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Biological Detoxification of Mercury Contaminated Soil

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BIOLOGICAL DETOXIFICATION OF MERCURY CONTAMINATED SOIL

by

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ABSTRACT

Biological Detoxification of Mercury Contaminated Soil

by

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This study examined biological mercury removal from soil using mercury-resistant bacteria in soil microcosms. Mercuric chloride was used to artificially contaminate Kidman soil to mercury concentrations of 5 ppm and 10 ppm. Soil moisture content was maintained at three levels, 20%, 30% and 50%. Mercury resistant-bacteria were added to soil samples and the mercury removal rate was compared to control samples without added bacteria. Mercury removal rate was initially enhanced by the addition of bacteria. After 30 days, no difference was observed between samples and controls with initial mercury concentration of 5 ppm when soil moisture content was 20%. At an initial mercury concentration of 10 ppm, soil samples had less mercury remaining than controls after 30 days. Autoclaved soil had a decreased mercury removal rate compared to soil not autoclaved. Addition of nutrient (sucrose) did not increase the mercury removal rate. A slurry-type bioreactor was

found to be more efficient than a non-stir type. After 30 days of continuous stirring, 85-90% of the added mercury (10 ppm) was removed, while under the same conditions except no stirring, only around 60% of the mercury was removed.

Overall, biological detoxification of mercury from contaminated soil can be achieved by using a slurry-type bioreactor with additon of mercury-resistant bacteria.

(72 pages)

CHAPTER I

INTRODUCTION

Toxic and potentially hazardous heavy metals from many industrial sources are now found in waste water, sludge, and soil. Among these metals, mercury is one of the most toxic and serious examples of such pollution. As industrial use of mercury has increased, the release of mercury into the environment has also increased. Sources of mercury waste include agriculture, mining, chlor-alkali, and paint and pharmaceutical industries (Nriagu, 1979). Attempts have been made to limit the amount of mercury released, but, because of unavoidable production of some mercurial waste at the present, and in the past from a number of major industries as mentioned above, an economical process of removing mercury from polluted water, sludge, and soil is of significant value.

A common treatment method for metal-contaminated soils has been to try to permanently immobilize the metal by either chemical or physical methods. But because physical and chemical mercury removal treatments from wastes have various problems that limit their application to industrial situations, biological treatment is receiving increasing interest. It is an attractive alternative to expensive physical and chemical cleaning methods when rapid degradation of the compounds concerned can be obtained. Mercury-resistant strains of bacteria have been shown to be

effective in the detoxification of mercury from waste water (Hansen et al., 1984). This is based on the functional property of mercury-resistant bacteria that can convert organic and inorganic mercurial compounds (Hg^{2+}) enzymatically to Hg^{0}, and then this can be volatilized from the growth medium. The effectiveness of the biological detoxification method depends on the design of the bioreactor (Ross, 1991) and various factors such as mercury contamination concentration, soil moisture content, and bacteria growth nutrients (Rogers, 1979).

Volatilization of elemental mercury by naturally occurring microorganisms in soil has been reported by several authors (Kimura and Miller, 1964; Rogers, 1979). Biological detoxification of heavy metals such as cadmium and copper has also been investigated (Campbell and Martin, 1990; Dunn and Bull, 1983). However, no approach has been made to increase the volatilization rate of mercury and thus more efficiently remove it from contaminated soil.

This research was a preliminary investigation to determine if mercury-contaminated soil can be detoxified biologically. Two methods were chosen to remove mercury from soil. A 250 ml flask containing mercury-contaminated soil with a continuous air flow through it was the first method. The second method was a slurry-type bioreactor with continuous air flow and a magnetic stirrer to keep water and soil in a slurry state.

CHAPTER II

LITERATURE REVIEW

Mercury pollution of the environment is a well-known phenomenon occurring throughout the world in recent years. The behavior of mercury as an environmental contaminant is intimately related to the special physical, chemical, and toxicological features of this heavy metal (Schroeder, 1982).

Finding an easy and efficient way of detoxification of mercury-contaminated waste has been the subject of much research (Hansen et al., 1984). Recently, the focus has been on using mercury-resistant bacteria that can change mercuric compounds to Hg^0, which then volatilizes (Summers and Lewis, 1973).

Uses of Mercury

Mercury is a dense (density 13.5 g/ml), silver-white liquid at room temperature. It is characterized by low electrical resistivity, high surface tension, and high thermal conductivity. At ordinary temperatures, mercury does not react with air, carbon dioxide, sulfur dioxide, hydrogen sulfide, nitrous oxide or oxygen, but is highly reactive towards ozone, halogens, hydrogen peroxide, nitric acid, concentrated sulfuric acid, ferric chloride and perchlorate, thionyl chloride, and liquid white phosphorus. Mercury has attracted more attention than many other trace elements because of its high toxicity and wide distribution

in the environment. It has been estimated that in the past 100 years or so, nearly 5 x 10^8 kg of pollutant mercury have been released into the atmosphere, about 1 x 10^8 kg have been discharged into natural waters, and about 5 x 10^8 kg have been deposited on the land (Andren and Nriagu, 1979). The world-wide historical production figures for mercury are summarized in Table 1. These figures further implicate human beings as a current major factor in the global dispersion of mercury (Andren and Nriagu, 1979).

Functional Properties of Mercury-Resistant Microorganisms

Bacteria Strains. As interest in biological detoxification of mercury contamination in the environment has increased, the mechanism and functional properties of mercury-resistant bacteria have attracted many studies (Barkay et al., 1989; Trevors, 1987). Microorganisms living in an environment with heavy metals are reported to play an important role in various biological interactions (Ben-Bassat and Mayer, 1975; Brunker and Bott, 1974; Campbell and Martin, 1990; Dunn and Bull, 1983).

Numerous mercury-resistant bacteria strains have been isolated and studied in the past 20 years (Trevors et al., 1985). Most of these organisms belong to the genera Pseudomonas, Mycobacterium, Bacillus, Acinetobacter, Staphylococcus, Escherichia, Thiobacillus, Arthrobacter, Citrobacter, Enterobacter, Flavobacterium and Vibrio (Trevors, 1987; Summers and Lewis, 1973). Nakamura et al.

Table 1. Worldwide Mercury Production and Release to the Environment (Andren and Nriagu, 1979)

Period	Mercury produced ($\times 10^6$kg)	Mercury release (10^6kg)		
		Air	Water	Soil
Pre-1900	200	232	37.0	252
1900-1909	34.3	39.8	6.3	43.3
1910-1919	37.6	43.6	6.9	47.5
1920-1929	37.8	43.8	7.0	47.8
1930-1939	35.9	41.6	6.6	45.3
1940-1949	61.5	71.3	11.4	77.8
1950-1959	65.0	75.4	12.0	82.2
1960-1969	78.5	91.1	14.5	99.2
1970-1979	87.6	102	16.2	111
Total	638	741	118	806

(1990) have recently reported that the organomercurial volatilizing strains of bacteria found in a mercury-polluted marine bay sediment were gram-positive *Bacillus* spp.

In the case of *Enterobacter*, it appears that during the lag phase most of the cells are lysed and mercury-resistant mutants selected. Then, when the mercury concentration is reduced to 1 μg/ml, via chemical and biological reduction of Hg^{2+}, the surviving cells initiate growth at a normal rate

(Vaituzis et al., 1975).

Coding for Mercury Resistance. Previous experiment have been done on the biological mechanism of mercury-resistance in bacteria. It has been shown that the mechanism of mercury resistance is an enzymatic reduction (Summers and Silver, 1972) by mercuric reductase (Silver, 1985). Tsai and Olson (1990) investigated two types of mercuric reductase under temperatures of 4^0C and 23^0C and found one type induced by Hg^{2+} at 4^0C can function at both temperatures, and the other type induced at 23^0C can only function at higher temperatures.

Coding for mercury resistance can be located on plasmids in certain bacteria (Trevors and Oddie, 1986). Olson et al. (1981) found a high frequency of plasmids conferring ability to volatilize mercury and heavy metal resistance in estuarine bacterial populations, and suggested that plasmids may be important in the rapid response of natural populations to metal stress. Plasmids can be transferred in sterile soil at temperatures ranging from 15 to 30^0C with additional nutrients such as standard broth added (Trevors and Oddie, 1986).

Barkay et al. (1989) found that the bacteria strains with alternative *mer* genes play an important role in the ecology of Hg^{2+} resistance and volatilization in aquatic environments. Strong positive correlations have been found between mercury concentration and the frequency of mercury resistance genes (*mer*) in bacteria isolated from sediments

(Barkay and Olson, 1986).

Reducing Ability of Mercury-Resistant Bacteria. The reducing ability of mercury-resistant bacteria has been found to transform organic and inorganic mercurial compounds into Hg^0, which is insoluble in water and rapidly volatilizes from growth medium (Clark et al., 1977; Nelson et al., 1973). The resulting Hg^0 is lost by volatilization owing to its high vapor pressure (Barkay, 1987). When the resistant strains volatilize mercury, the rate of loss of ^{203}Hg from the aqueous phase was much higher when the cells have been induced by growth in 10^{-5} M $HgCl_2$ than cells that have not been induced to $HgCl_2$ (Summers and Lewis, 1973).

Bacteria growth medium will also affect mercury adsorption. During exposure to elemental mercury for 48 hours, medium containing yeast extract adsorbed about twice as much mercury as did medium without yeast extract. The organisms growing in basal salts medium contained less mercury than those growing in media containing yeast extract (Holm and Cox, 1975). Because both media (yeast extract and basal salts) were incubated aerobically, the observation suggests that the nature of the organic carbon supplement is more important in the oxidation of elemental mercury than is the dissolved oxygen in the medium (Holm and Cox, 1975).

Not only mercury-resistant bacteria, but also a yeast of the genus Cryptococcus is capable of reducing mercury to the elemental state (Brunker and Bott, 1974).

A study on the presence of algae (Chlorella) cells in a

medium containing $HgCl_2$ showed a rapid decrease in the mercury content of the algae suspension (Ben-Bassat and Mayer, 1975). Results of this study also showed that the amount of volatilization of mercury depended on the algae cell concentration and that the maximal volatilization was obtained at initial cell concentrations between 300-1000 cells/mm^3. Ben-Bassat and Mayer (1975) thought it was possible that mercury taken up by the cells was not readily available for the conversion reaction. The conversion can occur in the medium and the cells excrete a chemical factor which reacts with the mercury in the medium.

Methylmercury. Because of the high toxicity of methylmercury, research has been carried out on the different conditions for methylation. One study showed that biological mercury methylation is related to overall microbial activity. Mercury is actively methylated in the presence of bound sulfide (Furutani and Rudd, 1980). Another study indicated that the methylation reaction is abiotic because, under the same conditions, more methylmercury was produced from sterile soil than from non-sterile soil (Rogers, 1976). It is possible to extract the methylating factor from soil (Rogers, 1977).

Biological Treatment of Waste Water. Hg^{2+} can be biotransformed to Hg^0 by freshwater and estuarine microbial communities. The activity is solely mediated by the bacterial component of both communities (Barkay et al., 1989). Research has shown that mercury-resistant strains of

bacteria have been effective in the detoxification of mercury from waste water at a rate of 2.5 mg/l h and at efficiencies exceeding 98% (Hansen et al., 1984).

Bacteria have been used not only for detoxification of mercury-contaminated water (Hansen et al., 1984, Hansen, 1990), but also for other metals such as Cd and Cu (Campbell and Martin, 1990; Dunn and Bull, 1983). Removal of poisons including heavy metal from soil using microorganisms has also been studied.

Mercury-resistant bacteria have also been used in petroleum degrading (Walker and Colwell, 1974). When the concentration of mercury in the oil was 4,000 times higher than in sediment and 300,000 times higher than in water samples, the mercury-resistant bacteria have been shown to degrade oil. With a Pseudomonas sp. being most resistant, the bacteria could resist mercury chloride ranging from 2 to 30 mg/l (Walker and Colwell, 1974).

