating the cells with lysozyme followed by the alkaline lysis method. Clear lysates were processed by isopycnic centrifugation in cesium chloride containing ethidium bromide. Strains nos. 2, 3 and 5 did not form a distinguishable plasmid band in the cesium chloride gradients when the SDS alkaline lysis method was used.

Subsequently, plasmid DNA isolation was done by the method of Kado and Liu (2) from 3 ml cell cultures and from 1 l cell cultures by treating the cells with protease and lysing the cells with 3% sodium lauryl sarcosinate in 50 mM Tris OH, pH 12.6. This was followed by phenol extraction. Plasmid DNA bands were collected in all strains after iso-pycnic centrifugation in cesium chloride containing ethidium bromide. The concentration of DNA was determined spectophotometrically at 260 nm. The plasmid DNA was digested with a variety of restriction enzymes for restriction mapping and in preparation for hybridization with a mer probe.

Chromosomal DNA was isolated from the different strains after growing them in an appropriate medium. Cells were harvested by centrifugation and DNA was extracted by a modified method of Marmur (4). Cells were resuspended in TES buffer (50 mM Tris-HCl, pH 8.0, 4 mM EDTA, 0.17 mM NaCl), treated with solid protease to a final concentration of 1 mg/ml, and Tris-SDS was added. Cells were incubated at 42°C for 1-2 hours, extracted with phenol, and centrifuged to separate layers. The aqueous layer was transferred to a sterile tube, then deprotenized repeatedly with chloroform-isoamyl alcohol extraction until very little protein was evident at the interface. The supernatant obtained after the last in the series of deprotenization steps was precipitated with 2 volumes of 95 % ethanol followed by incubation on ice for 20 minutes. This was followed by centrifugation at 4°C. The pellet was then resuspended in TES buffer. Pancreatic RNase (previously heated to destroy DNase activity) was added to a final concentration of 50 ug/ml and the mixture was incubated at 37°C for 30 minutes. This was followed by deproteinization with chloroform-isoamyl alcohol, then resuspended in TES buffer and dialysed against 3 changes of Tris acetate (10 mM) pH 8.0. The concentration of DNA was determined spectrophotometrically at 260 nm, and by estimation by comparison with known concentrations of marker DNA on a 1 % agarose gel. The DNA of different strains was cut with enzymes and ready for hybridization with mer probe. Small and large scale preparations of plasmid DNA and chromosomal DNA from each strain were digested with various restriction enzymes for varying time periods with appropriate controls.

From analysis of gel patterns in dozens of experiments, it is apparent that whenever the plasmid DNA preparations are nicked by a restriction enzyme, plasmid degradation occurs. It appears likely that an exonuclease copurifies with the plasmids and is activated by the assay conditions. We have tried treatment of DNA preparations with different proteolytic enzymes to destroy the contaminating nuclease activity, but we still observe a smeared gel pattern. Whereas this pesky nuclease has prevented some of the detailed characterization of plasmid structure that we planned to obtain, it will not likely interfere with hybridization analysis with the mer probe to locate genes for mercuric reductase. The plasmid DNA and chromosomal DNA of all strains will be hybridized with a mer probe from two sources to determine whether homologous sequences are of plasmid and / or of chromosomal origin.

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Final Report

for

Subcontract No. ORRI-85-2

between

Oak Ridge Research Institute

and

Tuskegee University

by

Julius E. Thomas

SUMMARY OF ACCOMPLISHMENTS

The specific aims of this project were to identify and characterize four independent bacterial isolates from soil sampled near an industrial plant along the East Fork Poplar Creek in Oak Ridge, Tennessee. We have successfully identified these isolates as a species of Micrococcus and species of Bacillus. We further characterized these strains for a number of growth parameters, including growth rate; optimum growth temperature; minimal nutritional requirements and rate of accumulation of informational macromolecules. We also determined that all strains are highly resistant to $HgCl_2$ but preliminary experiments showed no evidence for the presence of relaxed plasmids. This latter result is in agreement with observations by other investigators that mercury resistance in Gram-positive bacteria can be chromosome based.

