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LA THÈSE A ÉTÉ
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Microbial Action on Heavy Metals

by

Richard Tak-Cheong Cheng

A Thesis presented to the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in Department of Biology

Ottawa, Ontario, 1979

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ABSTRACT

Speciation of mercury under different conditions was studied.

Conversion of methylmercury by biota which included bacteria and algae, and by organic rich sediment in presence of Ottawa River water was studied. Methylmercury was converted to inorganic mercury by the biota and sediment. The decreasing order of conversion of methylmercury to inorganic mercury was Pseudomonas fluorescens, Anabaena flos-aquae, Escherichia coli, and sediment. The water column in all cases contained low levels of either methylmercury or inorganic mercury. The mechanism seems to be the initial sorption of added methylmercury by the biota or sediment, followed by conversion to inorganic mercury. The conversion rate was proportional to the biomass but varied inversely with the initial methylmercury concentration. For the systems studied here, growing Pseudomonas fluorescens could further volatilize the mercury species. However, thermally killed bacteria (both Pseudomonas fluorescens or Escherichia coli) methylated the inorganic mercury present in the stock solution rather than demethylated the methylmercury.

Speciation of metallic mercury in water and in air above the water column was studied. The effects of added

hydrogen peroxide, of bacterial growth medium, and of both living and dead bacteria (Pseudomonas aeruginosa or Escherichia coli)were tested. The bacteria chosen, either living or thermally killed, transformed metallic mercury from water into air. The amount of Hg^0 produced in the head space of an especially designed experimental chamber was almost the same in living or dead bacterial and hydrogen peroxide systems~7 ppb. Hydrogen peroxide stabilized Hg^{2+} in the aqueous phase and head space. It can be concluded from this study that the nature of the redox couple is important in mercury speciation.

Studies of the binding affinity of mercuric ions and cupric ions onto Pseudomonas aeruginosa indicated that the metal ions were mainly retained on the outer cell wall layer (for Cu^{2+}: cell wall layer, 75.3%; ribosome, 9%; intracellular fluid, 15.7% and for Hg^{2+}: cell wall layer, 78.6%; ribosome, 5.8%; intracellular fluid, 15.6%). The strength of binding of Hg^{2+} ions ($K = 2.3 \times 10^{-1}$, K = Dissociation Constant,) was two orders higher than the Cu^{2+} ions ($K = 9.4 \times 10^{-4}$). In fact, acid leaching experiments indicated that the cell might have proton exchangeable and proton nonexchangeable sites for Hg^{2+} binding sites while only proton exchangeable sites existed for Cu^{2+}.

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Chapter I

INTRODUCTION

1.1 HEAVY METAL POLLUTION OF THE ECOSYSTEM

The toxic effect of heavy metals on living organisms has long been known and considered, in the main, as a problem of occupational health including accidential poisoning of children. High levels of metals such as copper and mercury appear in the environment due to their widespread industrial use. They occur frequently in waste water effluents and have many toxic effects. A recent study in the Trondheimsfjorden area in Norway has shown that copper is one of the major pollutants in this area occurring in the effluents from base mining industries, causing a reduction of the marine fauna and flora (Lande, 1977). Similar results on aquatic communities caused by mercury have also been observed (Briand et al., 1978 ; Harriss, 1971; Rice et al., 1973 ; Sigmon et al., 1977). The selective poisoning of certain species of phytoplankton and not of others leads to an undesirable imbalance in plankton populations. Reduced competition may result in blooms of certain species which are less susceptible to heavy metal poisoning (Briand et al., 1978).

The hazards of heavy metals, especially mercury and its compounds, have been of concern for years. For mercury, the alkylmercurials have been shown to be more toxic than the inorganic forms. The first report on alkylmercurial poisoning caused by environmental pollution occurred in Japan, in the vicinity of Minamata Bay in 1953-6 and in Niigata in 1965 (Fujiki and Tajima, 1969; Krenkel, 1973; Matida and Kumada, 1969). It was proved that methylmercury accumulated by fish in Minamata Bay was discharged into the bay by an acetaldehyde plant (Fujiki and Tajima, 1969). Reports of Minamata disease for some animals such as cats have been reported in several areas in Quebec and Ontario, Canada (National Academy of Sciences, 1978; Takeuchi et al., 1977) and other parts of the world.

Another major occurrence of organic mercury poisoning was noted among the bird populations in Sweden in 1955 (Krenkel, 1973). A decrease of wild birds during the 1950's was found to be significant and some species were near extinction. Evidence showed that the increase of mercury level had an adverse effect on the bird population, and this prompted Sweden to prohibit the use of alkylmercury in seed dressings in 1966.

Excessive concentrations of mercury in Canadian wildlife were also reported in 1969 and major attention was also focussed on the fish population (Krenkel, 1973). The rise in mercury content was correlated with industrial water pol-

lution as well as the increased usage of organomercuric compounds in agriculture.

1.2 CHEMISTRY OF MERCURY

Mercury is the only metallic element existing in liquid state at room temperature. It has an atomic number of 80 and atomic weight of 200.59. It exists in three oxidative states: Hg^0, Hg^{1+}, and Hg^{2+}.

Metallic mercury (Hg^0) is a soft acid according to the Lewis concept of hard and soft acid-base (Jensen, 1974; Williams, 1972). Metallic mercury is soluble up to about 30 ppb (ug per liter) of water at room temperature. Solubilities of mercury and its salts depend upon the anionic species and the Eh-pH condition. In the mercurous state, it exists as dimeric cations in metal-metal pairs or in ionic bonds with its salts while the mercuric ion forms covalent compound (such as mercuric chloride) with most of its salts. A characteristics reaction of mercurous salts is the disproportionation involving simultaneous oxidation and reduction to form mercuric ion and elemental mercury.

$$Hg_2^{2+} \rightleftharpoons Hg^0 + Hg^{2+} \qquad (1)$$

The volatilization of elemental mercury may shift the equilibrium to the right. Interconversion of these three inorganic forms (Hg^0, Hg^{1+}, and Hg^{2+}) can also be mediated by microorganisms (Wood, 1974).

Organic mercury, such as methylmercury, has a great lipid solubility. The covalent nature and the dissociation constant of several forms of X in CH_3HgX are known: the pK for RS^- is 17; for I^-,9; for Br^-,7; and for Cl^-,5.7 (Petering and Tepper, 1976), thus it is not suprising that alkyl mercurials are bound strongly to the sulfhydryl groups. However, the pool of nitrogenous ligands, as well as Cl^- and OH^- will compete with RS^- for Hg^{2+}, as well as $ArHg^+$ (Ar=Aryl) and RHg^+ (R=Alkyl) in biological tissues.

1.3 SOURCES OF MERCURY POLLUTION

Two major sources of mercury contamination of the ecosystem include: 1) Natural sources and 2) Man made sources.

1.3.1 Natural Sources

Mercury is dispersed widely on the earth's crust at a concentration 0.08 ppm (Krenkel, 1973). It is found in rocks of all classes, especially the igneous and sedimentary rocks (Jonnasson and Boyle, 1972). It occurs in many forms in nature, and the most important primary mineral is cinnabar (HgS). Since mercury is present in various forms and various strata, a general cycle is involved wherein the mercury is transported to the hydrosphere from the lithosphere, through geochemical leaching and natural degassing as well as through biological activity. The pools of mercury in na-

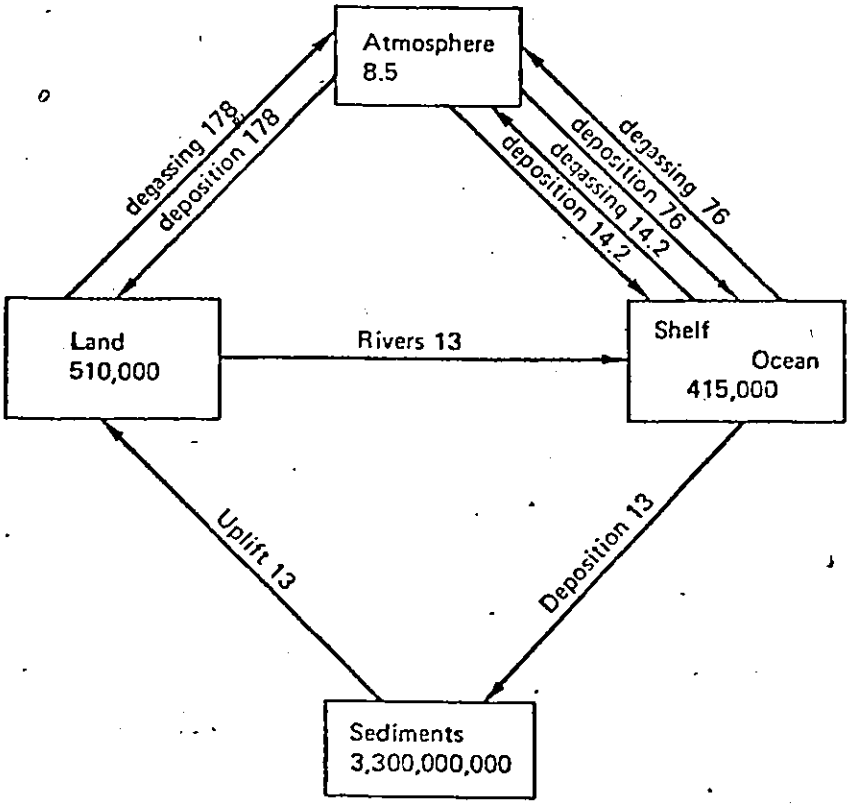
tural global cycling without involvement of human activity is shown in Fig.1.

The atmosphere plays an important role in the process of mercury mobilization, since lithosphere or hydrosphere-to-atmosphere flux is several times greater than those occurring between the lithosphere and hydrosphere. The lithosphere or hydrosphere -to-atmosphere flux is mainly in the form of the elemental mercury vapor, whereas the flux between lithosphere and hydrosphere involves inorganic divalent mercury associated with dissolved or particulate (particle size equal or greater than 0.45 μm) organic matter.

Relationships between mercury flux are shown in Fig.1. mercury flux between either lithosphere or hydrosphere and atmosphere is measured as degassing rate which ranges from 0.0014 to 10 $\mu g/M^2/day$. The degassing of mercury to the atmosphere from the lithosphere is higher than from oceans. Mercury in the atmosphere is removed via precipitation at the rate of 0.06 $\mu g/M^2$ /day. This deposition rate is only for polar, open ocean and oceanic shelf areas while the rate for the continents remains to be evaluated (National Academy of Sciences, 1978).

Figure 1: Tentative global cycle of mercury without Involvement of Human Activity.

Fluxes in unit of 10^8 gm Hg/yr.; pool masses in unit of 10^8 gm Hg.
Source: Modified from Wollast et al (1975) as described by National Academy of Sciences, 1978).


Atmosphere
8.5
degassing 178
deposition 178
degassing 76
deposition 76
degassing 14.2
deposition 14.2
Land
510,000
Rivers 13
Shelf
Ocean
415,000
Uplift 13
Deposition 13
Sediments
3,300,000,000

The residence time of mercury for the various compartments has been calculated to be 2.5 x 10^8 year for sediments, 3,200 years for oceans, 1,000 years for soils and 11 days for atmosphere (National Academy of Sciences, 1978).

1.3.2 Man Made Sources

The other major source of mercury contamination comes from human activities. Table 1 shows the amount of mercury consumed in metric tons over the years 1970-1973 in USA. It also projects the amount for the year 1985 which is more or less close to the figure for the year 1970. This table also lists the major users of mercury such as in chloralkali plants, electrical apparatus industry, etc. Table 2 shows the emissions of mercury from human activities into air, water and land in USA in 1973. A total of about 1,500 metric tons of mercury was lost from all industrial operations of which 31% was to air, 6% to water and 63% to land. More or less equal amounts of mercury were released by natural degassing processes which account for the substantial atmospheric mercury burden (National Academy of Sciences, 1978).

1.4 MERCURY IN VARIOUS COMPARTMENTS IN THE ECOSYSTEM

1.4.1 Mercury in The Atmosphere

The natural sources of mercury in air are soil vola-

TABLE 1

Mercury Consumption in United States, 1970-1973, and Projected for 1985

End Use Activity	1970	1971	1972	1973	1985
			(Metric tons)		
Agriculture	62.3	50.8	63.2	62.9	24.1
Catalysts	77.0	34.3	27.5	23.2	17.2
Dental preparations	78.6	64.4	102.6	92.2	129.0
Electrical apparatus	548.7	572.6	535.0	619.2	662.0
Caustic chlorine	516.4	421.5	396.2	449.6	481.6
Laboratories	62.1	46.7	20.4	22.6	?
Industrial instruments	166.2	134.4	225.0	246.1	447.2
Paints	355.9	296.0	282.8	261.5	106.6
Pharmaceuticals	23.7	23.0	19.9	20.8	17.2
Others	209.3	170.0	147.2	69.1	206.4
Total (rounded)	2100	1814	1820	1867	2091

Source: From National Academy of Sciences (1978).

TABLE 2

Mercury Losses in United States in 1973

Activity Description	Total Losses to			Total Hg lost	Total Hg recycled
	Air	Water	Land		
	(Metric Tons)				
Hg mining, smelting	7.85	0	0.42	8.27	0
Other mining activities	50.71	3.07	4.91	58.09	0
Energy activities	104.5	2.18	42.07	148.73	0
Manufacturing and processing	26	21.72	271.33	319.05	20.66
Commercial, industrial and consumer use	282.2	60.73	646	990	126.11
Total	471.26	87.70	964.73	1524.73	146.77
Sewage	4.01	19.92	22.88	46.0	
Natural degassing	1018.7			1018.7	

Source: From National Academy of Sciences (1978).

tilization, biological and anthropogenic inputs, and the suspended dust in air. The annual natural flux of mercury to the atmosphere from both terrestrial and oceanic sources is around 30,000 metric tons per year, besides the 20,000 metric tons from anthropogenic sources (National Academy of Sciences, 1978).

Depending on the degree of urbanization, background atmospheric mercury concentrations varying between 0.001 and 50 ng/M^3 are common. Elemental mercury is the major form, especially near geothermal and volcanic areas. Johnson and Braman (1974) developed a technique for distinguishing different forms of mercury vapor in air. Their data indicated that dimethylmercury was rarely detected in the atmosphere, but other forms were present in varying amounts. Mercury associated with particulate matter accounted for 4% of total atmospheric mercury, the other mercury vapor species were distributed as follows: Hg(II) 25%; Methylmercury 21%; Metallic mercury 49% and dimethylmercury 1%.

In mineralized areas, mercury occurs as cinnabar (HgS). Cinnabar, when mined, releases mercury which may undergo transformation to other forms into the environment.

In soil, the background mercury concentration level is usually between 10 and 150 ppb. However, higher mercury levels are associated with soil containing high humus or organic fraction.

1.4.2 Mercury in The Hydrosphere

Lakes, rivers and oceans of the world are the key modes of transport of mercury in the environment.

The normal background levels of mercury in natural waters are ranged from 0.03 to 0.2 ppb and are generally less than 0.1 ppb. The mercury content of natural waters is summarized in Table 3.

$HgCl_2$ or $Hg(OH)_2$ predominates in fresh waters while about 80% of the inorganic mercury in the sea water occurs as $HgCl_4^{2-}$ (Andren and Harriss, 1975). Hem and others (Gavis and Ferguson, 1972; Hem, 1970; Krenkel, 1974; Wollast et al., 1975) have constructed the Eh-pH stability diagrams for the aqueous mercury and concluded that almost all mercury in natural waters which are low in chloride or other complexing ligands should exist as Hg^0. In well oxygenated waters, metallic mercury may exist as Hg^{2+}. In moderately oxidizing to mildly reducing waters, metallic mercury, mercurous or mercuric species may predominate. In reducing condition, elemental mercury, HgS or HgS_2^{2-} may predominate. However, many other kinetic factors can change the above equilibria. For example the association with organic compounds, chelating agents or particulate matter may stabilize the mercuric forms.

Andren and Harriss (1975) observed a strong association between mercury and dissolved organic matter in fresh

TABLE 3

Mercury in Natural Waters (ppb)

Description	Range	Mean
Rainwater	0.05 - 0.48	0.2
Coal mine waters (Donets Basin, USSR)	1 - 10	
Normal stream, river, and lake waters	0.01 - 0.1	0.03
Stream and river waters near mercury deposits	0.5 - 100	
Oceans and seas	0.005 - 5.0	0.1
Hot springs and certain mineral waters	<0.01 - 50	0.1
Normal ground waters	0.01 - 0.1	0.05
Ground waters and mine waters near polymetallic sulfide deposits	1 - 1000	
Oil field and other saline waters	0.1 - 230	

Source: From D'Itri (1972).

interstitial and estuarine water and suggested that mercury could be removed in such forms in the transitional zone between fresh and salt water. Fitzgerald and Lyons(1973) have also reported 50-60% of the dissolved mercury in the coastal waters would associate with organic matter of unspecified composition. The nature of the association is not well understood at present although functional groups such as SH, COOH and N are assumed to be responsible.

By analysis of the mercury contents of great lakes waters, Chau and Saitoh (1973b) have found that significant amount of mercury can not be extracted by the dithizone solution from non oxidized water samples. This suggests that mercury might be strongly bound to suspended particulate matter. Smith et al (1971) have also reported that about 82-97% of mercury in the tidal water of the Thames River is on suspended matter. In the Ottawa River, 58% of all mercury transported downstream is on suspended matter (Kudo et al (1978).

Humic compounds are the most widely occurring natural complexing agents. Harriss (1971) has reported that a large fraction of the total mercury in natural water system exists in sediment as a humate or mineral complex. However, the actual effect of metal chelation on the biological cycling of toxic elements in the environment is not well understood (Saxena and Howard, 1977).

The nature of this dissolved organic matter and/or detrital organic matter on mercury cycling has been studied. Andren and Harriss (1975) have reported that fulvic acid in nature with molecular weight less than 500 has the highest mercury complexing ability. Ramamoorthy and Kushner's group (Mckenzie et al., 1976 ; Ramamoorthy and Kushner, 1975 and 1977b) have determined that the molecular weight of the binding component in Ottawa river is equal or less than 500 but claimed that fulvic acid is not the major binding component. It seems likely that different sources of fulvic acid may have different mercury binding capability.

The binding of mercury to sediments has been reported in literature. Loring (1975) has observed that in the St. Lawrence River, mercury is associated with the organic fraction of the fine grade sediment. The study of the Ottawa River agrees with this finding (Ramamoorthy and Rust, 1977). Thus the evaluation of the partition coefficients of mercury associated with this organic rich sediments has an important ecological significance in predicting the mercury distribution in the ecosystem (Kudo et al., 1977a, b and 1978 ; Miller et al., 1977).

Sorption of mercury by suspended solids in natural waters might contribute to the survival of phytoplankton species. The level of dissolved mercury in water will depend on the amount of suspended solids. This might result in the decrease of mercury uptake from water per unit of algal cell with the increase of suspended solids in water.

In order to understand more of the behaviour of mercury in the aquatic environment, the Ottawa River system has been under intensive study by Canadian scientists for the past five years (Environmental Management Service, 1977; Federal-Provincial Working Group on Water Quality in the Ottawa River, 1978; Ramamoorthy and Kushner, 1977a). Several significant findings have been reported. Firstly, it has been found that only the top 4 cm layer of bed sediment is active in mercury transport. However, the role of the bed sediment movement on mercury transport is insignificant although 96.7% of the total mercury and 97.8% of the total methylmercury is in bed sediment. It is only the bulk of water itself, as well as the suspended solids in water that plays a significant role in mercury transport because of their enormous mass. Secondly, the biota in Ottawa River contains 1 % of the total mercury distributed in the study area. It has been reported that the aquatic biota might concentrate the mercury from surrounding water thousands of times. Mortimer(et al., 1977) reported that the vascular aquatic plants have the ability to accumulate mercury from surrounding waters, but the uptake is not via roots as shown by analysis of different plant parts. The accumulation of mercury by different plant parts except root indicates that rooted plants may be the best indicators of mercury pollution in flowing water. Thirdly, the uptake of the methylmercury by fish seems to occur mainly from the water column

but not through the fish's invertebrate food. This indicates the importance of the water vector. Fourthly, sorption of mercury to sediments is rapid and most of the mercury is associated with the organic fraction of the suspended solids or sediments.

1.5 INTERCONVERSION OF MERCURY IN AQUATIC ENVIRONMENT

Sorption of mercury by sediments is rapid. The fine suspended solids undoubtedly play an important role in providing a large surface area for the initial sorption of mercury from solution, followed by sedimentation to the river bottom. The organic matter on sediments is a greater collector of mercury as well as suitable medium for its retention. Subsequently, the mercury might undergo interconversion to other forms, and some of these species might be released into the water column and bioaccumulated into organisms.

Figure 2 shows the interconversion of mercury in the aquatic environment. This figure concentrates on the movement of methylmercury and the bioaccumulation in fish and shellfish. The lower left hand part of this figure is amplified in Fig. 3 which indicates the methylmercury and demethylation reaction in natural waters.

Figure 2: Mercury cycle demonstrating the bioaccumulation of mercury in fish and shellfish.

Source: Modified from National Academy of Sciences, 1978.

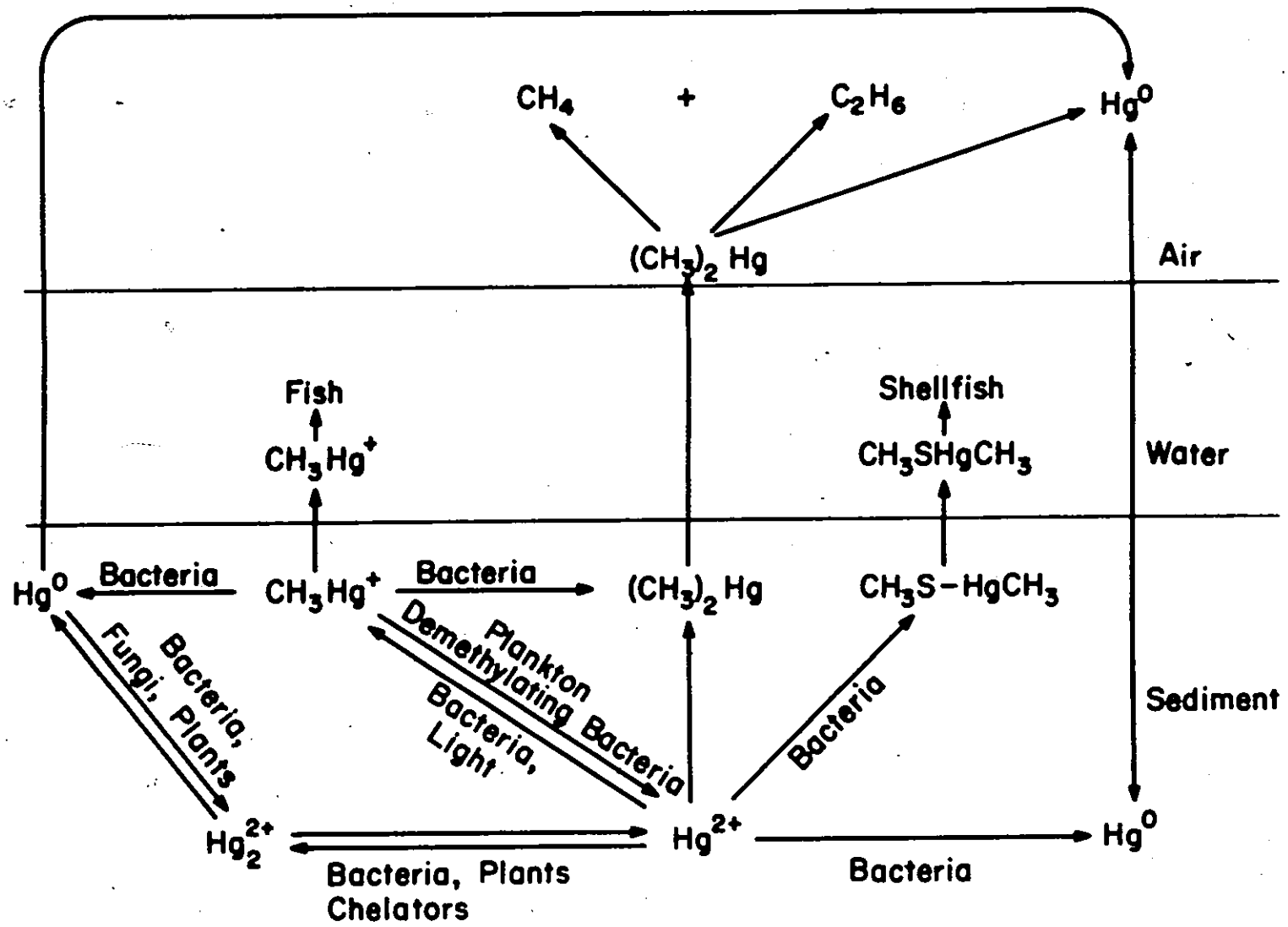
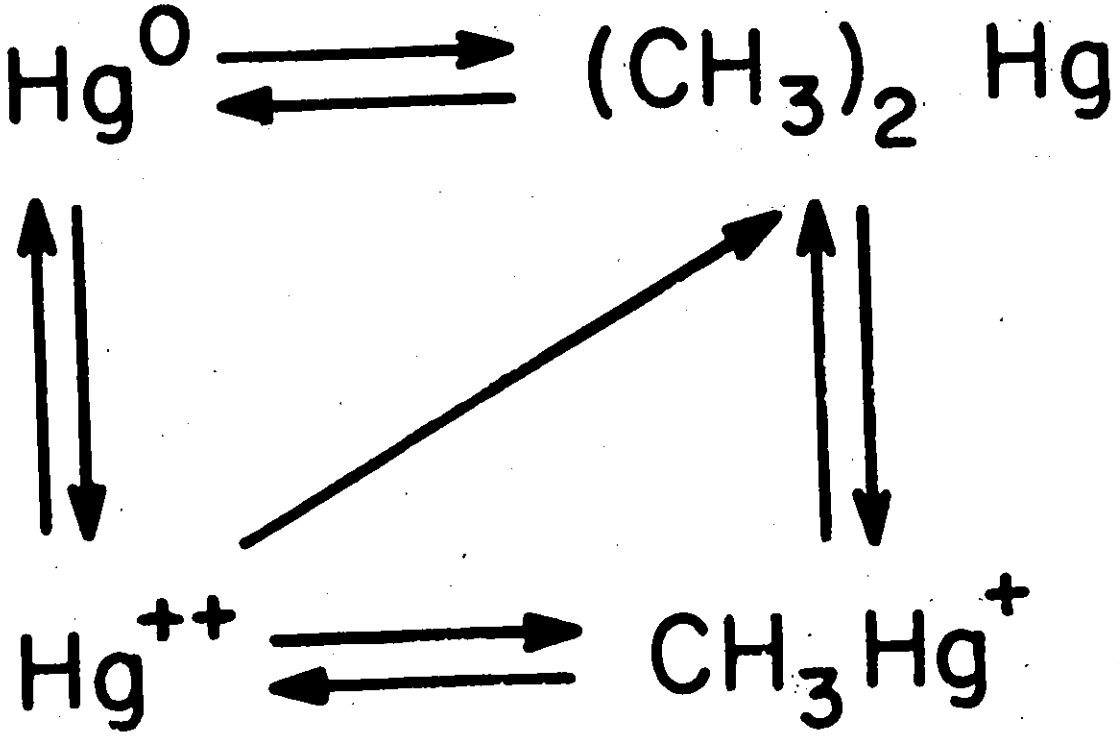

CH_4 + C_2H_6
Hg^0
$(CH_3)_2Hg$
Air
Fish
CH_3Hg^+
Shellfish
CH_3SHgCH_3
Water
Hg^0
Bacteria
CH_3Hg^+
Bacteria
$(CH_3)_2Hg$
$CH_3S-HgCH_3$
Bacteria, Fungi, Plants
Plankton Demethylating Bacteria
Bacteria, Light
Bacteria
Sediment
Hg_2^{2+}
Hg^{2+}
Hg^0
Bacteria, Plants Chelators
Bacteria

Figure 3: A hypothetical model of the conversion of mercury in the aquatic environment.

Source: Modified from D'Itri, 1972.


Hg^0
$(CH_3)_2$ Hg
Hg^{++}
CH_3Hg^+

1.6 METHYLATION OF MERCURY IN NATURAL WATERS

In the environment, methylation of mercury can be mediated by both nonbiological and biological processes. Biological methylation can be of two types, enzymatic and nonenzymatic.

Rogers (1977) has found that an alkaline extract from soil can methylate mercury. The methylating component is organic in nature and low in molecular weight ($\leq$ 12,000). This study is significant in identifying an abiological methylating component from environmental samples.