Factors Affecting Mercury Absorption to Soils

Depending on the redox conditions, mercury may occur in three different valence states, namely as Hg^0, Hg_2^{2+} and Hg^{2+}, that may change with changing redox conditions in soils. Moreover, Hg^{2+} normally does not occur alone but forms various complexes with Cl^- and OH^- ions; hence, the chemical form is also dependent on pH, salt content, and composition of the soil solution. These transformations are important because the chemical form influences the retention and

mobility in the soil material and in the soil profile, and also influences the transference to neighboring reservoirs and ecosystems (Anderson, 1979).

Studies have shown that mercury compound absorption to soil is based on several factors such as soil pH, temperature, the content of organic matter in the soil and the type of soil.

Farrah and Pickering (1978) studied three types of clay soil: kaolinite, illite, and montmorillonite. They found that the addition of small amounts of Cl^- (molar ratio $Cl^-:Hg^{2+} = 2:1$) produced great changes in adsorption behavior, increasing uptake on illite, and decreasing uptake on montmorillonite (at pH<7). Around pH 5 the results showed the amount of mercury absorbed was illite > montmorillonite > kaolinite.

Lodenius et al. (1987) studied sorption of mercury in soils with different humus contents. Their results indicate that mercury has leached further into deeper layers at 5^0C than at 20^0C in the peat and sand columns, and they found even a low humus content is enough to absorb significant amounts of mercury.

Soil organic matter has been found to play an important role in mercury adsorption. Semu and Singh (1987) discovered that in all soil samples they tested, there was a significant reduction in mercury adsorption of $HgCl_2$ solution following organic matter removal. Zvonarev and Zyrin (1983) found that most of the mercury in organic soils

is bound to sites with high bonding energies. This means the extractability of mercury is low.

Eichholz et al. (1988) found that standing metallic mercury could penetrate up to 3-4 cm into dry soil columns under its own head, and mercury moved further where wet soil was allowed to dry and crack. Normally, adsorption of $HgCl_2$ increased substantially with increasing pH in all soils. This may be the result of different mechanisms, including ion exchange, specific adsorption, and precipitation of mercury (Semu and Singh, 1987).

Hg^{2+} adsorption is also affected by the type of acid used for soil pH adjustment. Studies have shown the reduction in $HgCl_2$ adsorption because of high chloride ion concentration at lower pH was due to use of HCl for pH adjustment. On the other hand, concentrated HNO_3 used for soil pH adjustment may not be involved in the reduction of mercury adsorption because the formation of Hg^{2+}-nitrate was very weak (Semu and Singh, 1987). Strong acids alone have been variously described as effective or ineffective in stabilizing mercury. Mercury solution containing hydrochloric acid (pH = 0.06) is stabilized more effectively than with sulfuric (pH = 0.18) and nitric (pH = 0.06) acids. The reason is probably because the preponderance of mercury is present as $HgCl_2$ (Avotins and Jenne, 1975). Chloride concentration plays an important role in the amount of mercury absorbed to soils. In the absence of chloride, mercury absorption increased significantly in almost all

kinds of soils. With 10 g/l of chloride present no mercury was absorbed (Farrah and Pickering, 1978).

Semu and Singh (1987) found the effect of soil:solution ratio and ionic strength on adsorption of mercury depends both on the soil:solution ratio and the initial mercury concentration in solution. Their explanation for the variations in mercury adsorption was in terms of chemical equilibrium between mercury adsorbed on soil particles and that in the equilibrating solution. For example, increasing of soil:solution ($HgCl_2$ solution) ratio while maintaining the solution mercury concentration constant increased the initial quantity of mercury available for adsorption by the same amount of soil.

Factors Affecting Mercury Volatilization

Interest in the environmental cycling of mercury has turned to the transformation of mercury into forms other than organics. It is known that mercury applied to the soil in many chemical forms can be lost as volatiles (Landa, 1978; Rogers and Mcfarlane, 1979).

The mercury volatility rate from soil depends on several factors. The mercuric salts used as the mercury source is one of the factors. Frear and Dills (1967) found when mercuric salts $HgCl_2$, $Hg(NO_3)_2$ and $Hg(CH_3COO)_2$ were added to soils, $HgCl_2$ had a much higher volatility rate than the other two salts. Normally, the higher solubility of mercury salts in water, the higher mercury volatility rate (Rogers,

1979).

When Rogers and Mcfarlane (1979) chose loamy sand soil and a silty clay-loam soil as their samples and mercuric nitrate was used to amend the soil, they found that within one week, 20% of the applied mercury was lost from the silty clay-loam soil and 45% was lost from the loamy sand soil. They also found that steam-autoclaved sandy soil amended with $Hg(NO_3)_2$ at a concentration of 20 μg Hg/20 g soil (1 ppm) had a total volatile loss of only 10% of the applied mercury after 144 hours compared to non-sterilized soil that lost approximately 50% of the applied mercury in the vapor form from the sandy soil.

Initial moisture content in soil will also affect mercury volatilization. As soil moisture content increases, the production of gaseous Hg^0 is found to increase. No Hg^{2+} reduction was observed in air-dried soil at temperatures below 5^0C and at a pH below 5.2 (Anderson, 1979).

Kimura and Miller (1964) found that mercury vapor captured from contaminated soil increased with increasing initial moisture.

Addition of nutrient also affects the mercury volatility rate. The adding of glucose to mercury-contaminated soil can accelerate removal of mercury. The addition of a N source to the glucose has little effect on the loss of mercury, suggesting that a C/N ratio imbalance is not involved in the termination of mercury loss (Landa, 1978).

Volatilization of mercury is inducible by the addition of Hg^{2+} or organomercurials to bacteria strains with mercury resistance plasmids. Bacteria not previously exposed to mercury or mercurials have low volatilization rates which increased upon induction (Clark et al., 1977). According to Schottel et al. (1974), the initial rate of loss of $^{203}Hg^{2+}$ was five to ten times more rapid with bacteria that had been previously exposed to 10 μM $Hg(NO_3)_2$ or 1 μM phenylmercuric acetate.

The detection of bacterial volatilization of mercury can be done in several ways. The most common one is using radioactive ^{203}Hg along with a Hg^{2+} reducing reaction assay (Avotins and Jenne, 1975; Schottel et al., 1974). Another method reported by Nakamura and Nakahara (1988) used X-Ray film on which volatile mercury will develop foggy. This method agrees with the radioactive assay and has the advantages of being rapid, simple to perform, and inexpensive (Nakamura and Nakahara, 1988).

Furthermore, research showed that about 17 to 19% of the mercury added to soil was neither volatilized nor extracted. This indicates that the binding of mercury to soil is irreversible (Kimura and Miller, 1964).

Biological Detoxification of Contaminated Soil

Weaver et al. (1984) found that the plant *Cynodon dactylon* can grow on soil containing 50 mg/kg mercury, and can uptake from soil to its leaves, stems and roots.

Parathion-acclimated microorganisms were extremely effective in rapidly degrading concentrations of parathion of at least 5000 ppm in non-flooded soil within 3 weeks under laboratory conditions (Daughton and Hsieh, 1977).

Soil contaminated with metals such as Se, Cu and Cd can be detoxified biologically. Selenium compounds like Na_2SeO_3 are detoxified in the presence of mold (Abu-Erreish et al., 1968). The rate of Se evolution increased with organic matter additions and with increasing of the water soluble Se content, but decreased in autoclaved soil samples regardless of whether starch or wheat was added. Bacteria can bioaccumulate copper from solution (Dunn and Bull, 1983). Preliminary evidence suggested that the copper was bound to the outside of the bacteria. Fungi are found to tolerate and absorb high levels of heavy metals, especially cadmium (Campbell and Martin, 1990).

Environmental factors are very important for microbial activity. Since biodegradation of organic constituents is accomplished by enzymes produced by microorganisms, the amount of enzyme released by microbial cells is very important. Optimum soil water level (25 - 80%), oxygen for aerobic metabolism, redox potential of the soil, nutrient and optimum temperature (15 - 45^{0}C) are all factors affecting microbial activity. Biodegradation of organic constituents will stop at a soil temperature of 0^{0}C due to reduced microbial growth and metabolic activity (Sims et al., 1990).

Because references did not show that previous research has been on detoxification of mercury-contaminated soils, this research will be very important in this area.

Mercury Analysis

There are several methods available to determine mercury concentration in its various forms. Procedure selection is based on the form of existing mercury needing to be tested. The most common method is atomic absorption spectrophotometry (AA) to measure dissolved mercury in the parts-per-billion (ppb) range. This method is based on the use of a cold vapor cell apparatus, which is an adaptation of an EPA Procedure (Eichholz et al., 1988).

Digestion and extraction of the soil sample may be required before mercury analysis. Standard methods have been approved by various governmental agencies for these procedures. Total mercury in the soil also can be determined on the wet sieved pelitic fraction by digesting dry soil with an acid mixture of HNO_3/H_2SO_4 (1:1, V:V) in teflon vessels, under-pressure at 120^0C (Baldi, 1988).

Besides AA, gas chromatography is used for determining the type and concentration of specific organomercurial compounds. Homogenizing samples and using acids and organic solvents are required. The components are separated by chromatographic techniques and analyzed qualitatively and quantitatively (National Academy of Sciences, 1978).

CHAPTER III

OBJECTIVES

The objectives of this study were

1. Investigate biological detoxification of mercury-contaminated soil by adding mercury-resistant bacteria directly to soil and by using non-stir and slurry-type bioreactor with and without bacteria.

2. Compare the mercury removal rate among soils containing three different moisture contents.

3. Compare the mercury removal rate of soils amended with 5 ppm and 10 ppm mercury.

4. Compare the effect of adding bacterial nutrient (sucrose) versus no additional nutrient.

CHAPTER IV

MATERIALS AND METHODS

Soil

Soil used for this study was Kidman soil collected from Kaysville, a small town in northern Utah. Before use, the soil was air-dried, sieved (<2 mm) and stored at room temperature. Soil analysis was done by the Soil Testing Laboratory in the College of Agriculture at Utah State University. The physical and chemical properties of the soil are given in Table 2. Sterilized soil was prepared by autoclaving the soil for 3 hours at 121^{0}C and 110.32 KPa.

Mercury-resistant bacteria used in this study were identified as Enterobacter Cloacae and Klebsiella Oxytoca by the microbiology laboratory at the Logan Regional Hospital, Logan, UT.

Soil samples (50 g) with and without added bacteria were poured into flasks and then amended with mercuric chloride stock solution (1 μg Hg/ml, 1000 ppm), 0.25 ml or 0.50 ml which gave 5 ppm and 10 ppm of mercury contamination. Five ppm and 10 ppm mercury concentration were chosen because most naturally occurring mercury-contaminated soil sites fall within this range (Personal Communication with T. Barkay, 1999). Distilled, deionized water was added to all soil samples to adjust moisture contents to 20%, 30% and 50% (wt/wt).