Resistance to inorganic mercury is conferred by the acquisition of the capacity to synthesize an enzyme which reduces inorganic mercury to metallic mercury which is volatile. The period of this grant expired before extracts of the isolates could be assayed for inorganic mercury reductase activity; nor were the isolates tested for organomercury resistance. We will attempt to obtain funding to continue this investigation in order to answer these questions as well as to determine possible resistance to other heavy metals and to determine the genetic basis for the resistance.

DETAILED NARRATIVE

I. Identification of Bacterial Isolates

In our previous progress report of January, 1987, we reported the identification of three of the four isolates to the level of the genus *Bacillus*. We have subsequently determined that each of these isolates is of the species *Bacillus cereus*. We have also determined that the fourth isolate is of the genus [*Micrococcus* sp.]. A fifth strain believed to be a laboratory contaminant and previously characterized as a Gram-negative rod has not been further classified. These data are shown in Tables 1 and 2.

II. Regulation of Informational Macromolecules

We previously observed that the rate of the stable RNA accumulation is proportional to the rate of protein accumulation and thus the growth rate in all of the isolates. However, in the strains of bacilli, there appears to be an overaccumulation of DNA during exponential growth as measured by the incorporation of radioactive thymidine. This observation suggested the possibility of the presence of relaxed plasmids. Furthermore, resistance to heavy metals is known to be plasmid-borne, especially in Gram-negative bacteria.

Relaxed plasmids can be amplified in bacteria by addition of antibiotics that inhibit protein synthesis. We attempted to demonstrate plasmid amplification in the isolates by measuring the incorporation of radioactive thymidine in chloramphenicol inhibited cells. Our results were negative for all strains. These results could indicate stringent or low copy number plasmids or that heavy metal resistance is chromosomal.

III. Mercury Resistance in Isolates

Isolates were tested for ability to grow on LB/NCE agar supplemented with various concentrations of $HgCl_2$. A known strain of *Bacillus cereus* from Midwest Culture Service was also tested. The results, as shown in Table 3, reveal that all strains including TU101 show resistance to up to 825 uM $HgCl_2$. The control grew at 125 uM but not at higher concentrations.

IV. Future Directions

Heavy metal resistance has not been studied nearly as fully in Gram-positive as in Gram-negative bacteria. In addition, the level of resistance in these isolates appears to be several-fold higher than that commonly found in natural isolates. These isolates include both a Gram-positive coccus and Gram+positive bacilli. The genetic basis for the resistance should be thoroughly investigated and resistance to other heavy metals such as cadmium should be tested in these isolates.

Table 1

STRAIN LIST

Strains	Species
TU101	Unknown
TU102	Bacillus cereus
TU103	[Micrococcus sp.]
TU104	Bacillus cereus
TU105	Bacillus cereus

Table 2

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERISTICS

	TU101	TU102	TU103	TU104	TU105
Gram Stain	-	+	+	+	+
Cell Shape	Rod	Rod	Cocci	Rod	Rod
Lactose	B	A	A	A	A
Methyl Red	-	-	+	-	-
Dextrose	A	A	A	A	A
H_2S Production	-	-	-	-	-
Indole Production	-	-	-	-	-
Citrate Use	-	+	-	+	+
Gelrite Liquefaction	+	+	-	+	+
Catalase Activity	+	+	+	+	+
Mannitol	A	B	A	B	B
Sucrose	A	B	A	B	B
Species		Bacillus cereus	[Micrococcus sp.]	Bacillus cereus	Bacillus cereus

+ = positive: - = negative: A = Acidic: B = Non-acidic

Table 3

Mercury Resistance

Concentration $HgCl_2$ uM	Strains TU101	TU102	TU103	TU104	TU105	Control Bacillus cereus
125	+	+	+	+	+	+
225	+	+	+	+	+	-
325	+	+	+	+	+	-
425	+	+	+	+	+	-
525	+	+	+	+	+	-
625	+	+	+	+	+	-
725	+	+	+	+	+	-
825	+	+	+	+	+	-

Table 4

No Carbon E Medium (NCE)

50X NCE/liter

KH_2PO_4	197g
K_2HPO_4 (H_2O)	325.1g
$Na(NH_4)HPO_4$ (H_2O)	175g
H_2O	925ml

Luria Broth (LB)

Per Liter

Tryptone	10g
Yeast Extract	5g
NaCl	5g

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