Studies (Akagi and Takabatake, 1973; Brinckman and Iverson, 1975; Jewett and Brinckman, 1975; Springthorpe, 1977) have indicated clearly that photolysis might have an important role in mercury transformation in the environment. Acetic acid, propionic acid, methanol and ethanol are present, at least transiently, in aquatic ecosystems as end products of microbial metabolism. These products can photochemically induce methylation of inorganic mercuric ions. Acetic acid, a common fermentation product was an efficient donor of methyl radical, yielding about 0.1% methylmercury, when the aqueous solution of mercuric chloride (2×10^{-2}M), in the presence of acetic acid (1×10^{-1}M), was irradiated with ultraviolet ray at room temperature in an experimental period of twenty hours (Akagi et al., 1972).

Methylation of mercury may require the presence of a methyl donor and there are many chemicals in nature to ful-

fill this requirement. A well studied methyl donor is methylcobalamin, a methyl derivative of vitamin B12. Wood et al(1968) have demonstrated its relvence to methylmercury production. They found that cell free extracts of a methanogenic bacterium can use methylcobalamin for methane production, but in the presence of mercuric ions, this enzymatic reaction is inhibited and the methyl group is transferred to mercuric ions by a non enzymatic reaction. This study also indicates that at very low mercury concentration, dimethylmercury is the ultimate product of the methyl transfer reaction, whereas at higher mercury concentrations, monomethylmercury is formed. Monomethyl mercury is formed by a two step process with dimethylmercury as the intermediate. The existence of this nonenzymatic processes has been confirmed by other workers (Imura et al., 1971).

The possibility of transmethylation from one metal to another has been shown. Jewett and Brinckman (1975) previously determined that Me_3Sn^+ easily undergoes loss of one methyl group to form $MeHg^+$ quantitatively from Hg^{2+} as shown by the following equation:

$$Me_3Sn^+ + Hg^{2+} \xrightarrow{H_2O} Me_2Sn^{2+} + MeHg^+ \qquad (2)$$

$(Me=CH_3^-)$

Aquatic microorganisms can also participate in this process. Pseudomonas species, isolated from Chesapeake Bay,

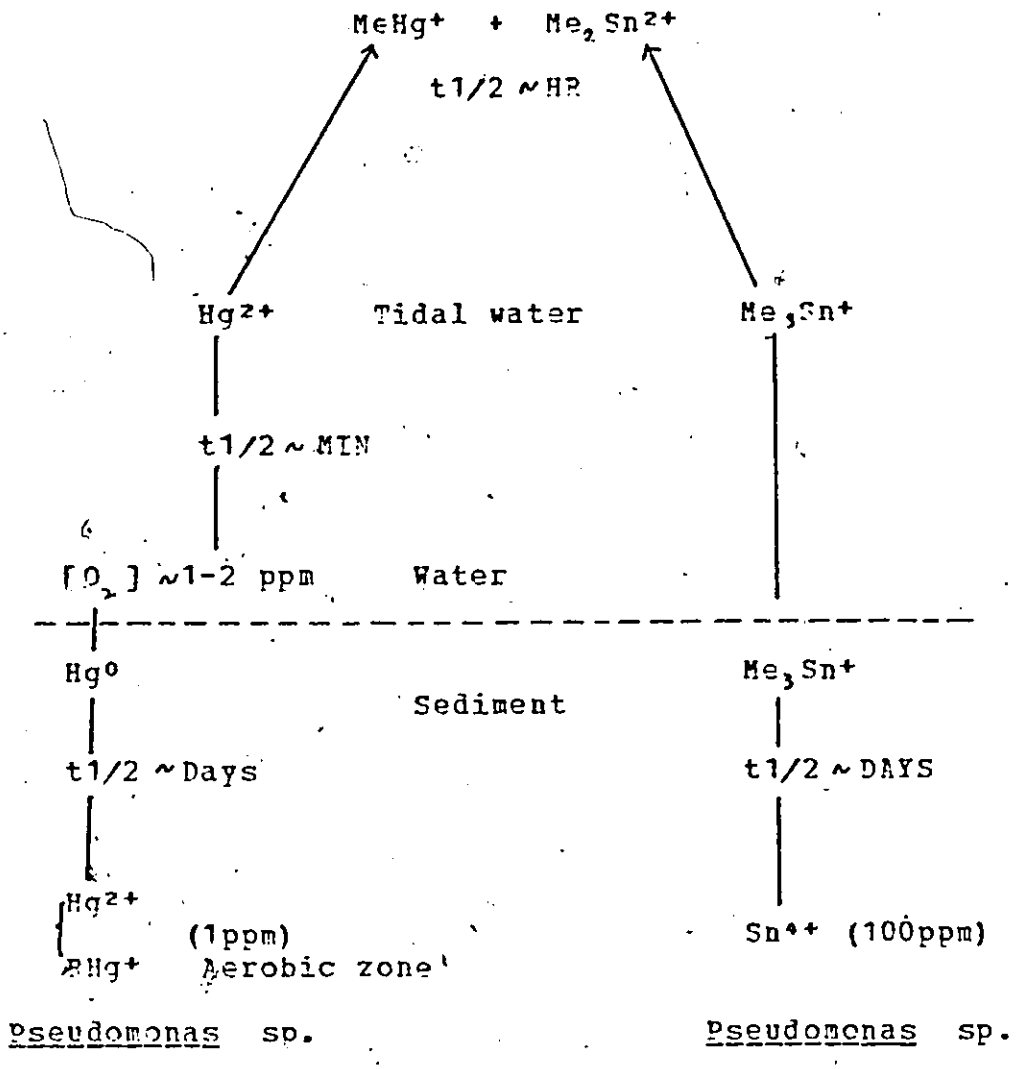
USA., can release mercury from sediments and also produce a methylated tin species which is capable of subsequently methylating available Hg^{2+} by an abiotic mechanism. A bimetallic model for the formation of methylmercury which has been demonstrated, with the known metal concentrations involved and estimated half life (t1/2) of tin and mercury for various transport processes is given in Fig. 4 (Brinckman and Iverson, 1975).

Thus it appears that the sediment bound mercury can undergo transmethylation involving anthropogenic alkylated pollutants. This process involves the biotic release of both mercury and tin from the sediment matrix into water, followed by transmethylation between the biomethylated tin species, Me_3Sn^+ and Hg^{2+} (Huey et al., 1974).

Mercury associated with sediments is commonly in the form of mercuric sulfide. The release of methylmercury from sediment depends on the oxidation of the sulfide moiety to sulfate. Jernelov's group (Pagerstrom and Jernelov, 1971; Jernelov, 1968 and 1972) have observed the formation of methylmercury from mercuric sulfide in sediments by aerobic bacterial activity. This finding is important since mercuric sulfide is common in ores and sediments. Brinckman and

Figure 4: Bimetallic model for the formation of methylmercury.

Source: From Brinckman and Iverson (1975).


MeHg+ + Me2 Sn2+
t1/2 ~HR
Hg2+
Tidal water
Me3Sn+
t1/2 ~MIN
[O2] ~1-2 ppm
Water
Hg0
Sediment
Me3Sn+
t1/2 ~Days
t1/2 ~DAYS
Hg2+
RHg+
(1ppm)
Aerobic zone
Sn4+ (100ppm)
Pseudomonas sp.
Pseudomonas sp.

Iverson (1975) have shown a correlation between sulfur and mercury content which increases proportionally with sediment depth in mercury contaminated areas. Since a free form of sulfur is present, a substantial bacterial sulfur cycle is suggested as shown in Fig. 5.

As shown in Fig. 5, under anaerobic conditions, sulfide ion is formed from the aqueous sulfate (pathway A). The mechanism of the oxidation reaction shown in pathway B has not been elucidated.

Not all sedimentary mercury is bound. Some labile mercury might exist at certain depths within the sediment matrix which is not insolubilized by large excesses of sulfide ion in interstitial waters. Although the molecular form of this labile mercury is not yet known, several estuarine facultative or anaerobic microorganisms isolated from these sites are capable of forming methylmercury from inorganic mercury(II) (Blair et al., 1974).

Enzymatic methylation processes of mercury are also reported in literature. Jensen and Jernelov (1969) have first reported the conversion of inorganic mercury to methylmercury in aquarium sediment, and later by microorganisms. Since then, numbers of researchers have studied the role of microorganism on methylation of mercury. The following table (Table 4) is the summary of the microbial

Figure 5: Outline of estuarine sulfur cycle.

Source: From Brinckman and Iverson (1975).

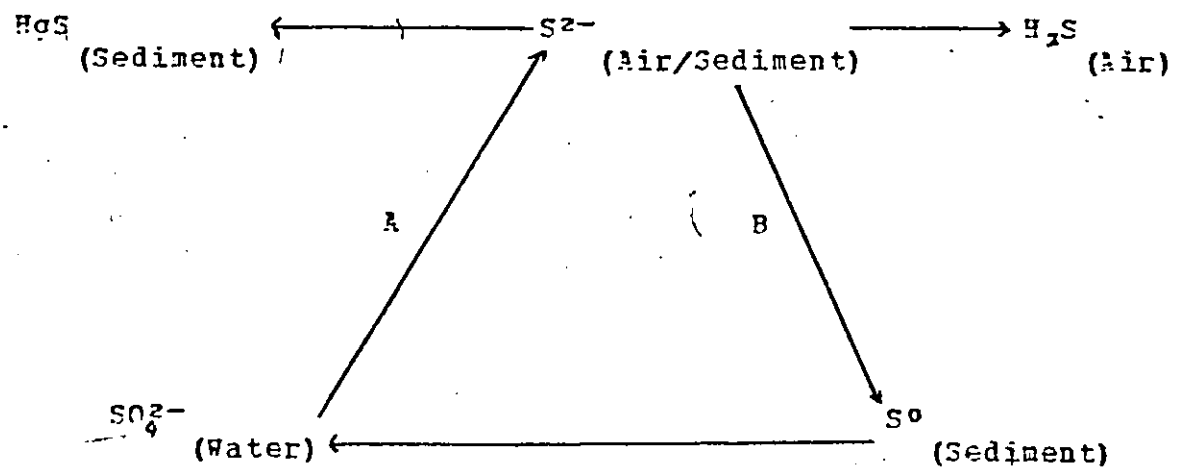

MnS
(Sediment)
S2-
(Air/Sediment)
H2S
(Air)
A
B
SO4 2-
(Water)
S°
(Sediment)

TABLE 4

Summary of Microbial Methylation Reported in the Literature

Author	Culture Condition		Mercury added (ppm)	% Conversion to MMC	
Edwards & McBride (1975)	mixed (human faeces)	anaerobic (1-6 days)	0.1 1.4	0.5 in 2 days 0.3 in 2 days	
Nandy (1977)	Enterobacter aerogenes	Aerobic (10 days)	50.0	cells 0.029	medium 0.05
		Unaerated (10 days)	50.0	0	0
Nandy & noyes (1975)	pure Enterobacter aerogenes			cells	medium
		3 days	50.0	0.4	0.5
		10 days	50.0	0.02	0.03
		20 days	50.0	0.26	0.09
Jensen & Jernelov (1969)	mixed (aquaria sediment)	aerobic ? (7 days)	0.1 1.0 5.0 10.0 50.0 100.0 500.0 1,000.0	- 1.0 0.8 0.5 0.2 0.1 0.01 0.001	
Landner (1971)	pure Neurospora crassa	aerobic (4 weeks)			
	original strain		40.0	0.6	
	Hg resistant mutant		40.0	0.2	
	original strain		80.0	0.1	
	Hg resistant mutant		80.0	0.1	

Rowland et al (1975)	puro strep-tococci	aerobic (44 hrs.)	5.0	0.05 -0.12
	Staph-ylococci		5.0	0.01 -0.1
	E. coli		5.0	0.02 -0.06
	yeasts		5.0	0.015-0.03
		annerobic (44 hrs.)		
	Lactobacilli		5.0	0.01
	BacterDides		5.0	0.01
Yonk & Sijpesteijn (1973)	pure Pseudomonas fluorescens	aeobic (7 days)	5.0	0.006
	Mycobacterium phlei		5.0	0.0015
	E. coli		5.0	0.004
	Enterobacter aerogenes		5.0	0.001
	Aspergillus niger	(14 days)	100.0	0.15
	Scopulariopsis brevicaulis		100.0	0.12
Yamada & Tonomura	puro Clostridium cochlearium	anaerobic (5 days)	10.0	0.35
			20.0	0.3
			30.0	0.15
			40.0	0.02

MMC=Methylmercury

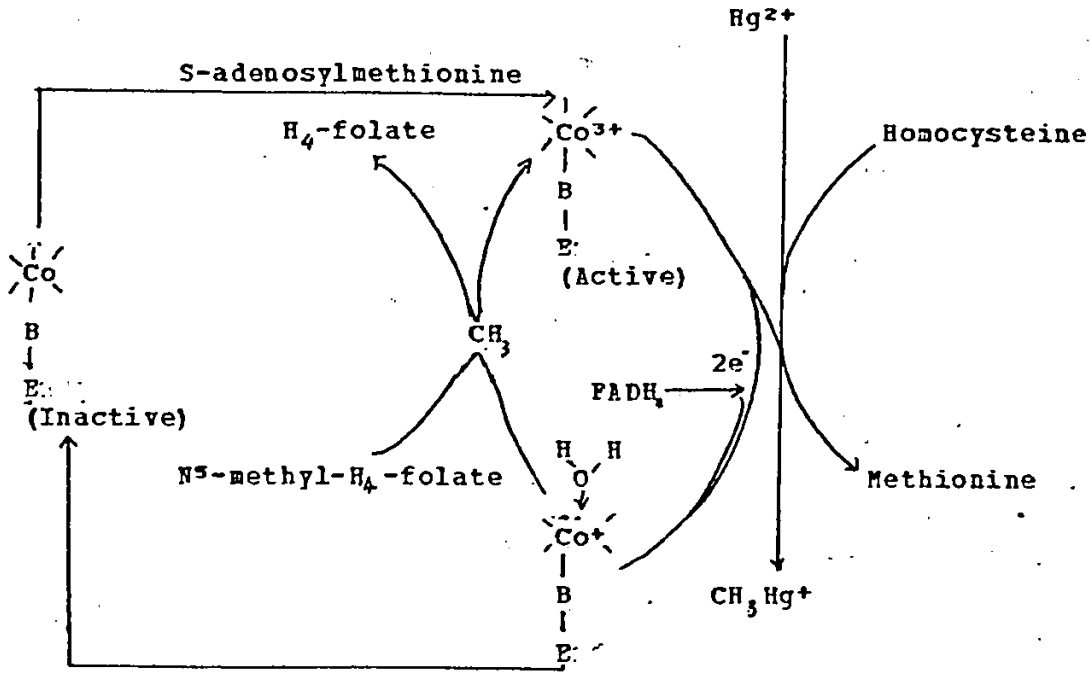
methylation reported in literature. This table indicates that methylation of mercury can be caused by many different microorganisms, is non specific, and can occur under both aerobic and anaerobic conditions.

The mechanism of enzymatic methylation of mercury is based on the reaction of mercuric ion with vitamin B12 (such as methylpentacyanocobaltate) and an appropriate enzyme system (Vonk and Sijpesteijn, 1973). So far, three coenzymes are involved in biomethylation reactions : 1) S-adenosylmethionine, 2) N^5-methyltetrahydrofolate derivatives and 3) methylcorrinoid, or methylcobalamin (Bartlett et al., 1977 ; Vallee and Ulmer, 1972; Wood, 1974). The first two are incapable of methyl group transfer to Hg^{2+} because for both of them, the methyl group is transferred in carbonium (CH_3^+) form. Therefore, the only known methylating agent capable of methyl transfer to Hg^{2+} is methylcorrinoid because it can generate methyl groups of carbanion CH_3^- , radical $CH_3\cdot$ in addition to carbonium ion CH_3^+.

Methylcobalamin can be isolated from microorganisms, and in its presence, enzymatic and nonenzymatic synthesis of the alkylated mercury occur. Bacteria, containing a cobalamin dependent N^5-methyltetrahydrofolate homocysteine transmethylase (methionine synthetase) (Taylor and Weissbach, 1969) can synthesize methylmercury. As shown in Fig. 6, methylcobalamin is the coenzyme here and is activated by S-a-

Figure 6: Enzymatic methylation of mercury.

B: 5,6-dimethylbenzimidazole
E: Methionine synthetase


Hg2+
S-adenosylmethionine
H4-folate
Co3+
B
E
(Active)
Homocysteine
Co
B
E
(Inactive)
CH3
2e-
FADH2
N5-methyl-H4-folate
H H
O
Co+
B
E
Methionine
CH3Hg+

denosylmethionine. The methyl group from this activated methylcobalamin is transferred to homocysteine, the enzyme-coenzyme complex is remethylated by the N^5-methyltetrahydrofolate (Burke et al., 1970). In presence of Hg^{2+}, electrophilic attack on CH_3^- of the enzyme-coenzyme complex should yield methylmercury, and the aquocobalamin enzyme complex which should be stored by reduction of Co^{3+} to Co^{1+} with $FADH_2$, is followed by remethylation from N^5-methyltetrahydrofolate (Landner, 1971; Vallee and Ulmer, 1972; Wood, 1971; Yamada and Tonomura, 1972). Methylmercury interacts with homocysteine to give methylmercurihomocysteine.

In summary, the production of methylmercury is determined by a number of factors including the microbial community, amount of soluble mercuric ions and nature of methylmercury group donors. Under many experimental conditions, the production of CH_3Hg^{1+} is usually less than 0.1% of the added mercury. Once methylmercury is formed and is released into water, it may be removed by bioaccumulation. Recently, it was reported that methylmercury can disfuse rapidly to the cells of higher organisms (e.g. fish) in a very short time (20×10^{-9} sec) (National Academy of Sciences, 1978). Since methylmercury is absorbed rapidly by biota (Batti et al., 1975 ; Saxena and Howard, 1977), this can explain why there is no detection of methylmercury in the Great Lakes waters while the other forms of mercury range from 0.13-0.18

ppb (Chau and Saitoh, 1973a and b). The following diagram (Fig.7) shows the bioaccumulation and concentration factors of methylmercury by aqueous species.

1.7 THE DESORPTION OF METHYLMERCURY

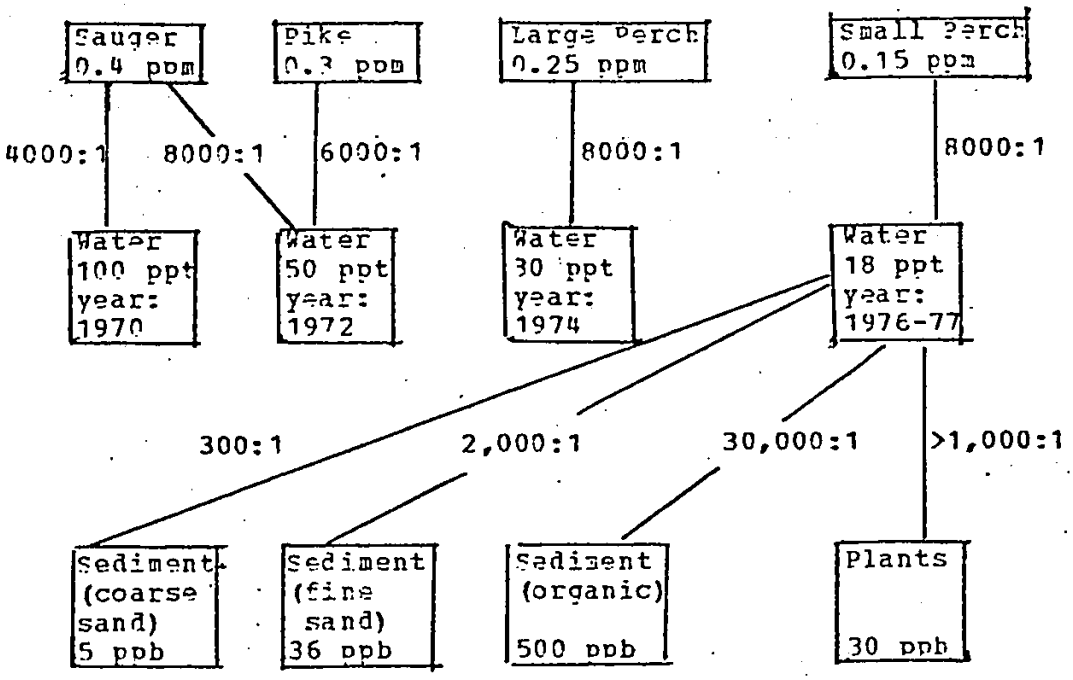
The desorption of methylmercury is governed by several factors such as pH, the organic content of the sediment/water, and microorganisms, although such processes are not necessarily related to methylmercury formation (Alberts et al., 1974 ; Matsumura et al., 1972 ; Shin and Krenkel, 1976).

In alkaline conditions, dimethylmercury is stable, but it is converted to monomethylmercury in acidic condition at pH $\leq$ 4.5. Jacobs and Keeney (1974) have observed differences in methylmercury production in sediments from different sources which are believed to vary in hydrogen ion concentrations and chemical composition.

Matsumura et al (1972) observed that some ligands such as EDTA and glutathionine, as well as glucose may increase the release of mercury from sediment to water. Ramamoorthy and Rust (1977) reported that sodium chloride and NTA have the same effect. It is believed that these results occur because 1) the EDTA and NTA are strong chelating agents, 2) glutathionine has the SH group that can bind mercury

Figure 7: Equilibrium values of mercury concentrations in different compartments of Ottawa River.

Source: Modified from Miller et al 1977.


Sauger
0.4 ppm
Pike
0.3 ppm
Large Perch
0.25 ppm
Small Perch
0.15 ppm
4000:1
8000:1
6000:1
8000:1
8000:1
Water
100 ppt
year:
1970
Water
50 ppt
year:
1972
Water
30 ppt
year:
1974
Water
18 ppt
year:
1976-77
300:1
2,000:1
30,000:1
>1,000:1
Sediment
(coarse
sand)
5 ppb
Sediment
(fine
sand)
36 ppb
Sediment
(organic)
500 ppb
Plants
30 ppb

strongly, and 3) chloride ion can form covalent complexes with mercury and 4) glucose can enhance microbial growth.

Jernelov (1970,1972) has reported that macroorganisms (tubificids) can release methylmercury to the water column from mercury enriched layers of sediment to a depth of 3 cm. On the other hand, freshwater mussels (Anodonta) can release mercury from a depth of 9 cm. It is believed that these species can release methylmercury by physical disturbance of the sediment. Bottom feeders can ingest the mercury containing sediment and move it up in the food chain.

1.8 THE DEGRADATION OF METHYLMERCURY

The alkylation of mercury has been intensively investigated but there are only a few reports on interconversions of such compounds. Because of the significant industrial and agricultural uses in the earlier years, the degradation of phenylmercuric acetate has been studied and it has been found to be degraded into benzene and metallic mercury by several aquatic organisms including green algae, guppy and snail (Pang, 1973) or by aquatic and soil microorganism (Matsumura, 1971; Nelson et al., 1973 ; Tonomura and Kanzaki, 1969). However little attention has been paid to the stability of methylmercury in natural waters. Kudo et al (1977) have reported that methylmercury in sand sediments might be degraded more rapidly than methylmercury from organic rich sediment. Within 25 days, 80% of the added me-

thylmercury is broken down in sand sediment, while only 14% is broken down in organic sediment. Billen et al (1974) have also observed that bacterial communities in sediment can degrade methylmercury which might reach an equilibrium with inorganic mercury. Spangler et al (1973a and b) have observed the biodegradation of methylmercury in mixed cultures of bacteria from sediments. The degradation reached 50% within 5 days.

1.9 AIMS OF THIS THESIS

In this thesis, experiments were designed to study the stability of methylmercury in aquatic environment under different conditions. The studies included the behavior of methylmercury in deionized water, Ottawa River water, microbial growth medium, organic rich sediment, biota in microbial growth medium as well as in deionized water. Organic rich sediment was chosen for this study because such sediment may be considered a source of mercury which can release mercury species into natural waters, where the methylmercury is bioaccumulated and moved up in the food chain. Pseudomonas fluorescens, Escherichia coli, as well as Anabaena flos-aquae, the common microorganisms in natural waters, were studied as possible biological mediators.

In addition to the study of stability of methylmercury in waters, the second part of this thesis deals with the transformation of metallic mercury in the aquatic envi-

ronment. metallic mercury was chosen because of its intensive industrial and laboratory uses; its high vapor pressure, high lipophilicity and toxicity to organisms; the little study of its fate in natural waters and also because it is a common pollutant in environment. The transformation of metallic mercury in water was thus studied. Mercury speciation in air overlying the water/metallic mercury system was also investigated, using Pseudomonas aeruginosa, and Escherichia coli as biological mediators.

The toxicity of mercury to organisms is due to its strong binding affinity to the cell surface and to intracellular fluid. Experiments were thus designed to study the affinity of mercuric ions for bacterial cells (Pseudomonas aeruginosa) as well as cellular locations of the mercuric ions. For comparsion, cupric ion was studied in the same way. The cupric ion was chosen as the reference for the study because 1) it is an environmental pollutant 2) it occurs in nature as sulfide ores similar to mercury 3) the coordination chemistry of copper is intermediate between covalent and ionic bonding and 4) technically, analysis of copper by atomic absorption spectrophotometry is convenient and accurate.

Chapter II

MATERIALS AND METHODS

Transformation of mercury, as with other environmental contaminants, is generally studied by following the formation of metabolites and endproducts. Volatility of some intermediate compounds and sorption onto particulate matter and surfaces of glassware may present a problem in low level detection. Thus the accuracy of the experimental results depends to a great extent upon the type and treatment of the glassware used in the experiments as well as the analytical methods employed.

All glassware except those contaminated with radioactive mercury, was washed with nochromix sulfuric acid. Glassware contaminated with radioactive mercury was soaked in 10% nitric acid for 48 hours. All the acid washed glassware was then rinsed thoroughly with distilled deionized water, and dried at 110°C for 48 hours.

2.1 PREPARATION AND STORAGE OF METAL STANDARDS

The standard mercuric nitrate (10,000 ppm in Hg) was prepared by dissolving 1.7131 gm of mercuric nitrate crystal (Fisher) in a minimal amount of nitric acid in a 100 ml volumetric flask, and made up to volume with distilled deionized water.

The standard cupric nitrate solution (6,354 ppm in Cu) was prepared as above with 2.4160 gm of cupric nitrate (Baker).

Other mercury stocks were obtained from commercial sources, these included mercuric chloride (1,000 ppm in Hg) (Fisher SO-M-114); metallic mercury (Engelhard, Canada); methylmercuric chloride (1,000 ppm in Hg) (Alfa ventron); methyl^{203}mercuric chloride (83 ppm in Hg) (New England Nuclear).

All stocks, except radioactive mercury, were kept in glass flasks or bottles and stored in the refrigerator at 4°C. The radioactive mercury was kept in a flask enclosed in lead blocks and kept at room temperature.

Stocks were kept in high concentration so that some losses by sorption to the glass walls did not appreciably change the total concentration.

All working standards were prepared fresh by dilution of the stocks for each experiment.

2.2 HEAVY METAL ANALYSES

The following methods were used to analyse mercury and copper species in this study.

2.2.1 Atomic Absorption Spectrophotometry (AAS)

2.2.1.1 Mercury Analyses

One of the conventional methods used for mercury analysis is Flameless Atomic Absorption Spectrophotometry (FAA). Hatch and Ott (1968) first introduced this technique. This method is based on the reduction of mercury salts in solution to the elemental form, which is then released by purging the solution with nitrogen. The liberated elemental mercury is then carried through a calcium chloride trap to remove water vapor. The elemental mercury is then passed through a quartz absorption cell positioned in the path of the monochromatic mercury line (2537 Å) emanating from a mercury hollow cathode lamp.

The conventional principle mentioned above is only for the detection of inorganic mercury. The detection of aryl and alkyl mercury present in samples is also possible since it has been found that organic mercury can be reduced directly with stannous chloride in the presence of sodium hydroxide and cadmium ion (Magos, 1971) or cupric ion (Matsunaga and Takahashi, 1976). However the organic mercury mentioned here and later refers only to methylmercury since it is the most predominant among organic forms of mercury in the environmental and biological samples. Magos (1971) introduced the method for detection of organic mercury in 1971 by adding stannous chloride, cadmium chloride and sodium hydroxide to the acidified biological samples for determination of total mercury. In the presence of stannous chlo-

ride-cadmium chloride reagents, the C-Hg bond is cleaved and both inorganic and organic mercury are reduced. The heat of neutralization of the acidic solution by sodium hydroxide increases the rate of reduction and release of volatile mercury. Magos' differential method is currently used in many laboratories for the analysis of total and organic mercury (Greenwood et al., 1977 ; Shishido and Suzuki, 1974).