Table 2. Soil Characteristics (Soil Testing Laboratory, College of Agriculture, Utah State University)

Soil		Kidman
Texture		Silt loam
Moisture (@1/3 bar)	(%)	20
pH		7.2
Organic Carbon	(%)	0.5
CEC	(meq/100gm soil)	11.7
Chelatable:		
Fe	(mg/kg)	4.5
Cu	(mg/kg)	1.6
Mn	(mg/kg)	6.3
Zn	(mg/kg)	0.6
Acid Digestion:		
Fe	(mg/kg)	1.4×10^4
Cu	(mg/kg)	14.3
Mn	(mg/kg)	3.5×10^2
Zn	(mg/kg)	45.7
Bacteria (unamended)	(c.f.u./gm soil)	6.7×10^6

Apparatus

Method 1. Erlenmeyer flasks (250 ml) were closed by two-hole rubber stoppers with inlet and outlet air lines as shown in Fig. 1. Results of preliminary experiments showed that air flow of 2.5 cm^3/min could carry volatilized mercury vapor from the soil flask outlet to a mercury absorption trap while preventing considerable moisture loss from the flasks. The trap solution contained 14% sulfuric acid and 3% potassium dichromate dissolved in distilled, deionized water (Wu and Hilger, 1985). Because of evaporation of moisture from the soil caused by the air flow, additional distilled, deionized water was added daily to maintain the specified moisture content. The amount of water added was determined by measuring weight loss from the soil. After the required amount of water was added each day, the flasks were vortexed to evenly distribute the moisture. Each trap solution was sampled and replaced daily with fresh solution to determine the amount of mercury trapped each day. After six days of this continuous operating and sampling from each flask, both samples and controls were sealed with Parafilm and set in an exhaust hood. In order to investigate the mercury removal rate over time, I analyzed these samples and controls once every six days for total mercury remaining in the soil. Flasks were unsealed for about 10 minutes before soil sampling. The total mercury analysis was carried out for an additional 24 days for a total of 30 days.

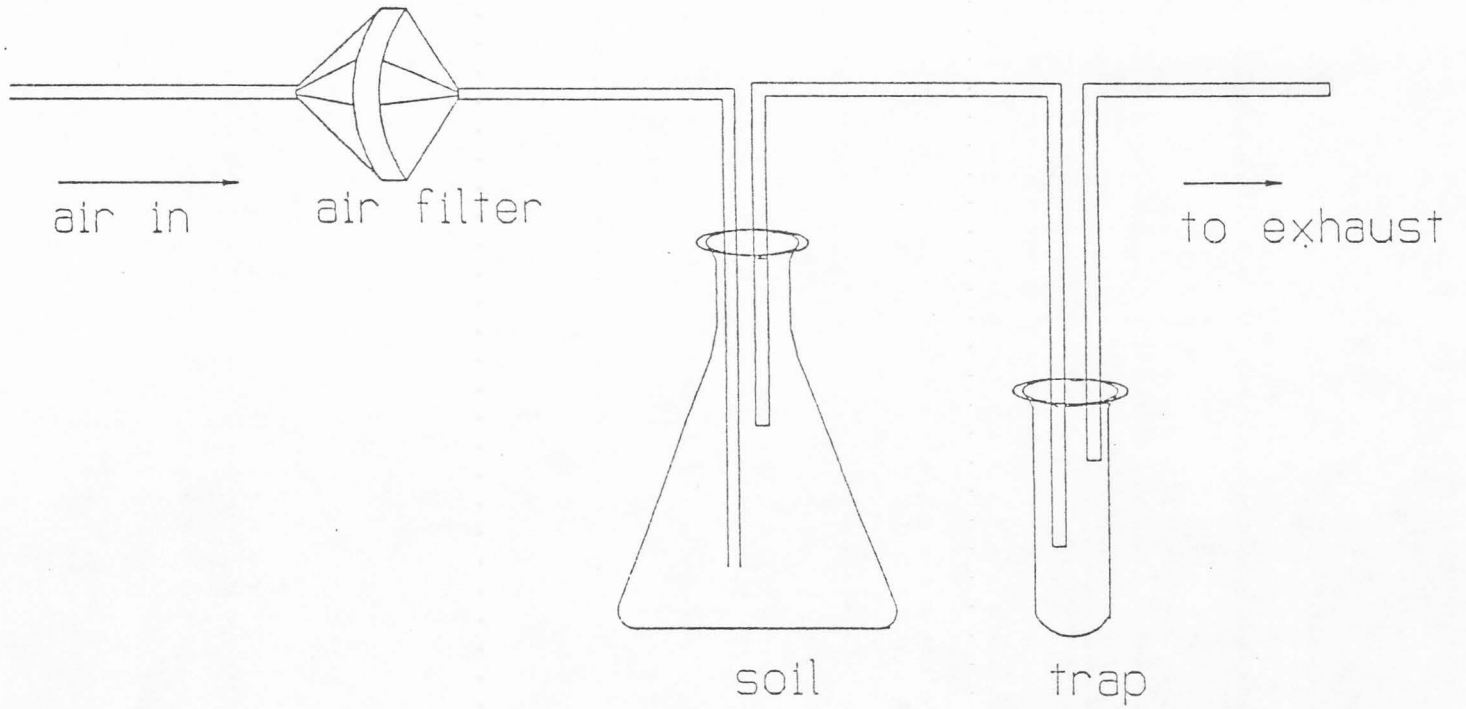

Fig.1--Non-stir bioreactor.

Method 2. A slurry-type bioreactor shown in Fig. 2 was used for several experiments to compare results with those carried out in the 250 ml flasks. This method used a vacuum Erlenmeyer (1000 ml) flask sealed by a one-hole rubber stopper with inlet air line. Air flow was 200 cm^3/min and carried volatilized mercury vapor to the same trap solution used in Method 1. A soil-water mixture of 50% soil moisture content was used and this was amended with 5 μg/g and 10 μg/g mercury (from $HgCl_2$) in separate experiments. A magnetic stir bar (0.5 inch diameter, 3 inches length) rotating at 120 rpm was used to mix the soil-water slurry.

Analyses

Mercury analysis of soil was carried out using the 1986 EPA standard method #7471 for mercury in solid or semisolid samples (USEPA, SW846). Five milliliters of distilled, deionized H_2O and 5 ml aqua regia (HNO_3:HCl = 1:3, v/v) (AR, Mallinckrodt Inc. Paris, Kentucky) was added to 0.2 g of anhydrous soil in BOD bottles. After heating at 95^{0}C for 2 minutes in a water bath, these BOD bottles were taken out of the water bath and set at room temperature until cool. Then 15 ml of 5% (wt/v) $KMnO_4$ (Mallinckrodt Inc. Paris, Kentucky) solution and 50 ml distilled, deionized water was added. This was again heated at 95^{0}C for another 30 minutes. Six milliliters of NH_2OH.HCl (AR, Mallinckrodt Inc. Paris, Kentucky) and NaCl (AR, Mallinckrodt Inc. Paris, Kentucky) solution (12 g of each dissolved in 100 ml distilled, deionized water) were added to reduce excess potassium

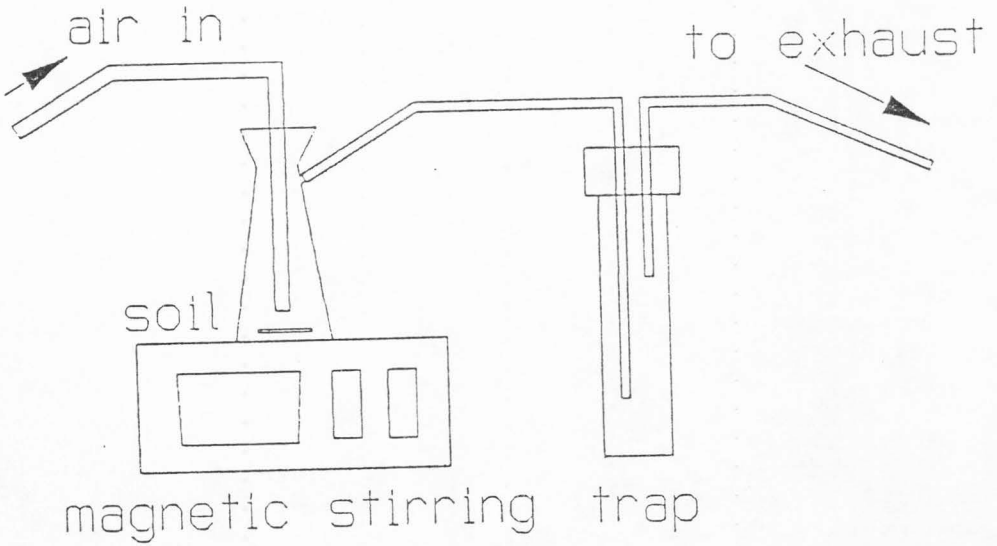

Fig.2--Slurry-type bioreactor.

permanganate, and then an additional 55 ml of distilled, deionized water were added to bring the total volume to 136 ml. Five milliliters stannous chloride (Sigma, St. Louis, Missouri) solution (10%, wt/v) was used to reduce the Hg^{2+} to Hg^{0} vapor which was analyzed by cold vapor atomic absorption. The presence of aqua regia produced chlorine gas when the BOD bottles were heated in the water bath. In order to prevent the laboratory being contaminated with chlorine, an autoclave digestion method was used in later analysis (USEPA, SW846). In this method, 5 ml of concentrated H_2SO_4 (AR, Mallinckrodt Inc. Paris, Kentucky) and 2 ml of concentrated HNO_3 were added to 0.2 g of dry soil. Then 5 ml of saturated $KMnO_4$ solution were added and the BOD bottle (Wheaton Scientific, Millville, New Jersey) was covered with a piece of aluminum foil (Reynolds Metals Company, Richmond, Virginia). The samples were autoclaved at 121^{0}C and 110.32 KPa for 15 min. When the sample cooled to room temperature, 90 ml distilled, deionized water were added to bring the total volume to 100 ml. Sodium chloride-hydroxylamine hydrochloride was added to reduce the excess permanganate. These samples were analyzed as soon as 5 ml of stannous sulfate were added.

Total mercury concentration was obtained by cold-vapor atomic absorption analysis. A standard curve (see appendix A) was made before each analytical run by using a commercial mercury standard stock solution containing 1000 ppm mercury (Millinckrodt Inc. Paris, Kentucky). The mercury stock

solution was diluted by adding distilled, deionized water to the mercury stock solution to a mercury working standard containing 1 ppm mercury. Acidity of the working standard was maintained at 0.15% nitric acid. Then, 0.0, 0.5, 1.0, 2.0-ml aliquots of the mercury working standard (0, 0.5 ppm, 1.0 ppm, 2.0 ppm) were transferred to a series of BOD bottles. These standards were treated the same as the dry soil samples through all digestion steps. Total mercury content in the standards and samples was measured by the percentage of light transmission which was recorded by a Linear 1200 recorder (Linear Instrument Corp., Reno, Neveda). Absorbance was obtained from the percent transmission by using the equation:

Absorbance=2-log(transmission)

There was a linear relation between absorbance and standard mercury concentration as shown in Appendix A, with regression between these two factors being obtained using a first order polynomial regression equation. The constant and absorbance values were obtained from a computer statistical software program (AXUM, TriMetrix Inc. Seattle, Washington) as shown in the example in Appendix B. Mercury concentration of the soil samples was calculated using the equation:

$$[Hg] = (A - k)/[Hg]_{std.}$$

where A = absorbance of samples

k = constant of statistical result (Appendix B)

$[Hg]_{std}$ = constant standard of statistical result

(Appendix B)

Trap solution digestion and mercury analysis followed the same steps as described above for soil samples.

All experiments were carried out at room temperature and with an initial soil pH of 7.2. Experiments were carried out in duplicate and soil mercury analyses were done in triplicate.

Sterilized soil was used for the comparison of mercury volatility rate to that of the natural soil. Sterilized soil was prepared by autoclaving for 3 hours at 121^{0}C and 110.32 KPa. After setting for two days at room temperature, 0.1 g of soil was put into 10 ml sterilized water and then placed in a petri dish containing nutrient agar. The plates were then incubated at 37^{0}C for 3 days.

During the first week of continuous running, bacteria plate counts of each autoclaved soil sample were made every day. Only sterilized water (autoclaved at 121^{0}C for 3 hours) was added to autoclaved soil to maintain the specific moisture content, and filtered air (0.3 μm hydrophobic air filter) was passed through the soil in an effort to maintain sterility.

An additional study was carried out using soil samples maintained under the same conditions as other experiments, except nutrient media was added. The nutrient media contained sucrose solution (2 g/l) with ammonium sulfate (4 g/l) and sodium citrate dihydrate (8 g/l). This gave an indication of whether or not mercury-contaminated soil

amended with bacteria and nutrient would improve mercury removal compared to bacteria amended soil without nutrient added.