In summary, the analysis of mercury may be represented by the following equations:

$$Hg(0) \longrightarrow Hg(0) \qquad (3)$$

$$Hg(0) + Hg(II) \xrightarrow{Sn^{++}} Hg(0) \qquad (4)$$

$$Hg(Inorganic) + Hg(Organic) \xrightarrow[NaOH]{Sn^{++}\ Cd^{++}} Hg(0) \qquad (5)$$

When the total mercury, inorganic mercury and elemental mercury are determined separatedly, by difference, the amounts of different forms of mercury in the sample are known.

Analytical Procedures by FAA

In this work, mercury analysis by FAA techniques were only performed for the study of transformation of metallic mercury in aquatic systems (Section 2.5) and for heavy metal ions binding onto Pseudomonas aeruginosa (section 2.6). Although organic mercury, if present, might be analysed by

Magos' differental method, prelminary analyses revealed the absence of organic mercury in these two studies (Section 2.5 and 2.6), hence the amount of inorganic mercury present represented the total mercury. The analytical methods for the mercuric ion as well as the elemental mercury are described as follows:

Jarrell Ash 801 AAS (Atomic Absorption Spectrophotometer) was used. A mercury analyzer apparatus (Jarrell Ash 82-731) (Fig.8) was installed for mercury analysis. The apparatus consists of a glass reaction vessel with a ground glass part of the aerator. The aerator is equipped with 2 three way glass stopcocks which manipulate the flow of nitrogen gas.

For actual mercury analysis, 2 ml of solution to be tested was pipetted into the reaction vessel and 0.3 ml freshly prepared 20% stannous chloride in concentrated hydrochloric acid was added. The vessel was then sealed immediately to the aerator, stopcock 1 was opened to let nitrogen flush in and the flow was adjusted to 2.5 psi. By opening stopcock 2, the liberated elemental mercury from the solution was passed through the calcium chloride trap and carried to the absorption cell which was mounted on the AAS burner head. After passing through the absorption cell, the gas was finally released to the atmosphere in the fume hood. The absorption peak as shown by the maximum reading on the

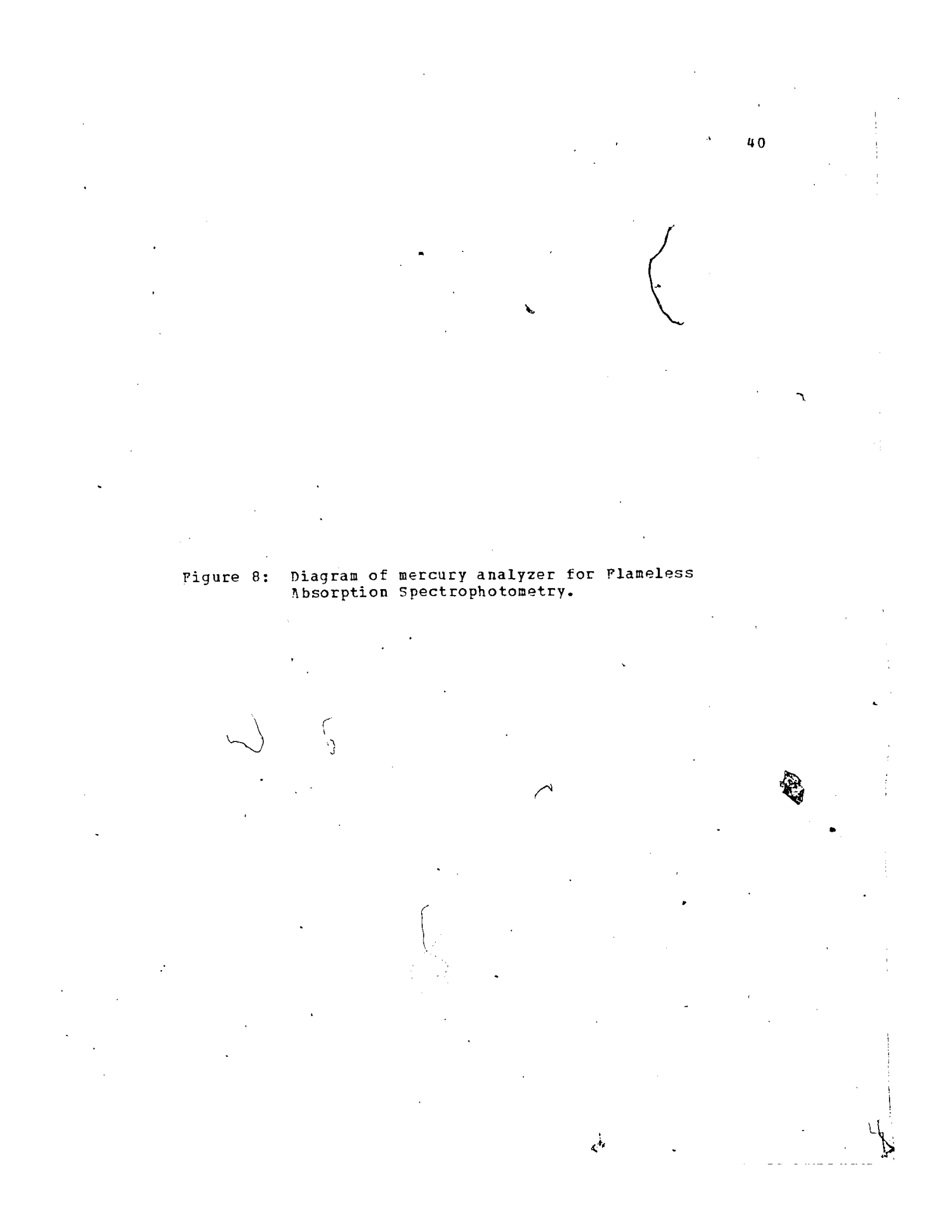
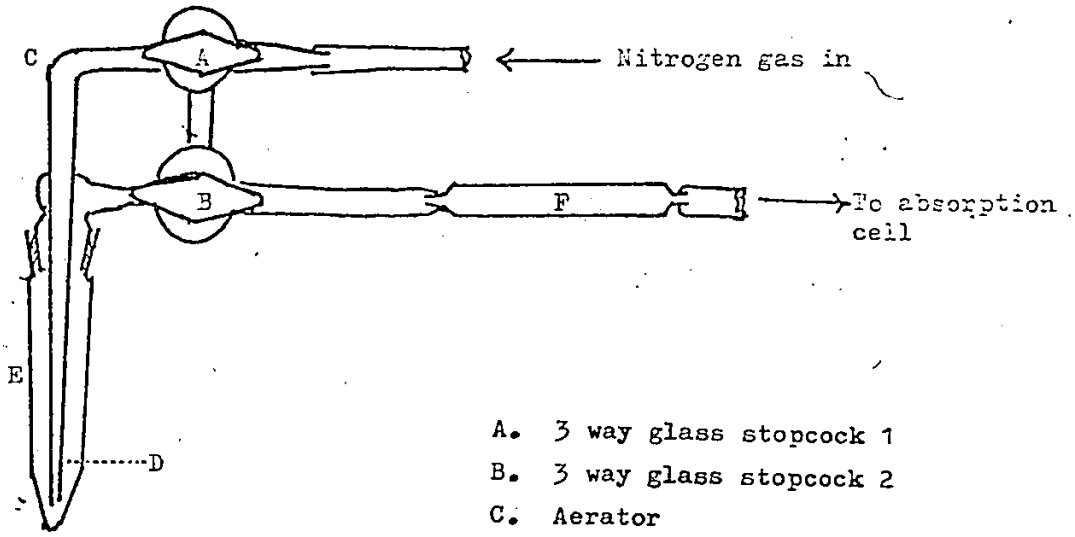


Figure 8: Diagram of mercury analyzer for Flameless Absorption Spectrophotometry.



A. 3 way glass stopcock 1
B. 3 way glass stopcock 2
C. Aerator
D. Aerator tip
E. Reaction vessel
F. Calcium chloride trap

screen of AAS was recorded. When the absorption reading dropped down to background level, the flow of nitrogen to the vessel was shut off by closing stopcocks 1 and 2. The vessel was then removed and the aerator tip was rinsed with deionized water.

Before the next test was started, a clean empty vessel was sealed with the aerator tip and flushed with nitrogen gas for about 10 minutes to ensure that no residual mercury vapor was left in the system.

A calibration curve of the standard was recorded before and after each set of sample assays, and a typical curve is shown in Fig. 9.

The measurement of elemental mercury present in the solution was done by the procedures described above except that 0.3 ml of concentrated hydrochloric acid, instead of the 20% stannous chloride solution was added to the test solution.

2.2.1.2 Copper Analyses

Copper compounds present in solution can be detected by conventional AAS. Unlike the measurement of mercury, copper metal was excited directly by an air/acetylene flame and absorbed at the resonance line 3,247 Å. By this method, only the total copper was detected. The calibration curve is shown in Fig. 10.

Figure 9: Calibration curve of mercury by technique of Flameless Atomic Absorption Spectrophotometry.

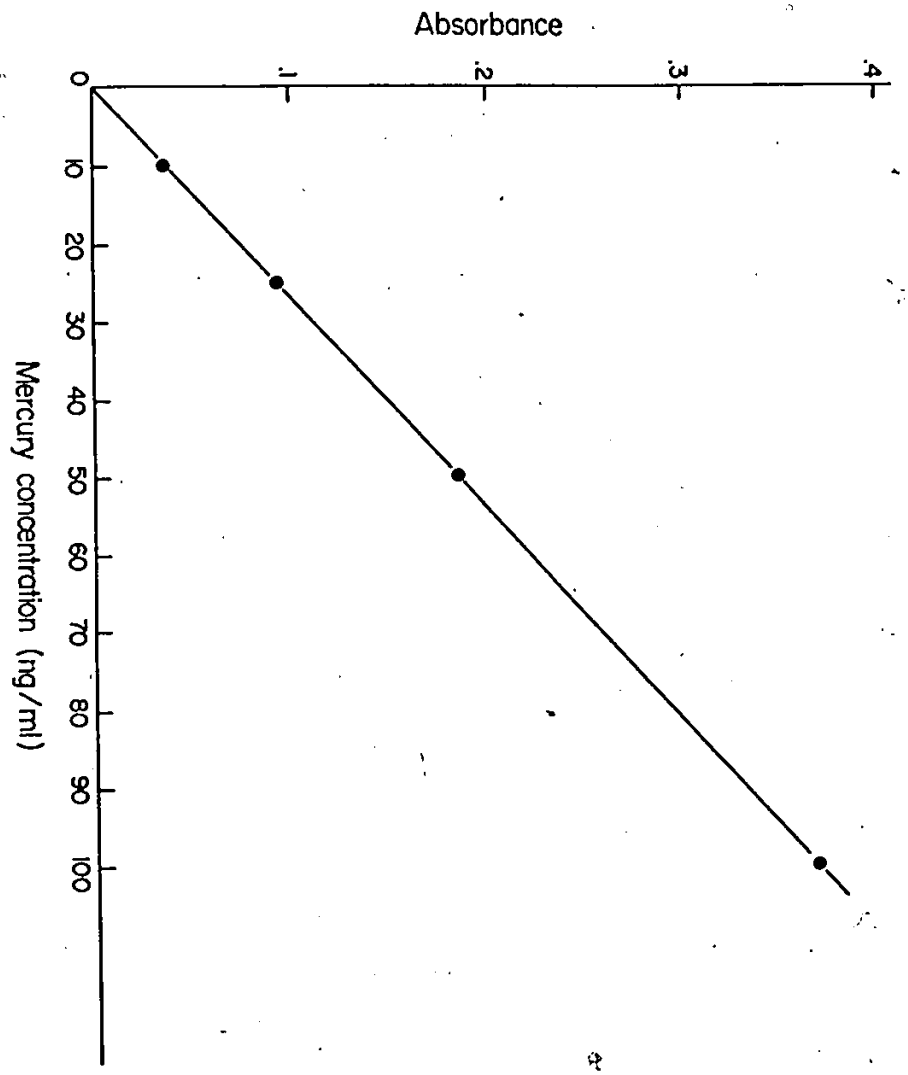
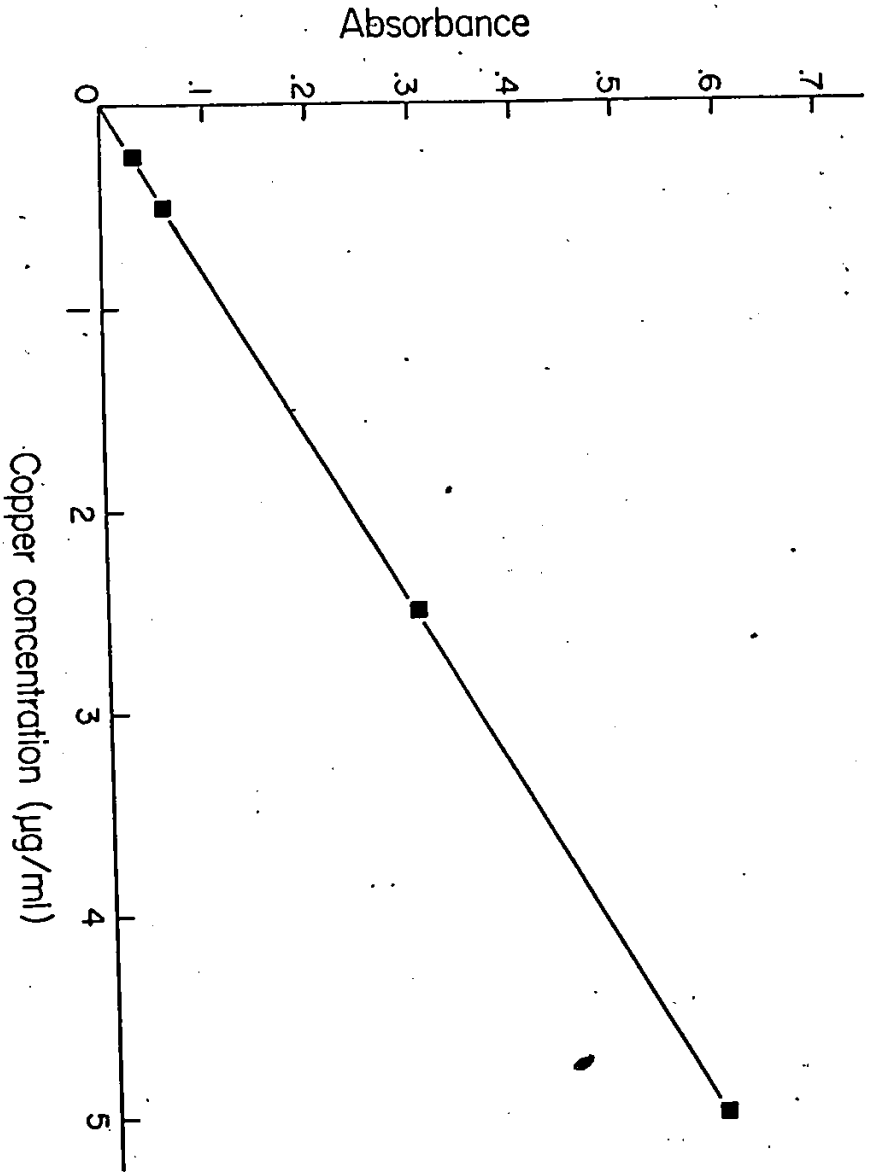

Absorbance
0
1
2
3
4
Mercury concentration (ng/ml)
0
10
20
30
40
50
60
70
80
90
100

Figure 10: Calibration curve of copper by technique of Atomic Absorption Spectrophotometry.


Absorbance
0
.1
.2
.3
.4
.5
.6
.7
Copper concentration (µg/ml)
1
2
3
4
5

2.2.2 Thin Layer Chromatography (TLC)

Only the principle of this method is mentioned here. Details of the analytical procedures of the application of these techniques are disscussed later in section 2.4.3 of this chapter.

The Thin Layer Chromatography (TLC) used in this study is for the separation and detection of inorganic (mercuric) and organic (methylmercuric) mercury present in environmental samples. The water sample is first acidified, then oxidized with potassium permanganate. Sedimental and microbial samples, on the other hand, are first treated with ethanolic potassium hydroxide in order to break down lipids and denature proteins. Mercury species in the samples are then extracted with 0.1% dithizone-benzene solution. Any lipid or fatty acid, present in the extract, can be removed by passage through a florisil-sodium sulfate column. Excess water in the extract is removed by sodium sulfate, and ammonium hydroxide removes the excess dithizone present in the extract. The extract is then dried by nitrogen and the residue is redissolved in a minimal amount of acetone. The acetone soluton is then applied on the TLC sheet (Polygram Sil N-HR) and is developed with a mixture of benzene and hexane mixture (1:1 in V/V) as solvent. Good separation of inorganic mercuric and organic methylmercuric compounds is achieved. When radioactive mercury compounds are present, the radioactivity of each mercury species can be monitored

by Gamma (γ) counting and the quantities can be estimated. Total radioactivity of mercury before, after dithizone extraction and after TLC development are usually consistent, and around 100% recovery is generally obtained.

The techniques of the combination of dithizone extraction, Thin Layer Chromatography separation, and radioactivity counting are recently developed and are suitable for the identification and quantification of mercuric and methylmercuric compounds in environmental samples.

Details of the analytical procedures of the application of these techniques are discussed in section 2.4.3 of this chapter.

2.3 GROWTH OF MICROORGANISM

The microorganisms used for this study were: Escherichia coli B/r, Pseudomonas aeruginosa NRCC5005, Pseudomonas fluorescens, and the blue green algae: Anabaena flos-aquae.

The Escherichia coli B/r was kindly provided by Dr. Iyer, Microbiology Department, University of Ottawa, Ottawa, Canada. Pseudomonas aeruginosa NRC5005 was obtained from National Research Council, Ottawa, Canada. Pseudomonas fluorescens was supplied by Carolina Biological Supply Company, Carolina, USA., while Anabaena flos-aquae was provided by Dr. Stanier, Paris, France.

2.3.1 Growth of Bacteria

Bacteria were maintained as stock cells on a semi solid medium as described by Breuil and Kushner (1975) at 4°C. Except where otherwise noted, the bacteria were grown in complex Peptone Yeast Extract Broth (PYEB) as decribed by Breuil et al (1978) and harvested. Before being cultured for experiments, stock cells were first grown for 48 hours in PYEB. Then a 1 ml aliquot was transferred to 100 ml of fresh PYEB medium. This later culture served as preculture and was incubated until an O.D. of 0.8 (measured at 610 nm) was reached, then 2 ml of this preculture was transferred to 100 ml of fresh PYEB medium in a 500 ml sidearm flask. Cell cultures were shaken at 100 rpm in an incubator shaker (New Brunkswick Scientific Inc. 3,002,895) at 37°C except for Pseudomonas fluorescens which was incubated at 25°C. The culture flask was fitted with an 18 mm sidearm so that the growth of bacteria, measured by monitoring the optical density (O.D.) at 610 nm on a spectrophotometer (Coleman Junior II), could be measured without removing the samples.

When the culture just reached the stationary phase, the cells were centrifuged at 0°C at 10,000 x g (Sorvell RC2-B) for 10 minutes. The cell pellets were washed three times with distilled deionized water and the wet weight of the cells was determined. Cells were then dried at 110°C until a constant weight was obtained (dry weight). The wet weight and dry weight ratio was calculated.

2.3.2 Growth of Blue Green Algae

Growth of *Anabaena flos-aquae* was basically the same as for the bacteria, with the following modifications: The stock organisms were maintained and grown in GO medium as described by Stanier *et al* (1971). The algae were incubated at 25°C on a gyratory shaker (New Brunswick SW) at 100 cycles per minute. Illumination of 1300 Lux intensity for 16 hours each day was provided by fluorescent light. Growth was monitored at 650 nm, and algal cells were harvested by centrifugation at 4,500 x g for 12 minutes at 0°C.

2.4 STABILITY OF METHYLMERCURY

2.4.1 In Natural Waters

The stability of methylmercury in natural waters was studied by adding known amounts of unlabelled and labelled methylmercuric chloride to 100 ml of fresh Ottawa River water contained in a stoppered 125 ml Erylenmeyer flask. The final concentration of mercury in the system was 0.1 ppm except where otherwise specified. Flasks were incubated at 25°C on a rotary shaker (Robbins and Muers) at 40 cycles per minute. The effect of adding sediment and different microorganisms were studied. Ten gm wet weight of organic rich clay type sediment (Ramamoorthy and Kushner, 1977a and b, Ramamoorthy and Rust, 1977) from which larger particles and debris had been removed was sampled from the Ottawa River and added to the water in the flask. Viable microor-

ganism studied here were Pseudomonas fluorescens, Escherichia coli as well as Anabaena flos-aquae. An amount of 0.45 gm wet weight (except where otherwise specified) of cell mass added to the water in the flask. The effect of thermally killed bacteria on the stability of methylmercury in the river water was also studied. In this case, 0.45 gm (wet weight) of Pseudomonas fluorescens or Escherichia coli was killed by boiling in the river water at 100°C for 30 minutes. The death of cells were confirmed by microscopic observation of motility as well as by the streak plate technique using Peptone Yeast Extract Agar (PYEA) (Peptone, 2%; Yeast Extract, 0.15%; and Agar, 1.25%; pH = 7). Controls with deionized water were also set up.

In order to set up experimental conditions similar to the environment, no extreme sterilization techniques were carried out. Samples were drawn at specific time periods for the determination of the stability of methylmercury as a function of time. For viable bacterial samples, surviving bacteria at various time intervals were counted by plating technique using Peptone Yeast Extract Agar (PYEA).

2.4.2 In Microbial Cultures

The stability of methylmercury in bacterial cultures was also studied. After trials, 10% and 50% of the diluted autoclaved PYEB media in 100 ml aliquots in 500 ml sidearm flasks were used. Labelled and nonlabelled methylmercuric

chloride were added to the culture three hours after introducing a 2% aliquot of the preculture of the bacteria: Pseudomonas fluorescens or Escherichia coli. The final concentration of methylmercury was 0.1 ppm. By plotting the growth curves, it was shown that this level of mercury did not interfere with growth of bacteria. Samples were withdrawn at specific time intervals for the determination of the stability of methylmercury as a function of time. A control of the sterilized medium alone was also set up.

2.4.3 Analyses of Mercury Species

2.4.3.1 Extraction of Mercury From Water Sample

The stability of methylmercury present in these systems was analysed by the combination of dithizone extraction, Gamma counting, and TLC techniques. The principles for the TLC techniques have been mentioned earlier in Section 2.2.2 of this chapter.

Mercury was extracted from water system, including deionized waters, river waters, sterilized PYEB media, water columns overlying sediments or biota, as well as the cell free supernatants of PYEB media. The water columns overlying sediments were withdrawn and filtered through 0.45 μm filters (Millipore Filter HA). The time of filtration was short, usually less than 2 minutes. The filter papers were put into glass counting vials and the radioactivity was monitored by Gamma counter (Nuclear Chicago Inc.,

1085-3-D4-R2-00-L3-00-00-00-0). Results indicated that the amounts of mercury absorbed onto the filter papers during filtration were insignificant. Water columns overlying biota or cell free supernatants of PYFB media were withdrawn and centrifuged at 10,000 x g for 10 minutes at 0°C.

The procedures for the extraction and analyses of mercury by these techniques are outlined in Fig. 11. An aliquot of 5 ml of water sample was pipetted into a counting vial and monitored for radioactivity. Another 50 ml of the water sample was pipetted into a ground glass stoppered volumetric flask and diluted to 100 ml with deionized water. The water was then acidified with 2 ml 2.4 N hydrochloric acid and oxidized with drops of 0.1 M potassium permanganate solution until pink color was persistent in the water sample. A minimal amount of hydroxylamine sulfate was then added to neutralize the color of the solution. After all these pretreatments, mercury in the water sample was extracted with 5 ml of 0.1% dithizone-benzene solution (appendix 1) by shaking with a mechanical wrist shaker (Burrell BB) for 10 minutes, and the aqueous layer was then discarded. To the dithizone-benzene solution, a minimal amount of anhydrous sodium sulfate was then added to remove any residual water.

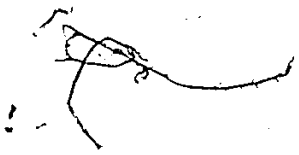
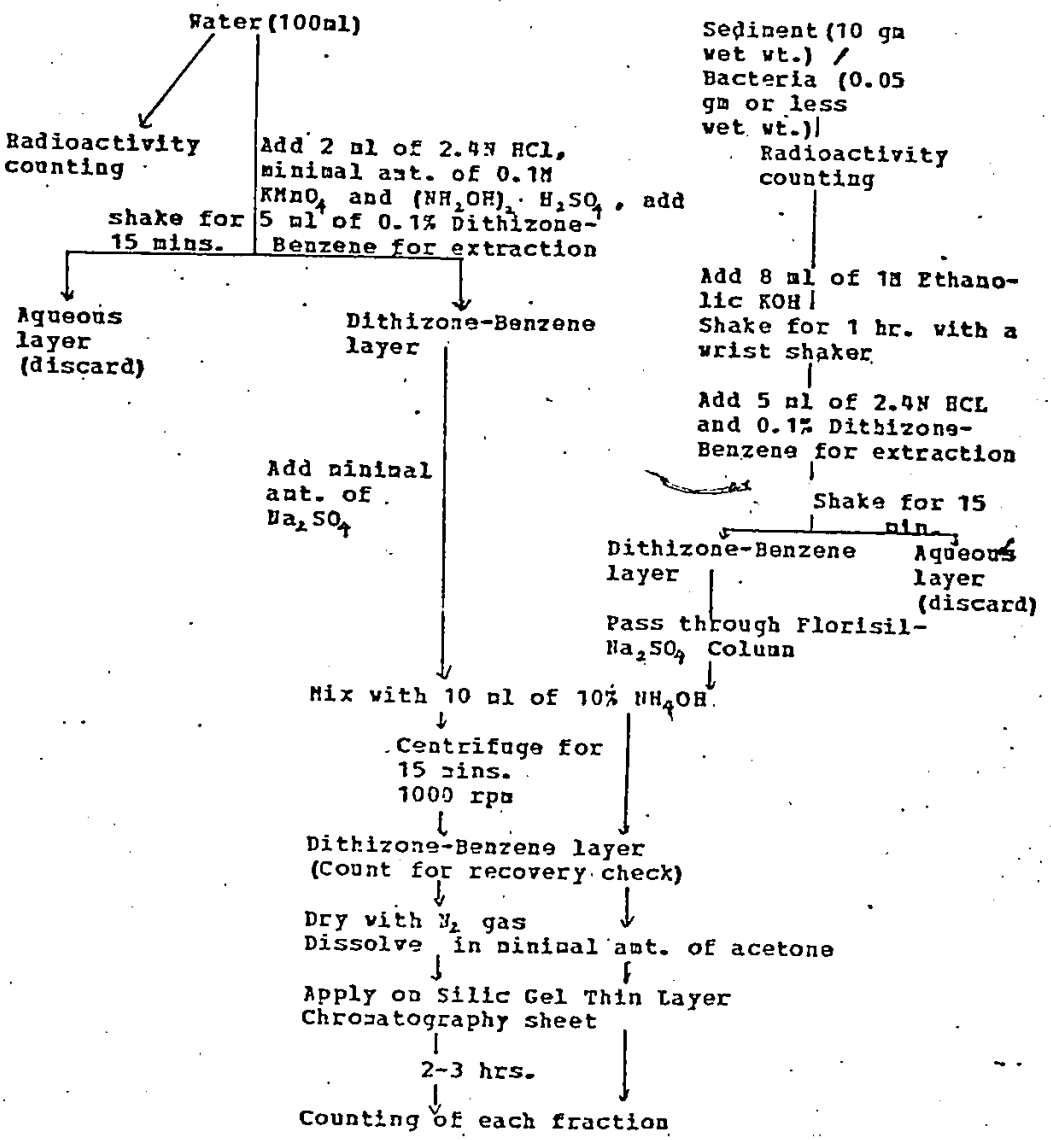


Figure 11: Flowchart for mercury analyses.

Methods used involved the combined techniques of Dithizone extraction, Thin Layer Chromatography as well as Gamma counting. (Detail see text)

Analytical Procedures



2.4.3.2 Extraction of Mercury From Sediments or Biota

The extraction procedures for sediments or biota were the same. Samples of sediments or biota were separated from the overlying water columns or cell free supernatants of PYPB media by filtration or centrifugation respectively. About 1 gm wet weight of sediment or 0.05 gm or less wet weight of biomass was put into the counting vial for radioactivity check. However, in the case of the bacteria grown in PYPB media, the total cell volume was used. An aliquot of 8 ml of 1M ethanolic potassium hydroxide was then added and shaken on a wrist shaker for 1 hour. An aliquot of 5 ml of 2.4N hydrochloric acid and 5 ml of 0.1% dithizone-benzene solution were added and the solution was reshaken for 15 minutes. An aliquot of 2 ml of the dithizone-benzene solution was passed through a florisil-sodium sulfate column and the effluent was collected.

The florisil-sodium sulfate column was constructed by packing 0.5 gm of florisil crystals into a 5.75 inch disposable Pasteur Pipet, 0.5 gm of anhydrous sodium sulfate crystals was then added to the column to overlay the florisil layer.

2.4.3.3 Analyses of Mercury Species by TLC Techniques

To 1 ml of the extract, obtained either from a water sample or from a sedimental or biological sample, 10 ml of 10% ammonium hydroxide was added. The solution was then vortexed and centrifuged at 100 rpm for 15 minutes at room temperature, the top dithizone-benzene was then pipetted into a counting vial and monitored for recovery test. After counting, the dithizone-benzene solution, containing the mercury species, was dried with nitrogen gas. The residue was redissolved in a minimal volume of acetone and the sample was applied onto TLC paper (Polygram Sil N-HR, Macherey-Nagel Co.).

With a soft lead pencil, a base line was drawn across a 5 x 20 cm Silica Gel Chromatography sheet approximately 3 cm from the base. Using a micropipet, both organic (methylmercuric) and inorganic (mercuric) mercury species standard were spotted onto the line at positions approximately 1 cm apart from each side. The sample acetone solution, containing mercury species was applied onto the line at the same time. The TLC sheet was developed at 20°C for 2-3 hours with a mixture of benzene and hexane (1:1 in V/V) as solvent. After development, the sheet was air dried at room temperature and cut into four sections for radioactivity measurement.

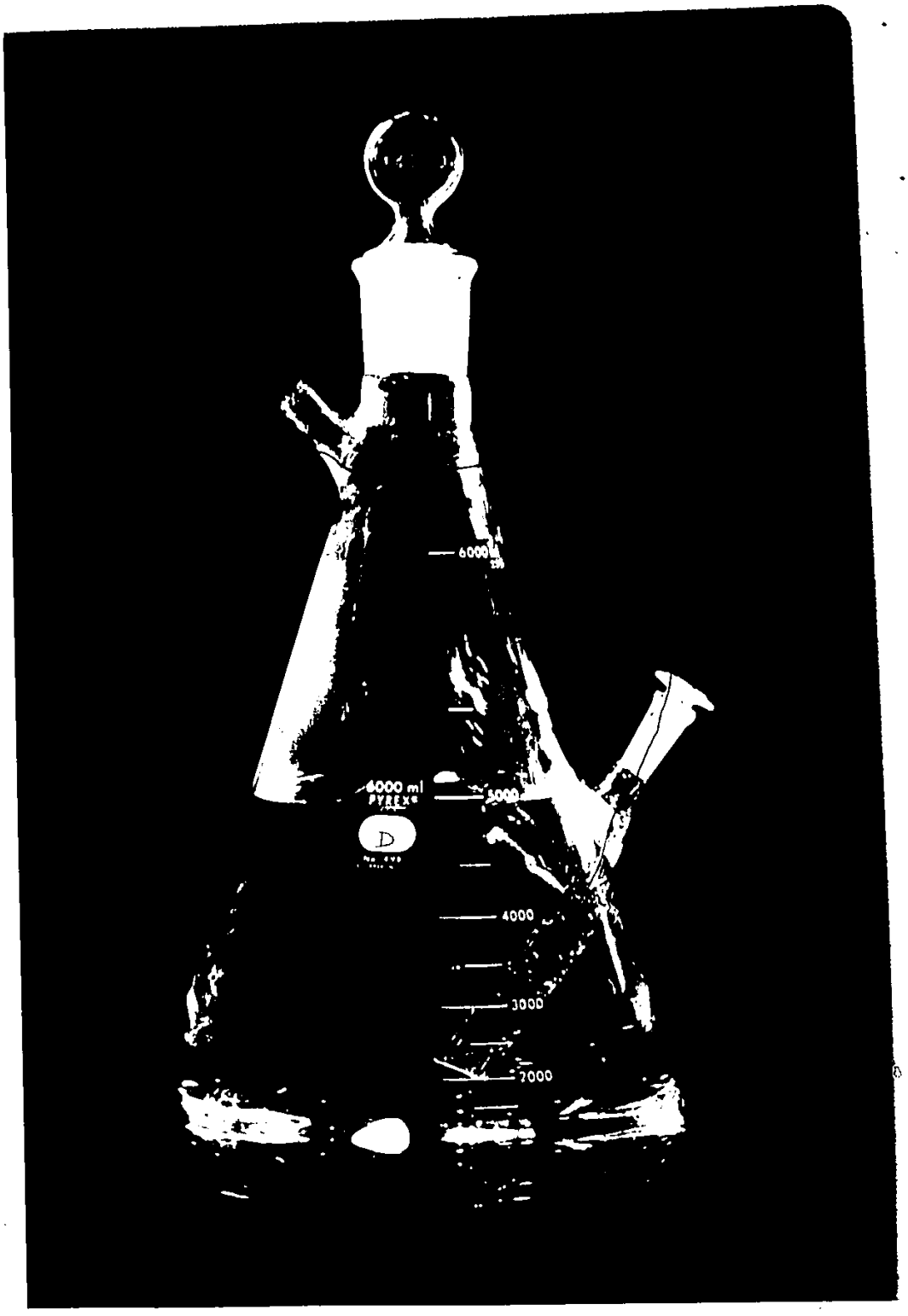
2.5 TRANSFORMATION OF METALLIC MERCURY IN AQUATIC SYSTEMS

Double distilled metallic mercury was used in this study. The oxidation-reduction potential (ORP) of the deionized water was adjusted with 0.3% hydrogen peroxide solution. The pH and ORP measurements were made with Orion Ionalyzer model 801 using the glass electrode and the combination redox electrode model 96-78 (Orion Research Inc.) respectively.

The experimental chamber was a six liter capacity pyrex conical flask with two sidearms (Fig.12). The upper shorter side arm (diameter 15 mm) was sealed with a Silicone Rubber Septum (Hamilton Septum-760-16) enclosed in a screw cap. The septum was coated with a layer of polymeric Silicone fluid (Dri-Film Sc-87, Chromatographic Specialities) which is highly inert chemically . The lower longer sidearm (diameter 18 mm) was closed by a ground glass stopper.

Sixty four microlitres equivalent to 870 milligrams of metallic mercury was introduced into five litres of deionized water in the reaction vessel. The amount of metallic mercury introduced into the system was well over the solubility of elemental mercury in water (~30 ppb) in order to maintain the mass flow following species transformation. The water was continuously stirred magnetically, except during sample withdrawal. The stirring was stopped 15 minutes prior to sampling in order that the undissolved metallic mercury would settle to the bottom of the flask. This proc-

Figure 12: Experimental apparatus designed for the study of transformation of metallic mercury.


6000
6000 ml
PYREX
5000
D
4000
3000
2000

edure avoided any possibility of trapping metallic mercury during sampling.

Aliquots of the water column were withdrawn at intervals up to 240 hours through the longer side arm and then analysed for the mercury species by Atomic Absorption Spectrophotometer simultaneously. Five ml of the air in the head space over the water column was withdrawn by a disposable syringe through the septum on the shorter arm for mercury species analysis. The air was emptied into 2 ml water, mixed well and then analysed for mercury species.

The systems studied in this work were metallic mercury in the presence of 1) deionized water, 2) deionized water with 0.3% hydrogen peroxide, 3) sterilized growth medium of Foot and Taylor (FT) (Foot and Taylor, 1949), 4) FT medium containing growing Escherichia coli B/r or Pseudomonas aeruginosa NRCC 5005, and 5) deionized water containing thermally killed Escherichia coli or Pseudomonas aeruginosa. The bacteria studied here are commonly present in natural waters.

For the study of transformation of metallic mercury by growing bacteria, the preculture of bacteria was grown in the FT medium to the end of logarithmic phase (O.D. = 0.08), a 25 ml aliquot was transferred to 5,000 ml of sterilized FT medium containing metallic mercury in the experimental chamber. Growth of bacteria in this system was observed by viable cell counts on Peptone Yeast Extract agar (PYEA).

In the case of transformation of metallic mercury by thermally killed bacteria, the bacteria were grown in PYEB medium to initial stationary and harvested as described in subsection 2.3.1. Two grams of the biomass were killed by boiling in deionized water at 100°C for 30 minutes. Non viability of the cells was confirmed by microscopic observation as well as by streak plateing technique on PYEA agar. The 2 gm of thermally killed cells were then added to the experimental chamber containing 5,000 ml of deionized water.

2.6 METAL BINDING ONTO PSEUDOMONAS AERUGINOSA

The binding of heavy metals onto Pseudomonas aeruginosa NRCC5005 was studied. After testing a number of media, Collin's minimal salt medium (Collin, 1963) with 0.5% peptone and 0.25% glucose added was selected. This medium provides suitable amounts of nutrients for bacterial growth, but still permits a resonable amounts of heavy metal ions to be taken up by the bacteria. Various amounts of mercuric nitrate or cupric nitrate solution were added to media with initial concentration ranging from 0-80 ppm. pH of the medium was adjusted to 7 with 45% sodium hydroxide (appendix 2) or 10% nitric acid. The medium, without glucose, but containing heavy metal ions, was autoclaved for 15 minutes at 121°C and 15 psi. Glucose solution filtered through a 0.22 um filter (Falcon 7103) was added to the autoclaved media to give a final concentration of 0.25%.

Growth of bacterial culture in modified Collin's medium was similar to that in PYPB medium as described in section 2.3.1 of this chapter. When the growth of culture entered into the initial stationary phase, 10 ml of the cell suspension was centrifuged at 0°C at 10,000 x g for 10 minutes. Cell pellets were washed three times with distilled deionized water and then digested with nitric acid for 20 hours in order to extract the heavy metals from the cells. Various concentrations of nitric acid were used for this purpose and the pH values of the digested solutions were measured. The total metal concentration of the autoclaved medium, cell free supernatant of culture medium, washing solution as well as the digested solution were measured by Atomic Absorption Spectrophotometry.

For the study of the cellular location of heavy metals, the cells were grown and harvested. After washing, the cells were suspended in deionized water and ruptured by a French Pressure Cell (Wabash) at 6,000 psi. Different cellular fractions were then obtained by ultracentrifuging of these mechanically broken cells. Outer cell wall layer was obtained by centrifuging at 30,000 x g for 30 minutes using ultracentrifuge (Beckman L5-50). In the supernatant, the ribosomal pellet was separated from the remaining intracellular fluid by centrifuging at 100,000 x g for 120 minutes. The various cellular fractions were then completely digested with nitric acid and tested for the amount of heavy metal present by Atomic Absorption Spectrophotometry.

Chapter III

RESULTS AND DISCUSSIONS

3.1 STABILITY OF METHYLMERCURY

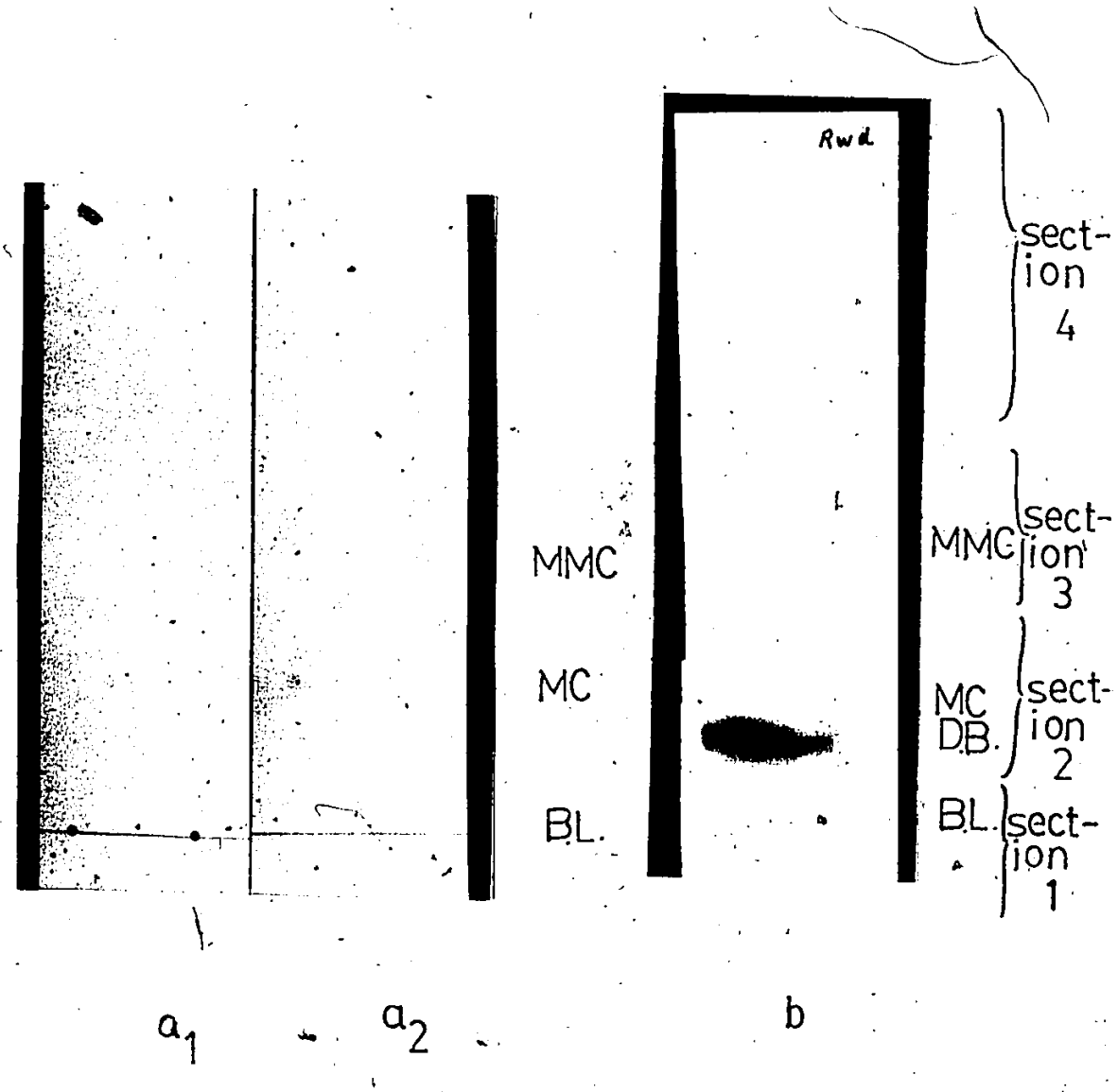
The production of methylmercury from inorganic mercury in natural waters by biological and nonbiological processes has been well established (Akagi and Takabatake, 1973; Brinckman and Iverson, 1975; Fagerstrom and Jernelov, 1969; Jernelov, 1968 and 1972; Jewett and Brinckman, 1975). Enzymatic as well as non enzymatic pathways have been implicated in the biological production of methylmercury (Bartlett, et al ., 1977 ; Langley, 1973; Vallee and Ulmer, 1972; Vonk and Sijpesteijn, 1973; Wood, 1974; Wood et al., 1968). The toxicity of methylmercury for aquatic organisms depends on the residence time in the organism and the stability of methylmercury in water. Studies on the half life of methylmercury in biota have been reported (Albert et al., 1973 ; Gerstner and Huff, 1977; Harbison et al., 1977), but information of the stability and residence time of methylmercury in the aquatic systems is limited (Billen et al., 1974 ; Kudo et al., 1977a ; Spangler et al., 1973a and b). This thesis reports the results of the stability and transformation of methylmercury in natural waters under a variety of ecological conditions as well as in microbial cultures. The

systems studied were as follows: 1) Methylmercury in deionized water; 2) Methylmercury in Ottawa River water; 3) Methylmercury in Ottawa River water with organic rich clay type sediment; 4) Methylmercury in Ottawa River water with living biota of Pseudomonas fluorescens, Escherichia coli or Anabaena flos-aquae ; 5) methylmercury in Ottawa River water with thermally killed biota: Pseudomonas fluorescens or Escherichia coli and 6) methylmercury in sterilized PYEB media and in growing bacterial cultures of Pseudomonas fluorescens or Escherichia coli in 10% and 50% Peptone Yeast Extract Broth (PYEB) media.

Radioactive $CH^{203}Hg^{+}$ was used in this study and the stability and transformation of methylmercury were analysed by the combined procedures of extraction of mercury species by dithizone solution followed by TLC plating and Gamma (γ) counting technique. There was a good separation of methylmercury from inorganic mercury by the TLC technique. A typical TLC separation is shown in Fig. 13. The TLC sheet was cut into four sections as marked. On Fig. 13 a and b, section 2 contained inorganic mercury and section 3 contained methylmercury while section 1 and 4 were the control background as shown by gamma counting. The extra colored band in Fig. 13b, in comparison with the standard (Fig. 13a), appearing below the inorganic mercury band seemed to be the excess dithizone remaining after treatment with ammonium hydroxide. This was proved by cutting and counting this

Figure 13: Separation of methylmercury and inorganic Mercury by TLC Techniques.

Figure 13a is the case of mercury standards 1. before development and 2. after development. 13b is the actual case, represented by Ottawa River water sample (for example), the extra dithizone band is shown. The sheet was cut into four sections for radioactivity check
B.L. = Base Line; D.B. = Dithizone band;
MC = Inorganic mercury; MMC = Methylmercury;
RWd = River water sample.


Rwd
MMC
MC
BL.
section 4
MMC
section 3
MC DB.
section 2
BL.
section 1
a1
a2
b

band alone for radioactivity check. The results were negative for mercury. In fact, by applying dithizone solution (with or without treatment with ammonium hydroxide) alone on TLC sheet, a band of the same color and position was obtained. However, the estimation of inorganic mercury was quantitative since the whole section 2 was counted. Since colorimetric method was not used in this study, there was no possibility of error in estimation.

The results of the various systems studied in this work are presented as follows:

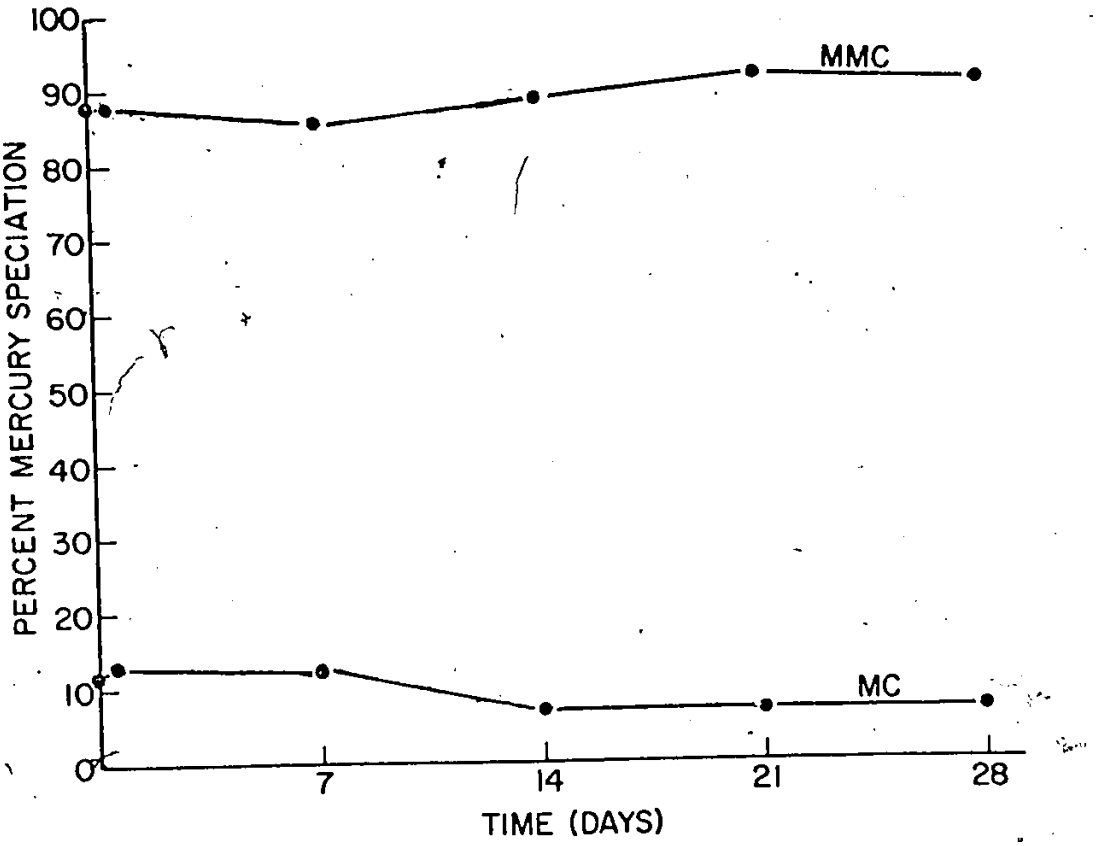
3.1.1 In Deionized Water

Figure 14 and the subsequent figures present the stability of methylmercury (MMC) and the production of inorganic mercury (MC). Systems containing heterogenous phase such as sediment or biota (discussed later on) present mercury speciation in both phases. In all these figures, the critical determinant is the stability of methylmercury as a function of time.

For the control of the deionized water and Ottawa River water, two sets of experiments with an interval of 10 months were done.

The first experiments were done as the control for the studies of 1) methylmercury in Ottawa River water with natural sediment and 2) methylmercury in Ottawa River water with living biota. The prelminary study indicated that

Figure 14: Stability of methylmercury in deionized water. MC = Inorganic mercury; MMC = Methylmercury.


100
90
80
70
60
50
40
30
20
10
0
PERCENT MERCURY SPECIATION
MMC
MC
7
14
21
28
TIME (DAYS)

there was about 12% of inorganic mercury (MC) and 88% of methylmercury (MMC) in the deionized water sample at 12 hours and this remained unchanged over the experimental period (Fig. 14).

Another set of experiments was done ten months later. In this case, the study of deionized water as well as Ottawa River water was the control of some later designed new experiments which were 1) methylmercury in Ottawa River water with thermally killed bacteria and 2) methylmercury in microbial cultures. In this study, it was found that the level of inorganic mercury species in deionized water was about 28% and methylmercury was about 72% over the experimental period.

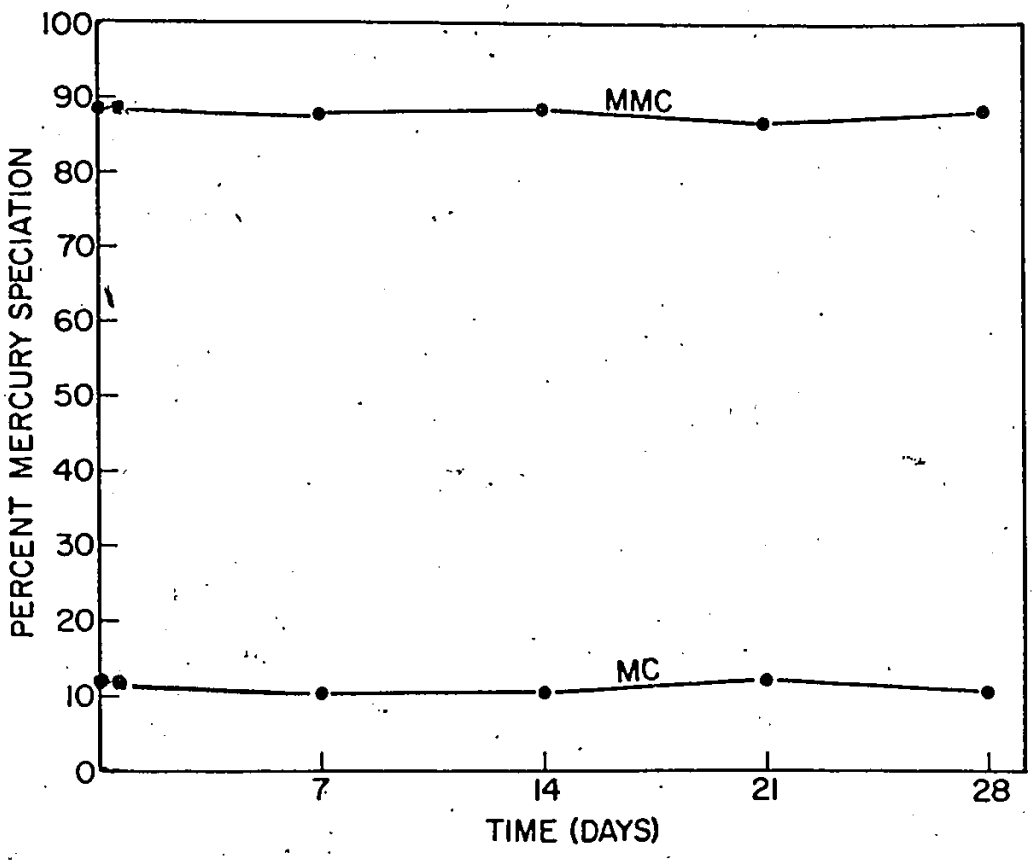
The appearance of inorganic mercury in the deionized water samples was suprising since deionized water was supposed to be chemically inert. The purity of the originial methylmercury stock was checked by using the same experimental techniques. It indicated that the orginial stock of methylmercury was contaminated with inorganic mercury. The amount of inorganic mercury increased slowly with time due to the breakdown of methylmercury.

3.1.2 Methylmercury in Ottawa River Water

The stability of methylmercury in Ottawa River is shown in Fig. 15. It indicates that about 12% of the methylmercury was degraded into inorganic mercury at zero time

Figure 15: Stability of methylmercury in Ottawa River water.

MC = Inorganic mercury; MMC = Methylmercury.


100
90
80
70
60
50
40
30
20
10
0
PERCENT MERCURY SPECIATION
MMC
MC
7
14
21
28
TIME (DAYS)

and remained unchanged thereafter. This result was similar to that of deionized water (Table 5 and 6). Thus it is interesting to note that the organic matter and microbial community at the levels present in Ottawa River water do not seem to play any significant role on breaking down the added methylmercury over the 28 day period, and the bulk of river water is not different from deionized water in altering the mercury species. This has important implications since methylmercury will have a fairly reasonable residence time in the river water which may contribute to bioaccumulation.