CHAPTER V

RESULTS AND DISCUSSIONS

Volatilization Rate and Bacteria Growth

When the soil was sterile, meaning no bacteria growth appeared on petri dishes of autoclaved soil samples, no mercury was volatilized. The results agree with those of Landa's (1978). Fig. 3 and 4 show that autoclaved soils with added bacteria reached their highest volatilization rate on the first day after amendment. This result was observed from soil samples with 30% and 50% soil moisture content and 5 ppm mercury. Plus, the volatilization rate of autoclaved soils without the addition of mercury-resistant bacteria increased after it became contaminated with microorganisms (Table 3).

Table 3. Mean Value of Total Bacteria Counts from Autoclaved Soil (CFU/g [wet wt.])

	Days				
Samples[a]	0	1	2	3	4
30% w/o	-	-	-	++[b]	+++
30% with	-	$3x10^7$	$4.3x10^7$	$7.6x10^7$	$3.7x10^7$
50% w/o	-	-	-	+++	+++
50% with	-	$3x10^7$	$6.6x10^7$	$1.1x10^8$	$1.0x10^8$

a: Percent soil moisture with or without added bacteria.
b: '++' means less bacteria growth compared to '+++'.

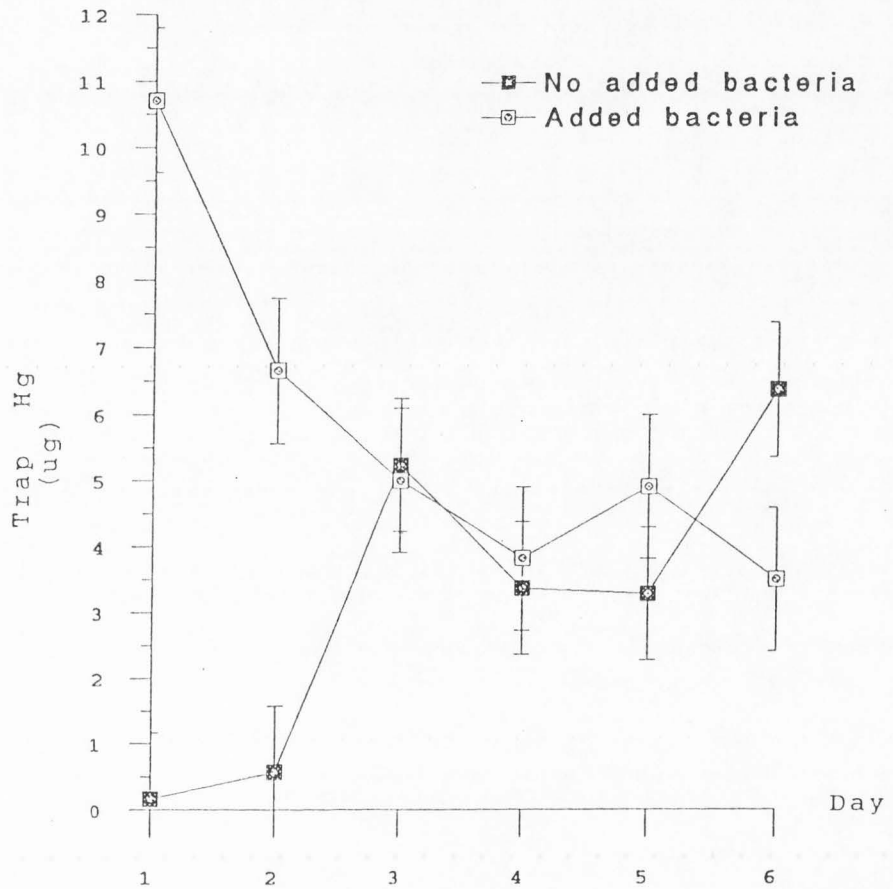

Fig.3--Volatilization of Hg from sterile soil amended to 5 ppm Hg (30% soil moisture, wt/wt).

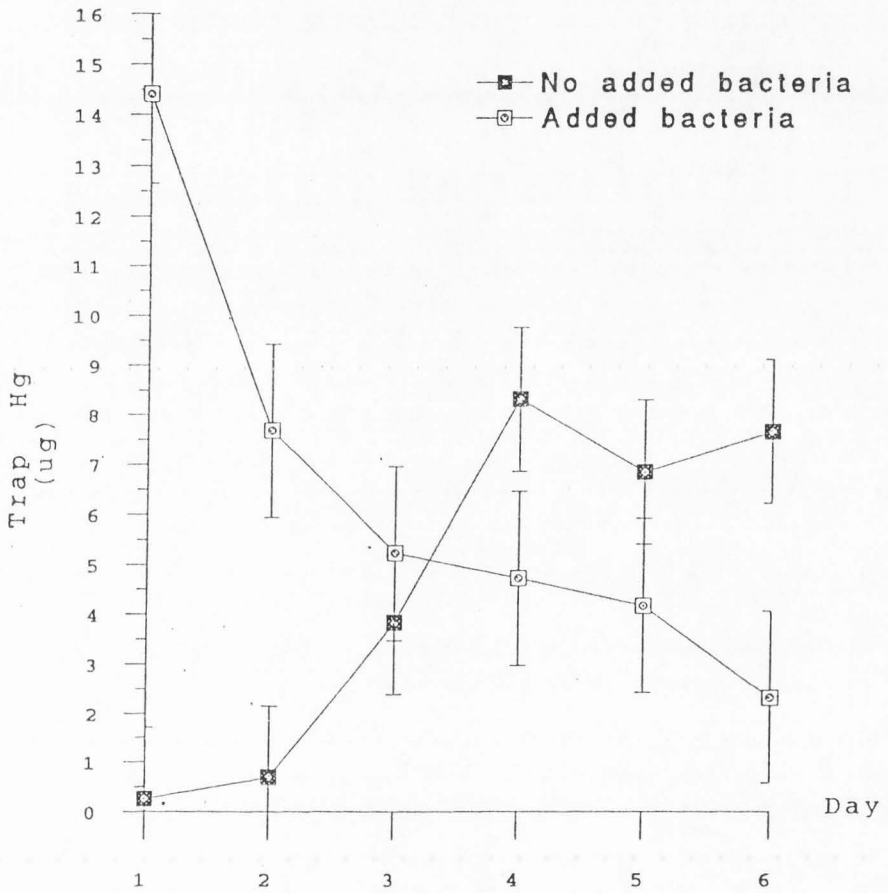

Fig.4--Volatilization of Hg from sterile soil amended to 5 ppm Hg (50% soil moisture, wt/wt).

This result indicates that microorganism are the major factor in reducing Hg^{2+} to Hg^{0} and thus making possible mercury volatilization. Whether there are some chemical or physical reactions or changes along with this biological process was not determined in this study. Soil samples could not be maintained sterile for the whole six-day experimental period because of air flow contamination or heat tolerant spores. This result agreed with Landa's (1978) and Abu-Erreish's (1968) observation that complete sterility could not be achieved. The contaminating microorganisms were mercury-resistant because they survived in mercury-contaminated soil. Mercury evolution was also observed.

Mercury began to be volatilized from the controls which had not been amended with bacteria after the third or fourth day from the start of the experiment. At the same time, bacteria growth was noted on the agar plates used to monitor soil sterility. The number of bacteria was not determined so that in Table 3, the '+' signs denote bacteria growth only. This bacteria contamination may have occurred the first day of the experiment, but the observed lag period may have occurred because these new bacteria may have needed to be exposed to mercury for a period of time to induce them to produce the mercury reducing enzyme (Clark et al., 1977).

Soil organic matter content and naturally occurring bacteria numbers are factors in mercury removal rate as noted earlier. Under the same experimental conditions, with

the same amount of mercury-resistant bacteria added, autoclaved soil had less mercury volatilized than non-autoclaved soil (Fig. 5 and 6). Autoclaving may not only have killed natural soil bacteria but may also have destroyed some organic matter. This also could explain why more mercury remained in autoclaved soil samples than in non-autoclaved soils 30 days after amendment, as shown in Fig. 7 and 8.

Moisture Content

When amended with the same amount of mercuric chloride, the mercury removal rate seemed generally to increase as soil moisture content increased during the first six days (Table 4). But statistical analysis showed there was no significant difference ($p>0.1$) among the three soil moisture contents on the mercury removal rate.

Mercury Concentration

Two soil mercury concentrations were used, 5 ppm and 10 ppm. Even though an equal number of mercury-resistant bacteria were added, a much higher rate of mercury volatilization was observed in the soil containing 10 ppm mercury than the 5 ppm mercury soil (Table 5). This difference was significant ($p<0.001$) and agreed with Olson et al. (1981) and Rogers and Mcfarlane (1979) that at progressively higher mercury concentrations, the bacteria developed a significantly increased resistance to mercury and thus increased mercury volatilization. A possible explanation may be that at higher mercury concentrations,

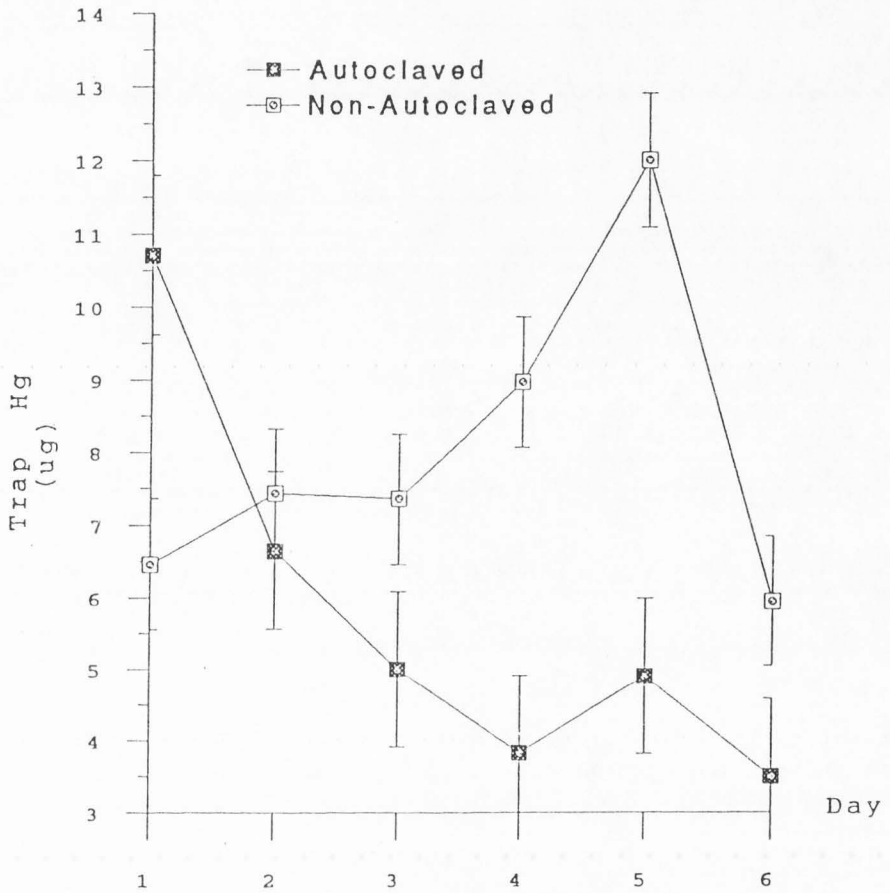

Fig.5--Volatilization of Hg from soil amended to 5 ppm Hg with added bacteria (30% soil moisture, wt/wt).