3.1.3 In Ottawa River Water With Sediment

Mercury speciation in Ottawa River water containing organic rich clay type sediment was totally different from the above results. This suggests that the microenvironments involving the interface of sediment and water are important in mercury interconversions. The results of demethylation of methylmercury in Ottawa River water-sediment system are given in Fig. 16 as well as Tables 5 and 6. Most of the added mercury (97%) present in the water column was lost to the sediment during the first 7 days and there was no further decrease with time in the 28 day period. However, the total amount of inorganic mercury species (or methyl-

TABLE 5

Distribution of Mercury Species in Different Phases of Natural Water Systems at 12 Hour

Sample		MC (%)	MMC (%)
Deionized water		12.6	87.2
Ottawa River water		11.8	88.0
Water column overlying Sediment		10.8	65.2
Sediment		1.8	20.1
	Total:	12.6	85.3
Water column overlying living *P. fluorescens*		13.1	4.8
Living *P. fluorescens*		41.3	40.0
	Total:	54.4	44.8
Water column overlying living *E. coli*		6.0	3.2
Living *E. coli*		6.8	82.8
	Total:	12.8	86.0
Water column overlying living *A. flos-aquae*		10.2	8.0
Living *A. flos-aquae*		11.4	70.4
	Total:	21.6	78.4

Amount of sediment=10.00 gm in wet weight
Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

TABLE 6

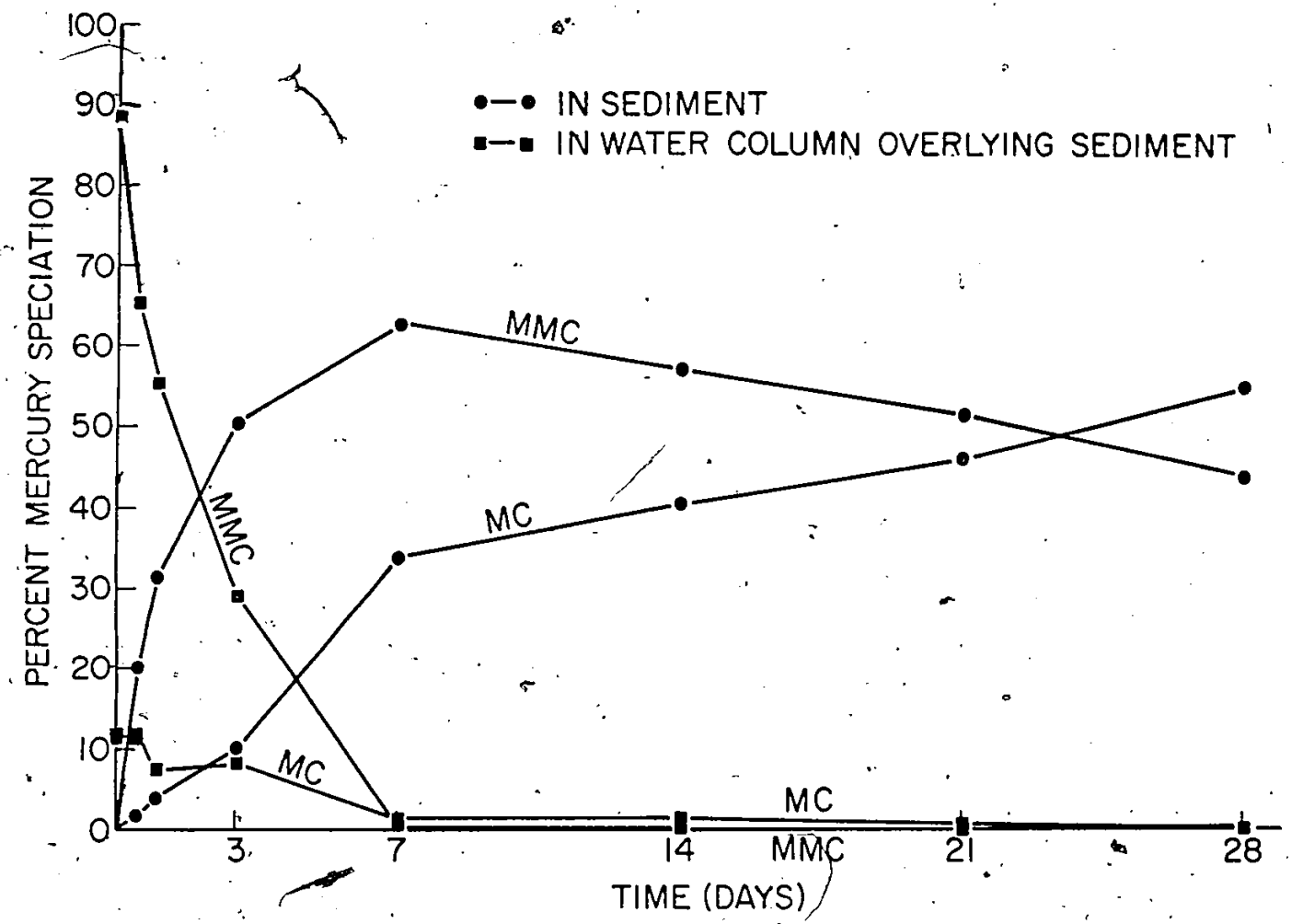
Distribution of Mercury Species in Different Phases of Natural Water Systems at 7 Day

Sample	MC (%)	MMC (%)
Deionized water	12.3	86.0
Ottawa River water	11.6	87.2
Water column overlying Sediment	1.2	0.5
Sediment	34.1	62.8
Water column overlying living *P. fluorescens*	4.6	1.5
Living *P. fluorescens*	90.0	3.7
Water column overlying living *E. coli*	2.0	2.0
Living *E. coli*	50.6	44.5
Water column overlying living *A. flos-aquae*	4.8	0.6
Living *A. flos-aquae*	83.4	8.7

Amount of sediment=10.00 gm in wet weight
Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

Figure 16: Stability of methylmercury in Ottawa River water-Sediment System.

IMC = Inorganic mercury; MMC = Methylmercury


IN SEDIMENT
IN WATER COLUMN OVERLYING SEDIMENT
PERCENT MERCURY SPECIATION
100
90
80
70
60
50
40
30
20
10
0
MMC
MC
MMC
MC
MC
MMC
3
7
14
21
28
TIME (DAYS)

mercury species) present in the first 12 hours was recovered and was similar to that of deionized water (Table 5), it thus indicated that there was no degradation of added methylmercury by sediment in 12 hours. Conversion only occurred after 12 hours in the sediment and about 35% of inorganic mercury appear in the sediment at 7 days, further increases were gradual, reaching as much as 55% in 28 days (Fig. 16). The decrease of methylmercury with time is also shown in the same figure. The contribution of methylmercury and inorganic mercury in the water column overlying the sediment reached equilibrium by the 7th day. The water column had only a very low level of methylmercury and inorganic mercury (~1.7%) by 7 days (Table 6).

This suggests that the added methylmercury is rapidly sorbed by the sediment and that further transformations occur in that phase. This organic rich clay type sediment has a great affinity for mercury and the desorption of mercury is insignificant over the experimental period, this finding agrees with the previous report of the Ottawa River project (Ramamoorthy and Kushner, 1977a and b).

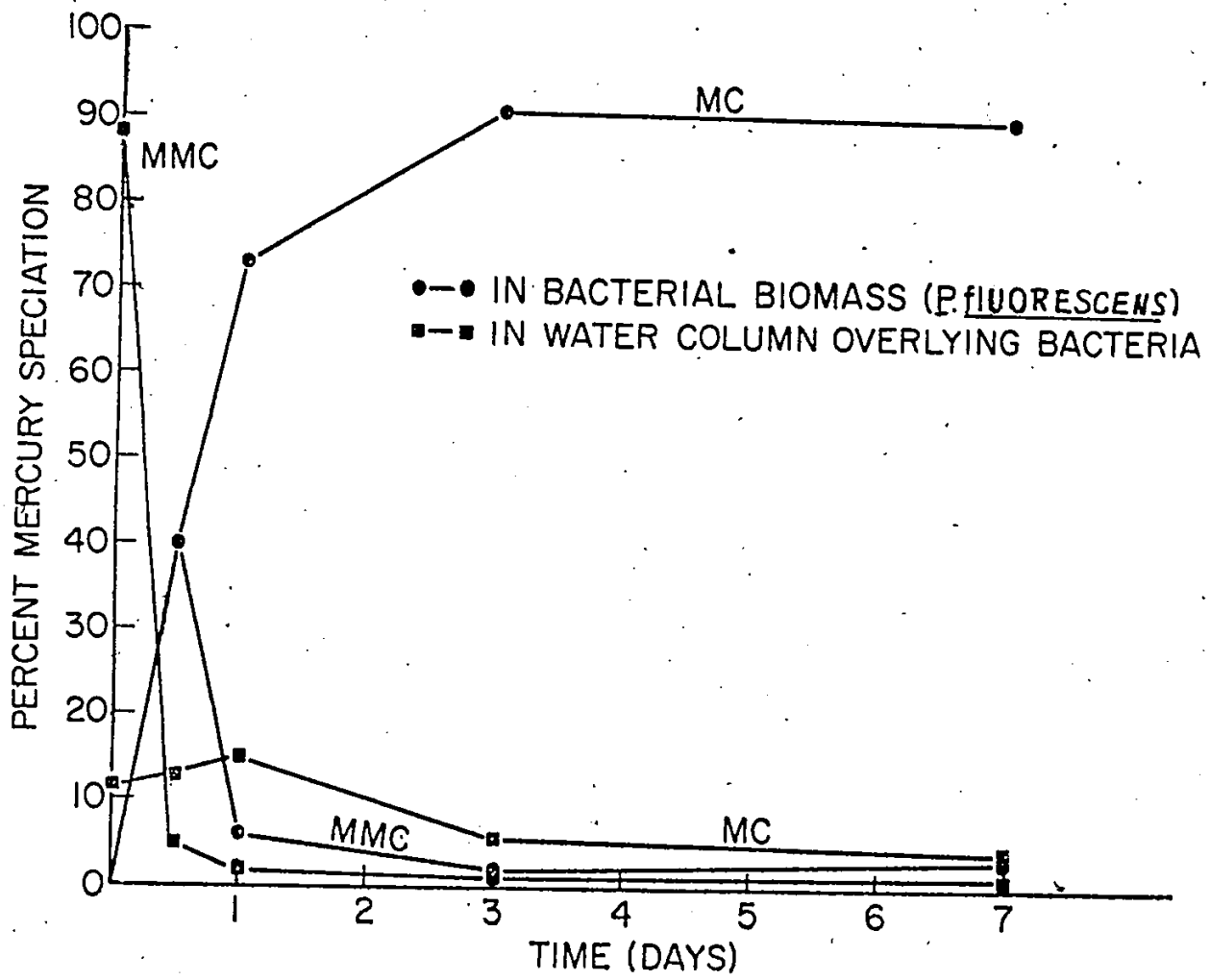
3.1.4 Methylmercury in Ottawa River Water With Viable Biota

3.1.4.1 Viable Pseudomonas fluorescens

The effect of biota on degradation of methylmercury was also studied. Fig. 17 presents the transformation of added methylmercury in the Ottawa River water-viable Pseudo-

Figure 17: Stability of methylmercury in Ottawa River water-living P. fluorescens system.

The biomass concentration = 0.45 gm wet weight per 100 ml of river water, the added mercury concentration= 0.10 ppm
MC = Inorganic mercury; MMC = Methylmercury.


100
90
80
70
60
50
40
30
20
10
0
PERCENT MERCURY SPECIATION
MMC
MC
MMC
MC
●—● IN BACTERIAL BIOMASS (P. FLUORESCENS)
■—■ IN WATER COLUMN OVERLYING BACTERIA
1
2
3
4
5
6
7
TIME (DAYS)

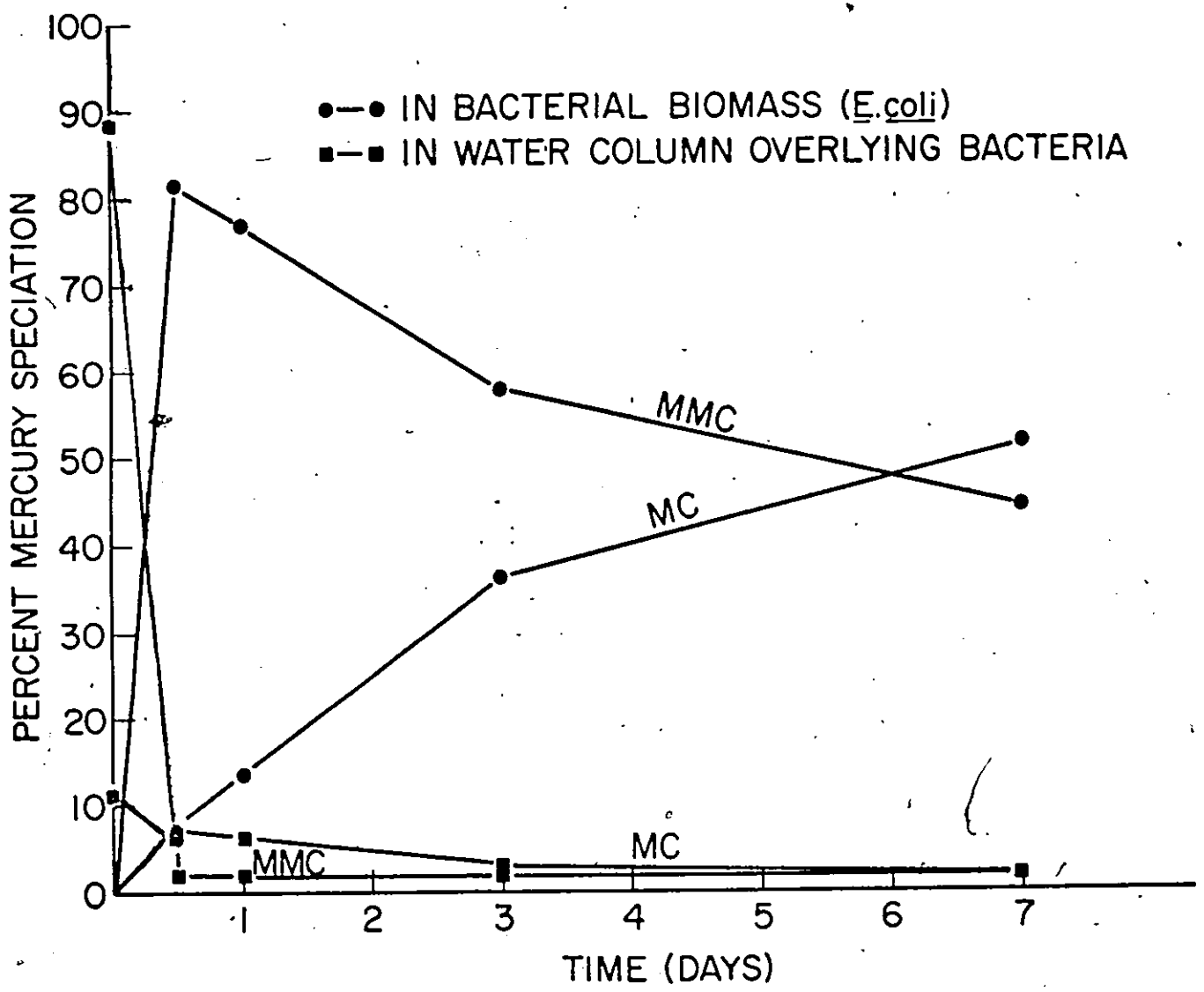
monas fluorescens system. Rapid sorption of methylmercury added to the water column by viable microorganisms was accompanied by rapid breakdown into inorganic mercury as shown in table 5. In the first 12 hours, 81.3% of added mercury was sorbed onto the cells. Pseudomonas fluorescens seems most efficient in converting methylmercury to inorganic mercury, as much as 73% in 24 hours, this increased to 90% in 72 hours, and remained unchanged for 7 days. The water phase of this system contained only about 6% of the total (methylmercury and inorganic mercury) at the end of 7 days (Table 6).

3.1.4.2 Viable Escherichia coli

The efficiency of Escherichia coli in conversion of methylmercury in Ottawa River water is shown in Fig.18. In contrast to Pseudomonas fluorescens, sorbed methylmercury was retained by the biomass of Escherichia coli with only slow gradual conversion to inorganic mercury. In fact, there was no degradation of methylmercury by Escherichia coli in the first 12 hours as the total percentage of methylmercury in this system was similar to that of deionized

Figure 18: Stability of methylmercury in Ottawa River water-living *E. coli* system.

The biomass concentration= 0.45 gm wet weight per 100 ml of river water, the added mercury concentration = 0.1 ppm MC = Inorganic mercury; MMC = Methylmercury.


●—● IN BACTERIAL BIOMASS (E.coli)
■—■ IN WATER COLUMN OVERLYING BACTERIA
PERCENT MERCURY SPECIATION
100
90
80
70
60
50
40
30
20
10
0
MMC
MC
MMC
MC
1
2
3
4
5
6
7
TIME (DAYS)

water (Table 5). Only 36.4% of inorganic mercury was found in Escherichia coli in 3 days and this increased to 50.6% at the end of 7 days. Of course, the amount of methylmercury in the bacterial mass decreased inversely with time. Once again, the water column overlying the bacteria contained insignificant amounts of mercury in the form of methylmercury and inorganic mercury at the end of 7 days (Table 6).

Viable cell counts under identical experimental conditions in the presence of added methylmercury showed that about 30% of Pseudomonas fluorescens and 37% of Escherichia coli died during the first six hours (Fig. 19).

3.1.4.3 Viable Anabaena flos-aquae

Figure 20 shows the role of viable blue green algae Anabaena flos-aquae in the conversion of methylmercury. This algae also sorbed the added mercury rapidly (Table 5). However, the production of inorganic mercury was slow at the start but increased rapidly to 83.4% in 7 days. The water column contained low level of mercury (Table 6).

In all systems, the biota seems to play a very dominant role in sorbing and converting the added methylmercury to inorganic mercury. Very little of the initial methylmercury or the converted inorganic mercury appeared in the water column. It is encouraging that biota cannot only sorb methylmercury quickly but can convert it to less toxic

Figure 19: The amount of viable cells as function of time.

The biomass concentration = 0.45 gm wet weight per 100 ml of river water, the added mercury concentration = 0.10 ppm
MC = Inorganic mercury; MMC = Methylmercury.

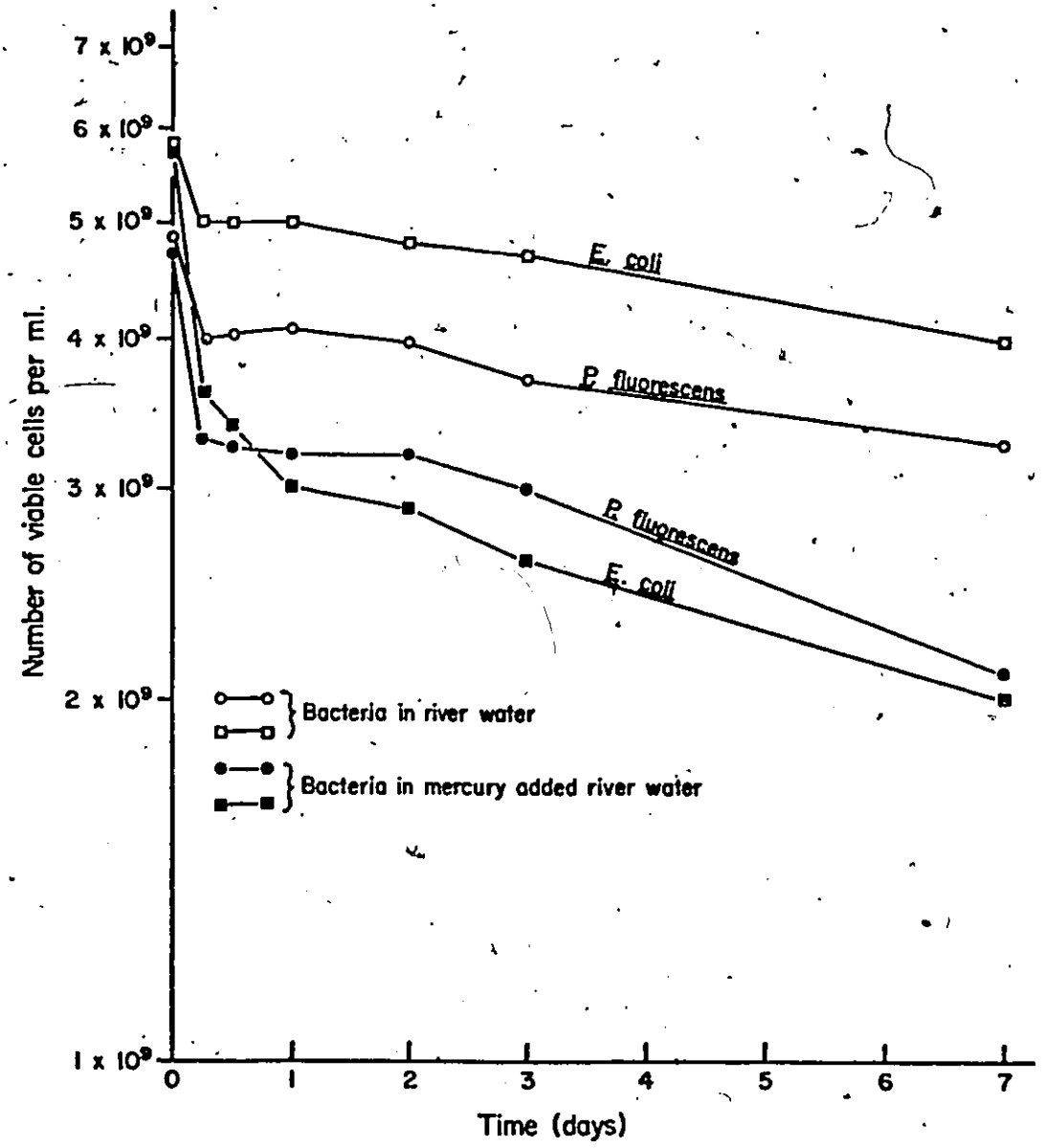
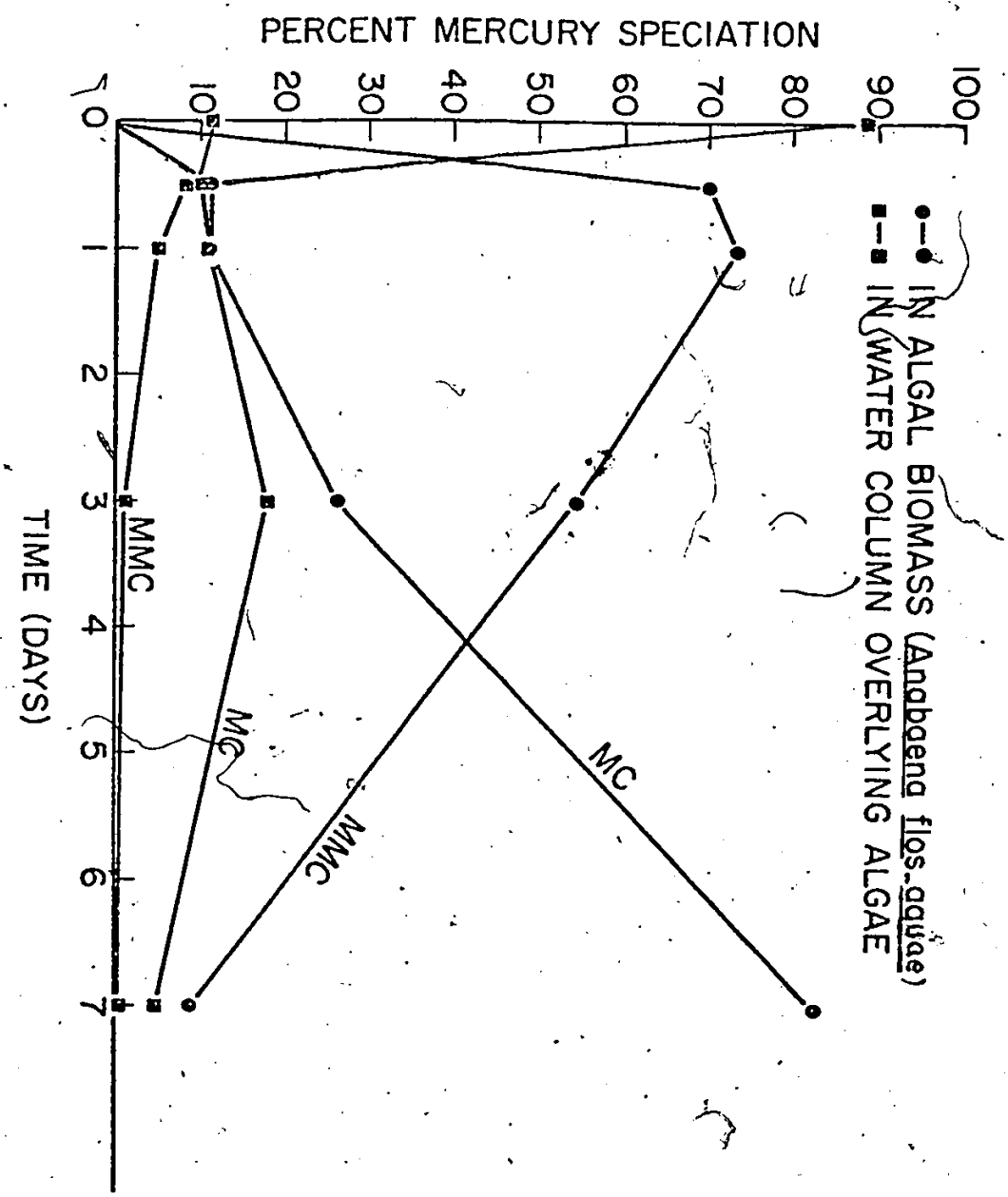

7 x 10⁹
6 x 10⁹
5 x 10⁹
4 x 10⁹
3 x 10⁹
2 x 10⁹
1 x 10⁹
Number of viable cells per ml.
E. coli
P. fluorescens
P. fluorescens
E. coli
Bacteria in river water
Bacteria in mercury added river water
0
1
2
3
4
5
6
7
Time (days)

Figure 20: Stability of methylmercury in Ottawa River water-living A. flos-aquae system.

The biomass concentration = 0.45 gm wet weight per 100 ml of river water, the added mercury concentration = 0.10 ppm
MC = Inorganic mercury; MMC = Methylmercury.


PERCENT MERCURY SPECIATION
0
10
20
30
40
50
60
70
80
90
100
TIME (DAYS)
0
1
2
3
4
5
6
7
MMC
MC
MMC
MC
IN ALGAL BIOMASS (Anabaena flos-aquae)
IN WATER COLUMN OVERLYING ALGAE

inorganic mercury forms which are retained by the biota itself. This means that the water vector will contain less than 2% of the total methylmercury burden at the end of 7 days. Of course, one has to remember that the cell concentration in these experiments is much greater than that found in the natural environment. In addition, physical factors such as turbulence, wind direction and suspended matter will play an important role in the sediment activity in the natural environment.

3.1.4.4 Effect of Bacterial Mass

Three concentrations of bacterial biomass were employed in this study. They were 0.45 gm, 0.2 gm and 0.02 gm wet weight (or 0.081, 0.035 and 0.0035 gm dry weight respectively) of Pseudomonas fluorescens in 100 ml of river water. The analysis of the 0.02 gm wet weight was abandoned since it was technically difficult to analyse the mercury in such a small bacterial mass, and to recover the biomass after centrifugation from the overlying river water column. The results for the other two concentrations are given in Table 7. It is clear that the biomass is critical in the demethylation processes. The higher the bacterial concentration, the more inorganic mercury is formed.

TABLE 7

Effect of Biomass (*P. fluorescens*) and Mercury Concentration on Degradation of Methylmercury in River Water.

100 ml of ORW containing		Time	Mercury in biomass	
Biomass (wet weight in gm)	Initial concentration of Hg (ppm)	Day	MC (%)	MMC (%)
0.45	0.1	1	73.0	5.9
0.20	0.1	1	53.7	29.2
0.45	0.1	3	90.5	1.9
0.20	0.1	3	62.7	26.0
0.45	0.1	7	90.0	3.7
0.20	0.1	7	64.2	21.4
0.45	0.1	1	73.0	5.9
0.45	1.0	1	16.1	49.2
0.45	0.1	3	90.5	1.9
0.45	1.0	3	46.7	27.2
0.45	0.1	7	90.0	3.7
0.45	1.0	7	68.4	3.8

MC=Inorganic mercury
MMC=Methylmercury
ORW=Ottawa River Water

3.1.4.5 Effect of Methylmercury Concentration

Table 7 also presents the results of the effect of 1 ppm and 0.1 ppm of initial mercury concentration on 0.45 gm (wet weight) of Pseudomonas fluorescens-Ottawa River water system. At the fixed biomass, the lower the initial amount of methylmercury, the larger the percentage of methylmercury is converted.

3.2 METHYLMERCURY IN OTTAWA RIVER WATER WITH DEAD BIOTA

The viable cell counts indicated that about 30% of added Pseudomonas fluorescens or 37% of added Escherichia coli were nonviable after 6 hours in the Ottawa River water-living biota system in the presence of methylmercury (Fig. 19). Thus it is interesting to know whether the dead biota can convert methylmercury to inorganic mercury. Experiments were designed and conducted with thermally killed bacteria (Pseudomonas fluorescens and Escherichia coli). As shown in Fig. 21, it was found that the effect of nonviable Pseudomonas fluorescens was similar to that of nonviable Escherichia coli. The total amounts of methylmercury or inorganic mercury in the river water-nonviable biota system were similar to the control of deionized water up to 3 days (Tables 8 and 9). This indicated that all the mercury in the water column was initially sorbed by the biomass without any conversion for the first 3 days. After 3 days, a certain fraction of the sorbed inorganic mercury (12.5% for Pseudomonas

Figure 21: Stability of methylmercury in Ottawa River water-thermally killed biota system

Ths biomass concentration = 0.45 gm wet weight per 100 ml of river water. The added mercury concentration = 0.10 ppm, MC = Inorganic mercury; MMC = Methylmercury.