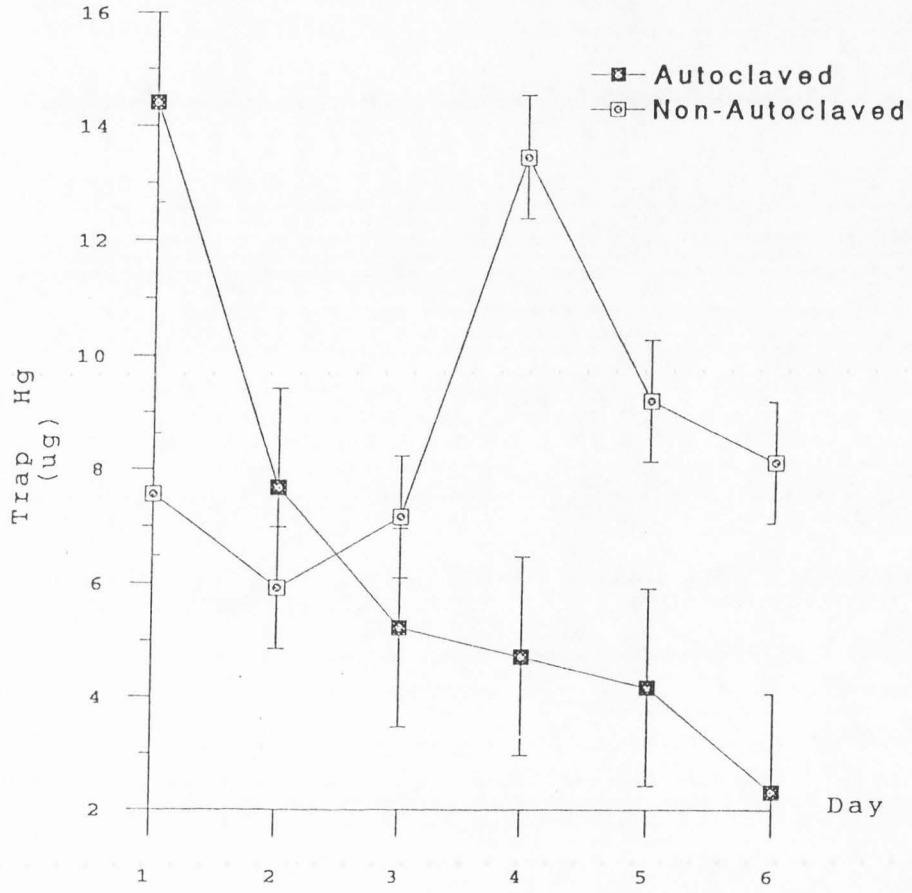

Fig.6--Volatilization of Hg from soil amended to 5 ppm Hg with bacteria added (50% soil moisture wt/wt).

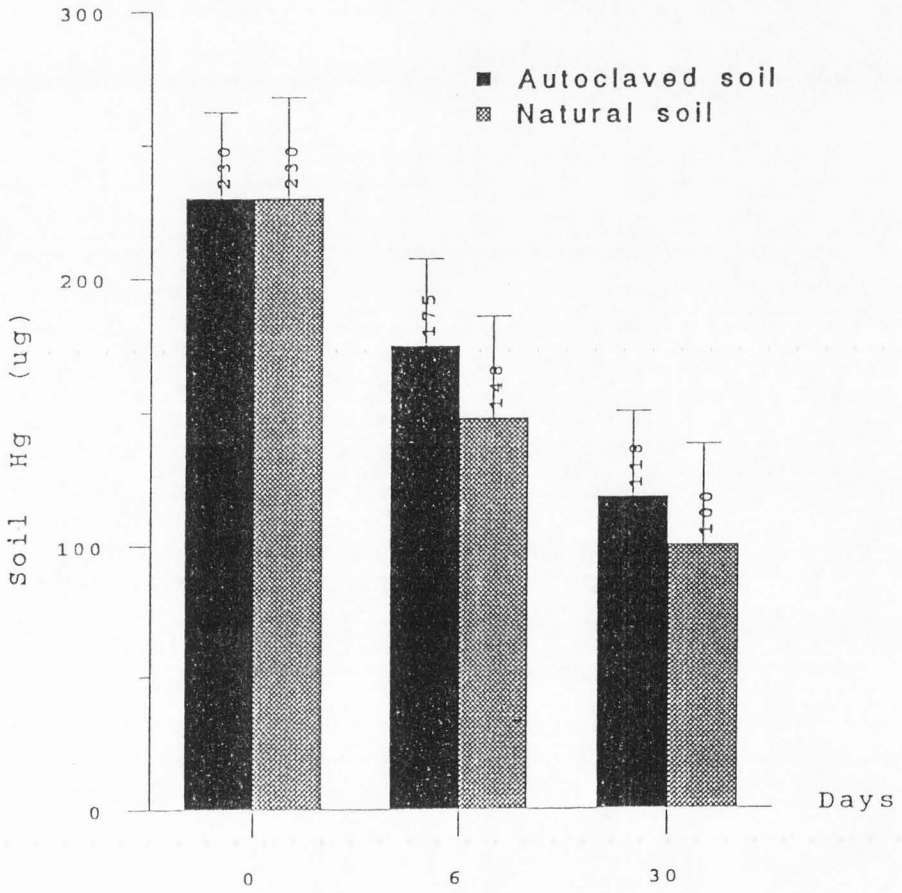

Fig.7--Comparison of Hg remaining in autoclaved and natural soil amended to 5 ppm Hg with added bacteria (30% soil moisture, wt/wt).

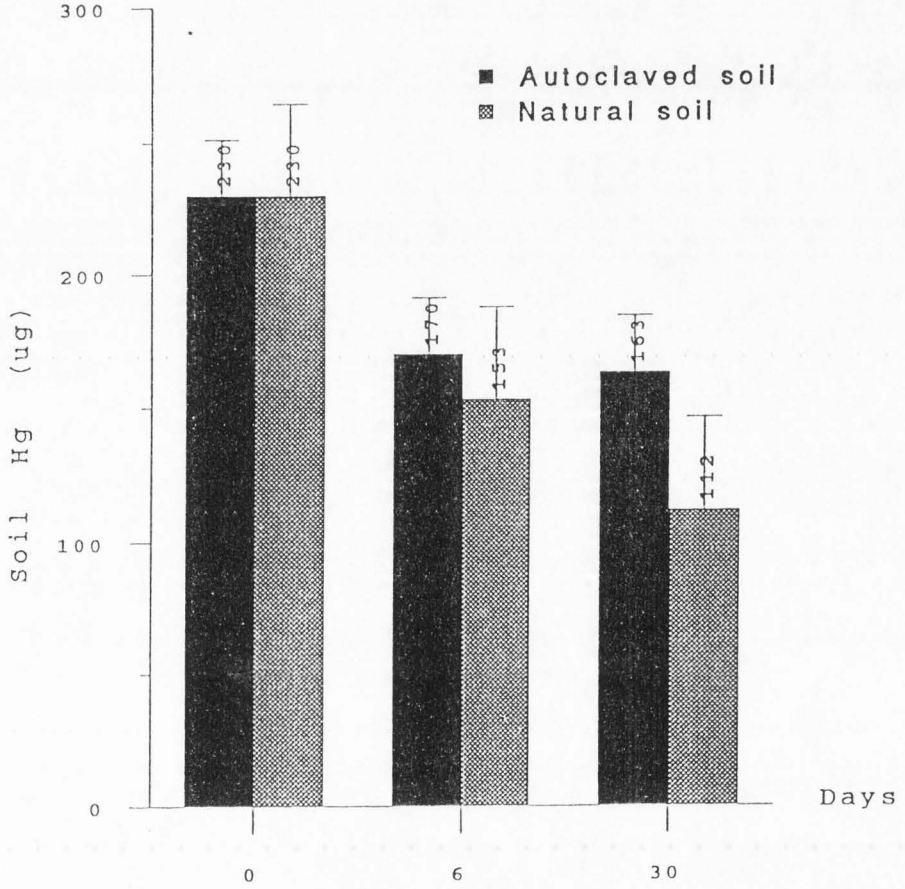

Fig.8--Comparison of Hg remaining in autoclaved and natural soil amended to 5 ppm Hg with added bacteria (50% moisture, wt/wt).

Table 4. Rate of Mercury (μg) Volatilization from Soil Amended to 5 ppm Mercury with Different Soil Moisture Contents

Day	20% Moisture	30% Moisture	50% Moisture
1	7.43 μg	6.64 μg	7.56 μg
2	8.83	7.43	5.92
3	8.77	7.36	7.17
4	5.33	8.97	13.46
5	6.07	12.00	9.22
6	5.60	5.95	8.14
Total	42.03	48.17	51.47

Table 5. Mean Value of Amount of Mercury (μg) Collected in Trap Solution (6 Days) from Silt Loam Soil Amended to 5 ppm and 10 ppm Mercury (Method 1)

Hg Concentration	Soil Moisture 20%	30%	50%
5 ppm *Control	31.54	23.11	21.70
5 ppm *Sample	42.03	45.35	48.78
10 ppm *Control	49.95	40.22	46.68
10 ppm *Sample	57.09	65.23	89.02

Control: Soil without added bacteria
Sample: Soil with added bacteria

mercury-resistant bacteria's metabolism increases, thus reducing mercury at a higher rate. On the other hand, at the lower mercury concentration condition, nearer to a level tolerated by the bacteria, the reducing activity was at a slower rate. The reason for the bacteria activity slowing down with decreasing mercury concentration was not addressed in this study.

Time Required for Removal

When soil moisture content was 20%, a significant difference was observed between samples and controls at 6 days at both 5 ppm ($p<0.05$) and 10 ppm ($0.001<p<0.005$) mercury concentration. Controls had less mercury remaining in soil than did samples. The reason for this result may be related to the mercury distribution in the soil. When soil moisture content was as low as 20%, even distribution of mercury was very difficult. At 30 days, no significant difference between them (Fig. 9 and 10) was observed. With 30% soil moisture and 5 ppm mercury concentration, no significant difference between samples and controls was observed at 6 days ($p>0.7$) or 30 days ($p>0.1$, Fig. 11). When mercury concentration increased to 10 ppm, soil samples had significantly less mercury left at both 6 days ($p<0.005$) and 30 days ($p<0.05$, Fig. 12). Also, there was no significant difference between controls and samples when

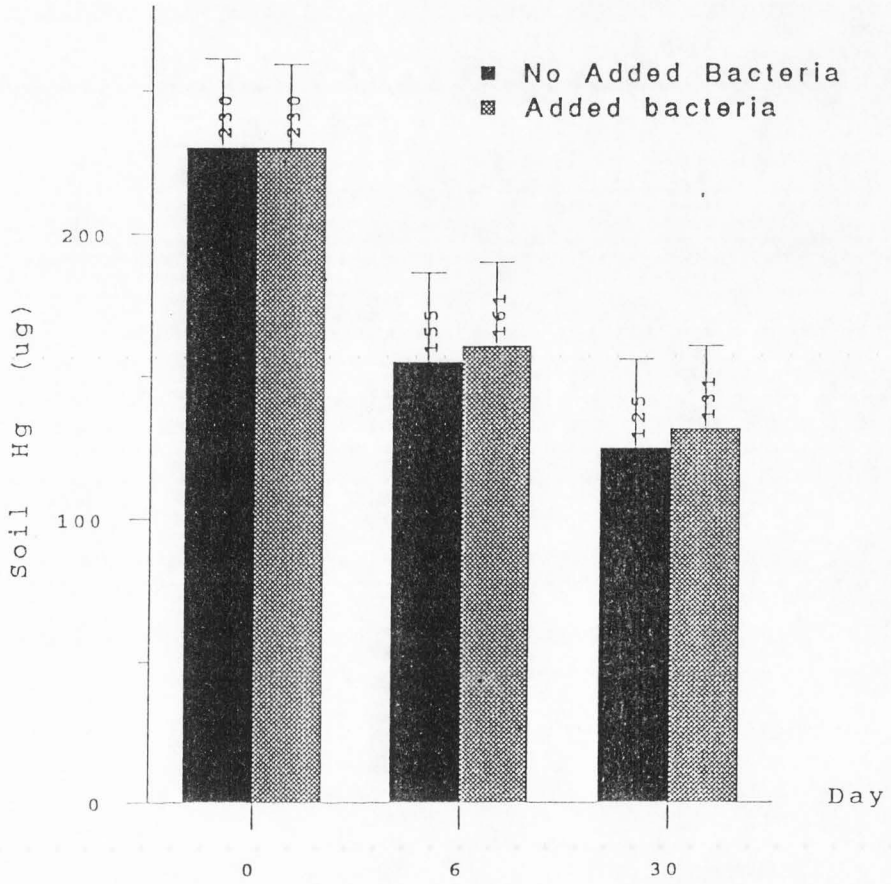

Fig.9--Hg remaining in soil amended to 5 ppm Hg (20% soil moisture, wt/wt).

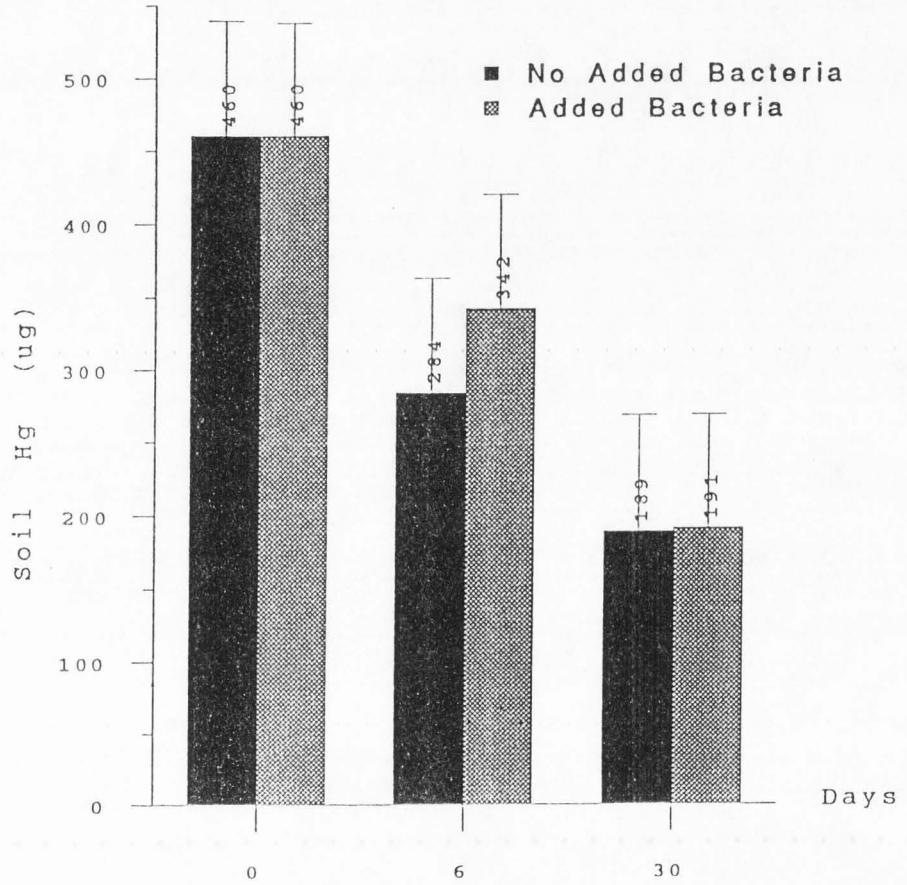

Fig.10--Hg remaining in soil amended to 10 ppm Hg (20% soil moisture, wt/wt).

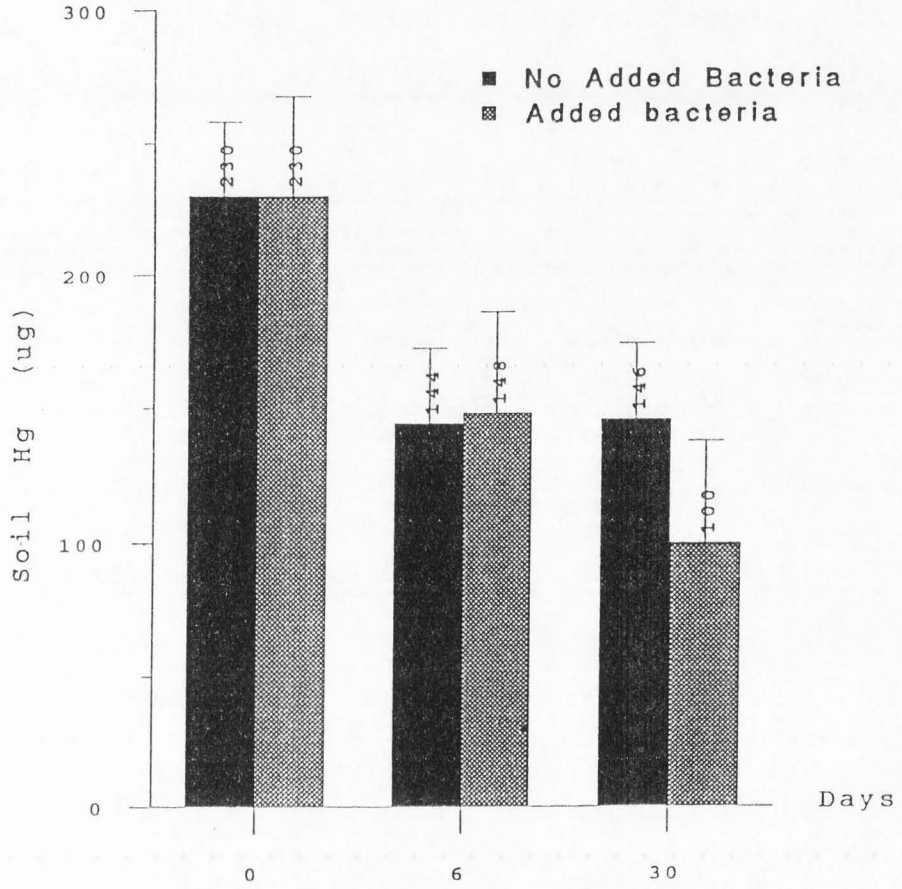

Fig.11--Hg remaining in soil amended to 5 ppm Hg (30% soil moisture, wt/wt).

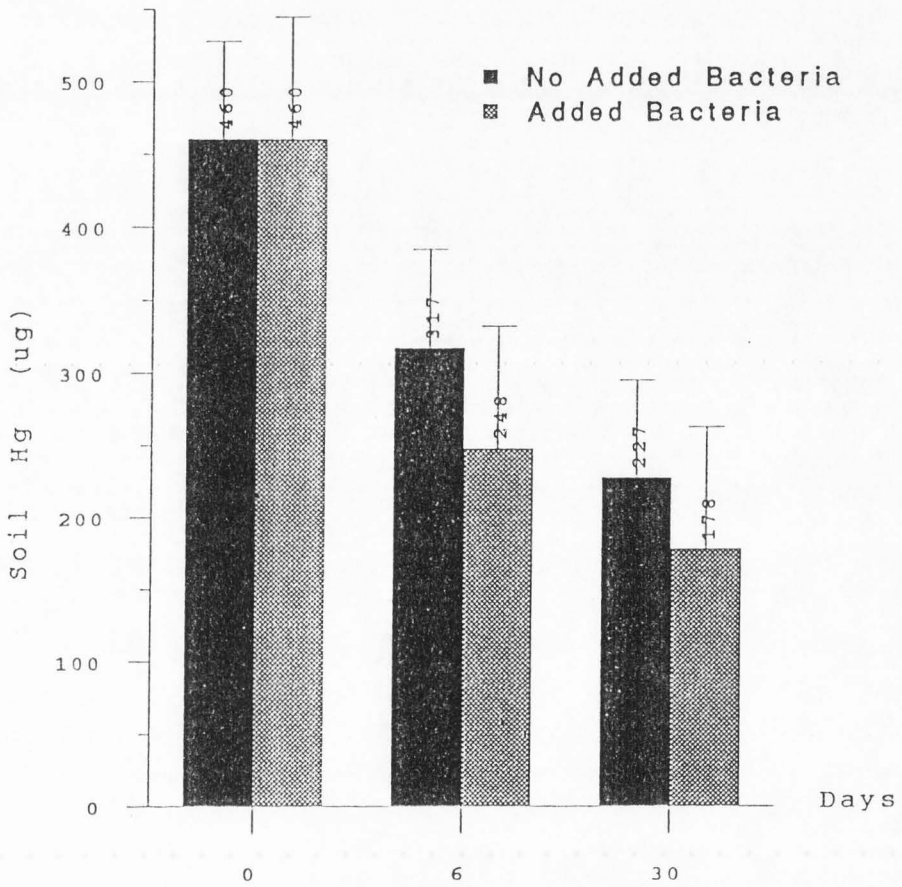

Fig.12--Hg remaining in soil amended to 10 ppm Hg (30% soil moisture, wt/wt).

mercury concentration was 5 ppm (50% moisture) after 6 days by using method 1 ($p>0.1$, Fig. 13). After 30 days, the difference was significant ($p<0.005$) showing that samples had less mercury remaining than controls. When mercury concentration was 10 ppm (50% moisture), difference between samples and controls was significant for both 6 days ($p<0.001$) and 30 days ($0.001<p<0.005$, Fig. 14).

Slurry-type Bioreactor

Slurry-phase biological treatment is a relatively new development for the remediation of hazardous wastes. It is highly effective for a variety of wastes and its rate of degradation is up to ten times faster than land treatment (Ross, 1991). This study found that the slurry-type bioreactor had a much greater mercury volatility rate when compared with the flask method (Fig. 15 and 16).

The t-test with a 95% confidence interval showed the slurry-type bioreactor had a significant difference compared to the non-stir reactor both at 6 days (5 ppm, $p<0.001$; 10 ppm, $p<0.001$) and 30 days (5 ppm, $p<0.001$; 10 ppm, $p<0.001$). With a mercury concentration of 5 ppm, the mercury volatilized from the slurry-type bioreactor was double that of the non-stir method. With the same soil moisture content (50%) and same mercury contamination concentration (5 ppm), 63% of the mercury was removed after 6 days from Hg^{2+} amendment. The same amount of mercury removed from the non-stir type required more than 30 days. The same result was also obtained with the initial mercury concentration at 10

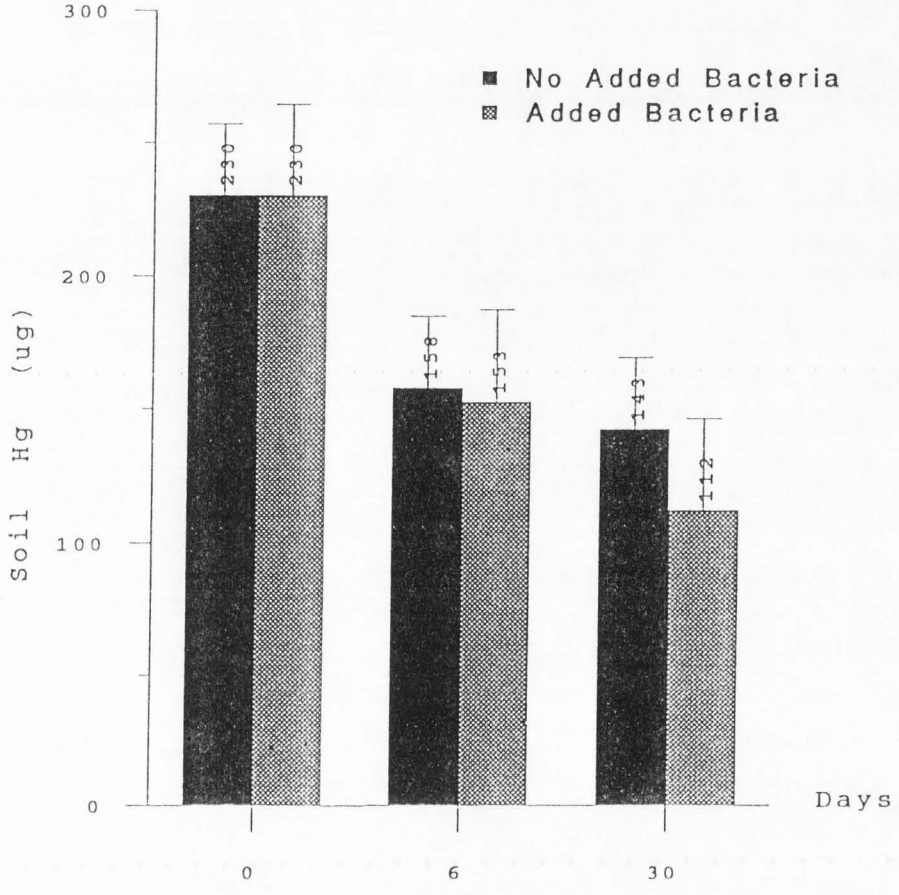

Fig.13--Hg remaining in soil amended to 5 ppm Hg (50% soil moisture, wt/wt).