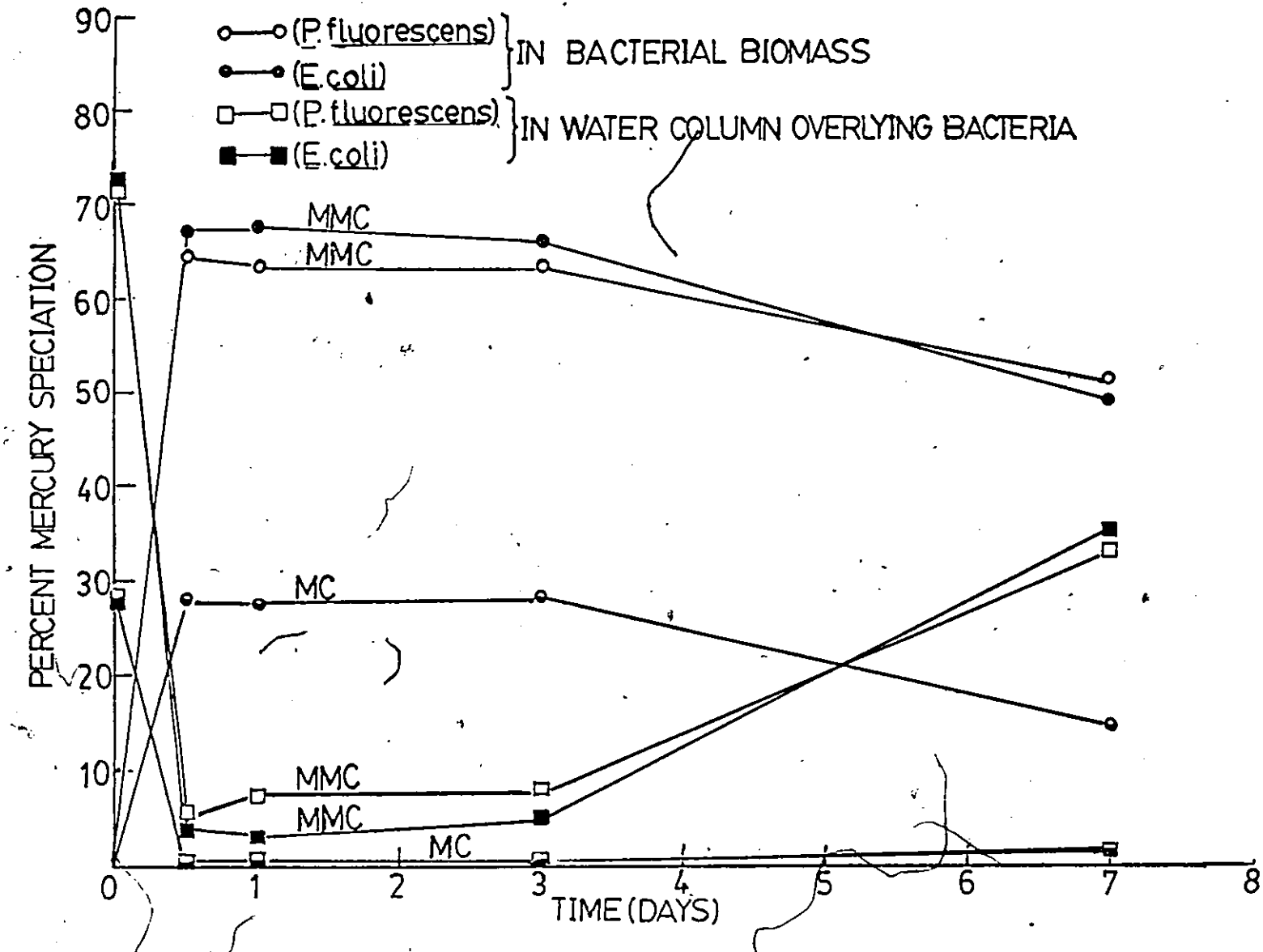

(P. fluorescens)
(E. coli)
IN BACTERIAL BIOMASS
(P. fluorescens)
(E. coli)
IN WATER COLUMN OVERLYING BACTERIA
MMC
MMC
MC
MMC
MMC
MC
PERCENT MERCURY SPECIATION
TIME (DAYS)
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8

TABLE 8

Distribution of Mercury Species in Different phases of Ottawa River Water-dead P. fluorescens System

Phase	Time (day)		MC(%)	MMC(%)
Deionized water†	-		27.9	71.6
Dead P. fluorescens	0‡		0.0	0.0
Water column overlying dead P. fluorescens	0‡		28.1	71.2
		Total:	28.1	71.2
Dead P. fluorescens	0.5		28.5	64.7
Water column overlying dead P. fluorescens	0.5		0.7	5.5
		Total:	29.2	70.2
Dead P. fluorescens	1		27.8	62.3
Water column overlying dead P. fluorescens	1		0.3	7.8
		Total	28.1	70.1
Dead P. fluorescens	3		28.2	63.3
Water column overlying dead P. fluorescens	3		0.4	8.0
		Total:	28.6	71.3
Dead P. fluorescens	7		14.5	51.4
Water column overlying dead P. fluorescens	7		1.7	32.4
		Total:	16.2	83.8

†Control, figures here are the average value of all individual controls at different time periods.

‡Zero hour

Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

TABLE 9

Distribution of Mercury Species in Different Phases of Ottawa River Water-Dead *E. coli* System

Phase	Time (day)		MC (%)	MMC (%)
Deionized water†	-		27.9	71.6
Dead *E. coli*	0‡		0.0	0.0
Water column overlying dead *E. coli*	0‡		27.4	72.1
		Total:	27.4	72.1
Dead *E. coli*	0.5		28.3	67.1
Water column overlying dead *E. coli*	0.5		0.3	4.0
		Total:	28.6	71.1
Dead *E. coli*	1		27.3	67.8
Water column overlying dead *E. coli*	1		0.3	2.7
		Total	27.6	70.5
Dead *E. coli*	3		28.4	65.9
Water column overlying dead *E. coli*	3		0.3	5.3
		Total:	28.7	71.2
Dead *E. coli*	7		14.2	48.7
Water column overlying dead *E. coli*	7		1.9	35.2
		Total:	16.1	83.9

†Control, figures here are the average value of all individual controls at different time periods.

‡Zero hour

Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

fluorescens or Escherichia coli) was converted to methylmercury. The appearance of significant amounts of methylmercury in the water column (32-35% for Pseudomonas fluorescens or Escherichia coli) in 7 days could probably be due to the release of some intracellular products binding methylmercury in the water column.

3.2.1 Methylmercury in Microbial Cultures

Experiments to study the demethylation of methylmercury by bacteria during the growth phase were conducted. Ten and 50% of the diluted Peptone Yeast Extract Broth (PYEB) were used to culture bacteria (Pseudomonas fluorescens and Escherichia coli). Methylmercury was spiked to cultures at 3 hours after introduction of the bacteria and the time of addition of mercury was taken as the zero time (Fig. 22 , 23 and Tables 10, 11). Demethylation of methylmercury at different time periods during the logarithmic and stationary phase of the bacterial growth was monitored.

3.2.1.1 Pseudomonas fluorescens

The results of the speciation study by the growing Pseudomonas fluorescens are shown in Fig. 22 and 23. It indicates that Pseudomonas fluorescens converted all the added methylmercury to inorganic mercury species which subsequently volatilized since the flasks were loosely capped with sterile cotton. Basically, there were little differences between the results of mercury speciation by growing

Figure 22: Stability of methylmercury in 10% PYPB medium-growing P. fluorescens system

The added mercury concentration = 0.10 ppm
MC = Inorganic mercury; MMC = Methylmercury.

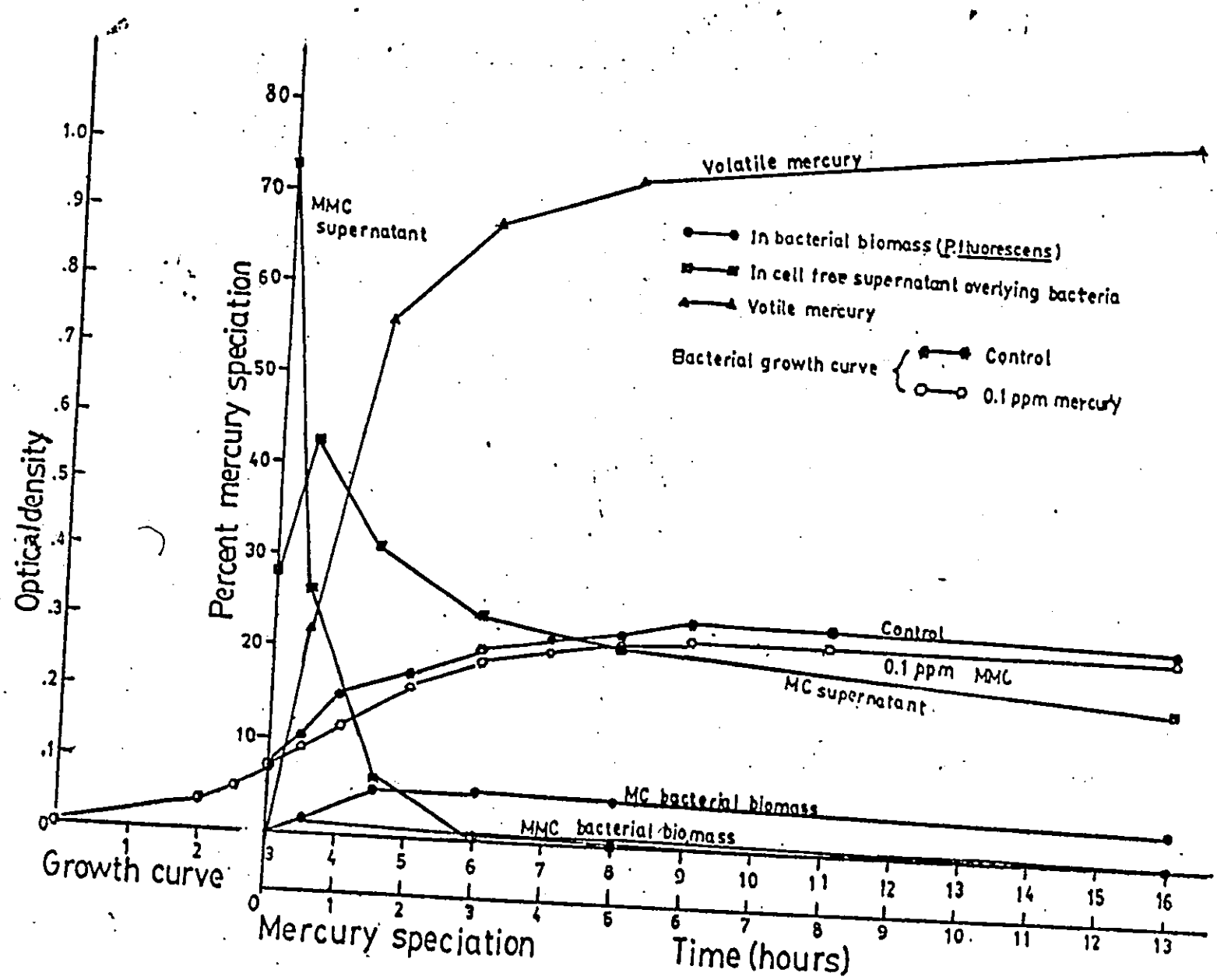

Optical density
Growth curve
Percent mercury speciation
Mercury speciation
Time (hours)
MMC supernatant
Volatile mercury
In bacterial biomass (P.fluorescens)
In cell free supernatant overlying bacteria
Votile mercury
Bacterial growth curve
Control
0.1 ppm mercury
Control
0.1 ppm MMC
MC supernatant
MC bacterial biomass
MMC bacterial biomass

Figure 23: Stability of methylmercury in 50% PYFB-medium growing P. fluorescens system.

The added mercury concentration = 0.10 ppm,
MC = Inorganic mercury; MMC = Methylmercury;
PYFB = Peptone Yeast Extract Broth

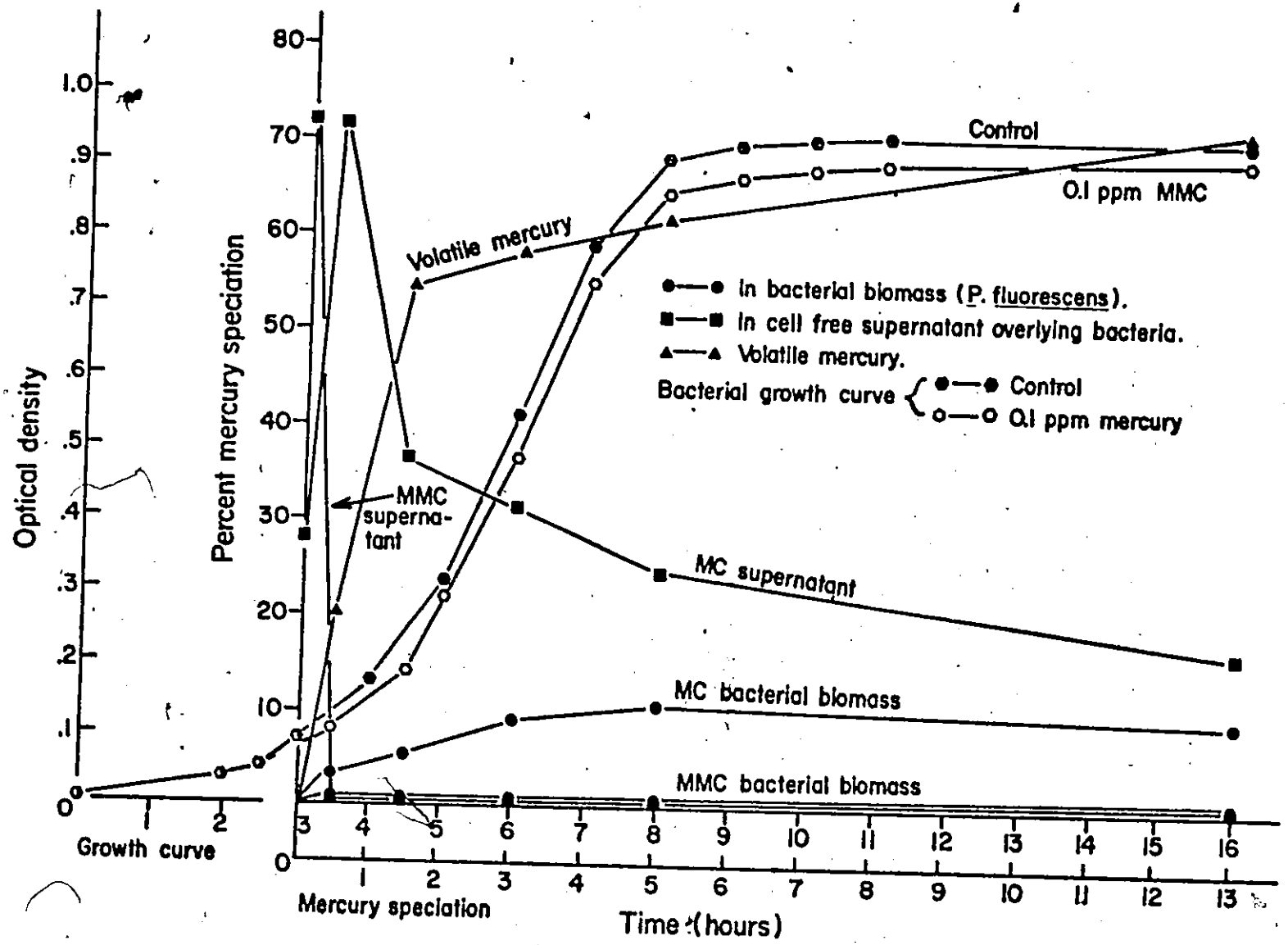

Optical density
Percent mercury speciation
Growth curve
Mercury speciation
Time (hours)
Control
0.1 ppm MMC
Volatile mercury
MMC supernatant
MC supernatant
MC bacterial biomass
MMC bacterial biomass
● — ● In bacterial biomass (P. fluorescens).
■ — ■ In cell free supernatant overlying bacteria.
▲ — ▲ Volatile mercury.
Bacterial growth curve
● — ● Control
○ — ○ 0.1 ppm mercury

TABLE 10

Distribution of Mercury Species in 10% Peptone Yeast Extract Broth with Growing *E. coli*

Phases	Time (hours)		MC (%)	MMC (%)
Deionized water†	-		27.9	71.6
Sterilized 10% PYEB†	-		26.7	71.3
E. coli in 10% PYEB	0‡		0.0	0.0
Cell free supernatant of 10% PYEB	0‡		28.6	70.4
		Total:	28.6	70.4
E. coli in 10% PYEB	0.5		9.0	13.0
Cell free supernatant of 10% PYEB	0.5		17.8	60.0
		Total:	26.8	73.0
E. coli in 10% PYEB	1.5		10.1	22.1
Cell free supernatant of 10% PYEB	1.5		15.4	52.4
		Total	25.5	74.5
E. coli in 10% PYEB	3		16.2	26.5
Cell free supernatant of 10% PYEB	3		11.7	44.0
		Total:	27.9	70.5
E. coli in 10% PYEB	5		21.3	27.5
Cell free supernatant of 10% PYEB	5		6.9	43.0
		Total:	28.2	70.5
E. coli in 10% PYEB	13		24.1	33.0
Cell free supernatant of 10% PYEB	13		3.3	39.5
		Total:	27.4	72.5

†Control, figures here are the average value of all individual controls at different time periods.

‡Zero hour

Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

TABLE 11

Distribution of Mercury Species in 50% Peptone Extract Broth With Growing *E. coli*

Phases	Time (hours)		MC (%)	MMC (%)
Deionized water†	-		27.9	71.6
Sterilized 50% PYEB†	-		27.1	71.6
E. coli in 50% PYEB	0*		0.0	0.0
Cell free supernatant of 50% PYEB	0†		28.2	71.7
		Total:	28.2	71.7
E. coli in 50% PYEB	0.5		13.6	12.3
Cell free supernatant of 50% PYEB	0.5		13.9	59.0
		Total:	27.5	71.3
E. coli in 50% PYEB	1.5		15.4	20.7
Cell free supernatant of 50% PYEB	1.5		11.8	52.1
		Total	27.2	72.8
E. coli in 50% PYEB	3		18.4	33.2
Cell free supernatant of 50% PYEB	3		8.5	39.8
		Total:	26.9	73.0
E. coli in 50% PYEB	5		23.3	41.2
Cell free supernatant of 50% PYEB	5		4.3	30.6
		Total:	27.6	71.8
E. coli in 50% PYEB	13		24.1	56.0
Cell free supernatant of 50% PYEB	13		2.8	16.9
		Total:	26.9	72.9

†Control, figures here are the average value of all individual controls at different time periods.

*Zero hour

Amount of biota=0.45 gm in wet weight
Added mercury concentration=0.10 ppm
MC=Inorganic mercury
MMC=Methylmercury

Pseudomonas fluorescens in 10% PYEB and 50% PYEB medium. Of course, the higher percentage of mercury species found in the biomass in 50% PYEB medium than in 10% PYEB medium could be due to the higher cell concentration in the former. On the other hand, higher percentage of mercury species in cell free supernatant and less percentage of volatilized mercury in 50% PYEB medium than that of 10% PYEB medium could be due to sequestration by increasing complexing. The possibility of more extracellular products present in the 50% PYEB medium retaining more mercury species could not be ruled out.

3.2.1.2 Escherichia coli

The results of mercury speciation by growing Escherichia coli is shown in Tables 10 and 11. By comparison with the control of deionized water, there was no interconversion of the sorbed methylmercury over the experimental period of 13 hours (Tables 10 and 11) and this agrees with the earlier results that there was no demethylation of methylmercury in the first 12 hours by viable Escherichia coli in natural river water (Table 5) However, it is important to remember that these experiments were done ten months apart and the stock solution of methylmercury contiained 12% of inorganic mercury which increased to 28% in ten months time and the remaining was methylmercury.

In summary, there is no doubt that all the viable microorganisms studied in this work possess the capacity to

demethylate methylmercury. The conversion rate is dependent on the biomass concentration as well as the initial methylmercury concentration present in natural waters. For the three organisms studied here, the increasing order of conversion of methylmercury to inorganic mercury is Escherichia coli, Anabaena flos-aquae, and Pseudomonas fluorescens. However, the thermally killed bacteria will methylate inorganic mercury rather than demethylate the methylmercury present in the stock solution.

3.3 TRANSFORMATION OF METALLIC MERCURY IN NATURAL WATERS

The determination of pathways by which heavy metals move in the ecosystem is important and this problem depends on establishing the reservoirs and the various species involved in the transport between compartments such as water, biota etc. In the study of mercury transport in the ecosystem, the identification of species is crucial since mercury undergoes several interconversions leading to the formation of Hg^0, Hg^{2+}, CH_3Hg^+ and other forms of organic mercury compounds. These interconversions of mercury depend on both the physico-chemical and biological environments. Of the species listed above, Hg^0 and CH_3Hg^+ are lipophilic and bioaccumulate in aquatic organisms, forming a reservoir in the ecosystem from which human resources and food supplies may be contaminated. It has been shown recently that atmospheric movement of mercury is an equally important pathway

in the transport of mercury in the ecosystem. The results of transformation of metallic mercury in natural waters are presented and discussed in this chapter.

The experimental chamber used for this work was a six liter capacity pyrex conical flask with two side arms as mentioned in section 2.5. The closed system described here is useful for the investigation of volatile mercury species in the air above the water surface, and the chamber was used to study the oxidation of metallic mercury in natural water as well as the transformation of metallic mercury by pure cultures of bacteria.

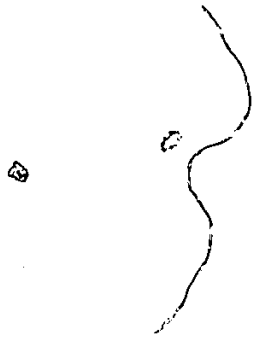
The systems studied in this work were metallic mercury in the presence of 1) deionized water, 2) deionized water with 0.3% hydrogen peroxide, 3) sterilized Foot and Taylor (FT) medium, 4) FT medium with growing Escherichia coli or Pseudomonas aeruginosa, and 5) deionized water with thermally killed Escherichia coli or Pseudomonas aeruginosa.

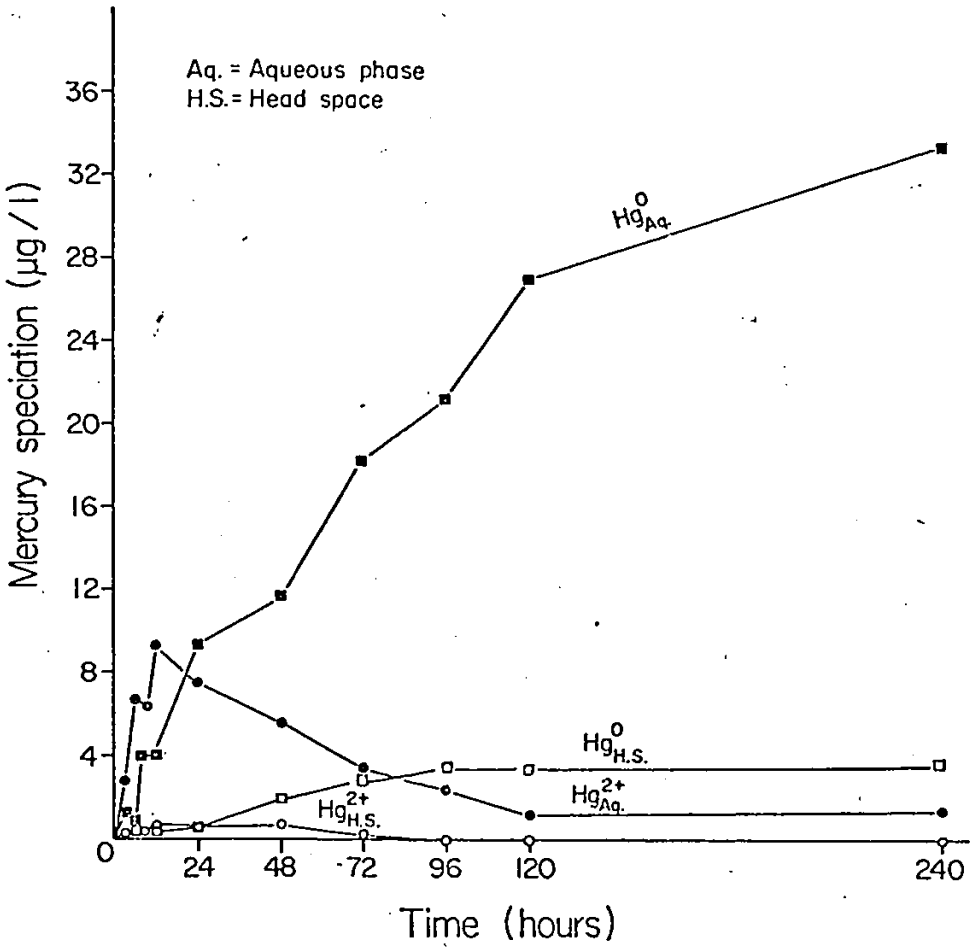
The results are presented in figures 24-30, showing mercury speciation (as Hg^0 and Hg^{2+}) as a function of time. No organic form of mercury was detected in any of the systems studied in this work.

3.4 METALLIC MERCURY IN DEIONIZED WATER

In deionized water, after approximately 24 hours, equal amounts of Hg^0 and Hg^{2+} species are found in the aqueous phase; later, the amount Hg^0 increased with time

Figure 24: Transformation of metallic mercury in deionized water.




Aq. = Aqueous phase
H.S. = Head space
Mercury speciation (µg / l)
36
32
28
24
20
16
12
8
4
0
24
48
72
96
120
240
Time (hours)
$Hg^{0}_{Aq.}$
$Hg^{0}_{H.S.}$
$Hg^{2+}_{Aq.}$
$Hg^{2+}_{H.S.}$

reaching 32 ppb at 240 hours (Fig 24). This amount corresponds to the solubility of Hg^0 in water (Glew and hames, 1972; Sanemasa, 1975; Wollast et al., 1975).

The formation of Hg^{2+} in deionized water could be due to the water reduction by Hg^0 since Hg is below hydrogen in the electromotive series. This might involve the formation of nascent hydrogen from H^+ radicals present in deionized water ($= 10^{-7}$ M) at pH = 7 ($Kw = 10^{14}$). The reaction became reversible when the $[Hg^{2+}] = 8$ ppb (or $= 4 \times 10^{-8}$ M) where the nascent hydrogen could in turn reduce Hg^{2+} ions to Hg^0, reaching a steady state at 120 hours. This could probably account for the initial rise and subsequently drop in $[Hg^{2+}]$. The Hg^0 in the head space (about 1/5 of the water column volume) reached 4 ppb. The amount of Hg^0 in the head space was three times the concentration of Hg^{2+} in the deionized water. Very little Hg^{2+} was present in the head space. The deionized water in a closed system produced more Hg^0 than Hg^{2+} in the aqueous phase and in the head space.

The redox potential (ORP) values and pH values with time for all systems is given in Table 12.

TABLE 12

The ORP and pH values of samples with Time

Samples		Time (hours) 0	3	6	9	12	24	48	72	96	120	240
Deionized water	ORP (meV)	475	475	450	454	458	424	378	424	409	400	414
	pH	6.8	6.8	6.5	6.4	6.7	6.8	6.6	6.5	6.4	6.6	6.8
0.3% H_2O_2 water		-	283	269	322	298	260	282	293	284	294	314
	pH	6.8	7.0	6.8	6.6	6.6	6.5	6.5	6.4	6.5	6.5	6.6
FT medium	ORP (meV)	488	-	488	-	462	452	440	448	454	429	441
	pH	7.0	7.0	7.0	7.0	7.1	7.0	7.1	7.0	7.1	7.0	7.0
FT-*P. aeruginosa* system	ORP (meV)	415	-	422	422	422	422	427	418	416	422	430
	pH	7.1	7.2	7.2	7.3	7.3	7.3	7.2	7.3	7.3	7.2	7.2
FT-*E. coli* system	ORP (meV)	420	-	438	-	432	433	438	433	426	432	469
	pH	7.0	7.0	7.1	7.1	7.2	7.3	7.2	7.2	7.2	7.0	7.1
Deionized water-dead *P. aeruginosa* system	ORP (meV)	442	-	404	390	390	386	408	413	441	417	407
	pH	7.1	7.1	7.4	7.3	7.2	7.4	7.5	7.1	7.3	7.2	7.1
Deionized water-dead *E. coli* system	ORP (meV)	412	395	404	391	434	388	369	405	390	413	392
	pH	7.1	7.1	7.1	7.2	7.3	7.1	7.2	7.4	7.3	7.1	7.2

FT=Foot and Taylor Medium
ORP=Oxidation-reduction potential (meV in unit, with reference to standard Calomel electrode)

3.4.1 Deionized Water Containing 0.3% Hydrogen Peroxide

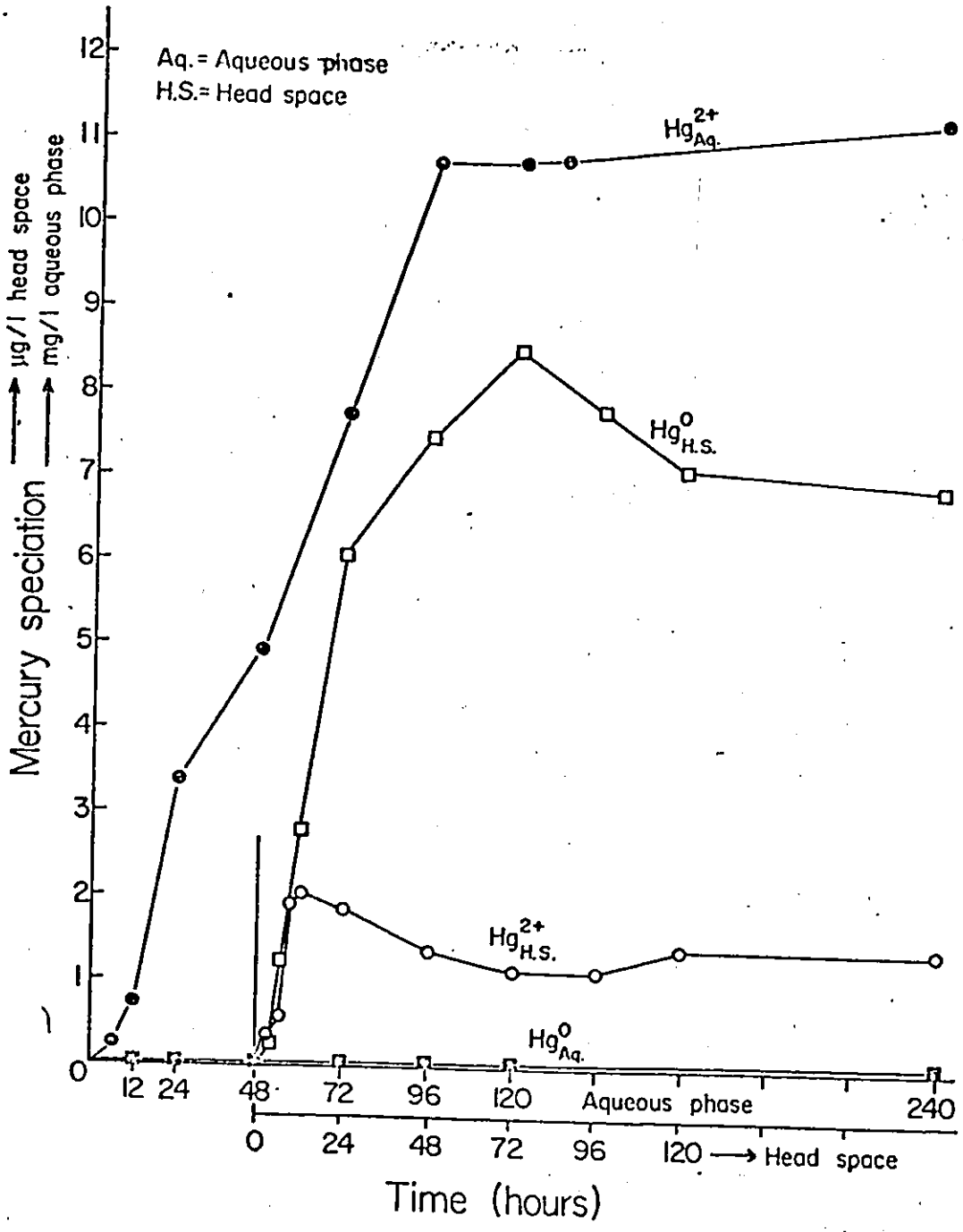
The speciation of mercury was quite different when 0.3% H_2O_2 was added to deionized water (Fig. 25). The level of Hg^{2+} in the aqueous phase increased 8700 times over that found in deionized water alone. The amount of Hg^{2+} in the head space also increased, but the ratio of $[Hg^{2+}]$ in water :$[Hg^{2+}]$ in head space was much higher (8,100 times) in H_2O_2 water than in deionized water (Fig. 24 and 25). This could be due to the fact that H_2O_2 has a higher oxidizing capacity than the deionized water. Even the reduced species Hg^0 was about 1.7 times higher in the head space (Fig. 24 and 25) in H_2O_2 water. The speciation seemed to reach a steady state in 120 hours.

By comparison with deionized water and other systems, the lower values of ORP in hydrogen peroxide water system might be due to its rapid oxidation of metallic mercury.

3.4.2 Sterilized FT Medium

The results were similar to those found in deionized water for the distribution of metallic mercury speices. As shown in Fig. 26, $[Hg^0]$ dissolved in the aqueous phase was 31 ppb and 4 ppb in head space, reaching a steady state in about 96 hours. The concentration of $[Hg^{2+}]$ species in the head space was below the limit of detection and thus needed not be considered in the mass balance. However, the $[Hg^{2+}]$ species in the aqueous phase reached 9 ppb at 'equilibrium'

Figure 25: Transformation of metallic mercury in deionized water with 0.3% hydrogen peroxide


Aq. = Aqueous phase
H.S. = Head space
Mercury speciation
µg/l head space
mg/l aqueous phase
$Hg^{2+}_{Aq.}$
$Hg^{0}_{H.S.}$
$Hg^{2+}_{H.S.}$
$Hg^{0}_{Aq.}$
0
1
2
3
4
5
6
7
8
9
10
11
12
12 24 48 72 96 120 Aqueous phase 240
0 24 48 72 96 120 → Head space
Time (hours)

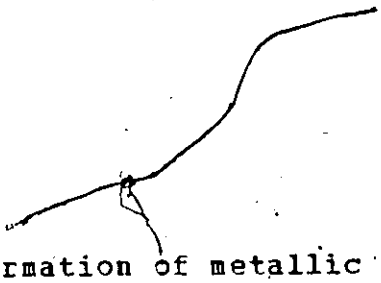
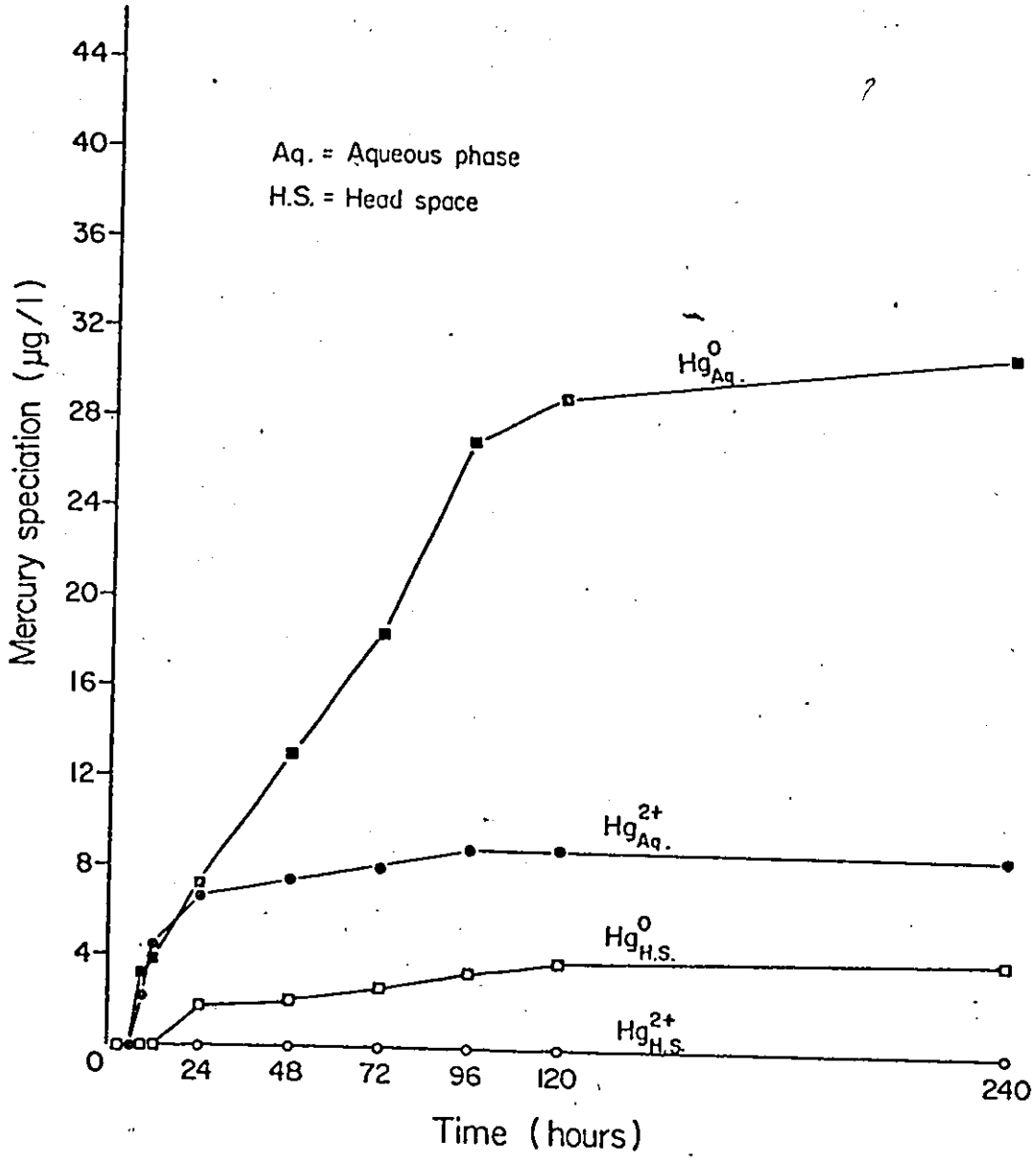


Figure 26: Transformation of metallic mercury in sterilized Foot and Taylor medium.


Aq. = Aqueous phase
H.S. = Head space
Mercury speciation (µg/l)
Time (hours)
$Hg^{0}_{Aq.}$
$Hg^{2+}_{Aq.}$
$Hg^{0}_{H.S.}$
$Hg^{2+}_{H.S.}$
0
4
8
12
16
20
24
28
32
36
40
44
24
48
72
96
120
240

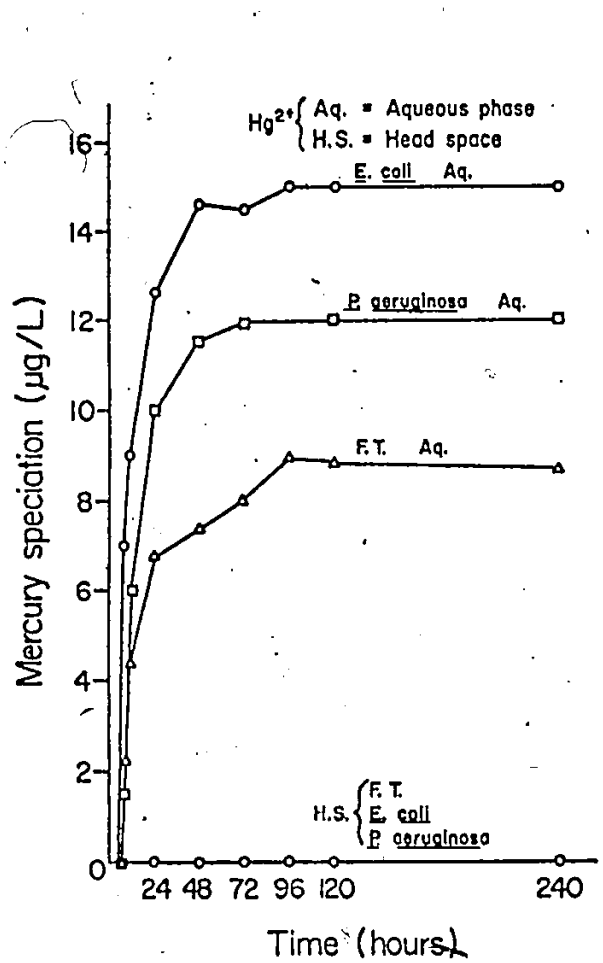
(96 hours) which was higher than that found in the deionized water system. This might be due to the fact that trace amounts of inorganic and organic compounds in FT medium can form complexes with Hg^{2+} ions and are stable in the aqueous phase.

3.4.3 FT Medium Containing Viable Bacteria

In the presence of growing bacteria, either Escherichia coli or Pseudomonas aeruginosa, $[Hg^{2+}]$ in the head space was below the limit of detection and hence was not considered in the mass balance of mercury. For the Hg^0 species in the head space, the amount was approximately the same for both bacteria and sterilized FT medium in the first 24 hours (Fig 27). However, the biological action on transformation of metallic mercury was obvious at 48 hours, and by 240 hours there was about 5 ppb of $[Hg^0]$ in the head space in the FT medium- Escherichia coli system and 8 ppb in the FT medium- Pseudomonas aeruginosa system. The level of $[Hg^{2+}]$ in the aqueous phase was slightly higher in the presence of growing bacteria, especially with Escherichia coli than in the FT medium alone. However, the level of Hg^0 in the aqueous phase was approximately the same as in the FT medium (Fig. 27). Viable cell counts showed that the bacteria had grown well in this medium. The number of viable cells was the same both in the control and in the mercury added FT medium for both organisms (Fig. 28 and 29).

Figure 27: Transformation of metallic Mercury in FT medium with/without growing bacteria

The control curves of sterilized FT medium system are redrawn from Fig. 26


Hg2+ { Aq. = Aqueous phase
H.S. = Head space
E. coli Aq.
P. aeruginosa Aq.
F.T. Aq.
H.S. { F.T.
E. coli
P. aeruginosa
Mercury speciation (µg/L)
0
2
4
6
8
10
12
14
16
24 48 72 96 120 240
Time (hours)

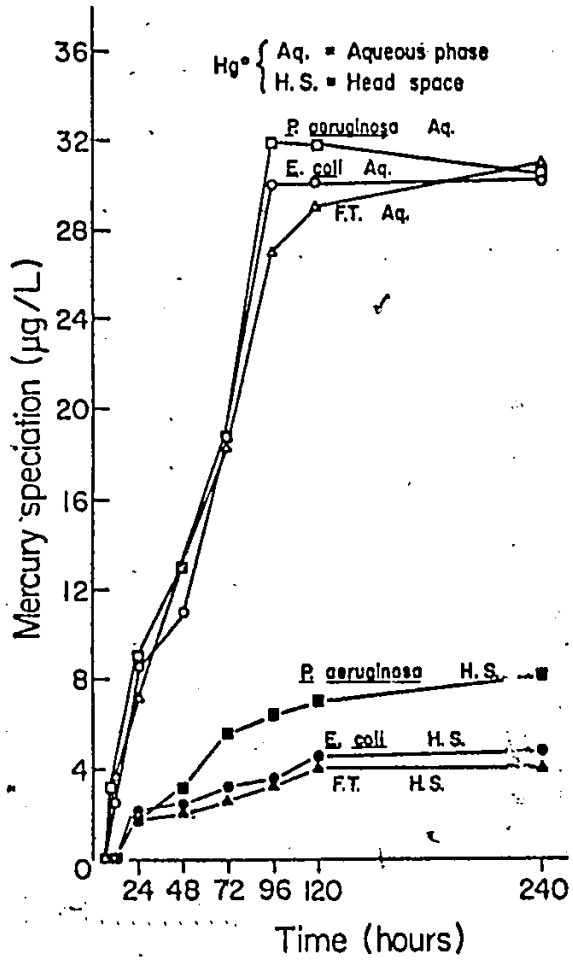

Hg° { Aq. = Aqueous phase
H.S. = Head space
P. aeruginosa Aq.
E. coli Aq.
F.T. Aq.
P. aeruginosa H.S.
E. coli H.S.
F.T. H.S.
Mercury speciation (µg/L)
0
4
8
12
16
20
24
28
32
36
24 48 72 96 120 240
Time (hours)

Figure 28: Growth of E. coli in FT medium with/without metallic mercury added

FT= Foot and Taylor medium

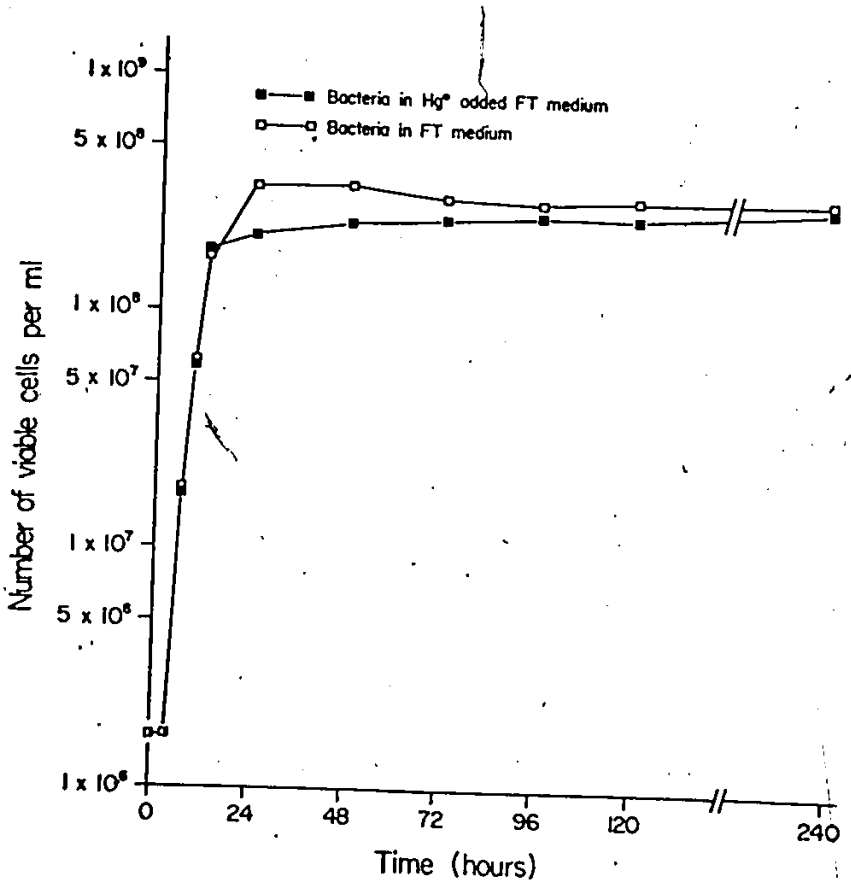
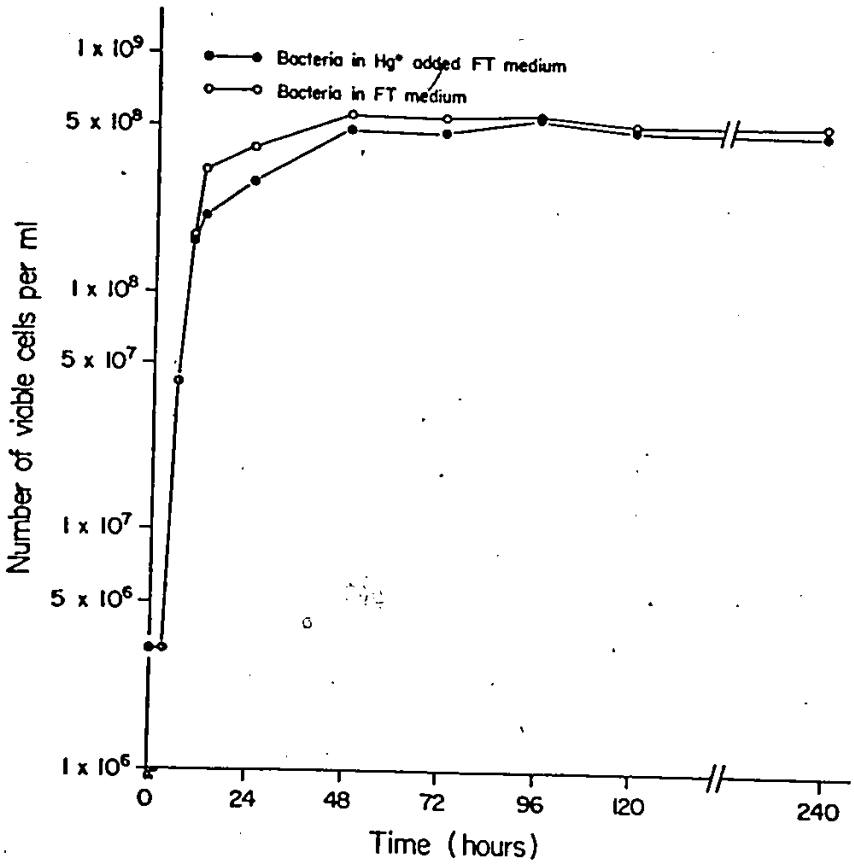

Bacteria in Hg° added FT medium
Bacteria in FT medium
Number of viable cells per ml
1 x 10⁹
5 x 10⁸
1 x 10⁸
5 x 10⁷
1 x 10⁷
5 x 10⁶
1 x 10⁶
0
24
48
72
96
120
240
Time (hours)

Figure 29: Growth of P. aeruginosa in FT medium with/without metallic mercury added

FT=Foot and Taylor medium


Bacteria in Hg° added FT medium
Bacteria in FT medium
Number of viable cells per ml
1 x 10⁹
5 x 10⁸
1 x 10⁸
5 x 10⁷
1 x 10⁷
5 x 10⁶
1 x 10⁶
0
24
48
72
96
120
240
Time (hours)

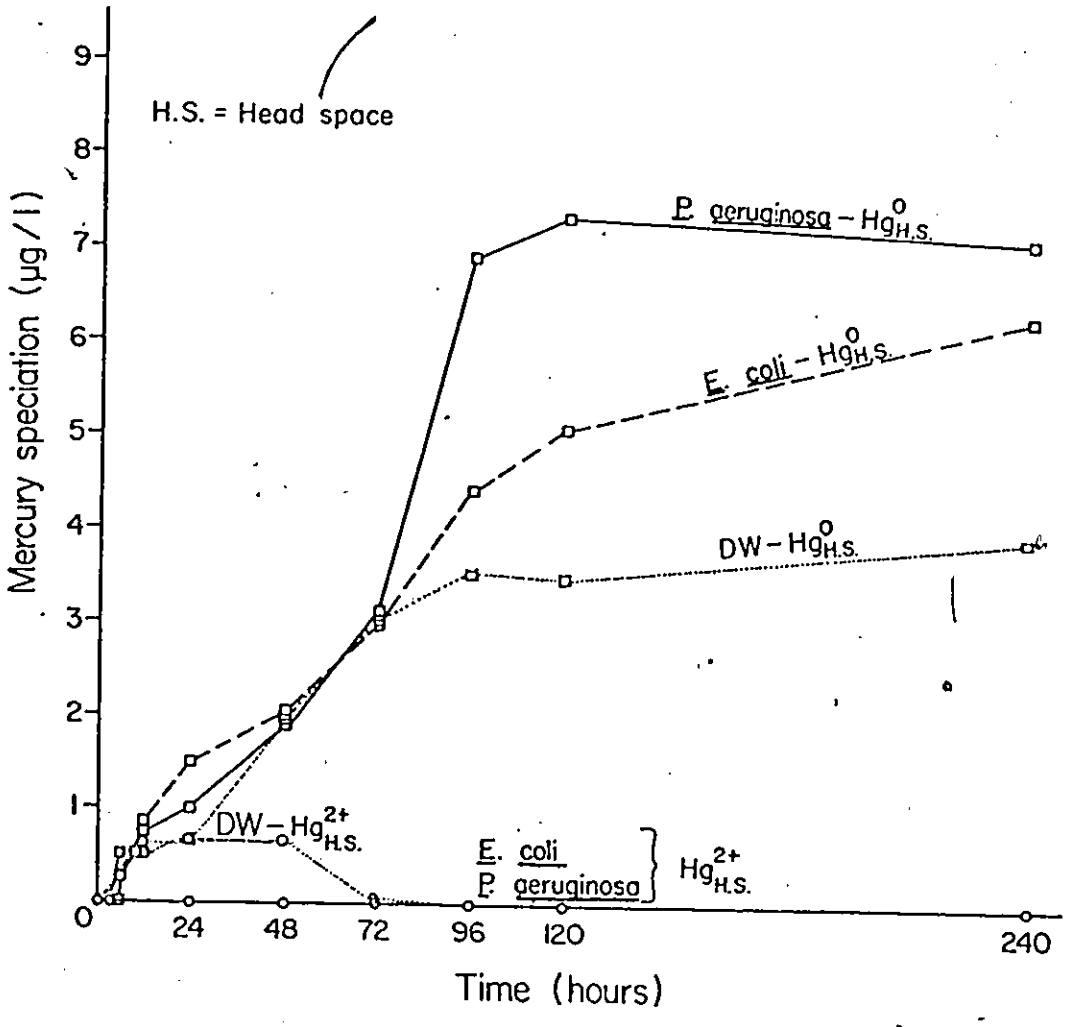
3.4.4 Deionized Water Containing Dead Bacteria

There is no doubt that living bacteria play a role in the transformation of metallic mercury. Therefore, it is important to study whether nonviable cells play the same role in the transformation of metallic mercury . Experiments were designed to study the transformation of metallic mercury in deionized water containing thermally killed Escherichia coli or Pseudomonas aeruginosa. Only the mercury species in the head space were monitored since these were to be the critical determinant species in mercury distribution in a closed system (i.e. water surface-head space) for the following reasons: The amount of dissolved Hg^0 in the aqueous phase in any system is limited by its solubility (~30 ppb). Hence the excess of Hg^0 produced by biological means should escape into the head space. Mercuric ions produced will be stabilized in the aqueous phase by the complexing components of the nutrient medium as well as the bacterial cell mass at the level of Hg^{2+} produced. Hence, determination of Hg^0 and Hg^{2+} in the head space will be sufficient to provide information on the speciation of Hg^0 in the system.

The results are shown in Fig. 30. Again, the concentration of Hg^{2+} in the head space was below the limit of detection, The amount of Hg^0 in the head space was approximately the same in both systems up to 72 hours; then it was higher in the flask with Pseudomonas aeruginosa at 240

Figure 30: Transformation of metallic mercury in deionized water containing thermally killed bacteria.

The control curves of deionized water are redrawn from Fig. 24.


H.S. = Head space
Mercury speciation (µg/l)
Time (hours)
P. aeruginosa – $Hg^0_{H.S.}$
E. coli – $Hg^0_{H.S.}$
DW – $Hg^0_{H.S.}$
DW – $Hg^{2+}_{H.S.}$
E. coli
P. aeruginosa
$Hg^{2+}_{H.S.}$
0
1
2
3
4
5
6
7
8
9
24
48
72
96
120
240

hours, about 7 ppb of metallic mercury in the head space was found for both bacterial systems.

At the 'equilibrium state' (about 120-240 hours), the amount of Hg^0 in the head space was about the same for both the dead or living bacteria (Fig. 27 and 30). However, the total amount of dead cells (2 gm wet weight) was less than that of living cells in the stationary phase (about 7.5 gm wet weight for Escherichia coli and 12.5 gm wet weight for Pseudomonas aeruginosa) at 240 hours. It seems that the nonviable cells are more efficient than living cells in accelerating the dissolution of metallic mercury in water and then volatilizing it into the head space. This suggests that thermal killing of bacteria might expose the internal structures releasing the intracellular Hg-binding components.

From the above studies, it is clear that the bacteria chosen, either living or thermally killed, possess the ability to transform metallic mercury from water into air. It is also clear that the amount of Hg^0 in the head space of the Pseudomonas aeruginosa system is slightly more than that of Escherichia coli. However, the amount of Hg^0 produced in the head space was also almost the same in viable or nonviable bacterial system as well as hydrogen peroxide system and was around 7 ppb. The pH values of systems all ranged from 6.4-7.5. The redox potential of the systems except the hydrogen peroxide system was 425 ± 25 mV with reference to stan-

dard calomel electrode. The drop in redox potential in the hydrogen peroxide system might be due to its rapid oxidation of metallic mercury (8700 times compared to that of deionized water). Hydrogen peroxide, however, stabilized Hg^{2+} in the aqueous and head space areas. This study suggests that the nature of the redox couple is important in the transformation of metallic mercury. The possibility of Hg^{o} being produced in natural water depends on the types of chemical and biological moities present which might provide favorable conditions.

3.5 BINDING OF HEAVY METAL IONS ONTO BIOTA

The toxicity of heavy metal ions such as copper and mercury on organisms has been well studied (Albert et al., 1973; Brown and KulKarni, 1967; Buhler, 1973; Gupta and Mukherji, 1977; Jugo, 1977; Krishnan Nambusan et al., 1977; lee et al., 1973; Mukherji and Nag, 1976; Rice et al., 1973; Sunda and Guillard, 1976; Tompkins and Blinn, 1976). At the cellular level, it is believed that heavy metal ions bind onto cells either at the surface layers (Brunker and Bott, 1974; Nakagami et al., 1969) or in the intracellular fluid (Norseth, 1969).

Binding of heavy metal ions may interfere with the biochemical reactions of the cell. Metal ions may produce structural changes in the cell membrane, affecting the permeability (Brunker, 1976; Passow and Rothstein, 1960;

Shieh and Barber, 1972; weed et al., 1962). However, studies with phytoplankton show that alternations in membrane permeability need not be the principle factor in determining inhibition of cell growth (McBrien and Hassall, 1967). The partitioning of metal ions among intracellular fluids may be of considerable importance, classes of proteins may change conformations by binding with particular metal ions; enzyme activities may become inhibited (Vallee and Ulmer, 1972) due to inactivation by metal ions; the interaction between metal ions and ligands within the cells or the changes in electrostatic charges along with a shift in the ionization constant also poison the cells.