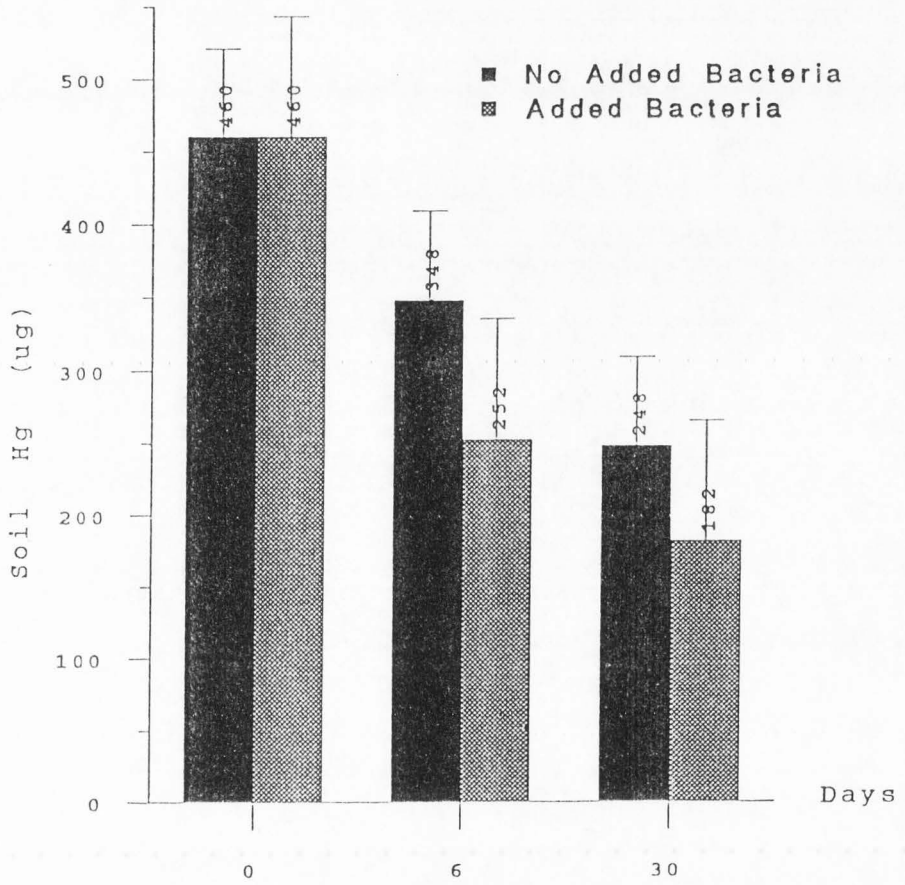

Fig.14--Hg remaining in soil amended to 10 ppm Hg (50% soil moisture, wt/wt).

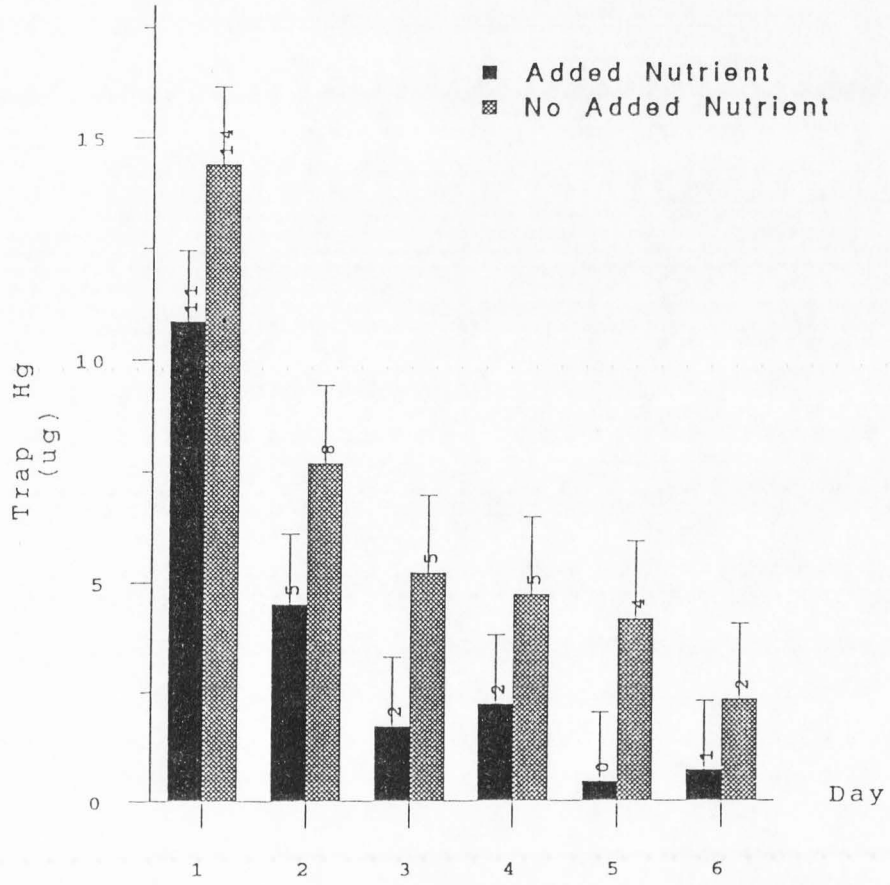

Fig.15--Volatilization of Hg ffrom autoclaved soil amended to 5 ppm Hg with 50% soil moisture.

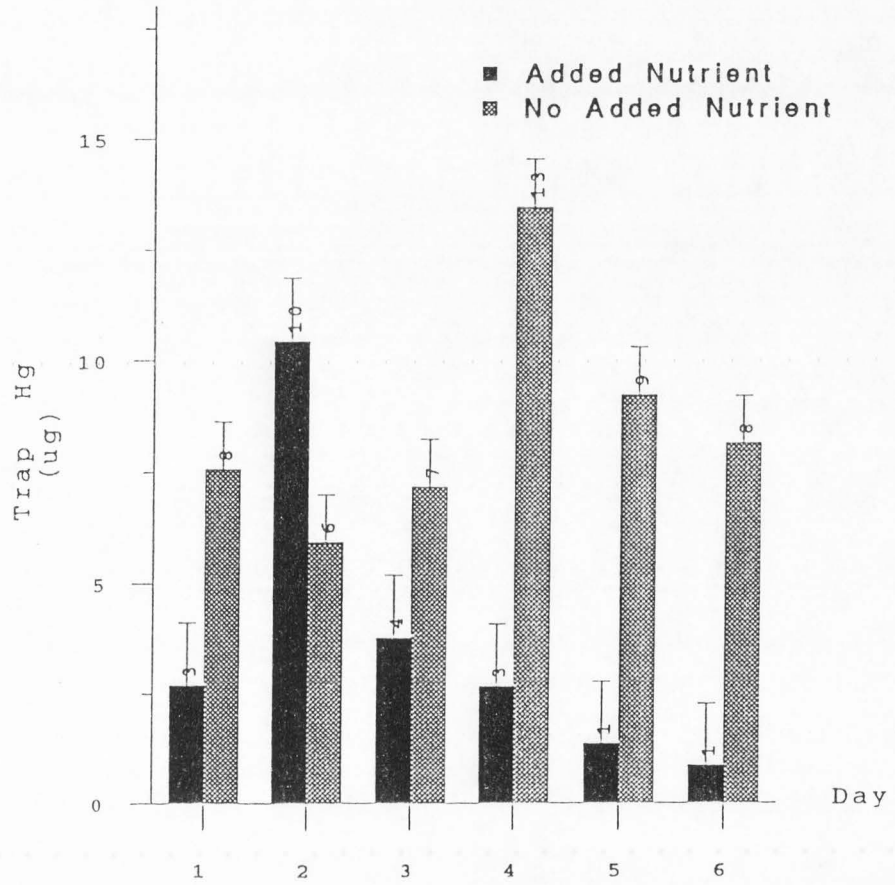

Fig.16--Volatilization of Hg from soil amended to 5 ppm Hg with 50% soil moisture.

ppm (Table 6).

Table 6. Percent of Mercury Removal from Soil (50% Moisture) After 6 & 30 Days with Bacteria Added

Mercury Con.	Method 1 (non-stir)	Method 2 (slurry reactor)	Control#
5 ppm (6 d)	33%	63%	31%
(30 d)	51	65*	38
10 ppm (6 d)	45	58	24
(30 d)	60	87+	46

* Stirring for 12 d, setting in the hood for 18 d.
\+ Stirring for whole 30 d.
\# Non-stirring, no added bacteria.

Because the important difference between the two methods was stirring and non-stirring of the bioreactor, a critical factor for mercury removal must be the close association of all the constituents (soil, water content, mercury, and bacteria) in the slurry mixture.

Nutrient Effect

According to Landa (1978), the addition of glucose increased the initial loss rate of applied Hg^{2+} from test soils. Under normal conditions we would not expect a natural soil to contain enough nutrient for the addition of a large number of mercury-resistant bacteria. A comparison was made of the mercury volatilization rate between nutrient

addition and non-addition to soil samples with the same mercury concentration and moisture content. Striking results were obtained showing that soil samples had a decreased mercury volatility rate soon after nutrient had been added to the flask (Fig. 17 and 18). An explanation may be that the mercury bound with the nutrients complexed with the sodium citrate dihydrate in the bacteria growth media.

Mercury Recovery

Recovery was obtained by totaling the amount of volatilized mercury trapped in the trap solution and the amount of mercury remaining in the soil after 6 days into the experimental run, divided by the amount of mercury amended to the soil (Table 7).

Autoclaved soil had a relatively higher recovery than non-autoclaved controls and samples. With 30% soil moisture content and 5 ppm soil mercury contamination, the autoclaved control had a 92% recovery and the sample had a 91% recovery. With the same mercury concentration, as soil moisture was increased to 50%, 86% and 91% recoveries were obtained from controls and samples. These recoveries were higher than the recoveries from non-autoclaved soil shown in Table 7.