Identification of the cellular location of the metal and the strength of binding are important in order to understand more about the toxicity of metal ions for organisms. Experiments were designed to obtain such information. Experimental procedures involved in the study are described in section 2.6. The heavy metal ions used were inorganic mercuric nitrate and cupric nitrate. The organism used was Pseudomonas aeruginosa.

3.5.1 Selection of Culture Media

In order to find out a suitable medium for this study, the following were used: 1) Peptone Yeast Extract Broth (PYEB) and 2) modified glucose basal minimal salt media: a) modified Finnerty's medium (Makula and Finnerty,

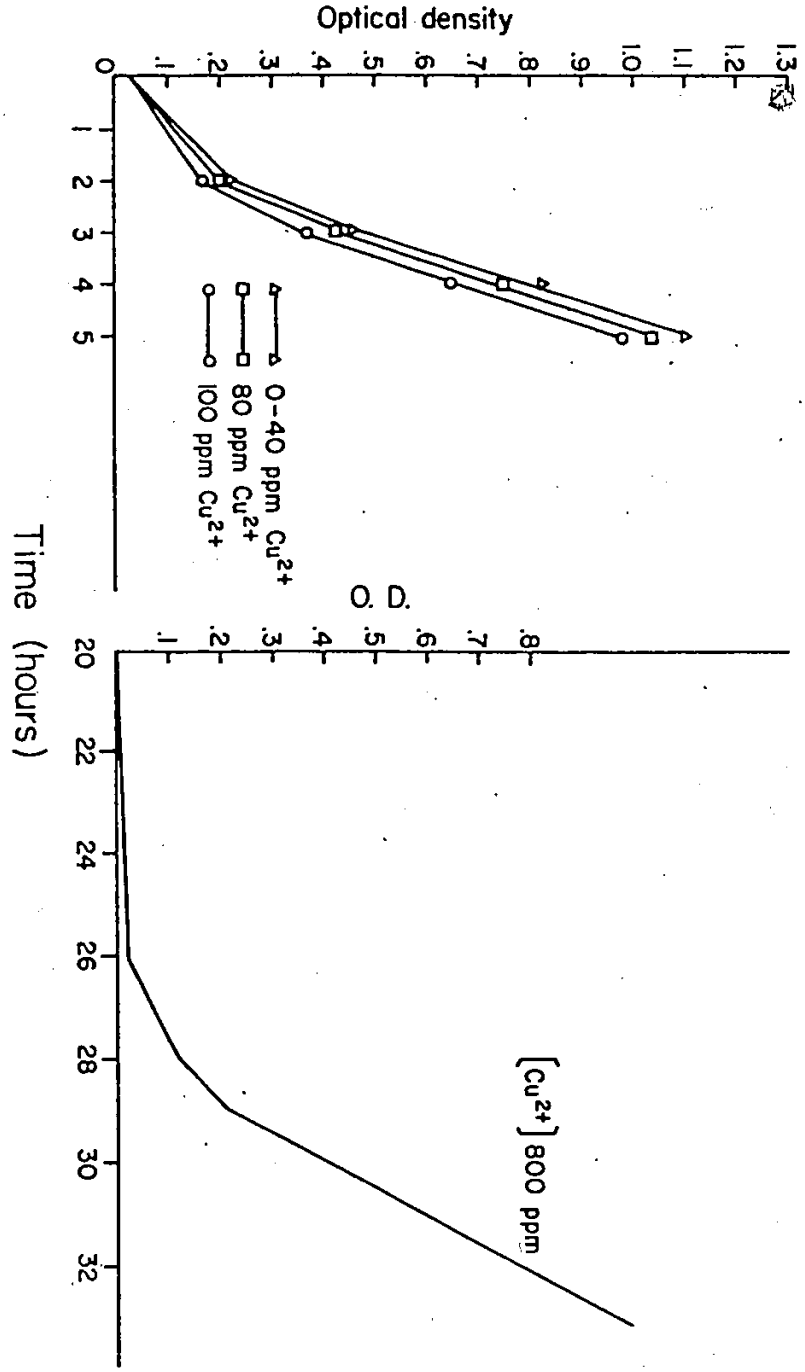
1968) as described by Breuil et al., (1978), and b) Modified Collin's medium.

3.5.1.1 Peptone Yeast Extract Broth (PYEB)

Peptone Yeast Extract Broth (PYEB), a complex medium containing organic as well as inorganic components, was first selected for the study. The growth of Pseudomonas aeruginosa in this medium with various concentrations of copper ions is shown in Fig 31. Bacteria grew well in this medium with copper ion concentrations up to 100 ppm. In fact, there was no difference in growth rate between the medium without copper ion or medium with 40 ppm copper ions. The bacteria even grew in PYEB medium with a copper ion concentration of 800 ppm . It is possible that not all the metal ions present in the medium were available for the cells. Ramamoorthy and Kushner (1975) have observed that heavy metal ions such as cupric and mercuric ions bind the yeast extract and peptone strongly. Gachter et al (1973) have found that only the ionic copper species, but not its organic complex species are toxic to algae. Hamdy (1977) has compared the growth of Enterobacter aerogenes in Glucose Basal Salt Broth spiked with mercuric ions with and without yeast extract, and reported that the bacteria grew faster if yeast extract was added, and most mercuric ions remained in the cell free medium containing yeast extract. Hannan and Patouillet (1972) have also reported that the toxicity of

Figure 31: Growth of *P. aeruginosa* in PYEB medium with copper ions

PYEB = Peptone Yeast Extract Broth.


Optical density
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1
2
3
4
5
0-40 ppm Cu2+
80 ppm Cu2+
100 ppm Cu2+
Time (hours)
O. D.
.1
.2
.3
.4
.5
.6
.7
.8
20
22
24
26
28
30
32
[Cu2+] 800 ppm

mercury for phytoplankton varies with the concentration of nutrients (phosphate, silicate, nitrate) present in the medium. Thus it can be concluded that PYEB medium is not a suitable medium for the study of toxicity and binding of cupric or mercuric ions onto microorganisms.

3.5.1.2 Glucose Basal Minial Salt Media

a) Modified Finnerty's Minimal medium and b) Modified Collin's Medium

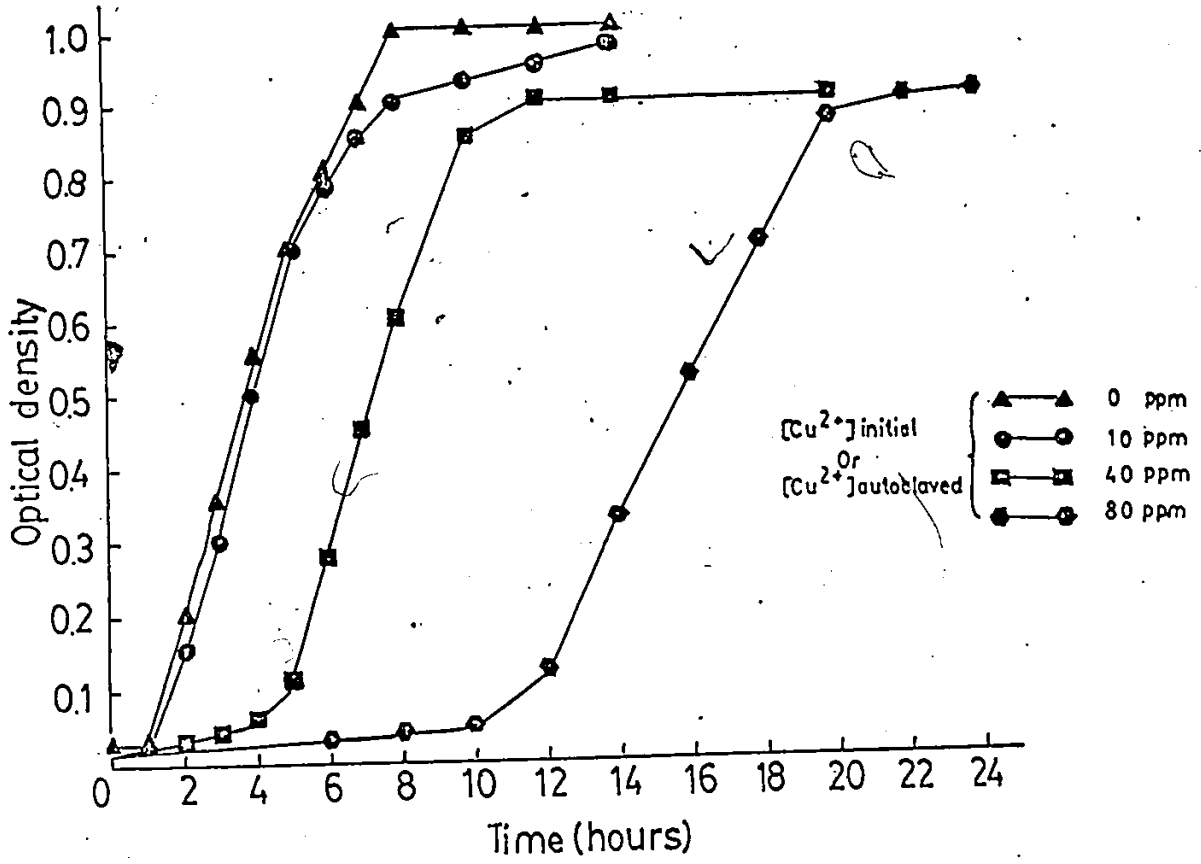
The next choice of medium was modified Finnerty's minimal medium (NH_4NO_3 2g/l; KH_2PO_4 4g/l; Na_2HPO_4 6g/l; $MgSO_4$ $7H_2O$ 0.2 g/l; $CaCl_2$ 0.05 g/l; $FeSO_4 7H_2O$ 0.01 g/l, pH = 7.4) with 0.25% filtrable glucose as the carbon source. Pseudomonas aeruginosa grew well in this medium, but when copper ions were added to the medium, this modified Finnerty's medium formed copper precipitate(s), this medium was thus abandoned and Collin's minimal salt medium (K_2HPO_4 0.21 g/l; NaH_2 PO_4 0.09 g/l; $FeSO_4 7H_2O$ 0.005 g/l; NH_4NO_3 1.0 g/l; $MgSO_4 7H_2O$ 0.05 g/l pH = 7.0) was used. The components of this medium are similar to those of modified Finnerty's medium except that the former does not contain $CaCl_2$ and the concentration of the other salts are much lower. In order to supplement the deficiency of nutrients, 0.25% filtrable glucose and 0.5% peptone were added. This modified Collin's medium was used for the subsequent study of heavy metal ions bound to Pseudomonas aeruginosa since this media supported good

growth of Pseudomonas aeruginosa. However, the bacteria had a lag phase of about 10-12 hours if 80 ppm of Cu^{2+} or 40 ppm of Hg^{2+} ions were added initially to the medium before autoclaving (Fig 32 and 33). (After autoclaving, 80 ppm of Cu^{2+} and only 5.15 ppm of Hg^{2+} remained in the medium (Table 13), (see detailed discussion later). No growth of bacteria was observed on the first day when autoclaved medium contained more than 80 ppm of Cu^{2+} or more than 5.15 ppm Hg^{2+}. This medium spiked with 80 ppm Cu^{2+} or 40 ppm (= 5.15 ppm after autoclaving) Hg^{2+} ions was used for the subsequent study of metal binding onto biota and the cellular location of the metal ions.

3.5.1.3 Heavy Metals Binding Onto Pseudomonas aeruginosa

Pseudomonas aeruginosa was cultured in modified Collin's medium with initial cupric ion concentration of 80 ppm or initial mercuric ion concentration of 40 ppm, and the cell pellets were obtained by centrifugation. The metals in washed cells were then extracted with varying concentrations of analytically pure nitric acid, and the concentrations of heavy metals in the fractions were estimated by Atomic Absorption Spectrophotometry as described in section 2.2.1. By determining the cupric concentration in the cell free medium, washing solution as well as washed cells, it was

Figure 32: Growth of P. aeruginosa in the modified Collin's medium with copper ions.


Optical density
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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)
[Cu2+] initial
Or
[Cu2+] autoclaved
0 ppm
10 ppm
40 ppm
80 ppm

Figure 33: Growth of P. aeruginosa in the modified Collin's medium with mercuric ions.

Loss of mercuric ions after autoclaving (detail see text).

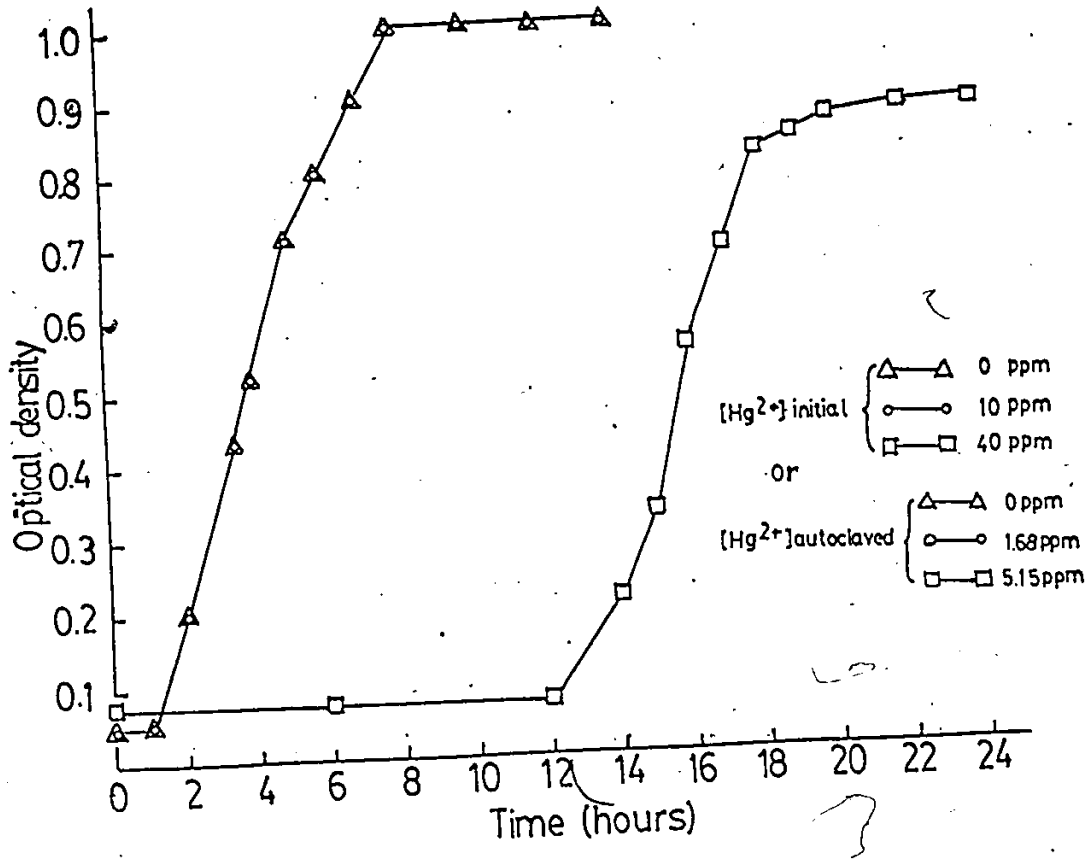
Optical density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)
[Hg2+] initial
0 ppm
10 ppm
40 ppm
or
[Hg2+] autoclaved
0 ppm
1.68 ppm
5.15 ppm

TABLE 13

Loss of Metal Ions During Autoclaving

	Modified Collin's Medium spiked with			
	Cu^{2+}		Hg^{2+}	
Initial concentration of metal ions in medium before autoclaving	10.00 ppm	80.00 ppm	10.00 ppm	40.00 ppm
Concentration of metal ions in autoclaved medium	10.00 ppm	80.00 ppm	1.68 ppm	5.15 ppm
Metal ions remained in autoclaved medium (%)	100.00	100.00	16.80	12.88
Loss of metal ions (%)	0.00	0.00	83.20	87.12

found that there was no loss of copper ions during the autoclaving and culturing process and the total amount of copper was recovered (Table 14).

In the case of mercury, the results were different. Added mercury in the 0-10 ppm range showed the same growth rate for Pseudomonas aeruginosa (Fig. 33). It is possible that there was a great loss of mercury from the medium during the autoclaving and culturing processes. In fact, the estimation of mercury in the autoclaved medium showed that about 83-87% of initially added mercury was lost (Table 13). More mercury was also lost during bacterial growth. It is shown (Summers and Lewis, 1973; Summers and Silver, 1972) that there are bacteria present in natural waters: e.g. Pseudomonas sp. which are capable of reducing mercuric ions to the volatile form of Hg^0. Based on the above observations and actual determinations of mercury content, only 5.15 ppm of mercury remained in the medium after autoclaving and about 1 ppm of mercury was left after growth when the initial concentration was 40 ppm of mercury. The loss of mercury after growth is due to the volatilization, and the remainder is in the cell-supernatant system (Table 14).

Tables 14 and 15 present the results of the extraction of cell bound metal ions by solutions of varying pH. The tables show the total concentration of cupric or mercur-

TABLE 14

Binding of Heavy Metal Ions onto P. aeruginosa

Modified Collin's medium spiked with

	Cu^{2+}	Hg^{2+}
Initial concentration of metal ions added to medium (ppm)	80.00	40.00
Concentration of metal ions in autoclaved medium (ppm)	80.00	5.15
Total concentration of metal ions in culturing cell-supernatant system after culturing (ppm)	80.00	1.14
Concentration of metal ions in cell free supernatant after culturing (ppm)	72.50	8.98×10^{-1}
Concentration of metal ions in water after washing the cells (ppm)	0.50	2.00×10^{-3}
Concentration of metal ions (out of the initial total amount) found on washed cells (ppm)	7.00	0.24
Concentration of cell bound metal ions leaching from cells under acidic condition (ppm)	3.60	0.02
Corresponding pH value of acid solution containing the metal ions leaching from cells	3.00	1.68
Dissociation Constant K of metal ions bound onto cells	9.44×10^{-4}	2.30×10^{-1}
Metal ions bound onto cells (%)	8.75	21.05

TABLE 15

Dissociation constant K of metal ions bound onto cells

I) Cu^{2+}

Concentration of metal ions (out of initial total amount) found on washed cells			7 ppm		
Concentration of metal ions leaching from cell under acidic condition (ppm)	7.00	6.80	5.40	3.60	1.60
Corresponding pH value of acid solution containing the metal ions leaching from cells	1.20	1.50	2.50	3.00	3.56
Dissociation constant K of metal ions bound onto cells	-	9.30×10^{-4}	9.34×10^{-4}	9.44×10^{-4}	9.30×10^{-4}

II) Hg^{2+}

Concentration of metal ions (out of initial total amount) found on washed cells			0.24 ppm		
Concentration of metal ions leaching from cell under acidic condition (ppm)	0.02	0.02	0.01	5.30×10^{-3}	2.50×10^{-3}
Corresponding pH value of acid solution containing the metal ions leaching from cells	1.00	1.68	2.00	2.31	2.69
Dissociation constant K of metal ions bound onto cells	-	2.30×10^{-1}	2.30×10^{-1}	2.17×10^{-1}	1.94×10^{-1}

ic ions bound, and leached from the cells by acid treatment. For example, for an initial concentration of 80 ppm Cu^{2+} ions, only 7 ppm was found in the washed cells. About half of the amount (= 3.6 ppm) of Cu^{2+} ions was released by acid extraction at pH 3. All the Cu^{2+} ions bound to the cells (=7 ppm) could be released by leaching with acid at pH 1.2. Similarily for mercury, 0.9 ppm was found in the supernatant and 0.24 ppm of Hg^{2+} in the cells for the medium spiked initially with 40 ppm of Hg^{2+} (= 5.15 ppm Hg^{2+} in autoclaved medium). However, only 0.02 ppm of Hg^{2+} could be extracted at pH 1.68.

For both Cu^{2+} and Hg^{2+}, the dissociation constant of the metal binding sites where the metal ions can be extracted with acid was calculated and shown in Tables 14 and 15. The dissociation constant K was calculated from the Hasselbalch and Henderson equation. The equation is modified since hydrogen ions displace heavy metal ions from the binding sites. The modified equation is defined as follows:

$$pH = pK + \log \frac{[M]\ \text{undissociated}}{[M]\ \text{dissociated}}$$

$$= pK + \log \frac{[M]\ \text{total} - [M]\ \text{dissociated}}{[M]\ \text{dissociated}}$$

and $pK = -\log K$

where K = Dissociation Constant for metal ions [M] from the binding sites

[M] total = the concentration of the heavy metal ions (out of the initial

total amount) found on washed cells
[M] dissociated = the concentration of cell bound heavy metal ions leached at a known pH
pH = negative log of [H^+] of the leaching solution

From the above calculation, it was found that the attachment of Hg^{2+} to mercury binding sites in cells was stronger than that of Cu^{2+} to copper binding sites. In fact, certain fractions of mercuric ions could not be extracted with the acid at any pH. This may be due to the presence of two types of binding sites for mercury in cells, one being the proton exchangeable sites and the other being nonexchangeable with protons. Beaufard et al (1977) have also observed these two types of binding sites in plant systems.

Besides the study of the strength of metal binding, the cellular distribution of metal ions was also investigated and the results are shown in Table 16. It is obvious that most of the heavy metal ions are retained on the cell wall layer of cells which may explain why the cells were able to grow in a heavy metal contaminated environment. Several bacterial strains carrying genes resistant to mercury and other metal ion effects on naturally occurring

TABLE 16

Cellular Distribution of Heavy Metal Ions in P. aeruginosa

component of cell	Cu^{2+}	Hg^{2+}
Outer wall layer (30,000 x g, 30 minutes, pellet)	75.3%	78.6%
Ribosome (100,000 x g, 120 minutes, pellet)	9.0%	5.8%
Intracellular fluid (100,000 x g, 120 minutes, supernatant)	15.7%	15.6%

plasmids have been reported (Nakahara et al., 1977a,1977b,1977c; Novick and Roth, 1968; Pinney, 1978; Richmond and John, 1964). These resistant cells may either protect themselves by retaining the ions outside the cells (Kondo et al., 1974), by converting the metal ions (such as mercuric ions) to a volatile form (Brunker and Bott, 1974;Summers and Lewis, 1973; Summers and Silver, 1972) or changing the ions incorporated in the cells into an innocuous form (Kondo et al 1974). The possibility of the presence of a pool of free intracellular SH moities which protect the enzyme and protein systems cannot be ruled out. Such pools of SH moities have been reported in a highly mercury resistant strain of Aspergillus niger (Ashworth and Amin, 1964). The synthesis of several heavy metal binding proteins as a regulatory mechanism to avoid intoxication by excess uptake of the heavy metal ions is possible too, since such types of proteins are common in invertebrate, vertebrate and plant systems (Talbot and Magee, 1978).

Chapter IV

CONCLUSIONS

Mercury emission from both natural and man-made sources, the hydrosphere as well as the atmosphere are important as far as mercury interconversions and bioaccumulation are concerned. Depending on the environmental condition, methylation and demethylation processes of mercury are reversible. For the systems studied in this work, sediment and viable microorganisms can demethylate the methylmercury in natural waters. The increasing order of demethylation of methylmercury into inorganic mercury (MC) is river water, sediment, Escherichia coli, Anabaena flos-aquae, and Pseudomonas fluorescens. The stability of methylmercury in Ottawa River water is not different from that of deionized water, suggesting that the dissolved micro- and macro- solutes and the microbial community at the level present in river water are not involved in the demethylation process. However, sediment and biota do alter the speciation of mercury in natural waters. In a natural water system, transformed inorganic mercury forms sink with the sediments through strong covalent bonding. Methylmercury is quickly sorbed onto the biota as well, with ensuring conversion to inorganic mercury while bound onto the biota. Thus very little of inorganic

mercury or methylmercury is found in the water column. The conversion rate is dependent on the biota concentration and the initial methylmercury concentration. the rate being proportional to the biota concentration but varying inversely with the initial mercury concentration. For the systems studied here, viable Pseudomonas fluorescens can further volatilize the mercury species into air. However, thermally killed bacteria (either Pseudomonas fluorescens or Escherichia coli) methylate the inorganic mercury rather than demethylate the methylmercury present in the stock solution. Although physical factors such as turbulence, wind velocity and direction, and the amount of suspended matter play an important role in the sediment activity in natural environments, the finding in this study are of environmental significance. It is tempting to suggest that the toxicity of methylmercury-contaminated waters might be reduced by dumping large quantities of these low cost, fast multipling bacteria either at the discharge sources or municipal waste water treatment plant. It is also recommended that the effect of microbial extracellular products should be investigated in further study.

Since metallic mercury, like methylmercury, is lipophilic to organisms, the transformation of metallic mercury in the natural water system was also studied. The solubility of metallic mercury in water is about 32 ppb. Both living or dead Escherichia coli and Pseudomonas aeruginosa play

a role in the transformation of metallic mercury . The study of the transformation of metallic mercury in deionized water containing 0.3% hydrogen peroxide indicates that hydrogen peroxide can stabilize the Hg^{2+} in both the aqueous and head space area. Either living or dead Escherichia coli and Pseudomonas aeruginosa transform metallic mercury from water into air. The amount of Hg^0 produced in the head space is almost the same in both the living or dead bacterial and hydrogen peroxide systems (7 ppb). The redox potential of the systems, except the hydrogen peroxide system, is about 425 ± 25 mV with reference to standard calomel electrode. The hydrogen peroxide system, compared to other systems, has a lower redox potential. The drop of redox potential in the hydrogen peroxide system might be due to the rapid oxidation of metallic mercury (8700 times as fast as in deionized water). The pH values range from 6.4-7.5 for all systems. The study thus suggests that the nature of redox couple is important in mercury speciation in natural waters. The possibility of Hg^0 being produced in natural waters depends on the types of chemical and biological moities which might provide the favorable reducing conditions. In the natural environment, the redox potential might vary from the bottom of the water (anaerobic) to the upper level of the water (aerobic) in a flowing water system. It is possible that Hg^0 could be produced in a microenvironment where conditions are favorable and further movement to air via wa-

ter or subsequent transformation of this Hg^0 will depend on the physico-chemical conditions of the bulk water.

In addition to the speciation of mercury, the studies show that the minimal salt medium is the only medium suitable for the study of binding of Cu^{2+} and Hg^{2+} by Pseudomonas aeruginosa. It was found that the heavy metal ions are mainly retained on the outer cell wall (for Cu^{2+}: cell wall layer, 75.3%; ribosome, 9%; intracellular fluid, 15.7% and for Hg^{2+}: cell wall layer, 78.6%; ribosome, 5.8%; intracellular fluid, 15.6%). For Pseudomonas aeruginosa, the strength of binding of mercuric ions ($K = 2.3 \times 10^{-1}$, K = Dissociation Constant) was two orders higher than that of cupric ions ($K = 9.4 \times 10^{-4}$). In fact, the acid leaching at different pH has revealed the presence of two types of mercury binding sites in cells, namely, the proton exchangeable and proton nonexchangeable sites. Thus, the study of the affinity of heavy metal ions to microorgansms gives predictive information about the behavior of heavy metal binding to cells. For further work, the study of the affinities of other metal ions for various microorganisms is recommended. The genetics of the microorganisms, the presence of any heavy metal resistant genes in the plasmid, and mechanisms of defence against heavy metal poisoning should also be studied.

In this work, a satisfactory system of techniques for the analysis of transformation of methylmercury has been de-

veloped. This involves the combination of the dithizone extraction of heavy metal ions, Thin Layer Chromatography separation, as well as Gamma counting. It is suggested that the application of these techniques should be extended to the study of other heavy metal ion pollutants that might undergo alkylation which seems to be the most toxic to organisms. Tin, arsenic and selenium are the other metals recommended for the future study by the use of these techniques.

Appendix A

1) Dithizone-benzene solution (0.1%): The solution was prepared as follows: a amount of 0.57 gm of dithizone crystal (Baker) was dissolved in 500 ml benzene. The solution was purified by shaking it three times with 100 ml of 10% ammonium hydroxide. The ammonium hydroxide solution, containing the pure dithizone, was then neutralized with 45 ml concentrated hydrochloric acid and back extracted with 500 ml fresh benzene.

2) Sodium hydroxide solution (45%): The solution was prepared by dissolving 900 gm of sodium hydroxide (Baker) in 2 litres deionized water. After adding 10 ml of 10% stannous chloride solution, nitrogen gas was bubbled vigorously through the solution for about one hour to remove the contaminating mercury in the sodium hydroxide crystal solution.

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