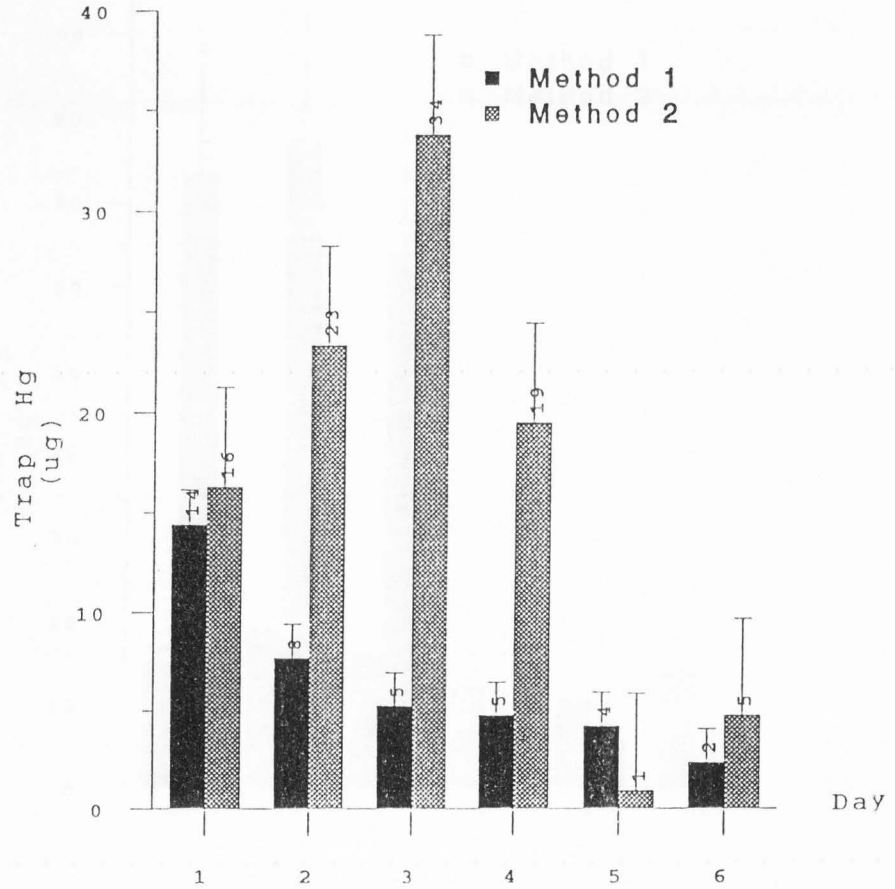

Fig.17--Volatilization of Hg from soil (50% moisture) amended to 5 ppm Hg comparing two methods of soil treatment. Method 1: flask with no stirring. Method 2: slurry-type bioreactor.

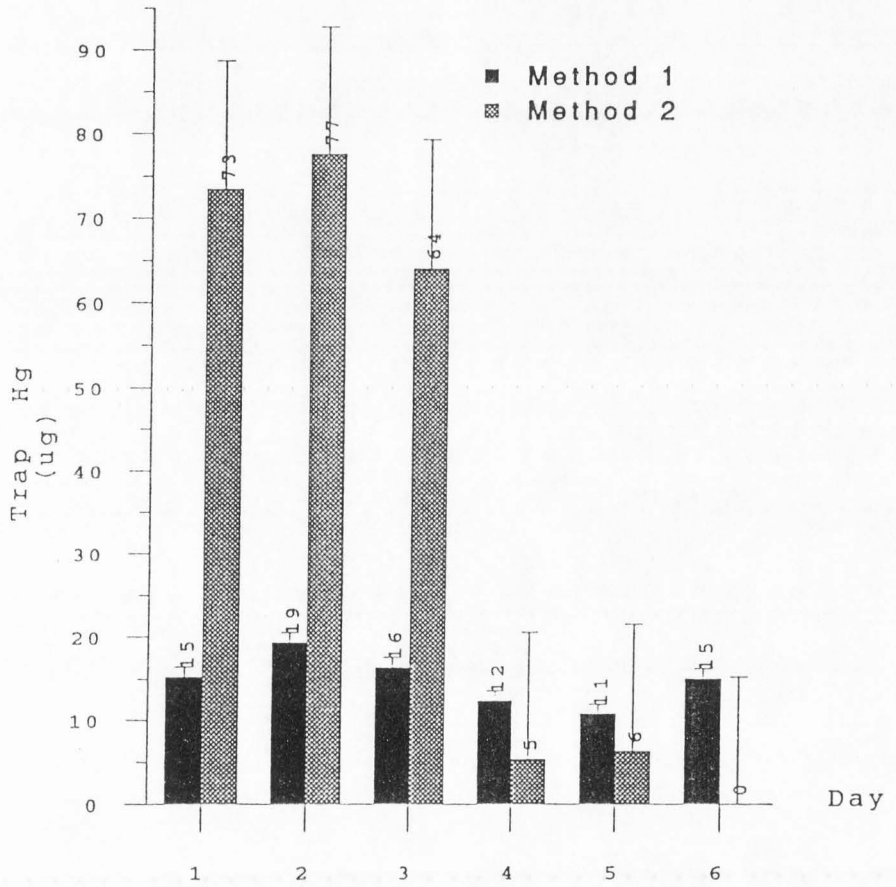

Fig.18--Volatilization of Hg from soil (50% moisture) amended to 10 ppm Hg comparing two methods of soil treatment. Method 1: flask with no stirring. Method 2: slurry-type bioreactor.

The recoveries for the slurry-type bioreactor were 82.15% at 5 ppm mercury and 98.55% at 10 ppm mercury with a moisture content of 50%.

Table 7. Percent Recovery from Non-stir Reactor Using Unautoclaved Soil After 6 Days into the Experiment

Hg Concentration		Soil Moisture Content 20%	30%	50%
5 ppm	Control	75%*	73%	78%
	Sample	80	84	88
10 ppm	Control	73	78	87
	Sample	85	68	73

Control: Soil without added bacteria
Sample: Soil with added bacteria
*: All values in this table were the average of two replicates

CHAPTER VI

CONCLUSIONS

This study found mercury added to soil as mercuric chloride was reduced to metallic mercury by microorganisms in soils of various moisture contents and various mercury concentrations. Mercury-resistant bacteria added to soil samples increased the mercury volatility rate, thus showing that the potential for bioremediation of mercury-contaminated soil does exist.

1. Initially (6 testing days), mercury-resistant bacteria added to mercury-contaminated soil (5 ppm) increased the mercury removal rate significantly ($p<0.001$) compared to the controls without the addition of mercury-resistant bacteria, but they had little effect on final (30 days) mercury content in the treated soil.

2. Mercury removal rate increased with increased soil moisture.

3. The mercury detoxification rate was proportional to the mercury concentration of the soil at a given soil moisture content.

4. There was no evidence that the addition of bacterial nutrient increased the mercury removal rate in a non-stir method.

5. The slurry-type bioreactor is more efficient than the non-stir type. After one month of experiment, about 90% mercury was removed from the soil with a moisture content of

50% and mercury concentration of 10 ppm.

This study indicated that biological detoxification of mercury-contaminated soil can be achieved by using a slurry-type bioreactor with mercury-resistant bacteria added.

CHAPTER VII

SUMMARY AND RECOMMENDATION

This preliminary study showed that mercury-contaminated soil could be detoxified biologically by using mercury-resistant bacteria. The bulk of the mercury (30-65%) was removed from soil in a short period of time (6 days), especially at the high mercury concentration (10 ppm). This was observed from both non-stir and slurry methods. Overall, the slurry-type bioreactor was more efficient than the non-stir bioreactor.

Future research may utilize a pull vacuum over the soil to achieve an increase in mercury removal efficiency. Because nutrient is necessary for bacterial growth, sucrose may be used to increase bacteria numbers in the soil without the addition of sodium citrate dihydrate, which may bind mercury inhibiting complete removal.

A slurry-type bioreactor may be more efficient the percent soil in the slurry is decreased to 20-40%.

CHAPTER VIII

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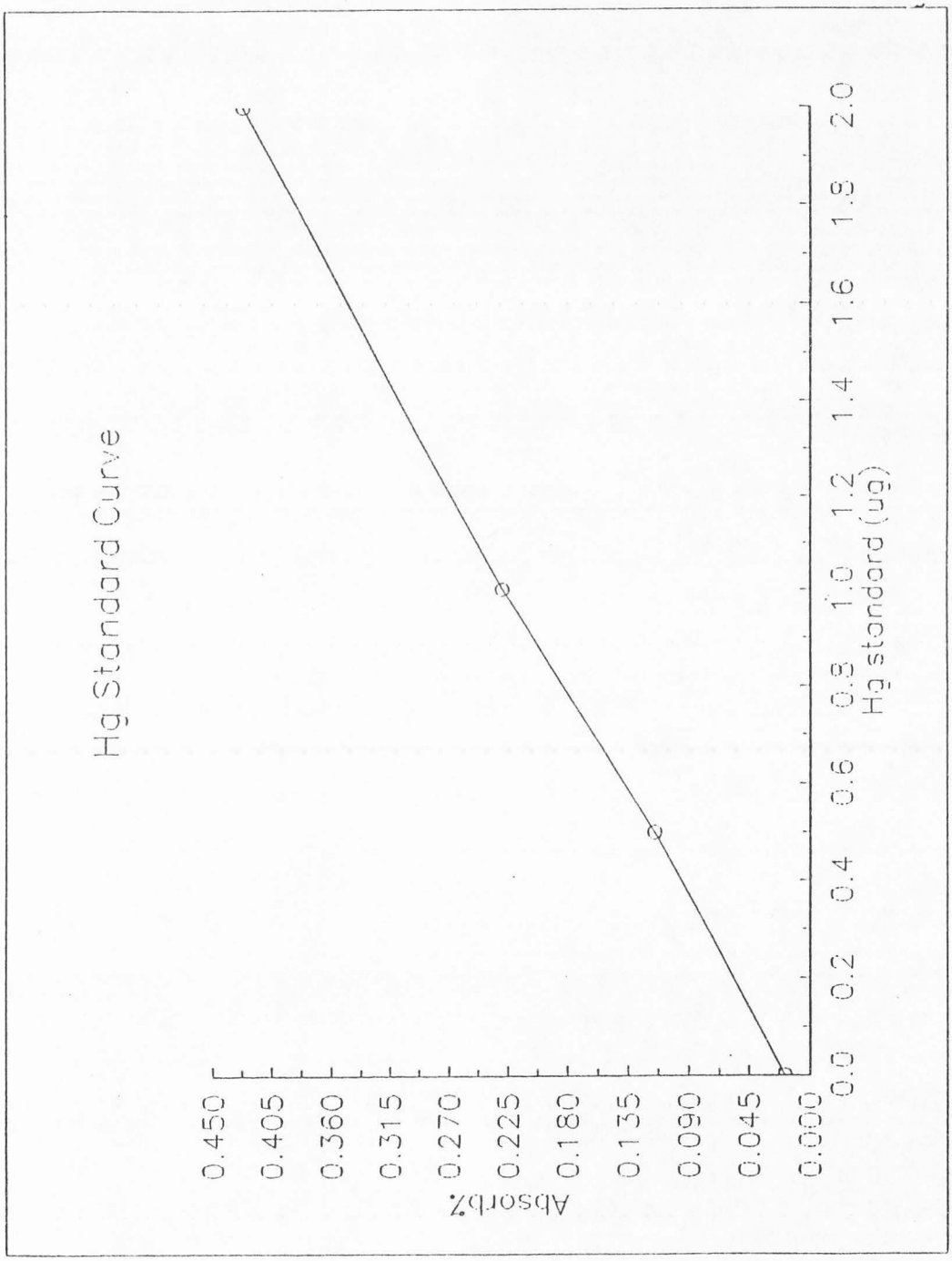
APPENDICES

Appendix A: Mercury Standard Curve



Appendix B: Statistical Calculation of Hg Standards

Std	Trans	Absorb	Con
0.00	97.90	0.01	-0.05
0.50	75.00	0.12	0.50
1.00	56.80	0.25	1.07
2.50	28.50	0.55	2.50

Mean of Dep. Var.	0.23
Number of Obs.	4.00
Number of Missing Obs.	0.00
Total Sum of Squares	0.16
Residual Sum of Squares	0.00
Std. Dev. of Estimate	0.01
R-squared	1.00
Adjusted R-squared	1.00
Degrees of Freedom (df)	2.00
Number of Ind. Vars. (K)	2.00
F(K-1, df)	993.12
Prob. Value of F	0.00
Constant	0.02
Std.	0.21